



Development and clinical evaluation of new bloodbased tests for improving the diagnosis of pulmonary tuberculosis and identification of candidate biomarkers for treatment monitoring : a multicenter study in moderate and high incidence settings

Rim Bayaa

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Development and clinical evaluation of new blood-based tests for improving the diagnosis of pulmonary tuberculosis and identification of candidate biomarkers for treatment monitoring: A multicenter study in moderate and high incidence settings

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Abbreviations

ADCC: antibody-dependent cytotoxicity

ADRs: Adverse drug reactions

AFB: acid-fast bacillus

AFB⁻: negative acid-fast bacillus smear microscopy

AG: arabinogalactan

Ag85: antigen 85

AlereLAM: Alere Determine TB LAM Ag

ATB: active tuberculosis

AUC: areas under the curve

BAL: broncho-alveolar lavage

BCG: bacille Calmette–Guérin

BMI: body mass index

CBC: blood cell count

cDNA: complementary DNA

CFP-10: 10-kDa culture filtrate protein

CFU: colony forming units

CI: confidence interval

CLT⁺: positive sputum culture

CM: central memory

Covid-19: coronavirus

CRP: C-reactive protein

CT: computed tomography

Ct: cycle threshold

CyTOF: cytometry by time-of-flight

DBS: dried blood spots

DCs: dendritic cells

DOTS: directly observed treatment

DR-TB: drug-resistant tuberculosis

DST: Drug susceptibility testing

DTH: delayed-type hypersensitivity

EC: early clearance

ELISA: enzyme linked immunosorbent assay

ELISPOT: enzyme-linked immunospot

EM: effector memory

EMB or E: ethambutol

ESAT-6: 6-kDa early secretory antigenic target

ESR: erythrocyte sedimentation rate

ESX1-5: five type VII secretion systems

FIND: Foundation for Innovative New Diagnostics

GPL: glycopeptidolipids

GSH: glutathione

GZB: granzyme B

HBHA: heparin-binding hemagglutinin antigen

HC: healthy controls

HD: healthy donors

HHCs: house hold contacts

HIV: human immunodeficiency virus

iDCs: infected dendritic cells

IFN- γ : interferon-gamma

IGRAs: interferon-gamma release assays

ILCs: innate lymphoid cells

ILTs: Ig-like transcripts

INH or H: isoniazid

IPT: isoniazid preventive therapy

IQR: interquartile range

KIR: killer immunoglobulin-like receptors

LAM: lipoarabinomannans

LIRs: leukocyte Ig-like receptors

L-J: Lowenstein–Jensen

LTBI: latent tuberculosis infection

LTFU: lost to follow-up

MAIT: mucosal-associated invariant T cells

MDR-TB: multidrug-resistant tuberculosis

MHC-I: major histocompatibility complex I

MM: mycomembrane

Mtb: *Mycobacterium tuberculosis*

MTBC: *Mycobacterium tuberculosis* complex

NAC: N-acetyl cysteine

NCAM: neural cell adhesion molecule

NCRs: natural cytotoxicity receptors

NK: natural killer

NKG2A: natural killer group 2A

NPV: negative predictive value

OL: outermost layer

ORD: other respiratory diseases

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffer saline

PG: peptidoglycan

PIM: phosphatidyl-myo-inositol-mannosides

PL: phospholipids

PM: plasma membrane

PMTs: photomultiplier tubes

POC: point-of-care

PPD: purified protein derivative

PPV: positive predictive value

PRF: perforin.

PZN or Z: pyrazinamide

QFN: QuantiFERON

QFT-G-IT: QuantiFERON TB-Gold-In-Tube

QFT-P: QuantiFERON TB Plus

qRT-PCR: quantitative real time PCR

rhIL-2: recombinant human interleukin 2

RIF or R: rifampicin

rmsHBHA: recombinant *Mycobacterium tuberculosis* heparin-binding hemagglutinin antigen expressed in *Mycobacterium smegmatis*

RNS: reactive nitrogen species

ROC: receiver operating characteristic curve

ROS: reactive oxygen species

RR-TB: rifampicin-resistant tuberculosis

SFC: spectral flow cytometry

SN-TB: Smear negative tuberculosis

TAG: triacylglycerols

TB: tuberculosis

TBF: Standard E TB-Feron ELISA

TB-LAMP: Tuberculosis- Loop Mediated Isothermal Amplification

TDM: trehalose dimycolates

TDR-TB: totally drug-resistant tuberculosis

TEMRA: terminally differentiated effector re-expressing CD45RA

Tgd: $\gamma\delta$ T cell

TLR2: Toll-Like Receptor 2

TMM: trehalose monomycolates

TNF: tumor necrosis factor

TPPs: target product profiles

Treg: regulatory T cells

TST: tuberculin skin test

ULBPs: UL16-binding proteins

UMAP: uniform manifold approximation and projection

UV: Ultraviolet

WBC: white blood cell

WGS: whole-genome sequencing

WHO: world health organization

XDR-TB: extensively drug-resistant tuberculosis

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Abstract

Background: Tuberculosis (TB) remains one of the major infectious causes of death worldwide. TB is generally treated with a four-drug regimen for 6 months, but treatment failure and relapse can occur, mostly driven by non-compliance leading to multi-drug resistance issues. Thus, it is essential to rely on early diagnosis and biomarkers assessing the outcomes during treatment monitoring. Currently, the most common methods used for diagnosing and monitoring TB treatment response are sputum-based tests including smear microscopy and culture. Patients' sputum samples are often difficult to collect, smear microscopy has limited specificity and sensitivity, and culture methods require a long time. In this context, the World Health Organization (WHO) has declared an urgent need for alternative non-sputum-based tests for TB diagnosis and treatment monitoring. Those new TB tests need to be faster, based on accessible biological samples and must be practical for field applications. For this purpose, blood-based host candidates represent promising surrogates for TB diagnosis and treatment monitoring.

Objectives and methods: Here, we aimed to evaluate novel non-sputum-based TB tests for TB diagnosis and to identify simple, rapid and potential biomarkers for monitoring TB treatment response in adults. In this way, we evaluated the performance of a 6 genes human-blood transcriptomic signature; called RISK6, in the context of TB diagnosis and monitoring treatment response. Besides, we focused on the identification of blood-based immune biomarker tests for TB treatment monitoring. First, by evaluating simple hematological parameters routinely performed using an inexpensive technique (complete blood counts). Then, by using a high-dimensional single cell technology; spectral flow cytometry, used to explore for the first time pulmonary TB, we conducted an in-depth phenotypic analysis to characterize specific immune subsets that could be used as potential biomarker for monitoring TB treatment response.

This work was conducted in the framework of a *multicenter prospective* study in five countries including Bangladesh, Georgia, Lebanon, Madagascar and Paraguay. In total 152 patients with pulmonary active TB (ATB), 26 individuals with latent TB infection (LTBI), and 71 healthy (HD) were enrolled. ATB patients were non-immunocompromised adults, with culture positive, drug-susceptible (DS) and drug-resistant (DR) TB. They were followed-up during the course of treatment in which whole blood and sputum samples were collected at baseline (T0), at two

months after treatment initiation (T1), at the end of treatment (T2) and two months after treatment completion (T3).

Results: RISK6 signature, assessed by quantitative real-time PCR, achieved the minimal WHO target product profile for a non-sputum-based TB screening and triage test for discriminating ATB from HD and for stratifying patients according to their TB infection status (e.g. ATB vs LTBI). Besides, examination of complete blood counts, revealed that the risk of treatment failure was associated with high white blood cell counts in addition to low proportions of lymphocytes detected in ATB patients at baseline. Finally, data obtained by high-dimensional spectral flow cytometry analysis highlighted a phenotypic signature of cured TB patients upon *Mycobacterium tuberculosis*-specific antigen stimulations. This was reflected by an increased terminally differentiated effector re-expressing CD45RA phenotype of CD8⁺ T cells, a decreased effector memory phenotype of CD4⁺ T cells, in addition of increased frequency of activated natural killer (NK) cell subset.

Conclusions and perspectives: Data from this work provide strong evidence that blood-based tests are promising surrogates for improving TB diagnosis and treatment monitoring. Subsequently, these findings support the efforts to incorporate these tools into a point-of-care test ensuring rapid and accurate detection of ATB cases, and prediction of TB treatment response. Moreover, novel immune features of T and NK cell associated with TB disease were identified. Further validation studies including LTBI individuals and uninfected household contacts are needed to characterize transcriptomic and immune factors associated with higher risk of progression to ATB, and, importantly, to validate the performance of such identified tests in other specific populations such as children.

Keywords: Tuberculosis, *Mycobacterium tuberculosis*, non-sputum-based tests, biomarkers, blood-based tests, diagnosis, treatment monitoring.

Résumé

Contexte: La tuberculose (TB) reste l'une des principales causes de décès dans le monde. La tuberculose est généralement traitée avec un régime à quatre médicaments pendant 6 mois, mais un échec du traitement et une rechute peuvent survenir, principalement en raison d'une non-observance entraînant des problèmes de multi-résistance. En conséquence, il est essentiel de s'appuyer sur un diagnostic précoce et des biomarqueurs évaluant l'efficacité du traitement. Actuellement, les méthodes les plus couramment utilisées pour le diagnostic et la surveillance de la réponse au traitement de la tuberculose sont les tests basés sur les expectorations, notamment l'examen microscopique des frottis et la culture. Les échantillons d'expectorations des patients sont souvent difficiles à prélever, l'examen microscopique des frottis a une spécificité et une sensibilité limitées et les méthodes de culture nécessitent beaucoup de temps. Dans ce contexte, l'Organisation mondiale de la santé (OMS) a déclaré un besoin urgent de tests alternatifs non basés sur les expectorations pour le diagnostic de la tuberculose et le suivi du traitement. Ces nouveaux tests de dépistage de la tuberculose doivent être plus rapides, basés sur des échantillons biologiques accessibles et doivent être pratiques pour les applications sur le terrain. À cette fin, les hôtes candidats à base de sang représentent des substituts prometteurs pour le diagnostic de la tuberculose et le suivi du traitement.

Objectifs et méthodes: Dans cette étude, nous avons cherché à évaluer de nouveaux tests de dépistage de la tuberculose non basés sur les expectorations pour le diagnostic de la tuberculose et à identifier des biomarqueurs simples, rapides et potentiels pour surveiller la réponse au traitement de la tuberculose chez les adultes. Nous avons évalué la performance d'une signature transcriptomique de sang humain à 6 gènes ; nommé RISK6, dans le contexte du diagnostic de la tuberculose et du suivi de la réponse au traitement. En outre, nous sommes concentrés sur l'identification de biomarqueurs immunitaires à partir des tests sanguins pour le suivi de l'efficacité traitement de la tuberculose. Tout d'abord, en évaluant des paramètres hématologiques simples réalisés en routine selon une technique peu coûteuse (numération sanguine complète). Ensuite, en utilisant une technologie monocellulaire de haute dimension; cytométrie en flux spectrale, utilisée pour explorer pour la première fois la tuberculose pulmonaire, nous avons effectué une analyse phénotypique approfondie pour caractériser des

sous-ensembles immunitaires spécifiques qui pourraient être utilisés comme biomarqueurs potentiels pour surveiller la réponse au traitement de la tuberculose.

Ce travail a été mené dans le cadre d'une étude prospective multicentrique dans cinq pays: Bangladesh, Géorgie, Liban, Madagascar et Paraguay. Au total, 152 patients atteints de tuberculose pulmonaire active (ATB), 26 personnes atteintes d'infection tuberculeuse latente (LTBI) et 71 donneurs sains (HD) ont été recrutés. Les patients avec la tuberculose active étaient des adultes non immunodéprimés, avec une culture positive, une tuberculose sensible aux médicaments (DS) et résistante aux médicaments (DR). Ils ont été suivis au cours du traitement au cours duquel des échantillons de sang total et d'expectorations ont été prélevés avant l'initiation du traitement (T0), deux mois après le début du traitement (T1), à la fin du traitement (T2) et deux mois après la fin du traitement (T3).

Résultats: La signature RISK6, évaluée par PCR quantitative en temps réel, a atteint le profil de produit cible minimal de l'OMS pour un test de dépistage et de triage de la tuberculose non basé sur les expectorations pour distinguer les patients avec une tuberculose active des donneurs sains et pour stratifier les patients en fonction de leur statut d'infection tuberculeuse (la distinction de la tuberculose active d'une infection latente). En outre, l'examen des numérations globulaires complètes a révélé que le risque d'échec du traitement était associé à un nombre élevé de globules blancs en plus de faibles proportions de lymphocytes détectés chez les patients avec une tuberculose active au départ avant l'initiation du traitement. Enfin, les données obtenues par analyse de cytométrie en flux spectrale à haute dimension ont mis en évidence une signature phénotypique des patients tuberculeux guéris lors de stimulations antigéniques spécifiques de *Mycobacterium tuberculosis*. Cela s'est reflété par une augmentation des lymphocytes T CD8⁺ Effecteur Mémoire ré-exprimant le CD45RA, une diminution des lymphocytes T CD4⁺ effecteur mémoire, en plus d'une fréquence accrue du sous-ensemble de cellules tueuses naturelles (NK) activées.

Conclusions et perspectives: Les données de ce travail fournissent des preuves solides que les tests sanguins sont des substituts prometteurs pour améliorer le diagnostic de la tuberculose et le suivi du traitement. Par la suite, ces résultats soutiennent les efforts visant à intégrer ces outils dans un point of care test assurant une détection rapide et précise des cas de la tuberculose active et la prédiction de la réponse au traitement de la tuberculose. De plus, de nouvelles

caractéristiques immunitaires des cellules T et NK associées à la maladie tuberculeuse ont été identifiées. D'autres études de validation incluant des personnes atteintes d'une infection latente et des contacts familiaux non infectés sont nécessaires pour caractériser les facteurs transcriptomiques et immunitaires associés à un risque plus élevé de progression vers la tuberculose active et, surtout, pour valider la performance de ces tests identifiés dans d'autres populations spécifiques telles que les enfants.

Mots-clés: Tuberculose, *Mycobacterium tuberculosis*, tests non basés sur l'expectoration, biomarqueurs, tests sanguins, diagnostic, suivi du traitement.

CHAPTER 1: GENERAL INTRODUCTION

Introduction

Tuberculosis (TB) is one of the leading infectious causes of death worldwide. According to the world health organization (WHO), one fourth of the world population is estimated to be infected with *Mycobacterium tuberculosis* (*Mtb*), the etiologic agent of TB. In 2020, it was responsible for nearly 10 million TB cases, resulting in 1.3 million deaths¹. Five to 10% of infected individuals develop the contagious, active form of TB disease (ATB), while most of them (90%) control the infection and develop asymptomatic latent TB infection (LTBI). However, a small proportion (< 10%) of LTBI individuals will develop active TB during their lifetime².

TB can be treated with a regimen of several antibiotics for a minimum of 6 months. In most patients, TB therapy provides cure, but treatment failure and relapse can occur³. These outcomes are associated with severe adverse effects and long treatment durations responsible for lack of patient adherence to the treatment regimen leading to multi-drug resistance issues⁴. Unfortunately, current data shows increasing emergence of drug-resistant (DR-TB), multidrug-resistant (MDR-TB), extensively (XDR-TB) and most recently totally drug-resistant *Mtb* strains¹. Emergence of these drug resistant strains, as well as the convergence of *Mtb* and human immunodeficiency virus (HIV) infections in several regions of the world, threaten the global TB control efforts. Notably, the problem is further complicated by the lack of vaccines able to confer significant and complete protection against TB. Indeed, the only authorized vaccine for the prevention of TB disease is the Bacille Calmette-Guerin (BCG) vaccine⁵, that protects against disseminated forms of TB in children⁶. However, there are currently no effective vaccines that prevent pulmonary and other forms of TB in adults^{6,7}.

Current ATB diagnostics rely on sputum-based tests including smear microscopy and culture which are also used for monitoring TB treatment response^{3,5}. Molecular tests like the GeneXpert MTB/RIF or ULTRA, are also performed using sputum samples⁸. However, sputum-based TB tests are associated with several limitations including the long-time of culture and the lack of sensitivity and specificity of smear microscopy⁹. Besides, although molecular tests are more sensitive for diagnosing pulmonary TB, they still have limited sensitivity in paucibacillary pulmonary TB patients^{10,11}. In addition, sputum samples may be difficult to obtain in some populations (e.g. children and HIV co-infected TB patients) as well as in ATB patients after

symptom improvement¹². Thus, it is essential to rely not only on early diagnosis but also on biomarkers assessing the outcome during treatment monitoring^{1,13}. In this context, the WHO has declared an urgent need for alternative non-sputum-based TB tests with a series of target product profiles (TPPs) which detailed the minimal and optimal criteria that should be met to diagnose and monitor TB treatment response^{14, 15,16}. Thus, developing new biomarker-based tests for TB from non-sputum samples are highly needed to develop rapid and inexpensive tools. These latter should be based on accessible biological samples (e.g. blood or urine), practical for field applications, and could be implemented in poor resource settings¹³. In this context, the aim of the present work is to evaluate the performance of novel non-sputum-based TB tests in the framework of TB diagnosis and to identify simple and rapid biomarkers for monitoring TB treatment response in adults. In this report, we will begin with a literature review presenting TB disease and its major challenges, followed by describing the objectives of this study, presenting the results and original publications, and finally discussing them to draw final conclusions and perspectives.

1. Tuberculosis: a global overview on the current situation

1.1. Worldwide epidemiology of tuberculosis

TB remains a global public health problem; in 2019, it was the 13th cause of death globally (Figure 1). Until the coronavirus (Covid-19) pandemic, TB was the top cause of death resulting from an infectious disease, ranking above human immunodeficiency virus (HIV). According to the WHO, one fourth of the world population is estimated to be infected with *Mtb*, the etiologic agent of TB. In 2020, approximately 9.9 million cases of TB were reported, of whom 1.3 million died from the disease, up from 1.2 million deaths reported in 2019. Overall, 56% of people with TB were man, 33% were women and 11% were children (aged <15). To note, trends in the epidemiology of TB reveal marked differences (Figure 2): indeed, most TB cases are reported in South-east Asia (43%) and Africa (25%). Eight countries accounted for two thirds of all TB cases globally: India (26%), China (8.5%), Indonesia (8.4%), the Philippines (6%), Pakistan (5.8%), Nigeria (4.6%), Bangladesh (3.6%) and South Africa (3.3%) (Figure 3). A slight decrease of the global rate of TB incidence was noted in the period 2015-2020 (11%), including a decline of 2.3% between 2018 and 2019¹.

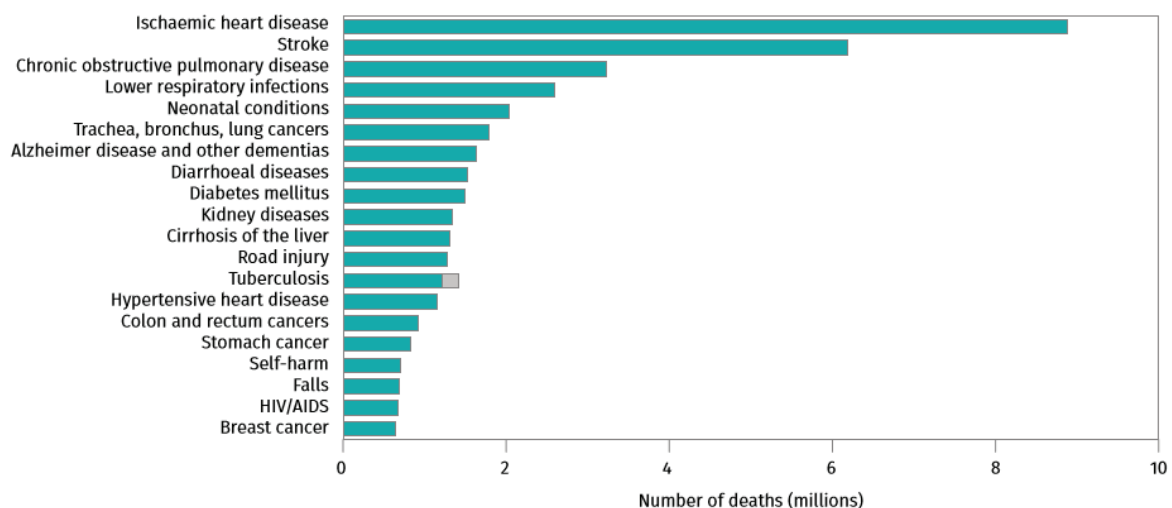


Figure 1: The leading causes of death worldwide reported in 2019. Deaths from TB among HIV-positive patients are shown in grey. Source: WHO¹.

Unfortunately, TB control efforts are being threaten by the increasing emergence of drug-resistant (DR-TB), rifampicin-resistant (RR-TB), multidrug-resistant (MDR-TB), extensively (XDR-TB) and most recently totally drug-resistant *Mtb* strains. Globally, 465 000 new cases of MDR/RR-TB were estimated to have occurred in 2019, resulting in 182 000 deaths. The three countries with the highest number of MDR/RR-TB cases are India (27%), China (14%) and the Russian Federation (8%) (Figure 4)¹.

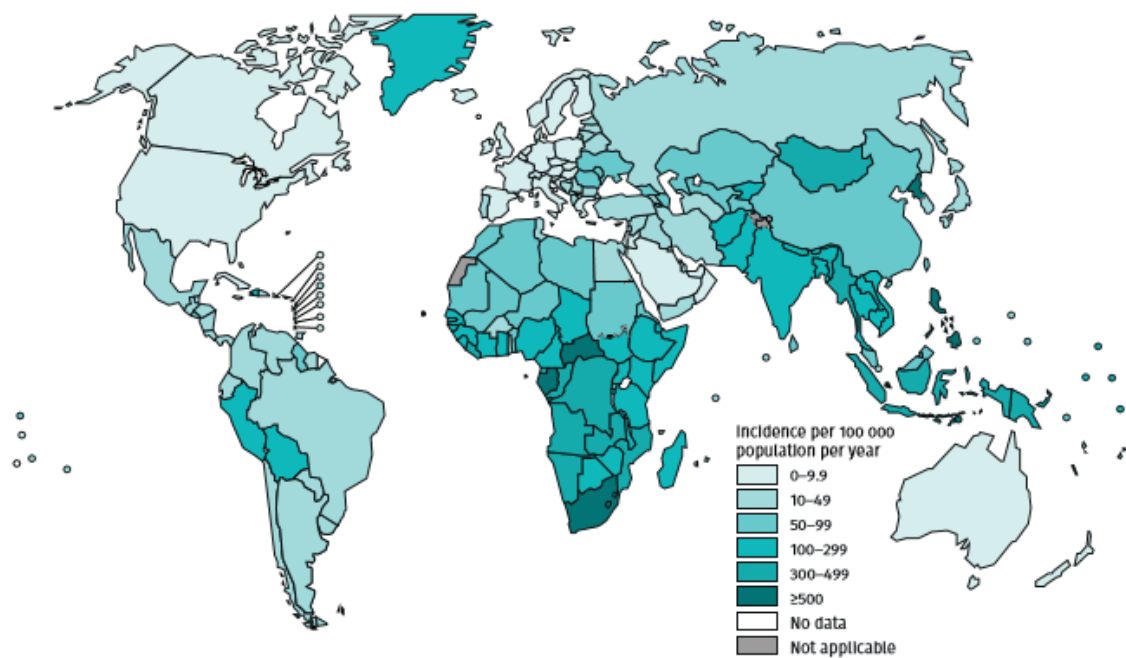


Figure 2: Global estimates of TB incident rate in 2020. The incidence of TB disease revealed marked disparities among countries in 2020. Source: WHO¹.

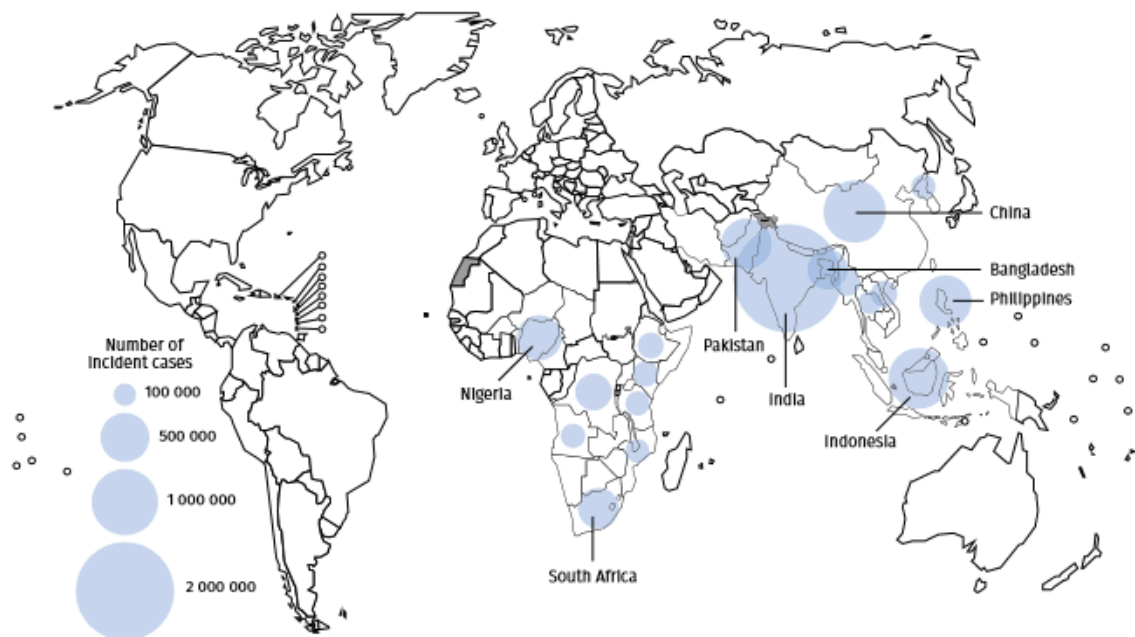


Figure 3: The high TB burden countries with at least 100 000 incident cases reported in 2020. Source: WHO¹.

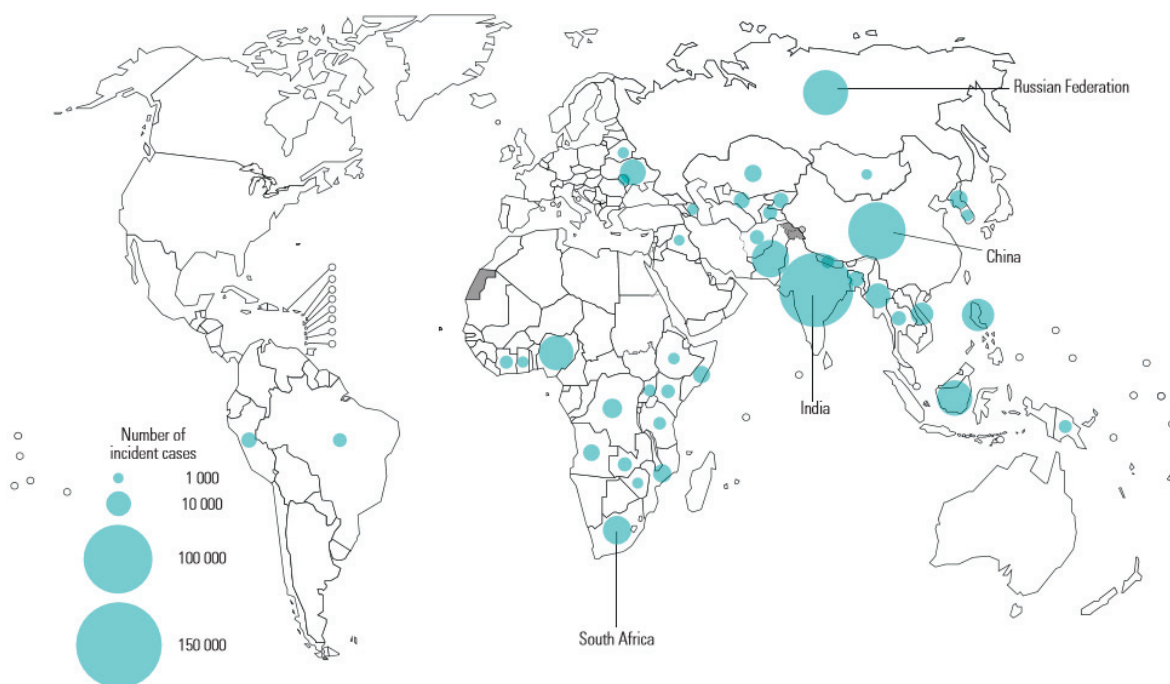


Figure 4: The geographical distribution of MDR/RR-TB cases in 2019. The three countries with the highest number of MDR/RR-TB cases are India, China and the Russian Federation. Source: WHO⁵.

1.2. Impact of the COVID-19 pandemic on TB epidemiology and services

At the beginning of 2020, the coronavirus disease 2019 (COVID-19) pandemic, a viral disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has affected TB epidemiology in addition to TB health services including TB diagnosis, care, and prevention. As reported by the WHO, an estimated 1.4 million fewer people received care for TB, in addition to a shortfall of 21% in TB case notifications in 2020 relative to 2019¹⁷. Moreover, the number of patients with DR-TB identified in the TB centers in 2020 was decreased due to the lockdowns, the limited access to TB care centers, and the restricted ability to pay care and transport costs. Unfortunately, an increase in TB deaths in May and July 2020 was reported due to deaths misattributed to COVID-19 and to other factors such as under-diagnosis¹⁸. The COVID-19 pandemic has also negatively affected the treatment of TB patients due to the reduced treatment support and stock outs. Subsequently, this increased the risk of treatment interruption and delay, and lack of treatment adherence^{19,20}. Similarly, COVID-19 pandemic has impacted the TB prevention services such as BCG vaccination and preventive treatment. Several TB centers in different countries reported a decrease of 30-70% in the preventive treatment enrolment^{21,22}. Meanwhile, data reported in India²³ and Pakistan²⁴ showed a decrease of up to 60% in BCG vaccination coverage, in addition to possible negative consequences on TB mortality, specifically among children²⁵. Overall, a rebound of TB diagnosis and case notifications is expected in the near future causing a massive increase in TB burden worldwide.

1.3. TB disease and attributable risk factors

Among major known risk factors for TB, the HIV infection is the strongest²⁶: Eight % of all those affected with TB disease and 214 000 deaths were among people co-infected with HIV in 2020. The remaining fraction of TB cases were attributable to undernutrition (15%), excessive alcohol use (8.1%), smoking (7.1%) and diabetes (3.1%) (Table 1). Notably, a strong correlation was detected between the prevalence of undernutrition and TB incidence rate (Figure 5)¹. Other risk factors for TB include poverty, indoor air pollution and active transmission in overcrowded hospitals and prisons²⁷.

Table 1: Global numbers of estimated TB cases in 2020 associated with selected risk factors. Source: WHO¹.

RISK FACTOR	RELATIVE RISK (UNCERTAINTY INTERVAL)		EXPOSED (MILLIONS)	POPULATION ATTRIBUTABLE FRACTION (%)	ATTRIBUTABLE TB CASES (MILLIONS, UNCERTAINTY INTERVAL)	
Alcohol use disorders	3.3	2.1–5.2	291	8.1	0.74	0.30–1.3
Diabetes	1.5	1.3–1.8	496	3.1	0.37	0.15–0.68
HIV infection	18	15–21	38	7.6	0.74	0.65–0.83
Smoking	1.6	1.2–2.1	1 050	7.1	0.73	0.25–1.5
Undernourishment	3.2	3.1–3.3	637	15	1.9	1.3–2.6

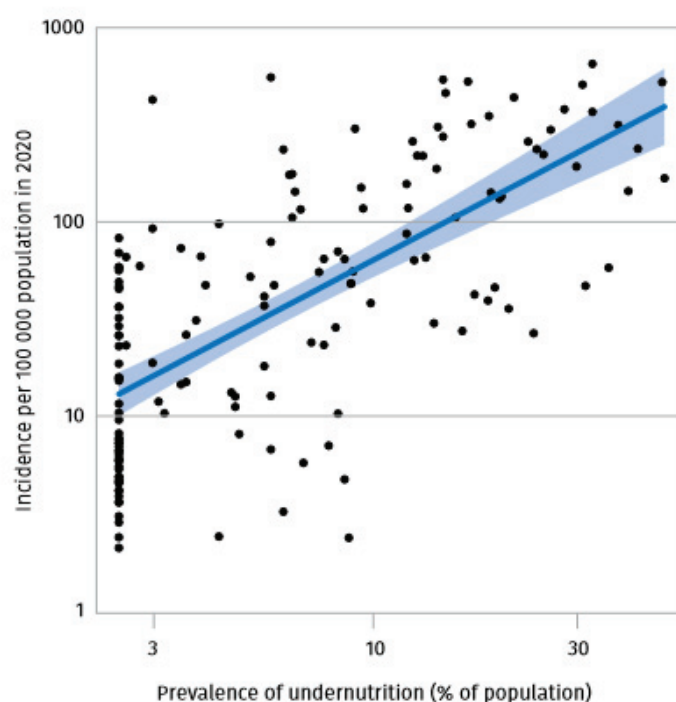


Figure 5: Correlation between the TB incidence rate and the prevalence of undernutrition. Source: WHO¹.

1.4. History of TB and its causal agents

In 1882, Dr. Robert Koch has discovered the *Mtb* bacillus, the causative agent of human TB²⁸. In addition to *Mtb*, the disease can be also caused by members of the *Mycobacterium tuberculosis* complex (MTBC) which includes several closely related species (*Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canettii*, *Mycobacterium microti*, *Mycobacterium caprae* and *Mycobacterium pinnipedii*) that can be classified into those that are human-host adapted and those that are able to propagate in a wide variety of animal hosts. *Mtb*, the etiological agent of TB in humans, is the most famous member of the MTBC.

Mycobacterium canettii and *Mycobacterium africanum* are also able to cause TB in humans only in certain regions of Africa. *Mycobacterium bovis* displays the widest spectrum of host infection, affecting domestic or wild bovines and goats but also causes TB in humans²⁹.

Mtb belongs to the *Mycobacterium* genus. It is a non-motile, aerobic, acid-fast and rod-shaped bacterium which is 1-4 μm in length and 0.3-0.6 μm in width (Figure 6)³⁰. These bacteria are characterized by a unique cell wall structure and composition. This feature is responsible for the complexity as well as the low permeability of this cell envelope, making *Mtb* bacilli resistant to most stains such as Zhiel-Neelsen acid-fast stain, thus playing a fundamental role in virulence³¹.



Figure 6: *Mycobacterium tuberculosis* scanning electron micrograph. *Mtb* appears microscopically as rod-shaped bacilli. Source: Jilani. *et al.*³²

1.5. Transmission of *Mtb* infection

TB mostly affects the lungs causing pulmonary TB (PTB) but can also affects other organs (bones, pleura, lymph nodes, abdomen, skin, joints genitourinary tract and brain) causing extra-pulmonary TB (EPTB)³³. Patients with PTB disease are considered as the most important source of TB infection. Indeed, TB spreads from person to person through aerosol transmission. Patients with contagious TB produce droplets that contain *Mtb* bacilli of 1-5 μm in diameter through coughing, sneezing or any other potent expiratory movement that produce respiratory secretions³⁴. The droplets persist in the air for several minutes to hours and could be inhaled by a new host reaching the lungs and causing infection³⁵ (Figure 7).

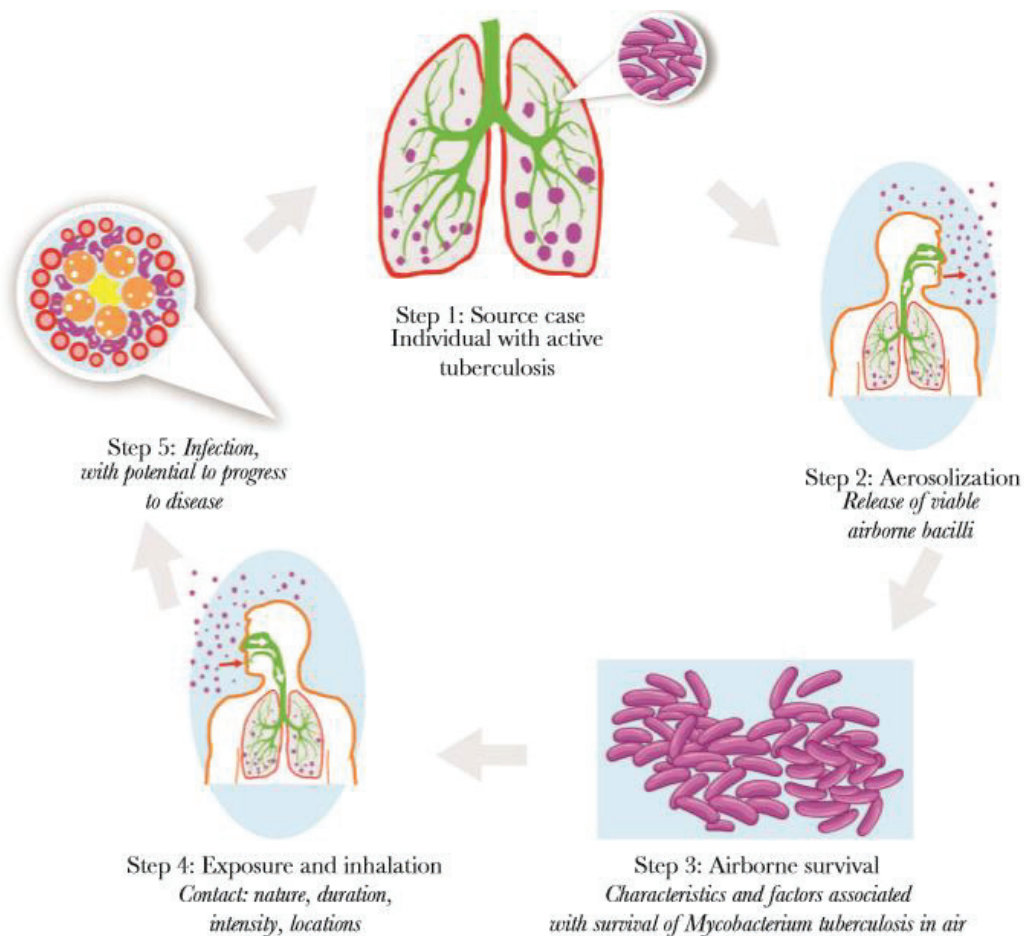


Figure 7: Tuberculosis transmission. Active pulmonary TB patients are the main source case of TB infection. Transmission occurs by releasing airborne droplets containing *Mtb* bacilli, which can survive in the air for several hours. Subsequently, these droplets can be inhaled by a healthy individual, and enter the lungs to reach the alveolar space where the process of infection evolves. Source: Churchyard. *et al.*³⁶.

1.6. Outcomes after *Mtb* infection

After the exposure to *Mtb*, approximately 5 to 10% of infected individuals develop ATB that leads to symptomatic disease including cough, fever, night sweats, fatigue, weight loss and hemoptysis³⁷. Notably, most individuals (90%) develop an effective immune response and remain clinically asymptomatic³⁸. This state is defined as a latent TB infection (LTBI) representing the natural reservoir of the TB pathogen. To note, subjects with LTBI carry a risk of approximately 10% to progress to ATB disease in the presence of weakened cellular immunity³⁷.

For example, patients with HIV are at very high risk of *Mtb* reactivation and development of ATB. Besides, the progression to ATB disease is also promoted by other immunosuppressive and clinical conditions such as diabetes mellitus, kidney failure, malnutrition and smoking³⁹.

1.7. Immune response to Mycobacterium tuberculosis

Following inhalation, *Mtb* enters the lungs and reaches the alveolar space where it encounters and infects the resident alveolar macrophages (Figure 8). Within macrophages, *Mtb* is able to prevent the phagosome fusion with the lysosome and thus multiply in the phagosome, ensuring its survival⁴⁰. Moreover, *Mtb* can escape from the phagosome membranes to the cytosol, and this translocation can serve as a virulence mechanism since cytoplasmic environment provides a more favorable milieu for mycobacterial replication by inhibiting the activation of autophagy which is critical for *Mtb* clearance by macrophages⁴¹. Subsequently, the macrophages will produce cytokines such as TNF, IL-6, IL-1 β , IFN- γ and chemokines that serve as a signal of infection, thus leading to the recruitment of an increasing number of immune cells including uninfected macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells and gamma delta ($\gamma\delta$) T cells to the site of infection⁴². In the meantime, either DCs⁴³ or inflammatory monocytes⁴⁴ transport *Mtb* and migrate to the pulmonary lymph nodes for T cell priming (both CD4⁺ and CD8⁺)^{43,45}. This induces a specific immune response that produces primed T lymphocytes against mycobacterial antigens which progressively migrate to the site of infection. This leads to the formation of cellular aggregates that contain many immune cell types including macrophages, DCs, adaptive immune cells (T cells and B cells) in addition to other host cells. These cellular events culminate in the formation of a well-organized structures called granulomas⁴⁶. These structures are also surrounded by other type of T cells such as Th2 and Treg cells that are recruited following the persistence of infection for a longer period⁴². The granulomas are essentially involved in the control of infection as they provide a protective function by preventing the bacterial spread in the rest of the lung tissue and of the body. However, in some cases they contribute to the bacterial persistence⁴⁷. Within the granuloma, CD4⁺ T cells produce the interferon-gamma (IFN- γ) cytokine playing a role in activating macrophages to inhibit *Mtb* growth by promoting phagosome-lysosome fusion, producing reactive nitrogen intermediates (RNIs), and activating autophagy pathways. CD4⁺ Th1 are the main source of IFN- γ during *Mtb* infections⁴³.

Notably, the granuloma can persist for many years leading to the containment of the bacteria in a dormant state; in this case infected individuals are recognized as having a latent TB infection (LTBI). However, failure to contain the infection can result when the pathogens multiply within it, resulting in abundant bacterial loads, leading to the breakdown of the granulomatous structure and to the spread of bacteria to other organs, in this case, active TB disease and transmission can occur in infected patients² (Figure 8).

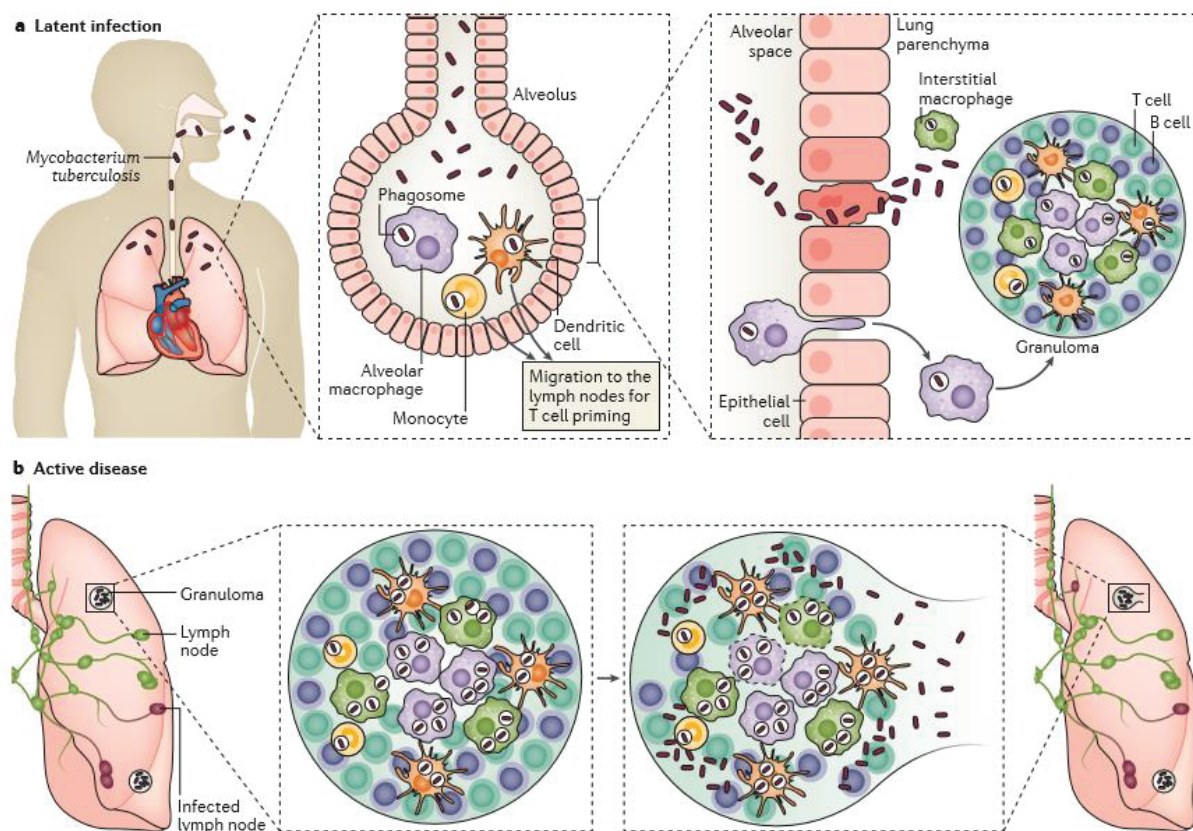


Figure 8: TB immunology. (a) *Mtb* enters the lungs of the host via inhalation of aerosols and reaches the alveolar space where it infects the resident macrophages. The exposure to *Mtb* can result in the immediate elimination of the pathogen by the pulmonary immune system. If this first line of immune defense fails, the *Mtb* gain access to the lung parenchyma. Thereafter, both the dendritic cells and monocytes migrate to the lymph nodes and prim T cells against mycobacterial antigens. This event leads to the recruitment of immune cells, including T and B cells to the site of infection to generate a granuloma. Therefore, the immune system of the host can eliminate the infection. If, *Mtb* pathogen is not eliminated, the bacteria could persist in a latent state and infected individuals are recognized as having a latent TB infection (LTBI). **(b)** However, the granuloma may fail to contain the infection when the *Mtb*

multiply within it, resulting in abundant bacterial loads. This will result in the breakdown of the granulomatous structure and *Mtb* bacteria will spread to other organs. Thus, the infected host is symptomatic and known as a patient with active TB disease. Source: Pai. *et al.*².

1.8. Virulence factors of Mtb

Mtb use various virulence factors and mechanisms to cause disease in their host. Mycobacterial virulence factors are defined as bacterial genes or cellular components that enable their replication and survival in the host. Well-established *Mtb* virulence factors include the surface-exposed lipids in the mycobacterial membrane, as well as the secreted proteins⁴⁸.

1.8.1. Mtb cell wall envelope

A characteristic feature of *Mtb* is the complex cell wall envelope that contains a high content of lipids and comprises 40% of the cell envelope weight. It contains a layer of arabinogalactan linked to a large amount of long chain fatty acids known as mycolic acids⁴⁹. This layer of mycolic acids is arranged in the form of a bilayer in association with various complex lipids such as glycolipids to form a strong hydrophobic barrier. This latter is responsible for the low permeability of the membrane that contributes to the infectiveness of many antibiotics against this bacteria⁵⁰. Additional components of the *Mtb* cell wall are illustrated in (Figure 9).

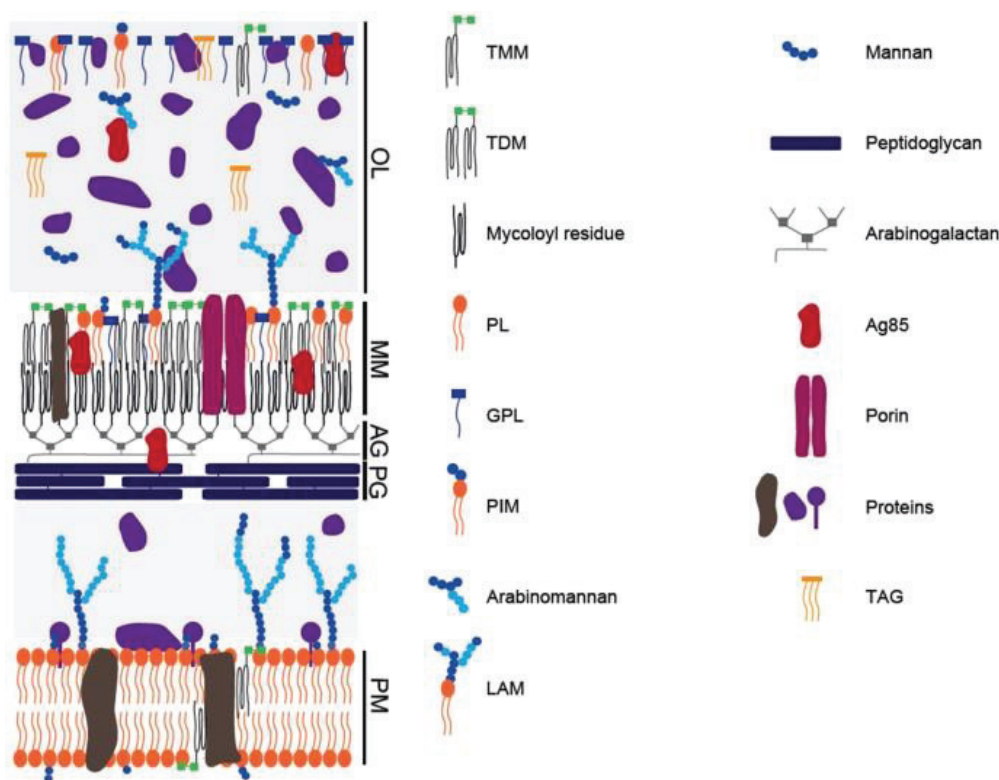


Figure 9: The mycobacterial cell wall components. A schematic representation of the arrangement of the complex mycobacterial cell envelope. Source: Daffé. *et al.*⁵¹.

OL: Outermost layer. MM: mycomembrane. AG: arabinogalactan. PG: peptidoglycan. PM: Plasma membrane. TMM: trehalose monomycolates. TDM: trehalose dimycolates. GPL: glycopeptidolipids. PL: phospholipids. PIM: phosphatidyl-myo-inositol-mannosides. LAM: lipoarabinomannans. TAG: triacylglycerols. Ag85: antigen 85.

1.8.2. Type VII secretion systems

Protein secretion systems play a major role in the virulence of *Mtb*. Indeed, this bacterium utilizes a series of five type VII secretion systems (ESX1-5); three of them (ESX-1, ESX-3 and ESX-5) are required for its virulence⁵². ESX-1, which is the best characterized among them, is considered as one of the most significant for pathogenesis of active TB disease⁵³. It is responsible of the rupture of phagosome membrane that induces a translocation of the bacterial products from the phagosome into the cytoplasm of infected macrophages, which constitutes a safe environment for the multiplication of *Mtb* bacteria^{54,55}. Essential components of the ESX-1 secretion system are the secreted antigens; 6-kDa early secretory antigenic target (ESAT-6) and

the 10-kDa culture filtrate protein (CFP-10) also known as EsxA and EsxB, respectively. Both of these proteins are major virulence factors. They are also highly immunogenic T-cell antigens and form the basis of several immunological diagnosis assays of *Mtb* infection⁵⁶ (see later).

1.8.3. Heparin-binding hemagglutinin antigen

New proteins and other molecular components involved in the pathogenesis of *Mtb* were identified in the last decade⁵⁷. Among them, the mycobacterial adhesin: heparin-binding hemagglutinin antigen (HBHA)⁵⁸, a surface-associated protein that mediate the adherence of *Mtb* bacteria to the lung epithelial cells^{59,60}. HBHA is involved in the dissemination of *Mtb* from the site of infection through binding of HBHA to proteoglycan that leads to an epithelial transcytosis, which promotes the dissemination of *Mtb* bacteria^{61,62}. Furthermore, it has been previously shown that HBHA is a mycobacterial antigen associated with LTBI⁶³. Indeed, large amounts of host interferon gamma (IFN- γ) were detected in response to *in vitro* stimulation with HBHA in LTBI individuals, while IFN- γ levels were significantly lower in ATB patients^{64,65,66}, suggesting that HBHA-specific T cell responses may participate in the containment of TB infection⁶⁷.

2. Tuberculosis: Diagnostic and Treatment Monitoring

2.1. TB diagnostic tests

Identifying *Mtb*-infected individuals is crucial for TB prevention and eradication. Thus, multiple advances have been made in the diagnosis of TB. Both direct and indirect TB diagnostic tests exist. The direct testing consists on the detection of *Mtb* bacteria and its products, while in indirect testing the host immune response against *Mtb* infection is detected.

2.1.1. Diagnosis of ATB disease

The diagnosis of ATB disease relies on the use of imaging techniques (chest X-rays and computed tomography), sputum-based tests such as smear microscopy, rapid molecular tests (GeneXpert MTB/RIF), mycobacterial culture-based methods in addition to urine-based tests⁵.

2.1.1.1. Imaging tools for ATB disease

The diagnostic of pulmonary TB disease first relies on signs and abnormal respiratory symptoms. Subsequently, imaging tests including chest X-rays⁶⁸ and computed tomography (CT) scan are used for the screening of ATB disease⁶⁹. These imaging modalities provide insights into the diversity of lung lesions. Nevertheless, chest X-rays lack specificity, hence abnormal X-rays findings need to be confirmed with microbiological tests⁶⁹. Notably, CT scan achieves better results than chest X-rays in the assessment of the disease activity^{70,71} (Figure 10). However, this imaging tool is too expensive and not recommended for routine use.

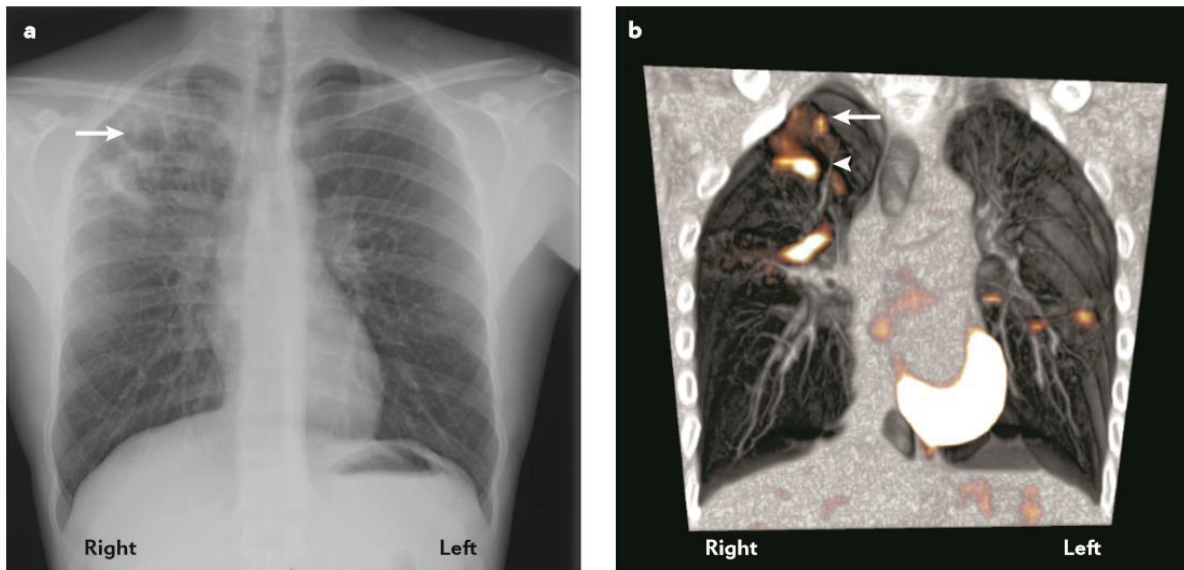


Figure 10: Imaging techniques for the diagnosis of ATB disease. (a) Chest X-ray. Pulmonary TB disease with a large cavity (arrow) in the right upper lobe of the lung **(b)** CT scan image showing abnormalities related to ATB disease using 18F-fluorodeoxyglucose (FDG) PET-CT scan. FDG is absorbed in regions with abnormalities (orange colors) and an air-filled cavity (arrow) appears in the right upper lobe. Source: Pai. *et al.*².

2.1.1.2. Sputum-based tests

Mtb bacteria can be detected by both microbiological and molecular tests using patients' sputum samples.

2.1.1.2.1. Microbiological tests

Microbiological tests used for the detection of *Mtb* bacteria include smear microscopy and culture tests. Although, those tests are essentially performed using sputum specimens, they can also be done using broncho-alveolar lavage (BAL) fluid samples.

2.1.1.2.1.1. Sputum smear microscopy

Sputum smear microscopy is the most widely used ATB disease test in low and middle-income countries⁷². To visualize the mycobacteria, light and fluorescent microscopy using Ziehl-Neelsen stain and auramine are used respectively. The respiratory specimen is smeared on a slide, stained, dried, and then treated with alcohol. Thereafter, the presence of any bacilli will be detected by a well red color following Ziehl-Neelsen staining⁷³ (Figure 11). Also, bright acid-fast bacilli can

also be visualized through fluorescence microscopy using fluorescent dyes such as auramine⁷⁴. Thus, a positive result is highly suggestive of ATB and represents an indication of a high risk of transmission of the disease. Sputum smear microscopy is fast, economical cheap, and represents a markers of patient's infectiousness³⁷. Nevertheless, this technique has many limitations: its sensitivity is variable (20 to 80%) and it is limited to samples with $\sim 10^4$ organisms/mL. Thus, a negative smear microscopy is frequently obtained because of the low number of bacilli in several populations such as children and HIV-positive patients⁷⁵. Furthermore, this test lacks of specificity, because it cannot distinguish *Mtb* from other mycobacteria, thus, more definitive testing should be performed³⁷.

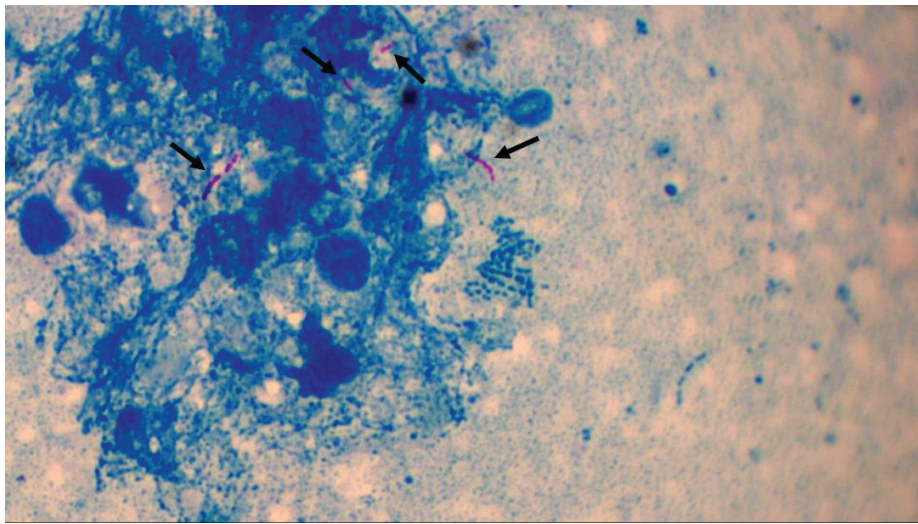


Figure 11: Ziehl-Neelsen stain showing positive *Mtb* bacilli detected by light microscope. Black arrows point to *Mtb* bacteria (red bacilli).

2.1.1.2.1.2. Culture

Mycobacterial culture is widely regarded as the gold standard confirmatory test for ATB diagnosis⁵. It remains the most sensitive test for the detection of *Mtb* in respiratory specimens including sputum samples. Indeed, it is able to detect $\sim 10^1$ to 10^2 organisms/mL exceeding the sensitivity of smear microscopy ($\leq 10^4$ organisms/mL)⁷⁶. Culture testing can be performed on liquid MGIT (mycobacterial growth indicator tube) and/or on solid L-J (Lowenstein-Jensen) media (Figure 12)⁸. *Mtb* grows slowly, thus three to six weeks are required for a positive result (visible colonies) on solid media. Liquid media are more expensive and disposed to

contamination than solid media⁷⁷. However, they are more sensitive, give better quality isolation and are more rapid, providing confirmation within four to fourteen days. However, although mycobacterial culture remains the gold standard for TB diagnosis, it is time consuming requiring a long time to confirm a positive result.



Figure 12: Lowenstein-Jensen (L-J) media showing the growth of *Mtb* colonies.

2.1.1.2.2. Molecular sputum-based tests

2.1.1.2.2.1. GeneXpert

Major advances have been recently made in the domain of molecular testing in order to improve the diagnosis of TB and for drug resistance. A molecular assay (Xpert MTB/RIF) based on the automated GeneXpert technology was developed⁷⁸. This assay has a higher sensitivity than sputum smear microscopy and possesses the additional advantage of yielding faster results than culture^{79,80}. Subsequently, the WHO recommended the use of Xpert MTB/RIF as a first-line diagnostic test in all adults and children who are suspected to have ATB disease⁸¹. This diagnostic test using the real-time PCR can detect the presence of the genetic material of *Mtb*; the DNA, in addition to the marker of rifampicin resistance^{78,82}. It is sensitive and specific for the diagnosis of TB disease, generating faster results within two hours⁸³. Moreover, the Xpert MTB/RIF is a low-cost diagnostic assay, and could be applied by a minimally trained health-care workers in the laboratories located in resource poor settings^{84,85}. However, this assay still have limited sensitivity in negative smears, paucibacillary and HIV co-infected TB patients⁵. To

overcome these limitations, the Xpert Ultra, as the newest version of the Xpert MTB/RIF, was developed by Cepheid. It uses the same GeneXpert platform as Xpert MTB/RIF, however, to improve the sensitivity in TB diagnosis, two different multi-copy amplification targets and a larger DNA reaction chamber than Xpert MTB/RIF was incorporated in Xpert Ultra. Consequently, it was reported that the limit of detection of using Xpert Ultra improved to 16 bacterial colony forming units (CFU)/mL compared to 114 CFU/ mL for Xpert MTB/RIF^{86,87}. In 2017, the WHO recommended also the use of Xpert Ultra as the initial test for TB diagnosis in individuals with signs and symptoms of TB, thereafter, several countries are currently using the Xpert Ultra instead of Xpert MTB/RIF⁸⁶.

2.1.1.2.2.2. Tuberculosis-Loop-mediated isothermal amplification

Another developed nucleic acid amplification technique, endorsed by the WHO and commercially available for the diagnosis of pulmonary TB in symptomatic adults is Tuberculosis- Loop Mediated Isothermal Amplification (TB-LAMP)⁸⁶. This assay have been designed to detect *Mtb* in sputum samples, in which the DNA fragment is amplified at a single temperature range, in contrast to the PCR where the amplification requires a thermocycler⁸⁸. Moreover, this assay can be performed with less than 1 hour and the detection of the amplicons is done with visual reading under UV light (Figure 13). TB-LAMP has several advantages; it is easy to perform, doesn't require complex instruments and is cost effective. Consequently, it can be implemented in clinical laboratories located in resource poor settings^{86,89}.

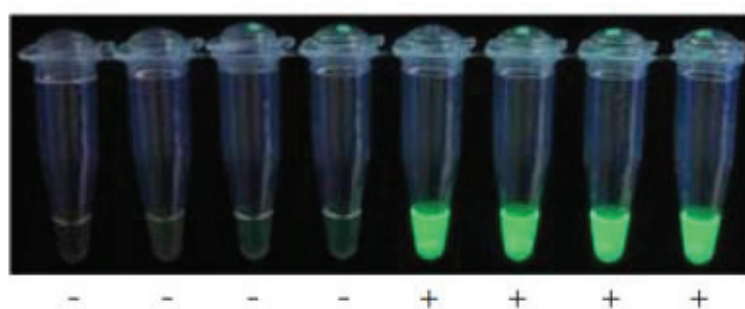


Figure 13: Visual detection of the amplified products of the TB-LAMP reaction using an UV light. Adapted from WHO⁷⁷.

TB-LAMP: Tuberculosis-Loop Mediated Isothermal Amplification. UV: Ultraviolet

2.1.1.3. Urine-based test

2.1.1.3.1. Urine lipoarabinomannan assay

A new, point-of-care, test for the diagnosis of TB among individuals co-infected with HIV, called (AlereLAM), is currently commercially available⁸⁶. This urine-based assay test detects the lipoarabinomannan (LAM) antigen, a component of the mycobacterial cell wall. This test is simple to use, requires little equipment and 25 minutes to generate results⁹⁰. Moreover, it relies on the use of urine which is a safe and an easily accessible sample compared to sputum. The use of AlereLAM makes advances among population at higher risk for TB and in which TB is hard to diagnose such as HIV-positive patients where the mortality has been reduced following the use of this assay^{91,92}.

2.1.2. Screening of latent TB infection

There are two available commercial tests included in the WHO guidelines and currently used for the diagnosis of LTBI: the tuberculin skin test (TST) and the interferon-gamma (IFN- γ) release assays (IGRAs)⁵.

2.1.2.1. Tuberculin Skin Test

The TST is based on a delayed-type hypersensitivity (DTH) host response that occurs within 48 to 72 hours, following an intradermal injection of tuberculin purified protein derivative (PPD). This latter is composed of a complex combination of *Mtb* antigens, shared with bacille Calmette–Guérin (BCG) vaccine and many non-tuberculosis mycobacteria. The interpretation of the TST results considers the size of induration, which represents the skin reaction resulting from the cell mediated immunity to these antigens. The induration of more than 5 mm is considered positive⁹³. The TST has several advantages: it is cost effective and requires limited laboratory equipment and skills, particularly in the low-resource settings². However, it lacks of specificity: false positive results can occur in BCG vaccinated subject or in those exposed to non-tuberculosis mycobacteria⁹⁴. Moreover, as the TST depends on a lymphocyte-induced delayed hypersensitivity reaction, it displays low sensitivity in immunosuppressed patients (e.g. HIV patients) in who false negative results can occur⁹⁵.

2.1.2.2. Interferon- γ release assays

Cell-mediated immunity based IFN- γ release assays (IGRAs) have been also developed for the diagnosis of LTBI⁹⁶. IGRAs are *in vitro* blood tests that measure the T cell release of IFN- γ following stimulation with the specific *Mtb* antigens: ESAT-6 and CFP-10⁹⁷. IGRAs are more specific than the TST as the antigens used in these assays are not encoded in the genome of the BCG vaccine strains and the majority of non-tuberculosis mycobacteria⁹⁷. Currently, there are commercially two available IGRAs. The first is a whole blood, enzyme linked immunosorbent assay (ELISA)-based QuantiFERON-TB Gold (QFN-TB Gold) assay (Cellestis Ltd., Carnegie, Australia), and the second is a peripheral blood mononuclear cell (PBMC) and enzyme-linked immunospot (ELISPOT) based-technology (T-SPOT.TB) (Oxford Immunotec, Oxford, UK)⁹⁸. These assays are based on the measurement of IFN- γ levels secreted in the plasma following the incubation of the blood with the specific *Mtb* antigens in QFN-TB Gold, or the quantification of the IFN- γ -producing T cells using T-SPOT.TB. Even though the IGRAs had higher specificity than the TST, both tests have a low sensitivity in immunocompromised patients. Besides, neither test can significantly discriminate between different stages of TB infection; ATB vs LTBI⁹⁹.

2.1.2.2.1. QuantiFERON-TB Gold assays

The next version of the QFN-TB Gold was called QuantiFERON TB-Gold-In-Tube (QFT-G-IT) that used in addition to ESAT-6 and CFP-10 an additional antigens the TB7.7, that induce a highly specific strong cell-mediated immune response¹⁰⁰. More recently, a new version of QFT-G-IT has been launched, the QuantiFERON TB Plus (QFT-P). It includes an additional antigen tube called TB2 in addition to TB1 tube and both positive (Mitogen) and negative control (Nil) tubes. In this assay, the TB1 tube contains the conventional ESAT-6 and CFP-10 antigens, designed to produce cell-mediated immune responses from CD4⁺ T-helper lymphocytes. However, TB2 contains in addition to ESAT-6 and CFP-10 antigens, new peptides that stimulate the IFN- γ production by both CD4⁺ and CD8⁺ T-cells¹⁰¹. Evidence now supports a role for CD8⁺ T cells participating in the host defense to *Mtb* by producing IFN- γ , which activate macrophages to suppress growth of *Mtb*, kill infected cells, or directly lyse intracellular *Mtb*^{102,103}. *Mtb*-specific CD8⁺ cells have been detected in subjects with LTBI and with ATB disease where IFN- γ producing CD8⁺ cells may be frequently found^{104,105}.

2.1.2.2.2. New IGRAs tests

2.1.2.2.2.1. The Standard E TB-Feron ELISA

Recently, a newly developed ELISA-based IGRA, the Standard E TB-Feron ELISA (TBF; SD Biosensor, Gyeonggi-do, Republic of Korea) was currently approved by the Ministry of Food and Drug Safety of the Republic of Korea for the diagnosis of LTBI¹⁰⁶. The principles of TBF assay are nearly like the QFT-GIT: three tube-based IGRAs including a single TB antigen tube. However, they predominantly differed by the structure of the TB-specific antigens. In TBF, the antigens contained in the TB tube are recombinant whole proteins of ESAT-6, CFP-10 and TB7.7, whereas those of QFT-GIT and QFT-P are recombinant peptide antigens¹⁰⁷. Notably, the use of recombinant whole proteins instead of peptides offers advantages in terms of increased sensitivity of TBF assay. Indeed, whole proteins induce the stimulation of TB-specific T cells in a more efficient manner, thus leading to higher detectable IFN- γ levels¹⁰⁸. Recently, it was demonstrated that (i) the TBF displays an excellent diagnostic performance comparable to that of QFT-GIT and QFT-P assays for the detection of LTBI, (ii) induce a strong stimulation of CD8⁺ T cells compared to those in QFT-P, and (iii) is cost-effective compared to both assays. Consequently, the TBF seems to be a promising alternative to the currently used IGRAs^{109,110}.

2.1.2.2.2.2. LIAISON QFT-Plus

The LIAISON QFT-Plus assay, is an *in vitro* diagnostic test for detecting LTBI. It is an improved version of QFT-P which is based on the detection of IFN- γ by a chemiluminescent immunoassay (CLIA) instead of ELISA¹¹¹. Recently, it was reported that the diagnostic performance of the automated LIAISON QFT-Plus test was comparable to that of QFT-P¹¹¹, it can also be used for the screening of LTBI with less time and labor¹¹².

2.1.2.2.2.3. LIOFeron TB/LTBI

The LIOFeron TB/LTBI test, is a new developed IGRA for the diagnosis of LTBI and TB¹¹³. This assay contains four blood stimulation tubes: negative control tube, positive control tube and two TB antigen tubes including TB A and TB B. This assays contains an additional antigen such as alanine dehydrogenase (Ala-DH) in addition to antigens used in previous IGRAs such as ESAT-6, CFP-10, and TB7.7. Ala-DH is an antigen absent in BCG that induces the production of IFN- γ by major histocompatibility complex (MHC) class I-restricted CD8⁺ T

lymphocytes^{114,115}. Recently, it was demonstrated that the LIOFeron TB/LTBI showed a higher sensitivity for the detection of LTBI when compared to QFT-P¹¹⁶. However, this assay as other IGRAs cannot discriminate between ATB and LTBI.

2.2. Treatment of tuberculosis

2.2.1. Treatment of drug-sensitive TB

The recommended treatment regimen for active drug-sensitive TB (DS-TB) disease is a six months of four first-line drugs: a combination of isoniazid (INH or H), rifampicin (RIF or R), ethambutol (EMB or E) and pyrazinamide (PZN or Z) given for a total of two months (the intensive phase of treatment), followed by a four months continuation phase of INH and RIF (noted 2HRZE/4HR)⁴. Although, this standard treatment regimen is highly effective with an approximately 85% treatment success rate⁵, it has also several limitations including the complexity and long duration of the treatment in addition to the potential toxicity¹¹⁷. Moreover, severe adverse events can occurs such as gastrointestinal intolerance (nausea, vomiting), severe hepatic toxicity, immune thrombocytopenia, hemolysis, renal failure, optic neuritis and ototoxicity¹¹⁸. The prolonged therapy is a main disadvantage of this regimen as it may contribute to bad treatment compliance. As a result, the lack of treatment adherence leads to treatment failure, relapse ensued by the emergence of DR-TB^{2 119,120}. The global treatment outcomes estimated by the WHO for new and relapse TB cases starting treatment between 2012 and 2019 are shown in Figure 14. In 2019, 86% of treated TB patients with first-line drugs had a successful TB treatment. For children (< 15 years), the treatment success rate was also higher (88%). However, the global treatment success rate was lower (77%) for people co-infected with HIV¹²¹. Subsequently, supportive tools are needed to ensure and improve treatment adherence. One of the most effective control strategy, recommended by the WHO is the directly observed treatment, short-course (DOTS)^{122,123}. It is a widely used process in which health care workers observe and record TB patients taking each dose of medication to ensure treatment completion, cure and limitation of the spread of drug resistance^{124,125}. Nowadays, several alternative tools are now being established to improve treatment adherence, such as phone-based technologies, video DOT and digital pillboxes¹²⁶.

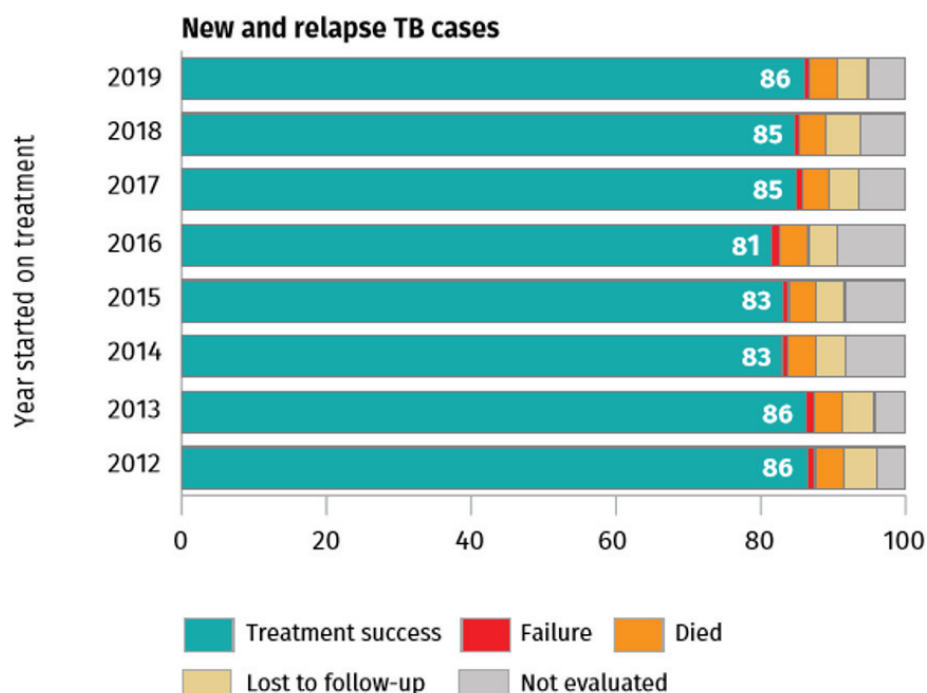


Figure 14: Global treatment outcomes for new and relapse TB cases starting treatment between 2012 and 2019. Source: WHO¹.

2.2.2. Treatment of drug-resistant TB and emergence of incurable strains

The occurrence of DR-TB strains includes: RR-TB which is defined as resistance to RIF, and MDR-TB, defined as caused by *Mtb* strains resistant to, at least, the two most important first-line drugs; RIF and INH⁵. The treatment of MDR-TB consists of a combination of multiple second-line drugs including fluoroquinolones, ethionamide, aminoglycosides such as kanamycin, amikacin and capreomycin, for at least 9 months and up to 20 months¹²⁷. Further, the emergence of MDR-TB strains with additional resistance to at least one fluoroquinolone and a least one second-line injectable drug is defined as extensively drug-resistant TB (XDR-TB) that shows a high mortality rate¹²⁸. XDR-TB patients could be treated with new drugs such as bedaquiline or delamanid¹²⁹. Currently, the recommended treatment regimen of these patients is very expensive, prolonged (up to 20 months), less effective than the first-line regimen, characterized by high toxicity and often associated with severe side effects such as ototoxicity, dysglycaemia, nephrotoxicity and hepatotoxicity. Indeed, there a poor adherence rates, resulting in a lower treatment success rate of only around 57% for MDR/RR-TB⁵. Most recently, the emergence of

totally drug-resistant TB (TDR-TB), referred as a TB caused by strains that are resistant to all first and second-line TB drugs, has been documented¹³⁰.

2.2.3. Detection of drug resistance in *Mtb*

Rapid and correct identification of patients with or at high risk of drug-resistant TB is a crucial requirement for TB management. Thus, tools that detect drug resistance has an important role in the identification of patients with DR-TB and to rapidly treat them¹³¹. In this way, several phenotypic and molecular approaches have been developed for the detection of DR-TB. Classical phenotypic culture-based test involves the culture of *Mtb* in the presence of anti-TB drugs to detect its ability to grow in the presence of these later¹³². Genotypic tests are based on the detection of genetic mutations associated with drug resistance. They include the molecular Xpert MTB/RIF, able, as cited above, to detect the mutations that causes the resistance to rifampicin^{78,132}. Most recently, whole-genome sequencing (WGS) is becoming a promising tool for the detection of drug resistant *Mtb*-strains. This approach is based on the detection of mutations in the genome of *Mtb*, and it shows a good performance to detect the resistance to the first-line TB drugs¹³³. Fortunately, a new assay relying on the use of the next generation sequencing and called the Deeplex Myc-TB® has been recently developed. This tool can target a deep sequencing leading to the detection of the resistance to 13 anti-TB drugs¹³⁴.

2.2.4. TB preventive treatment

Noticeably, the main health intervention for TB prevention is the treatment of people at high risk of TB. This preventive therapy has been shown to be highly effective in populations of all ages (both adults and children)⁵. The WHO recommended that selected high-risk groups, tested for LTBI, are targeted for preventive therapy. Such groups include people living with HIV, children and adults who had contact with patients with ATB disease (household contacts), patients initiating anti-TNF treatment, or dialysis, preparing for an organ or hematological transplant, or with silicosis. The preventive treatment regimens recommended by the WHO for LTBI individuals without HIV include: 6 or 9 months of daily isoniazid, or a 3-month regimen of weekly rifapentine plus isoniazid, or a 3-month regimen of daily isoniazid plus rifampicin. A 1-month regimen of daily rifapentine plus isoniazid or 4 months of daily rifampicin alone may also be offered as alternatives. In settings with high TB transmission, adults and adolescents living

with HIV should receive at least 36 months of daily isoniazid preventive therapy (IPT)¹³⁵. All regimens were found to be effective in preventing ATB disease¹³⁶. Subsequently, an increasing global number from 1 million in 2015 to 3.6 million in 2019 of individuals who have received the TB preventive treatment has been reported by the WHO. However, this rate was decreased to 2.8 million in 2020 due to the COVID-19 pandemic (Figure 15)¹.

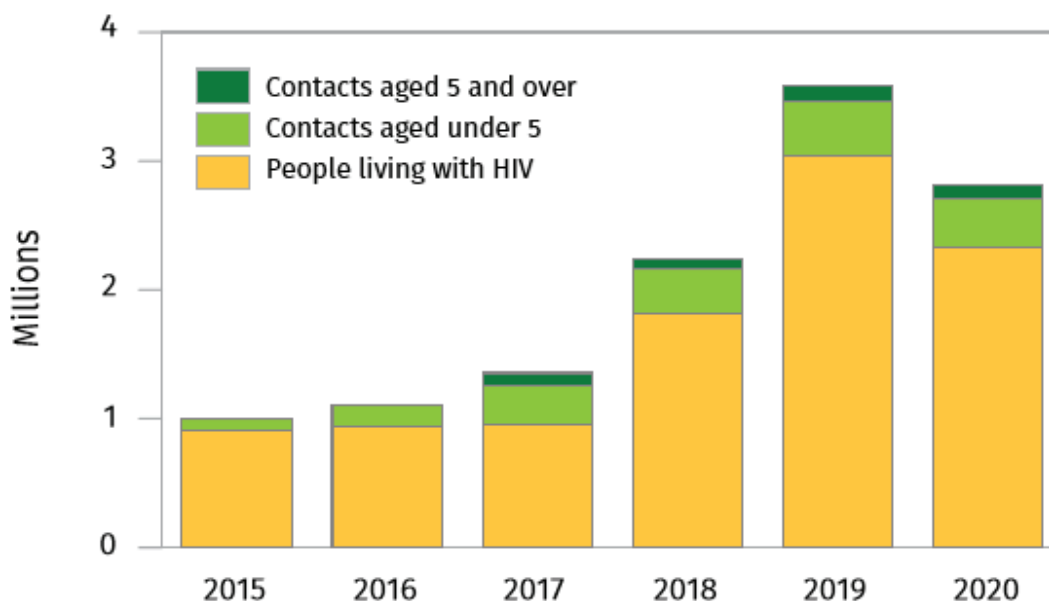


Figure 15: Global number of individuals provided with TB preventive treatment between 2015 and 2020^a.
Source: WHO¹.

^a The number of people living with HIV who were provided with TB preventive treatment in 2019 is lower than published in the Global tuberculosis report 2020. This is due to an update of the data reported by India.

2.3. *TB treatment monitoring*

Successful treatment is defined as completed TB therapy with a negative sputum smear and a negative culture result at the end of treatment. The definitions of treatment outcome for DS and DR-TB patients are detailed in Table 2. Currently, assessing *Mtb* treatment response is routinely performed by microbiological techniques using sputum samples, evaluating sputum conversion after at least 2 months of treatment initiation until the end of treatment. These tests include sputum smear microscopy and *Mtb* culture⁸. However, as cited before, limitations of these tests include the lack of sensitivity and specificity of smear microscopy and the longtime of culture⁹.

as well as the limited availability of sputum samples in several populations (e.g. children and HIV co-infected TB patients) and after symptom improvement. To note, imaging tools such as CT scan are also used for monitoring TB treatment response. However, although they are suggested as suitable tools to evaluate complications during treatment, they are too expensive and remain beyond reach for most patients especially in low-income countries¹³⁷. Therefore, the WHO has declared that there is an urgent need for rapid, quantitative, non-sputum-based biomarker tests to quickly assess the efficacy of treatment in all TB patients in an early stage of therapy^{5,15}. A biomarker designates a biological measurable element of the host or pathogen that indicates the presence of normal biological processes or the presence/severity of a disease. The availability of such novel biomarker-based tests would have significant impact on TB prevention and treatment especially if they are based on accessible biological samples (e.g. whole blood, urine, etc....) and are practical for field application¹³.

Table 2: Treatment outcome definitions for DS and DR-TB patients. Adapted from WHO¹³⁸.

Outcome	Definition in DS-TB	Definition in DR-TB
Cured	A pulmonary TB patient with bacteriologically confirmed TB at the beginning of treatment who was smear- or culture-negative in the last month of treatment and on at least one previous occasion	Treatment completed as recommended by the national policy without evidence of failure and three or more consecutive cultures taken at least 30 days apart are negative after the intensive phase ^a
Treatment completed	A TB patient who completed treatment without evidence of failure BUT with no record to show that sputum smear or culture results in the last month of treatment and on at least one previous occasion were negative, either because tests were not done or because results are unavailable	Treatment completed as recommended by the national policy without evidence of failure BUT no record that three or more consecutive cultures taken at least 30 days apart are negative after the intensive phase ^a
Treatment failed	A TB patient whose sputum smear	Treatment terminated or need for

	or culture is positive at month 5 or later during treatment	permanent regimen change of at least two anti-TB drugs because of: • Lack of conversion^b by the end of the intensive phase^a, or • Bacteriological reversion^b in the continuation phase after conversion^b to negative, or • Evidence of additional acquired resistance to fluoroquinolones or second-line injectable drugs, or • Adverse drug reactions (ADRs)
Died	A TB patient who dies for any reason before starting or during treatment	
Lost to follow-up	A TB patient who did not start treatment or whose treatment was interrupted for 2 consecutive months or more	
Not evaluated	A patient for whom no treatment outcome is assigned. (This includes cases “transferred out” to another treatment unit and whose treatment outcome is unknown)	
Treatment success	The sum of cured and treatment completed	

DS-TB: Drug sensitive tuberculosis. DR-TB: Drug resistant tuberculosis including RR-TB, MDR-TB and XDR-TB.

^a For Treatment failed, lack of conversion by the end of the intensive phase implies that the patient does not convert within the maximum duration of intensive phase applied by the program. If no maximum duration is defined, an 8-month cut-off is proposed. For regimens without a clear distinction between intensive and continuation phases, a cut-off 8 months after the start of treatment is suggested to determine when the criteria for Cured, Treatment completed, and Treatment failed start to apply.

^b The terms “conversion” and “reversion” of culture as used here are defined as follows:

- **Conversion (to negative):** culture is considered to have converted to negative when two consecutive cultures, taken at least 30 days apart, are found to be negative. In such a case, the specimen collection date of the first negative culture is used as the date of conversion.
- **Reversion (to positive):** culture is considered to have reverted to positive when, after an initial conversion, two consecutive cultures, taken at least 30 days apart, are found to be positive. For the purpose of defining Treatment failed, reversion is considered only when it occurs in the continuation phase.

2.4. TB available vaccine and new candidates

Currently, the bacille Calmette-Guérin (BCG) vaccine remains the only authorized vaccine for the prevention of TB disease¹. It is a live attenuated bacterial vaccine derived from *Mycobacterium bovis*, isolated from a cow affected with TB in 1902⁶³. BCG was first used in humans in 1921: it provides significant protection, especially from severe forms of TB in children⁶. The protective effect of BCG in children is likely up to 10 years¹³⁹. However, BCG is unable to prevent pulmonary TB disease in adults. Indeed, although BCG vaccine offers some protection to adults, its efficacy in this population is highly variable^{6,7}. Therefore, there is an urgent need for novel and highly effective vaccines that provide a protection against all forms of TB and in all population groups¹. Currently, 14 vaccine candidates are in clinical trials: two in phase I, eight in phase II and four in phase III. They include candidates that prevent TB infection and TB disease in addition to others that improve TB treatment outcomes¹. Recently, a novel TB vaccine candidate in a phase III trial, known as M72/AS01E, was found to provide a significant protection with an efficacy of >50% against TB disease among adults, over approximately three years of follow-up¹⁴⁰. Table 3 reviews the current vaccines under development.

Table 3: TB vaccine candidates in clinical phase. Adapted from Treatment Action Group¹⁴¹.

Vaccine	Type	Clinical phase
MIP	Whole-cell <i>M. indicus pranii</i>	Phase III
VPM1002	Live rBCG	Phase III
M72/AS01E	Protein/adjuvant subunit vaccine	Phase III
MTBVAC	Live, genetically attenuated MTB	Phase III
BCG (re) vaccination	Whole-cell <i>M. bovis</i>	Phase III
GamTBvac	Protein/adjuvant subunit vaccine	Phase III
DAR-901	Inactivated whole-cell <i>M. obuense</i>	Phase IIb
H56:IC31	Protein/adjuvant subunit vaccine	Phase IIb
ID93/GLA-SE (QTP101)	Protein/adjuvant subunit vaccine	Phase IIb
RUTI	Fragmented MTB Phase	Phase IIb
ChAdOx1 85A + MVA85A	Viral vector	Phase IIa

BCG (aerosol)	Whole-cell <i>M. bovis</i>	Phase I
AdHu5Ag85A (aerosol)	Viral vector	Phase I
AEC/BC02	Protein/adjuvant subunit vaccine	Phase I
TB/FLU-01L & TB/FLU-04L	Viral vector	Phase I

3. Biomarkers for TB diagnosis and treatment monitoring

3.1. Tuberculosis Diagnostics/ Treatment Monitoring and Biomarkers: Needs, Challenges and Recent Advances

Given the limitations of the available diagnostic and treatment monitoring tests, and while sputum culture and smear microscopy are recommended for the clinical use in TB diagnosis, sputum samples as cited above, may be difficult to obtain in several populations. In these latter, microbiological tests are not always feasible due to the limited ability to produce good quality sputum samples or due to low bacterial loads in their samples¹². In this context, the priority is to develop new, rapid, and low-cost non-sputum-based TB tests that could be integrated in the clinical laboratories mainly located in high-burden resource-poor settings. Indeed, a promising goal in this field is to develop inexpensive and sensitive point-of-care (POC) biomarker tests. For this purpose, the WHO and the Foundation for Innovative New Diagnostics has developed a series of target product profiles (TPPs) which detailed the minimal and optimal criteria that should be met to diagnose and monitor TB treatment response^{14, 15,16}. Table 4 lists the TPPs for non-sputum-based TB tests.

Table 4: The target product profiles (TPPs) set by the WHO for TB diagnostic tests. Source: WHO¹¹⁸ and Yerlikaya. *et al.*¹⁴².

Priority TPP	Description	Diagnostic sensitivity	Diagnostic specificity
Rapid biomarker-based non-sputum-based test for detecting TB	The majority of pulmonary TB cases are diagnosed by sputum smear microscopy. However, smear microscopy has suboptimal sensitivity, and children and HIV-infected individuals often have difficulties providing a good quality sputum sample. The unmet need is a rapid point-of-care test detecting characteristic biomarkers or biosignatures in non-sputum samples. The requirement is a very high specificity and moderate to high sensitivity for the purpose of initiating treatment.	Minimal: $\geq 65\%$ overall Optimal: $\geq 80\%$ overall ($\geq 98\%$ sputum smear-positive and $\geq 68\%$ in sputum smear-negative patients)	$\geq 98\%$ $\geq 98\%$
Community-based triage or referral test for identifying people suspected of having TB	Two weeks of cough is a widely used symptomatic indicator to identify individuals with presumed active pulmonary TB who require diagnostic testing. Since most individuals with suspected TB do not have TB, a triage test can help to narrow down the population that needs more costly and complex confirmatory testing. The needed point-of-care test has a high overall sensitivity and moderate specificity.	Minimal: $\geq 90\%$ overall Optimal: $\geq 95\%$ overall	$\geq 70\%$ $\geq 80\%$

Although several biomarkers and bio-signatures have been identified, only few biomarkers have been independently validated and approved as diagnostic tools to be translated into clinical use. To date, only a urine biomarker-based test (AlereLAM) has been recommended by the WHO for TB diagnosis⁸⁶ (Figure 16). To this end, the current TB research focuses on the discovery and the identification of new human based biomarkers^{14,143,144}. These markers need to be validated and

confirmed in several independent studies, then reviewed by the WHO, thus leading to the translation into new diagnostic tests (Figure 16)¹⁴².

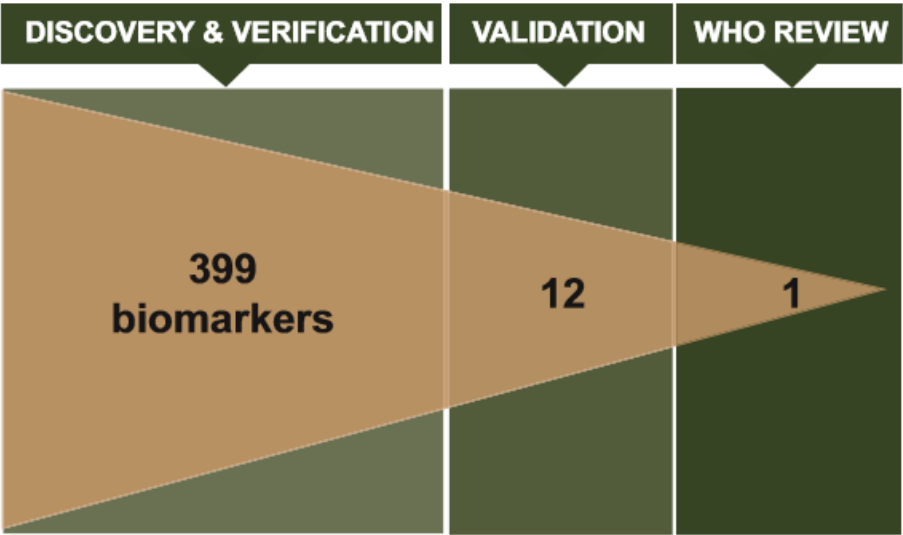


Figure 16: Tuberculosis Biomarker Pipeline. Source: Yerlikaya. *et al.* ¹⁴².

Among the first advancements in this context are the characterization of detailed *Mtb*-specific immune cell phenotypes and marker expression profiles^{145,146}. Changes in immune profiles, including the measurements of specific biomarkers in antigen-stimulated and unstimulated peripheral blood, were also evaluated. Furthermore, bio-signatures associated with TB diagnosis and treatment outcomes were defined¹⁴⁷. This latter field witnesses currently active research on a variety of TB biomarkers, including proteomic, and transcriptomic host TB markers. For this purpose, differentially TB-specific gene and protein expression levels, signatures, as well as proteome^{148,149} and genome¹⁵⁰ profiling, were investigated as predictive markers of TB disease and treatment outcomes¹⁵¹. On the other hand, a different strategy that holds promise is the metabolomics approach¹⁵², which relies on the identification of new metabolite as candidate biomarkers for TB disease and treatment response.

3.2. Targets for the discovery of host TB biomarkers

Based on the natural history of *Mtb* infection, biomarker targets for several key categories were identified. This includes the assessment of biomarkers for vaccine-induced immune protection, diagnosis of LTBI and ATB disease, monitoring anti-TB treatment response, and identification of prognostic markers including those who predict the risk of progression from LTBI to ATB disease or to predict TB relapse following the treatment. The targets for the discovery of host TB biomarkers are summarized in Table 5. Such markers, must be evaluated and validated in well-designed cohorts with appropriate selected patients groups including patients who are not able to produce sputum samples, and/or in who TB diagnosis is difficult (e.g. children, people living with HIV, and patients with extra-pulmonary and culture-negative TB)¹⁵³. Table 6 shows special populations that require a consideration in the validation of new markers.

Table 5: Unmet needs for tuberculosis biomarkers with respect to clinical end points and corresponding study populations. Source: Whitworth. *et al.*¹⁵³.

Unmet need	Study population	Clinical end point
Correlates of vaccine-induced immune protection	Individuals undergoing TB vaccination [†]	Progression to active TB
Correlates of sterilizing immunity	Close contacts of active TB cases, with recent exposure or repeated exposure	<i>Mycobacterium tuberculosis</i> infection (progression to active TB ^{†§})
Diagnostic biomarkers of LTBI	Individuals at risk for LTBI, including close contacts of TB and individuals from countries of high TB incidence	<i>M. tuberculosis</i> infection (progression to active TB ^{†§})
Prognostic biomarkers for TB activation	Individuals at risk for LTBI, including close contacts of TB and individuals from countries of high TB incidence	Progression to active TB
Biomarkers for LTBI treatment efficacy	Individuals with LTBI on prophylactic treatment	Progression to active TB
Diagnostic biomarkers of active TB	Patients with suspected active TB	Final diagnosis of active TB [¶]
Correlates of TB treatment response	Patients on treatment for active TB	Nonrelapsing cure of disease
Biomarkers predictive of TB relapse	Patients on treatment for active TB	Culture-positive TB following culture conversion [#]
[†] Healthy subjects with or without LTBI should be included. [‡] Progression to active TB should be measured in the absence of a gold standard for LTBI. [§] The absence of exogenous infection should be confirmed. [¶] A final diagnosis may include culture-confirmed or highly probable TB. [#] The same <i>M. tuberculosis</i> strain should be implicated pre- and post-treatment. LTBI: Latent tuberculosis infection; TB: Tuberculosis.		

Table 6: Special populations requiring consideration in the validation of new biomarkers. Source: Whitworth. *et al.*¹⁵³.

Special populations	Consideration
Extrapulmonary TB	Increased difficulty in diagnosis
Culture-negative TB	
HIV coinfectd Children	Increased difficulty in diagnosis and increased risk of infection and/or disease
Other diseases (e.g., diabetes or renal failure) Iatrogenic immunosuppression (e.g., high-dose corticosteroids, anti-TNF or organ transplantation)	Increased risk of infection and/or disease
Drug-resistant TB	Increased need for test of cure and biomarker of relapse

3.3. Blood-based immuno-assays used as TB diagnostic and treatment monitoring tests

The immune system plays a critical role in the control of *Mtb* infection¹⁵⁴. Therefore, blood-based immuno-assays have been identified and validated as a potential tool for TB diagnosis and treatment monitoring¹⁵⁵.

3.3.1. Blood cell count

Several components of blood that can be easily identified by blood cell count (CBC), was widely used to detect hematological changes associated with several diseases such as cancer¹⁵⁶ and TB¹⁵⁷. The white blood cell (WBC) count and numerous ratios of different white cells (e.g. the monocyte/lymphocyte ratio) have been frequently performed as a simple and rapid test in clinical practice, and acted as a potential markers associated with TB disease¹⁵⁶. Indeed, the simple marker of the increased monocyte to lymphocyte ratio, has been found to differentiate ATB from LTBI and also from healthy controls¹⁵⁸. Moreover, it was demonstrated that such an elevated ratio may be associated with the risk of ATB^{159,160} and may be beneficial to predict TB treatment outcomes¹⁶¹. Thus, a simple and rapid count of hematological parameters can be useful in improving TB diagnosis at a low cost, specifically when approved for clinical use¹⁶².

3.3.2. Interferon- γ release assays

(IGRAs) such as QuantiFERON-TB Plus tests, cannot discriminate ATB from LTBI. Moreover, this assay has also failed to show a clinical relevance and utility to monitor TB treatment response^{163,164,165,166}. However, the combined use of QFT-P with the HBHA-based IGRAs, that relies on the stimulation of whole blood with recombinant *Mtb* HBHA protein expressed in *Mycobacterium smegmatis* (IGRAs-rmsHBHA)⁶⁴, recently revealed the potential for the stratification of TB stages (e.g. ATB vs LTBI) (Figure 17). Results from these studies showed that the IFN- γ response to HBHA stimulation was significantly higher in LTBI subjects when compared to ATB patients and healthy controls, thus suggesting that the response to HBHA is associated with the LTBI stage^{65,66,167,168}.

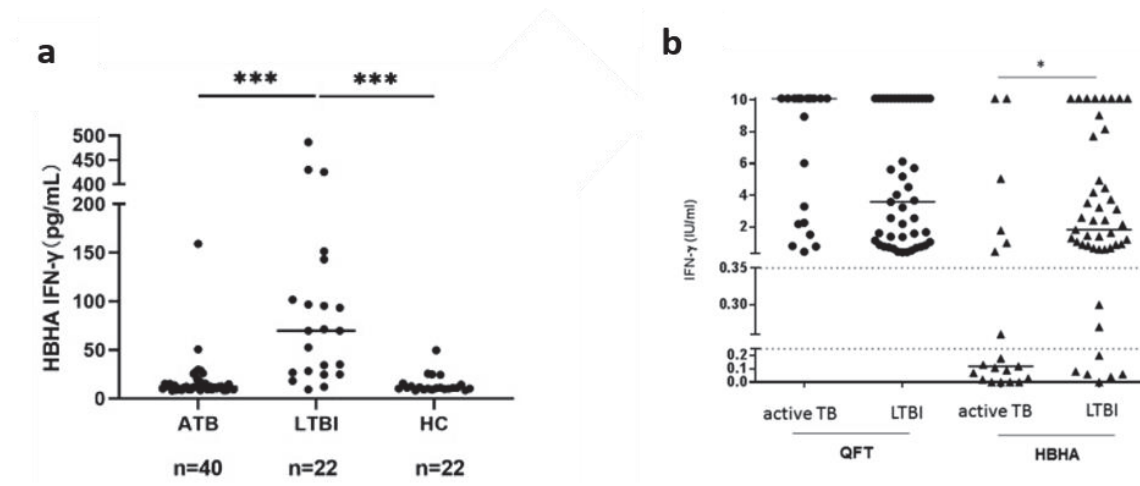


Figure 17: IFN- γ response to QFT and HBHA antigens. (a) IFN- γ response to QFT and HBHA antigens in adults with ATB disease, LTBI and HC. Adapted from Tang. *et al.*⁶⁵ **(b)** IFN- γ response to QFT and HBHA antigens in ATB patients and LTBI children. Adapted from Sali. *et al.*⁶⁶.

3.3.3. Candidate blood-based host immune markers

Recent advances in immunology research have highlighted the potential use of candidate blood-based host markers as a tool for TB diagnosis and treatment monitoring^{153,169}. While evaluating the abundance of *Mtb*-specific immune cells doesn't offer much value for TB diagnosis or to monitor TB treatment response, fortunately, evaluating the phenotypic profile of these cells seems to be promising for these purposes^{170,171}. Such biomarkers were identified based on

memory phenotypes, activation or cytokine expression profiles^{21,22 147}. Indeed, CD4⁺ T cells are considered as one of the principal immune cells involved in the immune response against *Mtb* infection. Consequently, several studies identified markers expressed by these cells in the context of TB disease. These include activation (CD38 and HLA-DR), and proliferation (Ki-67) markers expressed by *Mtb*-specific CD4⁺ T cells. Such markers could function as robust indicators to i) discriminate between the different stages of TB infection (ATB vs. LTBI)^{172,173} and (ii) to evaluate the effectiveness of the TB treatment response^{172,174 175}. In addition, recent studies suggest that the measure of a single activation marker (HLA-DR) generated an excellent diagnostic potential, distinguishing between recent and remote TB infection^{176,177}. Besides, other studies have focused on the evaluation of the differentiation profile of *Mtb*-specific CD4⁺ T cells based on the study of CD27; a maturation marker highly expressed by naïve and early effector T cells but down regulated during the later stages of the differentiation of effector T cells. Previous studies have demonstrated that the proportions of CD27 on IFN- γ -producing CD4⁺ T cells were significantly higher in ATB patients compared to LTBI individuals¹⁷³. In addition, a significantly higher proportion of CD27⁻ IFN- γ ⁺ CD4⁺ T cells was also detected in ATB patients when compared to healthy controls and cured successfully treated TB patients^{173,175}. On the other hand, it was recently demonstrated that the assessment of the expression of the CD40 ligand (CD154), an activation marker expressed on the surface of the central memory CD4⁺ T cells (CD4 T_{CM}), could be used as a potential tool to discriminate between three TB groups. Indeed, a higher expression of CD154 CD4⁺ T_{CM} was observed in ATB patients and LTBI individuals when compared to those without TB¹⁷⁸. Furthermore, in recent years, several studies have also evaluated the diagnostic potential of cytokine (IL-2, IL-4, IFN- γ ^{65,167} and TNF- α ^{11,12}) and chemokine (CXCL-10 (IP-10)^{14,15 179}, CXCL9, CXCL11¹⁸⁰) biomarkers and their changes throughout the course of anti-TB treatment. To note, efforts have also been made to study the possible involvement of CD8⁺ T cells in the immune response against TB infection. Noticeably, *Mtb*-specific CD8⁺ T cells responses were found to be predominately detected in patients with ATB disease when compared to LTBI individuals¹⁸¹.

On the other hand, the humoral immune response has gained an interest, suggesting that it could impact the host defense against *Mtb* infection. Subsequently, recent work has focused on the identification of candidate biomarkers based on the study of the frequencies of B cell subsets and the characterization of their different memory phenotypes. These markers were found to be able

to discriminate LTBI individuals from healthy controls¹⁸² or ATB patients¹⁸³ as well as to monitor the response to anti-TB treatment^{176,189}.

In addition, to the cells that contribute to the adaptive immune response, potential cell biomarkers were identified within the innate immune cells. Important cells involved in the innate immunity against TB include the natural killer (NK) cells¹⁸⁴, $\gamma\delta$ T cells¹⁸⁵ and the mucosal-associated invariant T (MAIT) cells¹⁸⁶. Recent studies suggest that the assessment of the frequencies of NK cells could be used for the discrimination between different stages of TB infection¹⁸². Moreover the study of the phenotypic and functional profile of these cells also revealed a potential to monitor the response to anti-TB treatment^{187,188}. More recently, it was also demonstrated that the levels of NK cells decreased in individuals who progressed from LTBI to ATB disease, whereas the non-progressors showed no significant changes in the frequencies of NK cells. Such data suggest that NK cells are involved in *Mtb* containment in the setting of LTBI and may be relevant candidates for predicting the risk of progression to ATB disease¹⁸².

The above mentioned immune-based biomarkers for TB diagnosis and treatment monitoring and their intended applications are summarized in Table 7.

Table 7: Immunological markers for TB diagnosis and treatment monitoring.

Marker	Function/Type	Intended application
T cell markers: Adaptive immunity		
CD38: activation Ki67: proliferation	Activation expression profile	▪ ATB vs. LTBI¹⁷² ▪ Treatment monitoring^{172,174}
Ki67: proliferation	proliferation expression profile	▪ ATB vs. LTBI¹⁷² ▪ Treatment monitoring^{172,174}
HLA-DR: activation	Activation expression profile	▪ ATB vs. LTBI^{172,173} ▪ Recent vs. remote infection^{176,177} ▪ Treatment monitoring^{172,174 175}
CD27: differentiation	Differentiation expression profile	▪ ATB vs. HC¹⁷³ ▪ ATB (SN-TB) vs. HC¹⁸⁹ ▪ ATB vs. LTBI¹⁷³

		▪ Treatment monitoring^{173,175}
IFN- γ (cytokine) response to HBHA	Cytokine expression profile	▪ ATB vs. LTBI⁶⁵ ▪ Treatment monitoring¹⁶⁷
IL-2: Th1 cytokine IL-4: Th2 cytokine	Cytokine expression profile	▪ Treatment monitoring¹⁹⁰
TNF- α : cytokine	Cytokine expression profile	▪ Treatment monitoring^{191,192}
CXCR3 ligands: include 3 chemokines (CXCL9, CXCL-10, CXCL11)	Chemokine expression profile	▪ Treatment monitoring¹⁸⁰
CXCL-10: chemokine	Chemokine expression profile	▪ ATB vs. HC^{193,194} ▪ Treatment monitoring¹⁷⁹
CD4 ⁺ T _{CM}	T-cell memory phenotypes	▪ ATB vs. LTBI ▪ ATB vs. HC¹⁷⁸
CD154 ⁺ CD4 ⁺ T _{CM} CD154: activation	T-cell memory phenotypes and cytokine expression profile	▪ ATB vs. HC ▪ LTBI vs. HC¹⁷⁸
CD8 ⁺ T cells	T-cell response	▪ ATB vs. LTBI¹⁸¹
Monocytes/Lymphocytes (M/L) ratio	Monocytes to lymphocytes count	▪ ATB vs. LTBI¹⁷⁸ ▪ Treatment monitoring¹⁹⁵
B cell markers: Adaptive immunity		
B cell frequencies	Immune cell frequencies	▪ LTBI vs. HC¹⁸² ▪ Treatment monitoring¹⁹⁶
B cell subsets	Frequencies of B cell subsets and memory phenotypes	▪ ATB vs. LTBI¹⁸³ ▪ Treatment monitoring^{183,196}
NK cell markers: Innate immunity		
NK cell frequencies	Immune cell frequencies	▪ LTBI vs. HC ▪ Risk of progression to ATB¹⁸² ▪ Treatment monitoring¹⁸⁷
CD57: differentiation marker of NK cells	Phenotypic and functional profiles of NK cells	▪ Treatment monitoring¹⁸⁸

SN-TB: Smear negative tuberculosis. CD4⁺ T_{CM}: Central memory CD4⁺ T cells. Recent infection: Recently acquired TB infection. Remote infection: Those with persistent (remote) infection. ATB: Active TB. LTBI: Latent TB infection. HC: Healthy controls.

3.4. Human blood transcriptomic signatures

3.4.1. Gene expression profiling using peripheral blood

Since 1990, whole genome expression microarrays have been developed in an effort to advance our understanding of the complex human response occurring as a response of a disease process^{197,198}. This approach includes transcriptomics, defined as the study of the transcriptome or the genes transcribed from the genomic DNA that enables the profiling of the expression levels of many hundreds or thousands of genes¹⁹⁹. The study of the gene expression profiles can be done using several high-throughput methods such as RNA sequencing, microarray analysis and quantitative real time PCR (qRT-PCR)²⁰⁰. Consequently, perturbed transcriptional regulatory pathways as well as the most differentially regulated genes can be determined as a consequence of a disease^{197,198}. Gene transcription profiling has been used to identify biomarkers or bio-signatures (an assembly of genes or transcripts), that enables a better understanding of host response to disease. Hence, it can potentially be used as diagnostic and prognostic tests as well as for treatment monitoring and the evaluation of response to vaccination^{15,201}.

The use of peripheral blood for transcriptional profiling has several advantages. First, it is an easily accessible sample, although, in some diseases, it may not be easy to access the site of primary infection. Second, reproducible blood transcriptional profile could be obtained using a small volumes of these samples^{202,203}. Third, the blood represents a reservoir of chemokines, cytokines and immune cells including: neutrophils, eosinophils, basophils, T and B cells, trafficking to and from the sites of active disease and the lymphoid organs²⁰⁴. The gene expression profiles using the peripheral blood was first presented in studies focusing on cancer and then on autoimmune diseases^{205,206}. Recently, blood transcriptomic gene expression has advanced the immunological knowledge that succeeded in measuring the host immune response to infection²⁰⁷. Notably, blood transcriptional signature changes observed in response to cancer, autoimmune or infectious diseases are highly influenced by the changes in absolute cell numbers, the proportions of cell types, and the changes in transcription within specific cell populations²⁰⁸.

3.4.2. Overview on blood transcriptional studies in infectious diseases

Several blood transcriptional signatures have been identified and showed their potential in distinguishing different infectious diseases, such as bacterial and viral infections^{209,210,211}. Early identification of the causative agent of a disease is one of the challenges in infectious diseases domain. The routinely used approach includes the direct visualization or the culture of the pathogen, the amplification of the pathogen-specific DNA or the quantification of specific antibodies. However, these tools are not always feasible in infectious diseases. Therefore, identifying a specific blood transcriptional response associated with the disease may be an alternative efficient tool for use in the diagnosis of infectious diseases^{209,210,211}.

The integration of experimental and computational models that include human and animal models provides system biology approaches for understanding the immune pathogenesis during TB infection and other infectious diseases. Data obtained from several transcriptional profiling studies contributes hugely to biological discovery such as developing new efficient diagnostic tools, drug regimens and new vaccines (Figure 18)²¹².

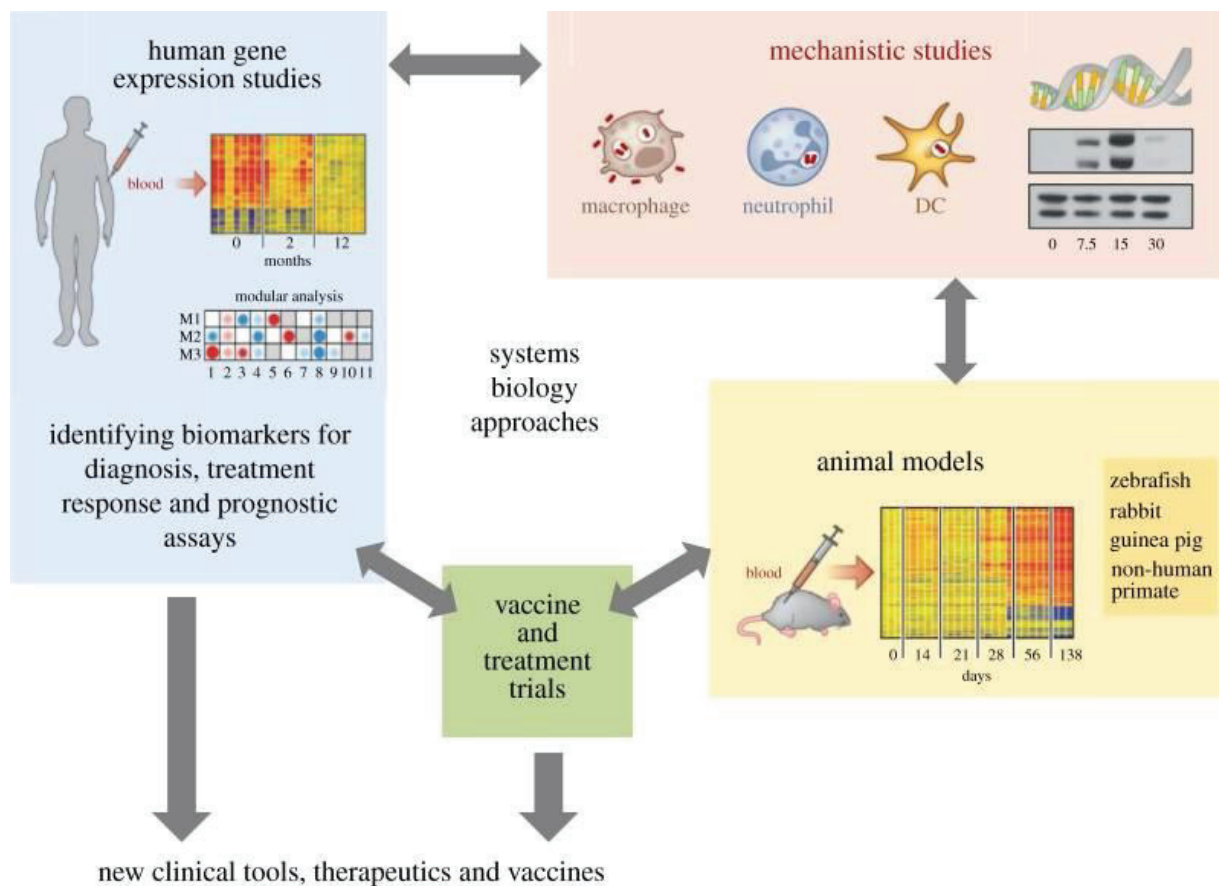


Figure 18: Systems biology approach used in infectious diseases. Source: O'Garra. *et al.*²¹².

3.4.3. Transcriptomic studies investigating the host response to *Mtb* infection

Transcriptomic approaches have focused on the identification of blood transcriptional signatures that can discriminate between the different stages of TB infection: the active form of TB disease from the LTBI as well as from healthy controls^{213,214, 215,216}. These advances have enabled the characterization of several immunological pathways associated with ATB disease such as an upregulation of IFN-inducible genes (both type I and type II) and a downregulation of transcripts encoding T and B cell genes²⁰⁷. It has also been shown that ATB patients and healthy controls have differentially expressed interferon-inducible and chemokine gene profiles^{217,218}. In 2010, a neutrophil-driven interferon signature has been identified in ATB patients, and this signature was absent in both healthy controls and the majority of LTBI individuals. This signature was validated in other independent cohorts from different countries, using independent validation tests²⁰⁷. On the other hand, other identified transcriptional signatures also showed significant

changes following successful anti-TB treatment^{219,220,221}. Further studies have also identified transcriptional signatures of LTBI such as the 16-gene (Zak)²²² and the 4-gene (RISK4) signatures²²³ which, respectively identified people at high risk of developing ATB up to 6 months and 2 years before the start of the disease. More recently, an 11-gene transcriptomic signature (RISK11)²²⁴, derived from the 16-gene Zak signature²²² also showed a good performance for short-term prediction of incident tuberculosis.

Importantly, the use of a gene signature as a diagnostic test for TB depends on its ability to distinguish between ATB and other diseases. Pulmonary TB and selected other inflammatory and infectious diseases were differentiated using a 86-gene signature²⁰⁷. Similar transcriptional profiles (interferon-signaling pathway) were detected in granulomatous disease affecting the lungs such as sarcoidosis and TB^{225,226}. Thus, there is a crucial need for alternative and complementary approaches to select the highly sensitive and specific signature of genes to diagnose TB from other diseases. Therefore, studies were designed to identify a blood transcriptional signature able to discriminate between diseases affecting the same organ with similar pathological outcomes such as sarcoidosis and TB as well as between TB and pneumonia or lung cancer^{227,228}. Currently, a new approach was applied, leading to the detection of the gene expression changes across the global immune response observed in TB and other diseases (Figure 19). The most discriminative genes were selected allowing for a more specific signature with a small set of genes, which allow the development of a more efficient diagnostic tool for TB disease²²⁹.

Collectively, these findings support the potential use of blood transcriptomic signatures as a diagnostic test for TB. However, it is important to validate such findings in large cohorts of patients with different ethnicities and in different geographical locations to insure the reliability of these tools.

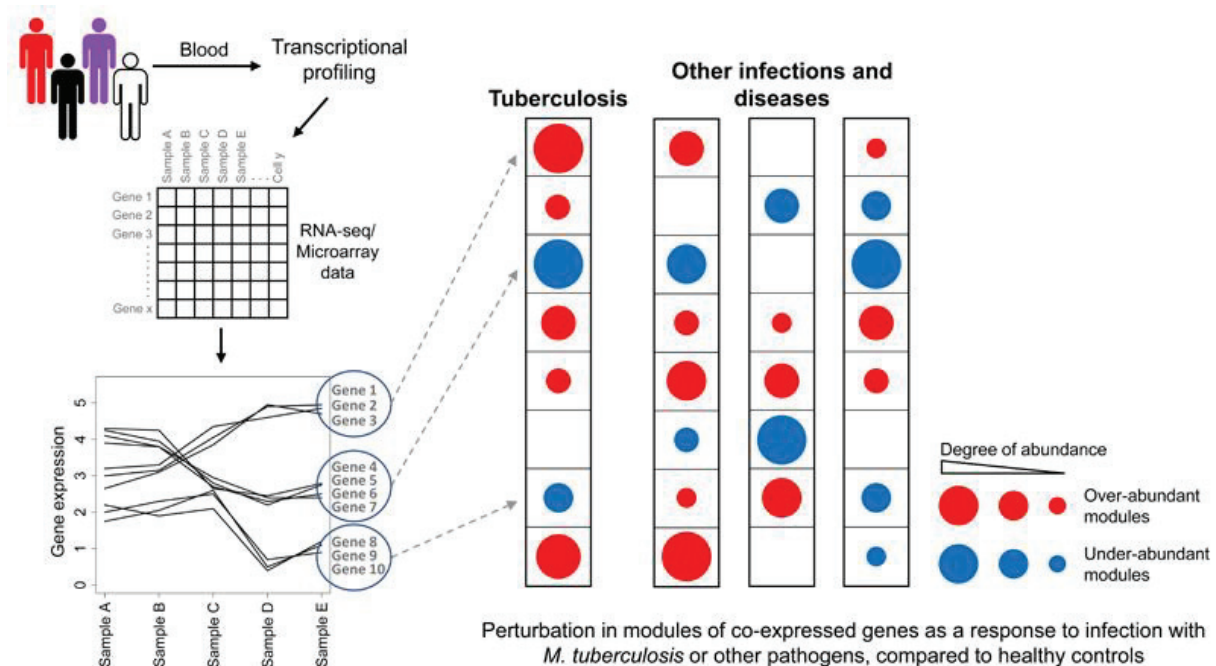


Figure 19: Modular host gene signatures in TB and in other diseases. The difference between TB and other disease can be detected using a modular approaches, this can be done by transcriptomic profiling of blood samples collected from patients. Each gene is expressed at a particular level and genes coding molecules involved in similar biological pathways are also expressed. Moreover, the perturbation of gene expression as consequence of TB or other disease can be measured relative to the expression of those genes in healthy controls. Source: Singhania. *et al.*²²⁹.

3.4.4. Human blood transcriptomic TB signatures

Several studies have shown that host blood transcriptomic signatures show promise as a powerful tool for the discovery of potential TB biomarkers. These latter can be used to i) predict the risk of progression to active TB in patients with latent infection^{222,230,223} ii) diagnose active TB disease and discriminate these patients from whom with a latent TB infection and /or healthy individuals^{155,207} and iii) monitor TB treatment response^{220,231}. The recent advances in research in human blood transcriptomic signatures as a tool for TB diagnosis and treatment monitoring are summarized in the Table 8. Notably, several transcriptomic signatures are currently in the pipeline set by the WHO for non-sputum-based TB diagnostic tests¹⁴. It is unknown if these signatures had the same accuracy to predict TB and treatment response, thus had the potential to replace sputum based assays²³². While all of these tests are in the early stages of development, Sweeney: a three genes signature, and RISK6; a six genes signature are the most advanced in the

pipeline to predict the risk of progression to ATB disease and for treatment monitoring¹⁴ (Table 9).

Table 8: Summary of blood transcriptional gene signatures for tuberculosis.

Signature	Number of genes	Population	Intended application
Penn-Nicholson6²³³ (RISK6)	6	Adolescents & Adults	▪ Risk of progression to ATB ▪ ATB vs. HC & ORD ▪ Treatment monitoring
Scriba11²²⁴ (RISK11)	11	Adults	▪ Risk of progression to ATB
Suliman4²²³ (RISK4)	4	Adolescents	▪ Risk of progression to ATB
Warsinske3²¹³ (Sweeney3)	3	Adolescents & Adults	▪ Risk of progression to ATB ▪ ATB vs. HC ▪ Treatment Monitoring
Sweeney3²¹⁹	3	Adults	▪ ATB vs. LTBI & HC & ORD ▪ Treatment monitoring
Sambarey10^{214, 215}	10	Adults	▪ ATB vs. LTBI & HC & ORD ▪ Treatment Monitoring
Berry20²³⁴	20	Adults	▪ ATB vs. LTBI & HC
Berry393²⁰⁷	393	Adults	▪ ATB vs. LTBI & HC ▪ Treatment Monitoring
Berry86²⁰⁷	86	Adults & Children	▪ ATB vs. ORD ▪ Treatment monitoring
Maertzdorf4²¹⁶	4	Adults	▪ ATB vs. LTBI & HC

Kaforou ²⁷ ²³⁵	27	Adults	▪ ATB vs. LTBI
Kaforou ⁴⁴ ²³⁵	44	Adults	▪ ATB vs. ORD
Bloom ⁶⁶⁴ ²³⁶	664	Adults	▪ Treatment monitoring
Bloom ³²⁰ ²³⁶	320	Adults	▪ Treatment monitoring
Bloom ¹⁴⁴ ²²⁶	144	Adults	▪ ATB vs. HC & ORD
Darboe ¹¹ ²³⁷ (ACS COR)	11	Adults	▪ Risk of progression to ATB
			▪ Treatment monitoring
Francisco ² ²³⁸	2	Adults	▪ ATB vs. HC & ORD
			▪ Treatment monitoring
Lu ³ ²³⁹	3	Adults	▪ ATB vs. LTBI
BATF ² ²⁴⁰	1	Adults	▪ ATB vs. LTBI & HC
Roe ³ ²⁴¹	3	Adults	▪ Risk of progression to ATB
Satproedprai ⁷ ²²¹ (TB Sick score)	7	Adults	▪ ATB vs. HC
			▪ Treatment monitoring
Zak ¹⁶ ²²²	16	Adolescents	▪ Risk of progression to ATB
Anderson ⁴² ²⁴²	42	Children	▪ ATB vs. LTBI
Anderson ⁵¹ ²⁴²	51	Children	▪ ATB vs. ORD
Gjoen ⁷ ²⁴³	7	Children	▪ ATB vs. HC & ORD
Jenum ² ²⁴⁴	2	Children	▪ Treatment monitoring
Thompson ⁵ ²²⁰ (RESPONSE5)	5	Adults	▪ Treatment monitoring

The gene signatures were named by combining the last name of the first author suffixed with the number of genes in the signature. The gene signatures with a given name in the publication are also represented in Red.

ATB: Active TB. LTBI: Latent TB infection. HC: Healthy controls. ORD: Other respiratory diseases.

Table 9: Tools that are currently in the pipeline set by the WHO, for the detection of incipient TB and for treatment monitoring. Adapted from WHO¹⁴.

Test/tool (Manufacturer)	Type	Potential use case	Sensitivity*/ Specificity*/ AUC**	Time to results	Price per test	Stage of development
Xpert-MTB-HR (Cepheid)	Host blood RNA response/PCR	Incipient TB/ Treatment monitoring	Incipient TB triage (PLWHA): SE: 90% SP: 55.8% AUC: 0.89 ¹¹³	Not yet available	Comparable to pricing of other Xpert tests ¹¹⁴	Early development; Expected launch in 2023 ¹¹⁵
RISK6 signature assay (QuantuMDx)	Host blood RNA response/PCR	TB infection/ Incipient TB	Not yet available	< 30 min ¹¹⁶	PCR-based test pricing	Early development/ Projected year of WHO review: 2022 ¹¹⁷
FilmArray Assay (bioMérieux)	Host blood RNA response/PCR	Treatment monitoring	Not yet available	< 1 hour ¹¹⁸	PCR-based test pricing	Early development ¹¹⁹

Incipient TB: a stage in the spectrum of TB infection indicating the progression from LTBI to ATB disease.

The Cepheid's Xpert-MTB-HR tests is for the Sweeney3 RNA gene signature.

4. High-throughput single cell analysis technologies

4.1. Evolution of tools for single cell analysis

Understanding the complexity of the human immune response requires the use of high-throughput single cell analysis technologies. Cytometry based assays has sought to address this crucial need as they enable the identification of immune subsets at a single cell level by binding fluorochrome-labeled antibodies to specific markers of interest. Conventional flow cytometry has been an important immuno-profiling technology used for cell analysis (Figure 21). In the 1980s, three markers were analyzed at a time²⁴⁵. However through the incorporation of additional lasers and detectors, and the usefulness of additional fluorophores, the number of parameters analyzed per cell has increased over the years, leading to a 17-color assay reported in 2004²⁴⁶, and then to a 28-color assay²⁴⁷. The arrival of mass cytometry (also called cytometry by time-of-flight or CyTOF) in 2009, has revolutionized the study of cellular and functional immune subsets²⁴⁸. This high dimensional single-cell analysis tool overcomes major limitations of conventional flow cytometry. Indeed, CyTOF uses metal-conjugated antibodies, limiting spectral overlap issues occurred in flow cytometry^{182,249}. Therefore, this property has increased the abilities of flow cytometers to assess wide phenotypic changes of several immune populations using more than 40 parameters at once²⁵⁰ (Figure 20). Nevertheless, high costs, cell loss, and high throughput of cells needed have limited the use of this technology in clinical studies and in some laboratories^{251,252}.

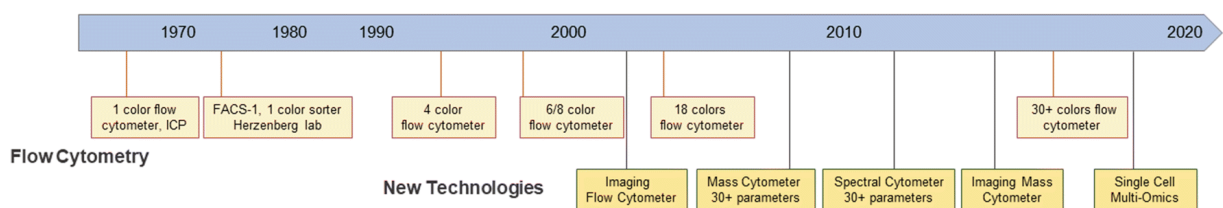


Figure 20: A schematic representation showing the evolution of technologies used in cytometry. Adapted from Mitra-Kaushik. *et al*²⁵³.

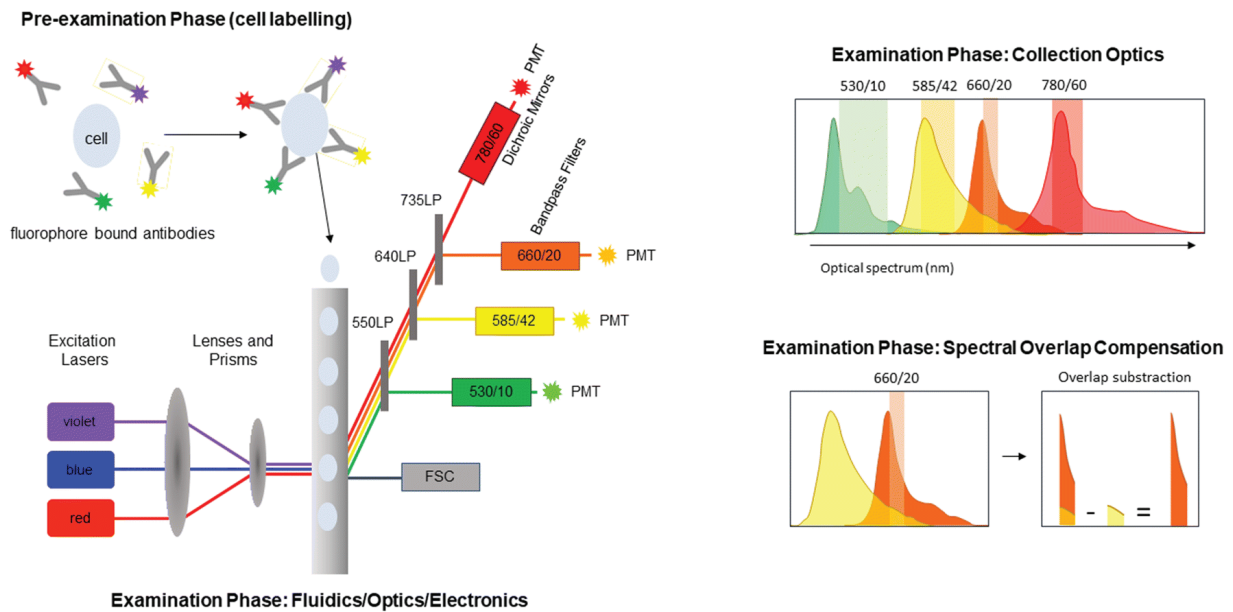


Figure 21: Conventional flow cytometry. Cells are stained with fluorescent-tagged antibodies during conventional flow cytometry. Within the flow cell, samples flow one cell at a time through a laser beam resulting in scattered light and excitation of the fluorophores. The fluorescence emitted are detected by photodetectors (photomultiplier tubes (PMTs)) and then converted into electric signals. The spectral overlap resulting from fluorophores present on the same cell is subtracted by applying the compensation. Source: Mitra-Kaushik. *et al*²⁵³.

4.2. Spectral flow cytometry

In recent years, improvements in flow cytometry optics, detectors and fluorophore availability have enabled the development of spectral flow cytometry (SFC)²⁵⁴, which improved the detection sensitivity, and differentiated fluorophores with very similar emission peaks thus reducing the requirement of compensation (Figure 22)^{255,256}. In contrast to conventional flow cytometry that identify only the peak emission of each fluorophore, SFC measures the full emission spectrum for every fluorophore across all lasers. In addition, it uses spectral unmixing technology with non-square compensation matrix to discriminate single marker²⁵⁷. Overall, spectral flow cytometry allows a greater flexibility in terms of highly multi-parametric panel design and fluorophore detection with high resolution²⁵⁸. Currently, a 40-color OMIP-069²⁵⁹, a full spectrum flow cytometry panel has been developed, and most recently a 43-color panel²⁶⁰ has been also published. This allows a deep immune profiling of distinct cell populations in human peripheral blood using more than 40 florescent markers in the same panel. Subsequently, Cytex Biosciences released the Aurora spectral flow cytometer in 2017, which has gained the

interest of researchers to perform deep immune-phenotyping and analyzing high dimensional data²⁶¹.

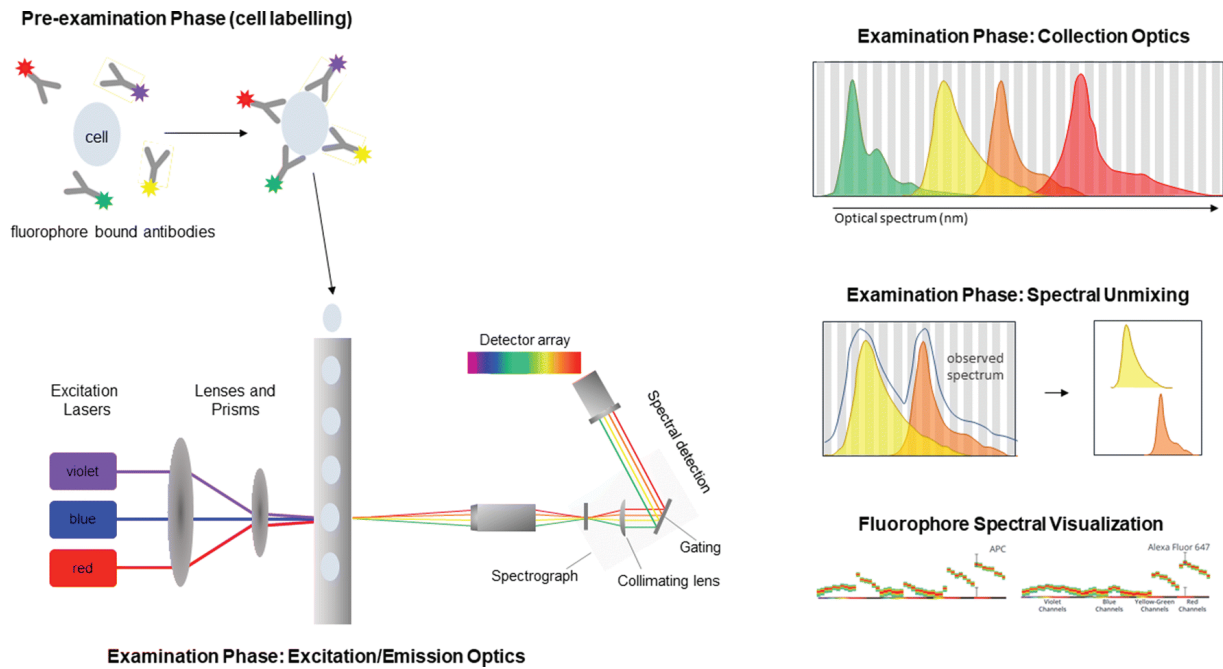


Figure 22: Spectral Flow Cytometry. Similar to conventional flow cytometry, cells are stained with fluorescent-conjugated antibodies. Within the flow cell, samples flow one cell at a time through a laser beam resulting in scattered light and excitation of the fluorophores. The fluorescence emitted is then detected by a detector array. The spectral overlaps between multiple fluorophores present on the same cell are separated using the spectral unmixing that enables the detection of fluorophores with a spectral overlap emission. Source: Mitra-Kaushik. *et al*²⁵³ and emission spectra from www.cytekbio.com

4.3. Characterization of the immune response against TB using spectral flow cytometry

Spectral flow cytometry represents a powerful tool for in-depth analysis of a wide variety of immune cell subsets in the context of different domains²⁶². Recently, it was applied in cancer research²⁵⁸ and drug discovery²⁶². Moreover, this technique is starting to become more common in the field of infectious diseases such as COVID-19 research²⁶³. However, to date, only few studies have highlighted the potential use of SFC technology to explore infectious diseases in multi-centric clinical cohorts and none specifically in TB. Subsequently, the development of a clinically applicable spectral flow cytometry model in the field of TB as a diagnostic and

treatment monitoring tool would be novel and interesting. It will enhance our understanding of the specific immune response against TB due to its potential to combine phenotypic and functional analysis of a wide variety of immune cell subsets of interest.

Research objectives

This project was coordinated by the Mérieux Foundation as a part of a multicenter cohort study conducted in partnership with five members of the GABRIEL Network (Global Approach to Biological Research, Infectious diseases and Epidemics in Low-income countries)²⁶⁴. The general aim of the project was to evaluate and to develop new non-sputum-based TB diagnostic tools according to the TB research priority needs advised by the WHO. Thus, we focused on the evaluation of novel blood-based TB tests in the framework of TB diagnosis and the identification of simple and rapid biomarkers for monitoring TB treatment response in adults. For that matter, we evaluated the performance of a new human-blood transcriptomic signature; RISK6, in the context of TB diagnosis and treatment monitoring. Thereafter, we identified blood-based immune biomarker tests for TB treatment monitoring. First, by evaluating simple hematological parameters routinely performed using an inexpensive technique. Then, by using a high-dimensional single cell technology; spectral flow cytometry, to perform in-depth phenotypic analysis to characterize *Mtb*-specific immune subsets.

Overall, the aim of this project was to report new advances within the TB research field, by offering new, simple, and rapid tools for TB diagnosis and treatment monitoring that could be translated from clinical research to clinical applications.

Now, moving on to the last part of this study evaluating the use of a novel high-dimensional single cell technology; spectral flow cytometry.

CHAPTER 2: RESULTS AND ORIGINAL PUBLICATIONS

1. Description of the research project: the HINTT study

This project was a part of a multicenter prospective cohort study: HINTT (HBHA Interferon- γ Release Assay Test for Tuberculosis) that aimed to evaluate the prognostic value of blood-based immunological biomarkers for monitoring TB treatment outcomes. It was conducted within the GABRIEL Network²⁶⁴ in five different lower-income countries including Bangladesh, Georgia, Lebanon, Madagascar and Paraguay. The study protocols were implemented, reviewed and approved by the human research ethics committees in each country. All study participants provided written informed consent. All research was performed in accordance with relevant guidelines/regulations. As cited before, the overall aim of this project was to monitor the efficacy of anti-tuberculosis treatment. For this purpose, patients with ATB disease were recruited at primary healthcare TB clinics in each country. The enrolled ATB patients were aged ≥ 15 years old, newly diagnosed with pulmonary ATB: scoring positive for TB following bacteriological (culture positive and/or sputum smear microscopy positive) and/or molecular analysis (GeneXpert positive results). Patients with negative cultures at inclusion, ATB patients with Human Immunodeficiency Virus (HIV) or with diabetes mellitus comorbidities and patients under immunocompromising treatment were excluded. Enrolled ATB patients were followed-up during the treatment course at four different time points and classified as follow: (i) ATB at baseline T0: patients who didn't start TB treatment; (ii) treated active TB at T1 and T2: patients with ATB followed-up during the treatment and tested after 2 months of the start of the treatment (T1), and at the end of treatment (T2); (iii) treated active TB at T3: treated TB patients tested at 2 months after treatment completion. At each time point, sputum and blood samples were collected from patients. ATB diagnosis was based on both bacteriological and molecular parameters. At least one sputum sample was collected at inclusion (T0) for culture testing (liquid culture media: MGIT mycobacterial growth indicator tube, BD BioSciences, NJ, USA and/or solid culture media: L-J (Lowenstein–Jensen) and also tested by microscopy for the presence of acid-fast bacilli (AFB) using the Ziehl-Neelsen staining method and/or Auramine O staining. In addition to positive culture, active TB status was defined by positive Xpert MTB/RIF (Cepheid). Patients

were re-evaluated by sputum smear and culture during the intensive phase of treatment (T1) thereafter at the end of treatment (T2) and 2-months after treatment completion (T3) to confirm that they were successfully treated and cured. Drug susceptibility testing (DST) methods were performed according to standard protocols¹²². A minimum of 10 ml of whole blood was also drawn from study subjects and divided as following for subsequent analysis (Figure 23):

- 3 ml of blood collected in tempus blood RNA tubes for the evaluation of the blood transcriptomic signature; RISK6
- 2 ml collected in EDTA and dry tubes for white blood cell counts
- 4 ml collected in 4 different QuantiFERON tubes for the QFT-P test and for in depth immune cell phenotypic analysis by spectral flow cytometry
- 1 ml collected in a heparin tube for the IGRA-HBHA test and for in depth immune cell phenotypic analysis by spectral flow cytometry

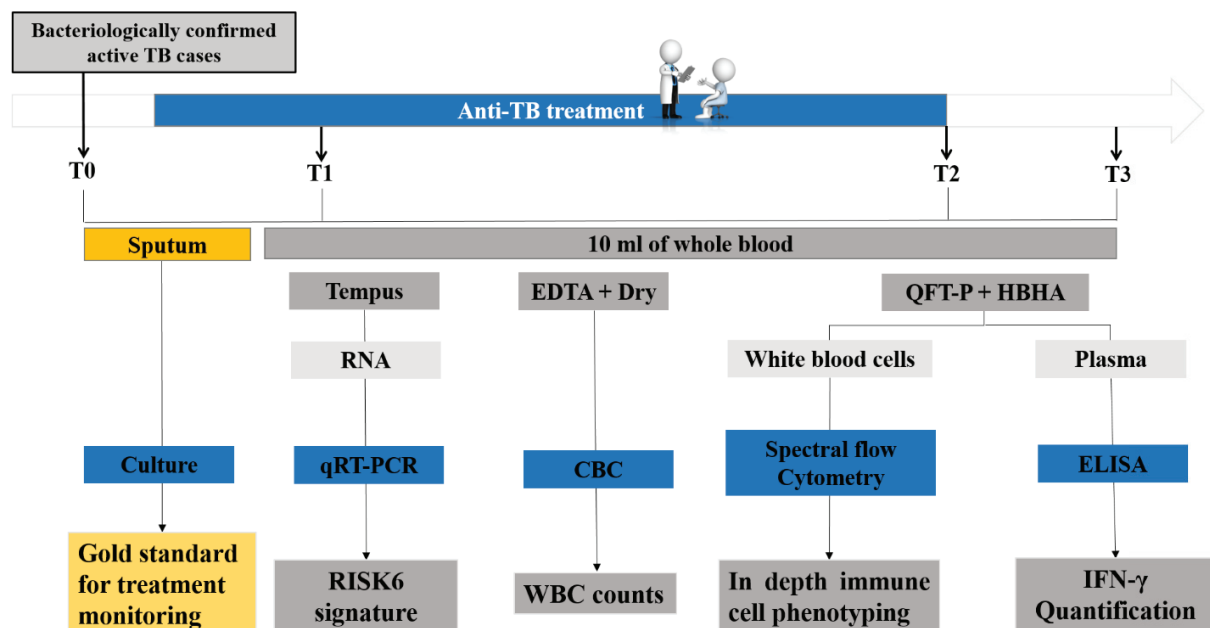


Figure 23: The overall workflow methodology of HINTT study.

The evaluation of the combination of QFT-P and HBHA assays for monitoring TB treatment response is not within the scope of this thesis, and the associated original publication was added to the Annexes. qRT-PCR: quantitative real-time PCR. CBC: complete blood count. WBC: white blood cells. QFT-P: QuantiFERON-TB Gold Plus.

2. Publication 1

Multi-country evaluation of RISK6, a 6-gene blood transcriptomic signature, for tuberculosis diagnosis and treatment monitoring

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Article summary

Current sputum-based tests for TB diagnosis and treatment monitoring are associated with several limitations. In this context, the WHO highlights the urgent need for alternative non-sputum-based TB tests, and sets a series of target product profiles (TPPs) which detailed the minimal and optimal criteria that should be met to diagnose and monitor TB treatment response. Those new TB tests need to be based on accessible biological samples such as whole blood or urine, and must be practical for field applications. Currently, there is much active research on human blood transcriptomic TB biomarkers. In the present study, we evaluate the performance of a six whole blood gene transcriptomic signature (RISK6), for TB screening, triage and treatment monitoring. In addition, RISK6 performance was also compared to that of two IGRAs: one based on RD1 antigens (QuantiFERON-TB Gold Plus, QFT-P, Qiagen) and one on recombinant *M. tuberculosis* HBHA expressed in *Mycobacterium smegmatis* (IGRA-rmsHBHA). The evaluation of RISK6 was done in a *multicenter prospective nested case-control* study conducted in Bangladesh, Georgia, Lebanon and Madagascar. In total, 238 participants were recruited and followed-up between August 2018 and September 2020. Participants included adult non-immunocompromised patients with bacteriologically confirmed active pulmonary TB (ATB; n = 141), latent TB infection (LTBI; n = 26) and healthy donors (HD; n = 71). Moreover, the enrolled ATB patients were followed-up during the treatment course at four different time points and classified as follow: at T0, T1, T2 and T3. Blood RISK6 scores were assessed using quantitative real-time PCR (qRT-PCR) and evaluated by area under the receiver-operating characteristic curve (ROC AUC).

In all cohorts, RISK6 scores were significantly higher in ATB patients at baseline compared to HD ($p < 0.001$). Moreover, the performance of RISK6 to discriminate ATB from HD reached an AUC of 0.94 (95% CI 0.89 to 0.99), with 90.9% sensitivity and 87.8% specificity, thus achieving the minimal WHO TPPs for a non-sputum-based TB screening test. Remarkably, in the Madagascar cohort that includes LTBI individuals, we also observed a significant difference for the RISK6 scores between ATB and LTBI groups ($p < 0.001$) but not between the LTBI group

and the HD group. Interestingly, the RISK6 yielded an AUC of 0.93 (95% CI 0.85-1) with 90.9% sensitivity and 88.5% specificity for discriminating different stages of TB infection (e.g. ATB from LTBI). Remarkably, RISK6 also showed higher performance (AUC 0.90, 95% CI 0.85-0.94) than IGRA-rmsHBHA (AUC 0.75, 95% CI 0.69-0.82) to differentiate ATB from LTBI. Furthermore, we found that ATB patients with low- or high-grade positive smears had significantly higher RISK6 scores ($p < 0.001$) compared to those with negative smears. Finally, we demonstrated that RISK6 signature scores significantly decreased after two months of TB treatment and continued to decrease gradually until the end of treatment reaching scores obtained in HD.

Data from this study provide strong proof that RISK6 can be applied as a non-sputum-based screening and triage test that met the WHO TPP benchmarks. This host response-based gene signature may be used for stratifying patients according to their TB infection status (e.g. ATB from LTBI), as well as for monitoring patients over the course of treatment. Fortunately, RISK6 signature is applicable using a robust and simple qRT-PCR platform which facilitates its implementation in the clinical laboratories located in resource-poor settings. Moreover, a key advantage of RISK6 is that it is a blood-based test, which is an easily accessible sample. Blood transcriptomic tests will improve the diagnosis of TB allowing faster treatment and thus reduction of transmission, especially in children, HIV co-infected TB patients and paucibacillary pulmonary TB patients. In such populations, microbiological tests are not always feasible due to the limited ability to produce good quality sputum samples or due to low bacterial loads in their samples. Overall, our findings support the efforts to incorporate RISK6 signature into a point-of-care test ensuring rapid and accurate detection of ATB cases. Indeed, such simple tests are highly needed to reduce TB spread and transmission especially in areas with high TB burden that are usually disturbed with poverty.



OPEN

Multi-country evaluation of RISK6, a 6-gene blood transcriptomic signature, for tuberculosis diagnosis and treatment monitoring

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There is a crucial need for non-sputum-based TB tests. Here, we evaluate the performance of RISK6, a human-blood transcriptomic signature, for TB screening, triage and treatment monitoring. RISK6 performance was also compared to that of two IGRAs: one based on RD1 antigens (QuantiferON-TB Gold Plus, QFT-P, Qiagen) and one on recombinant *M. tuberculosis* HBHA expressed in *Mycobacterium smegmatis* (IGRA-rmsHBHA). In this multicenter prospective nested case-control study conducted in Bangladesh, Georgia, Lebanon and Madagascar, adult non-immunocompromised patients with bacteriologically confirmed active pulmonary TB (ATB), latent TB infection (LTBI) and healthy donors (HD) were enrolled. ATB patients were followed-up during and after treatment. Blood RISK6 scores were assessed using quantitative real-time PCR and evaluated by area under the receiver-operating characteristic curve (ROC AUC). RISK6 performance to discriminate ATB from HD reached an AUC of 0.94 (95% CI 0.89–0.99), with 90.9% sensitivity and 87.8% specificity, thus achieving the minimal WHO target product profile for a non-sputum-based TB screening test. Besides, RISK6 yielded an AUC of 0.93 (95% CI 0.85–1) with 90.9% sensitivity and 88.5% specificity for discriminating ATB from LTBI. Moreover, RISK6 showed higher performance (AUC 0.90, 95% CI 0.85–0.94) than IGRA-rmsHBHA (AUC 0.75, 95% CI 0.69–0.82) to differentiate TB infection stages. Finally, RISK6 signature scores significantly decreased after 2 months of TB treatment and continued to decrease gradually until the

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end of treatment reaching scores obtained in HD. We confirmed the performance of RISK6 signature as a triage TB test and its utility for treatment monitoring.

One fourth of the world population is estimated to be infected with *Mycobacterium tuberculosis* (*Mtb*) that causes approximately 10 million cases of tuberculosis (TB) yearly. This disease ranks among the leading causes of death worldwide, resulting in 1.4 million deaths in 2019¹. Five to 10% of infected individuals develop the contagious, active form of TB (ATB) disease, while most of them (90%) control the infection and develop asymptomatic latent TB infection (LTBI). However, a small proportion (10%) of LTBI individuals will develop ATB during their lifetime². TB can be treated with a regimen of several antibiotics for a minimum of 6 months. In most patients, TB therapy provides cure³ but treatment failure and relapse can occur. These outcomes are associated with severe adverse effects and long treatment durations that induce a lack of patient adherence to the treatment regimen thus promoting the emergence of drug-resistance⁴.

Current ATB diagnostic tests include sputum-based culture and acid-fast Bacillus (AFB) smear microscopy which are also used for monitoring TB treatment response^{1,3}. Molecular tests like the GeneXpert MTB/RIF or ULTRA, are also performed using sputum samples⁵. Interferon (IFN)- γ release assays (IGRAs) such as QuantiFERON-TB Plus (QFT-P; Qiagen) are blood-based tests used for the detection of *Mtb* infection, yet cannot discriminate ATB from LTBI^{6–9}. However, the combined use of QFT-P with the heparin-binding hemagglutinin antigen; HBHA-based IGRAs, that relies on the stimulation of whole blood with recombinant *Mtb* HBHA protein expressed in *Mycobacterium smegmatis* (IGRAs-rmsHBHA)¹⁰, recently revealed the potential for the stratification of TB stages (e.g. ATB vs LTBI)^{11–14}.

Sputum-based TB tests are associated with several limitations including the long-time of culture and the lack of sensitivity and specificity of smear microscopy¹⁵. Besides, although molecular tests are more sensitive for diagnosing pulmonary TB, they still have limited sensitivity in paucibacillary pulmonary TB patients^{16,17}. In addition, sputum samples may be difficult to obtain in some populations (e.g. children and HIV co-infected TB patients) as well as in ATB patients after symptom improvement¹⁸. In this context, the World Health Organization (WHO) has declared an urgent need for alternative non-sputum-based TB tests with a series of target product profiles (TPPs) which detailed the minimal and optimal criteria that should be met to diagnose and monitor TB treatment response^{19–21}. Those new TB tests need to be based on accessible biological samples such as whole blood or urine, and must be practical for field applications²².

Currently, there is much active research^{23,24} on human blood transcriptomic TB biomarkers²⁵. A six whole blood gene transcriptomic signature (RISK6) has been recently described and validated in 7 independent cohorts, demonstrating its utility to predict the risk of progression from TB infection to ATB disease, as a screening test for TB, and to monitor TB treatment response^{19,26}. The present study aims: to evaluate the robustness of the RISK6 signature in four additional independent cohorts from different countries and ethnicities; to assess its performance for TB screening and triage; to compare its performance to that of two IGRAs (QFT-P and IGRAs-rmsHBHA); and to evaluate its utility for monitoring treatment outcome.

Results

Sociodemographic and clinical characteristics. A total of 141 patients with bacteriologically confirmed pulmonary ATB were included in the study. Their sociodemographic and clinical characteristics were compared at baseline. The median age was 28 years, 66% were male, and 51.8% were smokers. Among them, 48.2% had a positive sputum smear microscopy with a high grade at baseline (2+ or 3+). 97 of these patients were followed at least until the end of treatment and have been successfully treated for TB. The remaining participants included 26 individuals with LTBI and 71 healthy donors (Table 1).

Performance of the RISK6 signature as a screening and triage test for pulmonary TB disease. To investigate the use of RISK6 score as a screening and triage test for TB, we compared RISK6 scores between patients with ATB disease ($n = 141$), treated TB patients who have been successfully treated for TB (TREATED, $n = 97$, with negative sputum culture at T2 and/or T3), the individuals with LTBI ($n = 26$), and healthy donors (HD, $n = 71$). In all cohorts, RISK6 scores were significantly higher in ATB patients at baseline compared to HD ($p < 0.001$) and TREATED TB patients ($p < 0.001$) (Fig. 1a). Moreover, RISK6 score levels of TREATED patients became indistinguishable from HD. Remarkably, in the Madagascar cohort that includes the enrolled LTBI individuals, we observed a significant difference for the RISK6 scores between ATB and LTBI group ($p < 0.001$) but not between the LTBI group and the TREATED TB patients or the HD group. Remarkably, when we compared the RISK6 scores levels between study sites, we found that the RISK6 scores levels in ATB, TREATED TB patients and the HD recruited from Bangladesh were higher than the levels observed in the other study sites (Fig. 1a).

We then generated a receiver operating characteristic curve (ROC) and the respective areas under the curve (AUC) for each cohort to evaluate, by country, the performance of RISK6 signature as screening or triage test (Fig. 1b). First, we assessed the performance of RISK6 as a screening test for the discrimination between ATB patients and HD. Remarkably, the performance of the RISK6 signature was similar in the four different cohorts, with outstanding AUC values ranging from 90.1% (Bangladesh; 95% CI 80.7–99.4) to 96.4% (Georgia; 95% CI 90.5–100) (Fig. 1b). Secondly, ROC analysis was also performed to determine the potential of RISK6 signature as a triage test to discriminate between different stages of TB infection. Results demonstrated a powerful classifying potential to discriminate patients with ATB from LTBI or TREATED TB patients with an AUC of 92.8% (95% CI 85.6–100) and 96.1% (95% CI 91.7–100) respectively (Fig. 1b). Remarkably, we also found that the discrimination between ATB and HD was lowest in the cohort of Bangladesh when compared to other study sites (Fig. 1b).

	Georgia	Madagascar	Lebanon	Bangladesh	Total
ATB (N)	32	44	21	44	141
ATB patient demographics					
Age (years)	33.5 (26.75–44.5)	29.5 (21.75–43.25)	30 (22–37)	23.5 (20.75–30.5)	28 (22–39)
Gender (male)	81.2% (26/32)	59.1% (26/44)	47.6% (10/21)	70.5% (31/44)	66% (93/141)
BMI at baseline	20.06 (18.65–21.67)	17.19 (16.31–18.67)	20.94 (19.59–21.41)	18.28 (16.2–20.79)	18.68 (16.89–20.95)
Vaccination					
BCG vaccination	40.6% (13/32)	88.6% (39/44)	19% (4/21)	75% (33/44)	63.1% (89/141)
Risk factors					
Smoking habit	59.4% (19/32)	43.2% (19/44)	57.1% (12/21)	52.3% (23/44)	51.8% (73/141)
Alcohol consumption	9.7% (3/31)	45.5% (20/44)	9.5% (2/21)	11.4% (5/44)	21.4% (30/140)
Injecting drug users	–	–	–	9.3% (4/43)	2.9% (4/138)
Jail detention history	6.2% (2/32)	2.4% (1/42)	14.3% (3/21)	4.5% (2/44)	5.8% (8/139)
Other pathologies					
HCV positive	9.4% (3/32)	2.3% (1/44)	–	–	2.8% (4/141)
Other underlying disease	–	9.1% (4/44)	9.5% (2/21)	2.3% (1/44)	5.5% (7/127)
Sputum smear microscopy at baseline					
Low grade (1+ or scanty)	37.5% (12/32)	25% (11/44)	28.6% (6/21)	27.3% (12/44)	29.1% (41/141)
High grade (2+ or 3+)	25% (8/32)	54.5% (24/44)	38.1% (8/21)	63.6% (28/44)	48.2% (68/141)
Negative	34.4% (11/32)	20.5% (9/44)	19% (4/21)	9.1% (4/44)	19.9% (28/141)
Not evaluated	3.1% (1/32)	–	14.3% (3/21)	–	2.8% (4/141)
TB treatment					
Treated	26	33	15	23	97
LTBI (N)	–	26	–	–	26
Healthy donors (N)	7	23	25	16	77

Table 1. Baseline sociodemographic and clinical characteristics of ATB patients in the four cohorts. *TB* Tuberculosis, *BMI* Body Mass Index, *LTBI* latent TB infection, *IQR* interquartile range. Data were given as % (N) or median (IQR).

Performance of RISK6 signature benchmarked against the WHO TPP for a non-sputum based diagnostic test. Our findings were then benchmarked against the WHO TPP for a screening/triage test for TB that should have a minimum sensitivity of > 90% and specificity of ≥ 70%^{19,27}. At a sensitivity set to > 90%, the performance of RISK6 signature as screening/triage test demonstrated specificity scores of > 70% in all cohorts, except for Bangladesh (Table 2). This shows that RISK6 signature achieves the minimal WHO TPP for non-sputum-based screening and triage tests discriminating patients with ATB from both HD and LTBI groups.

Performance of RISK6 as a confirmatory test for pulmonary TB disease. Our next aim was to evaluate the performance of RISK6 signature in sputum smear-negative and culture-confirmed TB individuals. Based on the TPP criteria set by the WHO as a reference^{19,27}, we found that RISK6 achieved the minimal sensitivity of > 60% with 100% specificity for an initial TB diagnostic test for sputum smear-negative TB to replace smear microscopy in the cohort from Georgia (Table 2). Similarly, in the same cohort, RISK6 signature also reached the minimum criteria of 65% sensitivity and 100% specificity for a confirmatory test. However, RISK6 signature detection failed to meet these WHO requirements in the other study sites (Table 2).

As most ATB patients had a positive sputum smear microscopy with a high grade at baseline, we wondered if RISK6 scores and mycobacterial loads were correlated. We therefore performed a sub-analysis on stratified sputum smear microscopy results among ATB patients, defined as follow: negative smears, low-grade positive smears (1+ or scanty) and high-grade positive smears (2+ or 3+). RISK6 scores in the negative smear group showed a significant difference ($p < 0.001$) compared to HD (Fig. 2). Moreover, RISK6 scores were significantly lower (median = 0.31, IQR 0.22–0.40) in negative smears than in individuals with low- or high-grade positive smears ($p < 0.001$). While not statistically different ($p > 0.05$), RISK6 scores in the high-grade smear group were higher (median = 0.5, IQR 0.40–0.56) than in the low-grade mycobacterial load group (median = 0.46, IQR 0.38–0.52).

Performance of RISK6 signature compared to IGRAs. Next, we assessed the performance of RISK6 signature compared to two assays based on IFN- γ release: the commercial QFT-P, and the non-commercial IGRAs-rmsHBHA. Compared to the QFT-P assay, the RISK6 signature achieved better performance in AUC (94.1% vs 57.2%), sensitivity (90.9% vs 50.9%) and specificity (87.8% vs 57.2%) to discriminate ATB patients from an asymptomatic population (LTBI + HD) (Table 3). However, a comparative sub-analysis indicated a lower positive (79.7%) and negative (50%) predictive values of the RISK6 signature when compared to QFT-P assay (100% and 63.9%, respectively) in detection of *Mtb*-infected individuals (ATB + LTBI) from uninfected ones (HD). Notably, the RISK6 signature showed a higher performance (AUC 90.9%, 95% CI 87.2–94.5), with 90.1% sensitivity and 72.2% specificity than the IGRAs-rmsHBHA (AUC 75.3%, 95% CI 68.6–82) that achieved

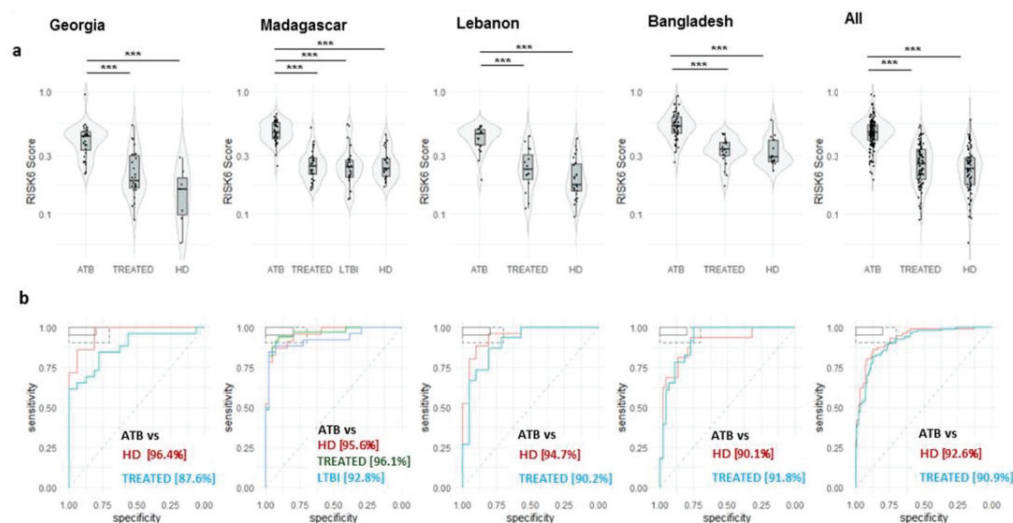


Figure 1. Validation of the performance of a multi-cohort 6-gene signature; RISK6 as a screening and triage test in patients with pulmonary TB. **(a)** Violin plots showing the differences in the levels of RISK6 signature scores from patients with active TB at baseline (ATB, $n = 141$), treated TB patients (TREATED, $n = 97$; patients with a negative sputum culture at T2 and/or T3), individuals with a latent TB infection (LTBI, $n = 26$), and healthy donors (HD, $n = 71$) from Georgia, Madagascar, Lebanon, Bangladesh and in all sites. Horizontal lines designate medians, boxes represent the inter-quartile ranges (IQR) and the ranges are represented by whiskers. Single patient results are represented by each dot in the graph. Statistical significance was calculated using Mann–Whitney U test. *Indicates a p -value < 0.05 , **indicates a p -value < 0.01 , and ***indicates a p -value < 0.001 . **(b)** Receiver operating characteristic (ROC) curve analysis and the respective areas under the curve (AUC) with 95% confidence intervals showing the performance of the RISK6 signature to discriminate between ATB patients at baseline, HD and LTBI. In the top left box, the solid and dashed lines represent the respective optimal and minimum criteria set by the WHO in the target product profile (TPP) for a screening/triage test for TB.

lower sensitivity and specificity (83.8% and 59.8% respectively) to differentiate *Mtb*-infection status (i.e. ATB vs TREATED TB patients) (Table 3).

RISK6 as a biomarker for TB treatment monitoring. Patients with successful treatment (defined as negative sputum culture at T2) were selected to determine whether RISK6 signature was a clinically relevant biomarker for TB treatment monitoring. Overall, in all cohorts combined, we observed a significant drop in RISK6 scores after two months of treatment (T1, $p < 0.001$) and until treatment completion (T2, $p < 0.001$). Moreover, RISK6 scores were significantly higher in cured TB patients (T2, $p > 0.05$) when compared to HD, however, in each of the four cohorts, there were no significant difference between these two groups ($p > 0.05$) (Fig. 3a). Similarly, analytical performance demonstrated capacity of RISK6 signature to significantly discriminate patients at baseline and two months after treatment initiation (AUC 69.7%, 95% CI 57.1–79.6) (Fig. 3b and Supplementary Table 6). Noticeably, by the end of treatment, the majority of patients had lower RISK6 score levels, further enhancing the discriminatory power between ATB patients at T0 and T2 (AUC 87.1, 95% CI 77.6–94.3) and at T3 (AUC 90.4, 95% CI 82.6–96.6) (Fig. 3b and Supplementary Table 6).

Furthermore, we evaluated whether RISK6 allows the discrimination of cured TB patients ($n = 104$) from those with a treatment failure (defined as positive sputum culture at T2, $n = 2$). Thereafter, patients were stratified into drug-sensitive (DS) and drug-resistant TB (DR-TB) cases and the RISK6 signature scores were compared within these groups. We found that RISK6 scores decreased throughout treatment among DS-TB patients independently of treatment outcome (Supplementary Fig. 1). In contrast, the RISK6 score remained stable at baseline and during treatment in a DR-TB patient with a treatment failure. Importantly, RISK6 score levels during TB treatment seem to be higher in patients with treatment failure among both DS and DR-TB cases. However, in a univariate or multivariate analyses, no significant association of the RISK6 score at baseline with treatment failure was found (Supplementary Table 8).

TPP requirement	Cut-off	Sensitivity%	Specificity%	Cases, n	Controls, n	AUC	AUC 95%CI
Screening test (ATB vs HD)							
Georgia	Sensitivity > 90%	> 0.2583	90.6	85.7	32	7	96.4%
Madagascar		> 0.3697	90.9	87	44	23	95.6%
Lebanon		> 0.3171	90.5	88	21	25	94.7%
Bangladesh		> 0.3625	90.9	68.8	44	16	90.1%
All		> 0.3209	90.1	80.3	141	71	92.6%
Triage test (ATB vs LTBI)							
Madagascar	Sensitivity > 90%	> 0.3697	90.9	88.5	44	26	92.8%
Initial TB diagnostic test to replace smear microscopy (ATB (CLT+ AFB-) vs HD)							
Georgia	Sensitivity ≥ 60%	> 0.3514	63.6	100	11	7	94.8%
Madagascar		> 0.4298	66.7	95.7	9	23	96.1%
Lebanon		> 0.3217	75	88	4	25	90%
Bangladesh		> 0.3541	75	68.8	4	16	79.7%
All		> 0.3823	60.7	88.7	28	71	87.7%
Confirmatory test (ATB (CLT+ AFB-) vs HD)							
Georgia	Sensitivity ≥ 65%	> 0.3131	72.7	100	11	7	94.8%
Madagascar		> 0.4298	66.7	95.7	9	23	96.1%
Lebanon		> 0.3217	75	88	4	25	90%
Bangladesh		> 0.3541	75	68.8	4	16	79.7%
All		> 0.3674	67.9	87.3	28	71	87.8%

Table 2. Receiver operating characteristic curve analysis of the performance of the RISK6 signature to distinguish active TB cases (ATB) from healthy donors (HD) and from latent TB infected individuals (LTBI) in cohorts from Georgia, Madagascar, Lebanon, and Bangladesh. The performance of the signature is benchmarked against the WHO TPP for a non-sputum based screening/triage test (at a sensitivity of > 90%, the minimum specificity as set out in this TPP should be ≥ 70%), for an initial TB diagnostic test to replace sputum smear (at minimum 60% sensitivity, the minimum specificity as set out in this TPP should be > 98%) and for a confirmatory test (at minimum 65% sensitivity, the minimum specificity as set out in this TPP should be > 98%)¹⁹. ATB active TB, LTBI latent TB infection (were only recruited from Madagascar), HD healthy donors, CLT+ positive sputum culture, AFB- negative AFB smear microscopy, AUC area under the curve, CI confidence interval, Vs versus.

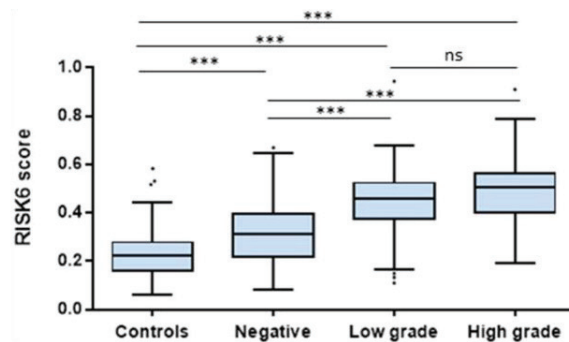


Figure 2. Correlation between RISK6 signature scores and mycobacterial loads determined by sputum smear microscopy in ATB patients. Boxplots comparing the RISK6 score levels stratified according to sputum smear grade: Negative smears, low grade positive smears (1+ or scanty) and high grade positive smears (2+ or 3+). Horizontal lines designate medians, boxes represent the inter-quartile ranges (IQR) and the ranges are represented by whiskers. Individual dots represent the results of patients with a RISK6 scores out of IQR. Statistical significance was calculated using Mann-Whitney U test. Ns non-significant, ***indicates a p-value < 0.001. HD Healthy donors.

Intended application	Test	Sensitivity%	Specificity%	PPV%	NPV%	Cases, n	Controls, n	AUC	AUC 95%CI
ATB vs (LTBI + HD)	RISK6	90.90	87.7	87	91.5	44	49	94.1	89.3–98.8
	QFT-P	67.50	46.9	50.9	63.9	40	49	57.2	45.2–69.1
(ATB + LTBI) vs HD	RISK6	90.00	30.4	79.7	50	66	23	77.8	68.5–87.1
	QFT-P	80.30	100	100	63.9	70	23	90.1	83.9–96.3
ATB vs treated TB	RISK6	90.1	72.2	82.5	83.3	141	97	90.9	87.2–94.5
	IGRAs-rmsHBHA	83.8	59.8	70.3	76.5	136	87	75.3	68.6–82

Table 3. Performance of RISK6 signature compared to Interferon- γ release assays: QuantiFERON-TB Gold Plus (QFT-P) and recombinant *Mtb*-HBHA expressed in *Mycobacterium smegmatis* (IGRAs-rmsHBHA). ATB active TB, LTBI latent TB infection, HD healthy donors, vs versus, rmsHBH recombinant *Mtb* HBHA expressed in, PPV positive predictive value, NPV negative predictive value.

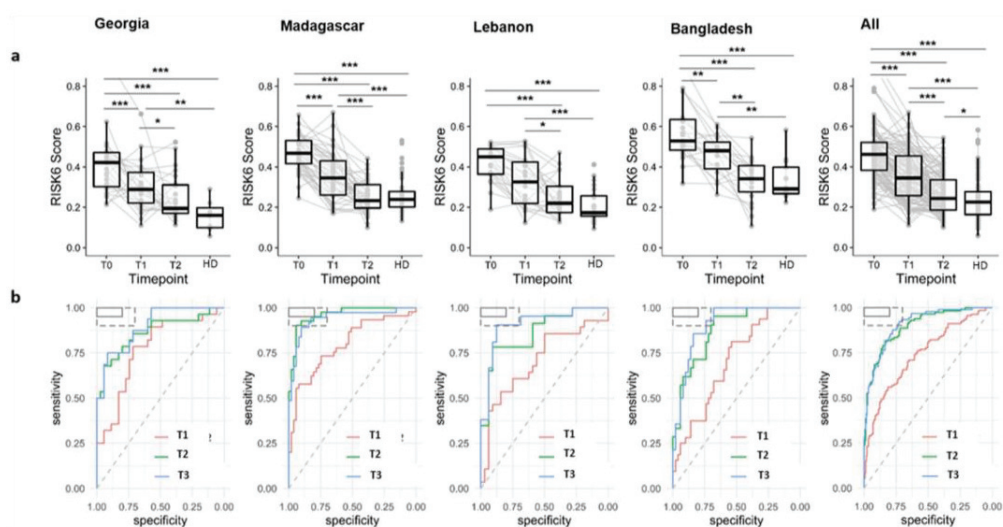


Figure 3. Validation of the performance of RISK6 signature as a biomarker for monitoring TB treatment response in four distinct geographical countries. (a) RISK6 scores were evaluated in whole blood of patients with active TB who had successfully completed their TB treatment until T2 ($n = 104$). Evaluation was done at baseline (T0), 2 months after treatment initiation (T1), and at the end of treatment (T2). RISK6 scores in healthy donors (HD: $n = 71$) were also evaluated. Horizontal lines designate medians, boxes represent the inter-quartile ranges (IQR) and the ranges are represented by whiskers. Single patient results are represented by each dot in the graph. Plotlines (grey) represent the RISK6 scores of the same patient at the three different time points. Statistical significance was calculated using Mann–Whitney U test. *Indicates a p -value < 0.05 , **indicates a p -value < 0.01 , and ***indicates a p -value < 0.001 . (b) Receiver operating characteristic (ROC) curve analysis and the respective areas under the curve (AUC) with 95% confidence intervals (CI) showing the ability of the RISK6 signature to discriminate between active TB patients at baseline (T0, $n = 141$) and at month 2 after treatment initiation (T1, $n = 117$), at the end of treatment (T2, $n = 104$) and 2 months after treatment completion (T3, $n = 79$).

Discussion

TB remains one of the major infectious causes of death globally. In this study, we aimed to evaluate the relevance of RISK6, a PCR-based six-gene blood transcriptomic signature²⁶, in the context of TB diagnosis and treatment monitoring. This was conducted in four independent cohorts enrolling ethnically and geographically diverse participants, including ATB patients, LTBI individuals, and HD, in both high- and low-TB incidence settings.

We first evaluated the performance of RISK6 signature as a screening test for TB and showed that it displayed similar performance in the four different cohorts with excellent near-identical ROC AUC values ($> 90.1\%$). Furthermore, RISK6 signature satisfied the minimum criteria set by the WHO TPP for a non-sputum-based screening test¹⁹. Notably, our findings suggest that, compared to IGRAs, the RISK6 signature showed a better performance as a screening test for discriminating between ATB patients and HD. Importantly, compared to previous RISK6 results reported by Penn-Nicholson et al.²⁶, our study found similar data in terms of score range

and score changes over time despite the heterogeneity of both cohorts and study designs. In addition, marked technical differences are also apparent between our studies: we performed the RISK6 scores measurements on RNA manually isolated from whole blood collected directly in Tempus Blood RNA tubes and from blood samples first collected in lithium heparin tube and then transferred in Tempus Blood RNA Tubes, while this measurement was done by Penn-Nicholson et al. using RNA extracted manually or by an automated processes from whole blood collected in PAXgene Blood RNA tubes. Collectively, these results highlight the robustness of this PCR-based host-blood transcriptomic signature.

Besides, the higher RISK6 score levels detected in the cohort of Bangladesh compared to the other study sites was a remarkable result. We hypothesized that these RISK6 scores observed in Bangladesh may be influenced by the differing epidemiology, geographical locations as well as differences in gene expression levels between ethnic populations that may have contributed to a stronger transcriptomic signal in Bangladesh.

Our AUC data showed that RISK6 scores had a powerful ability to distinguish ATB from HD, with better or equal results to what was found with other transcriptomic signatures^{28–34}. Moreover, while these previous signatures have shown promise as diagnostic tests, it should be noted that results of a three gene signature were not generalizable^{28,34}, while other signatures³³ require measurement of a high number of genes, thus limiting their possible application in resource-limited settings. Moreover, while RISK6 signature seems to meet or exceeded the TPP criteria based on each of our four cohorts, only two among the previous signatures (Sweeney³²⁸ and Sambarey¹⁰³²) satisfied the sensitivity and specificity TPP criteria set by the WHO for a triage test³⁵. However, it would be interesting to validate those signatures in other independent cohorts^{28,36}.

An important finding of our study is that RISK6 signature allowed to stratify TB patient's stages. Thus, when applied to the cohort of Madagascar, the only one including LTBI cases, the RISK6 signature demonstrated a significantly higher score in ATB individuals at baseline compared to those with LTBI. This is consistent with a previous study showing that a 3-gene transcriptomic signature was significantly higher in ATB patients versus LTBI²⁸ individuals, in addition to a 20-gene signature set that also discriminated ATB patients from LTBI and healthy controls¹⁸. In the same way, some gene-signatures were also evaluated¹⁸ and showed high specificity and sensitivity to distinguish ATB patients from those with LTBI^{23,28,31,33}. In our study, at >90% sensitivity, RISK6 signature discriminated ATB from both LTBI and HD with a specificity > 70% which met the WHO TPP for a triage test for TB. Besides, no significant differences in the classification performance of RISK6 signature were observed between LTBI and HD, in line with recent transcriptomic studies demonstrating failure in discriminating LTBI from HD^{18,28}. Moreover, while no previous studies has compared the levels of a transcriptomic signature between LTBI and treated TB patients, our data showed that the RISK6 signature reached the same score levels in treated TB patients when compared to LTBI individuals. Hence, it will be of interest to validate RISK6 signature in cohorts with larger number of latently infected individuals.

An additional finding of our study is that RISK6 signature also achieved the minimal WHO criteria in the Georgia cohort, for (i) an initial TB diagnostic test for sputum smear-negative TB to replace smear microscopy, using culture-confirmed TB as a gold standard (ii) and a non-sputum-based confirmatory test for sputum smear-negative TB. In this context, Turner et al.³⁷ reported a comparison of 27 signatures in cohorts of 181 patients for discriminating TB and no TB disease. They found that no previously published signatures achieved the minimal WHO sensitivity (65%) and specificity (98%) performance for a non-sputum-based confirmatory test for sputum smear-negative TB. Thus, our results are promising but further validation of RISK6 signature in larger cohorts will allow testing such performance. Furthermore, we found that ATB patients with low- or high-grade positive smears had significantly higher RISK6 scores compared with those with negative smears. Similarly to previous reported results with either Xpert MTB/RIF test or the C-reactive protein (CRP) concentration measurements^{38,39}, our findings suggest that RISK6 signature scores directly correlate with sputum smear grade, and may possibly represent a useful tool in the identification of patients with high transmission risk.

In the present study, we also attempted to compare the performance of different TB blood-based tests; RISK6 versus two IGRAs (QFT-P and IGRAs-rmsHBHA). Our results indicate that the performance of RISK6 was greater than that of QFT-P assay for ATB case-finding. Given that QFT-P was not recommended for the diagnosis of ATB but for LTBI diagnosis, we and others have shown that this assay is a better indicator for the detection of *Mtb* infection^{12,40,41}.

Our next aim was to evaluate variations in the RISK6 scores throughout successful treatment. We found that the RISK6 signature scores were significantly higher in ATB at baseline compared to HD, and continued to decrease progressively until the end of treatment reaching scores obtained in HD. Moreover, we also demonstrated that the RISK6 signature enables discrimination with high accuracy between untreated (T0), treated (T1 and T2), and post-treated (T3) TB patients who achieved a clinical cure. Taken together, these results showed the RISK6 genes might be modulated during anti-TB treatment as early as 2 months. Notably, the well-established data by Penn Nicholson et al.²⁶ also included additional earlier time points (week 1 and week 4) and found that RISK6 signature scores decrease over the course of successful treatment as early as 1 week. Data obtained with RISK6 is consistent with previous studies showing that transcriptomic signatures could be used as a powerful tool to monitor TB treatment response^{40,42–46}. In this context, it has been previously reported that reduced gene expression levels occurred rapidly during the first and the second weeks of TB treatment^{47,48}. An additional report showed that ATB gene set decreased after 4 months of anti-TB treatment, however, no tests were performed at earlier time points, or during TB treatment course⁴⁹. To note, we showed that the RISK6 signature scores returned to normal levels (compared to HD) after 6 months of treatment, which confirmed previous data²⁶ but contrasted with another transcriptomic study showing that normal levels were reached 12 months after the treatment initiation³⁰. Subsequently, these results indicate that RISK6 scores significantly stratified end of treatment from pre-treatment baseline. Taken together, our findings suggest that RISK6 signature could be used as a useful tool to monitor the response to anti-TB treatment. It may represent a potential alternative of the current tests used

to assess TB treatment efficacy and used comparing its result with those obtained by sputum culture that are crucial to evaluate drug resistance occurrence.

Remarkably, RISK6 relies on the use of qRT-PCR that could detect low levels of gene expression⁵⁰ and could be integrated into clinical poor settings in contrast to other complex methods. Besides, this signature requires the measurement of a small number of genes with subsequent reduced complexity and costs. Moreover, a key advantage of RISK6 is that it is a blood-based test, which is an easily accessible sample. Blood transcriptomic tests will improve the diagnosis of TB allowing faster treatment and thus reduction of transmission, especially in children, HIV co-infected TB patients and paucibacillary pulmonary TB patients. In such populations, microbiological tests are not always feasible due to the limited ability to produce good quality sputum samples or due to low bacterial loads in their samples. In the future, it will be of interest (i) to evaluate if RISK6 is able to predict the risk of progression to TB as demonstrated by the RISK11 signature²⁵ and (ii) to assess the diagnostic performance of RISK6 signature as a prototype cartridge assay as it has already been evaluated for the 3-gene signature against a microbiological reference standard⁵¹.

This study was subject to several limitations. Indeed, the sample size was relatively small and LTBI individuals were recruited from only one country. Hence, validation of our findings in cohorts with larger number of LTBI individuals is required to better estimate specificities and sensitivities for a triage test. Moreover, only two patients had failed treatment. Therefore, further validation is required to better understand how RISK6 signature tracks with response to treatment. Additionally, we excluded diabetic and HIV-positive patients and immunosuppressed individuals in general and our study was restricted to adults. Thus, similar validation studies are needed for children and HIV-positive patients. Moreover, in future studies, it would be relevant to evaluate the specificity of the RISK6 scores in comparison to other respiratory diseases than TB, which is considered as most difficult to distinguish with.

In conclusion, data from this study provide strong proof that RISK6 can be applied as a non-sputum-based screening and triage test that met the WHO TPP benchmarks. This host response-based gene signature may be used for stratifying patients according to their TB infection status, as well as for monitoring patients over the course of treatment. RISK6 signature is applicable using a robust and simple qRT-PCR platform which facilitates its implementation in the clinical laboratories located in resource-poor settings. Our overall findings support the efforts to incorporate RISK6 signature into a point-of-care test ensuring rapid and accurate detection of ATB cases. Indeed, such simple tests are highly needed to reduce TB spread and transmission especially in areas with high TB burden that are usually disturbed with poverty.

Methods

Study design and population. This evaluation of the RISK6 signature was a nested case-control multi-center prospective cohort study evaluating the prognostic value of blood-based immunological biomarkers for monitoring TB treatment outcome. It was conducted within the GABRIEL Network⁵² in four different countries including Bangladesh, Georgia, Lebanon and Madagascar.

In total, 238 participants were recruited and followed-up between August 2018 and September 2020. Participants included patients with ATB disease ($n = 141$), HD ($n = 71$) and individuals with LTBI ($n = 26$). Enrolled ATB patients aged ≥ 15 years old, newly diagnosed with pulmonary ATB: scoring positive for TB following bacteriological (culture positive and/or sputum smear microscopy positive) and/or molecular analysis (GeneXpert positive results) were recruited at primary healthcare TB clinics in each country: National Center for Tuberculosis and Lung Diseases (NCTLD) in Tbilisi, Georgia; Tuberculosis screening and treatment center (CHUSSPA) related to National Tuberculosis Programs (NTPs) in Antananarivo, Madagascar; NTP centers in Tripoli and Akkar, Lebanon and International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) in Dhaka, Bangladesh. Clinically asymptomatic healthy donors; who do not have a previous TB history and who have no recent TB contacts were also recruited in all sites. In Madagascar, participants with positive QFT-P results (IFN- γ production ≥ 0.35 IU/mL) were defined as latently *Mtb* infected individuals. Patients with negative cultures at inclusion, ATB patients with Human Immunodeficiency Virus (HIV) or with diabetes mellitus comorbidities and patients under immunocompromising treatment were excluded (Fig. 4).

Enrolled ATB patients were followed-up during the treatment course at four different time points and classified as follow: (i) ATB at baseline T0: patients who didn't start TB treatment; (ii) treated active TB at T1 and T2: patients with ATB followed-up during the treatment and tested after 2 months of the start of the treatment (T1), and at the end of treatment (T2); (iii) treated active TB at T3: treated TB patients tested at 2 months after treatment completion.

Ethics statement. The study protocols were reviewed and approved by the human research ethics committees in each country; Georgia, the Institutional Review Board of the National Center for Tuberculosis and Lung Diseases (NCTLD) (Reference number: IORG0009467), Madagascar, the Ministry of Public Health and the Ethical Committee for Biomedical Research (Reference number: n°099-MSANP/CERBM), Lebanon, the institutional review board of NINI hospital (Reference number: IRB-F-01) and Bangladesh, the Research Review Committee and the Ethical Review Committee of International center for diarrheal diseases and research (icddr,b). All study participants provided written informed consent. All research was performed in accordance with relevant guidelines/regulations.

Diagnostic assessment and follow-up. ATB diagnosis was based on both bacteriological and molecular parameters. At least one sputum sample was collected at inclusion (T0) for culture testing (liquid culture media: MGIT mycobacterial growth indicator tube, BD BioSciences, NJ, USA and/or solid culture media: L-J (Lowenstein-Jensen) and also tested by microscopy for the presence of acid-fast bacilli (AFB) using the Ziehl-Neelsen

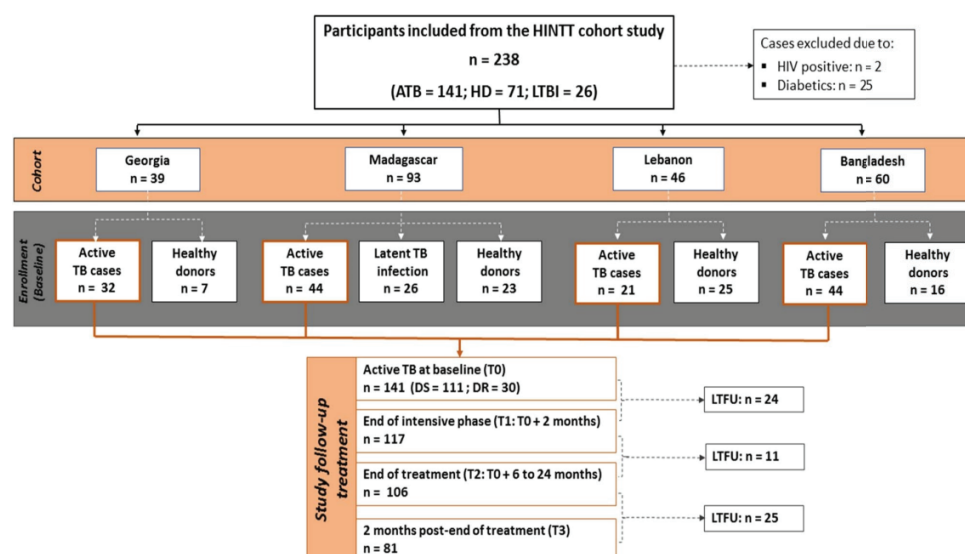


Figure 4. Flow diagram describing the enrollment and exclusion of participants with active TB, latent TB infection, and healthy donor participants from the different cohorts. ATB patients were followed-up at four different time points: at baseline (T0), ATB patients who didn't their TB treatment and followed throughout antibiotic therapy: at month 2 (T1), at the end of treatment (T2), and 2 months after treatment completion (T3). TB Tuberculosis, ATB active TB, LTBI Latent TB infection, HD healthy donors, HIV human immunodeficiency virus, DS drug-susceptible, DR drug-resistant, LTFU lost to follow-up.

staining method and/or Auramine O staining. In addition to positive culture, active TB status was defined by positive Xpert MTB/RIF (Cepheid). Patients were re-evaluated by sputum smear and culture during the intensive phase of treatment (T1) thereafter at the end of treatment (T2) and 2-months after treatment completion (T3) to confirm that they were successfully treated and cured. Drug susceptibility testing (DST) methods were performed according to standard protocols⁵³.

Demographic and clinical data collection. At enrollment and at each follow-up visit, medical history, clinical and demographic data were collected using standardized questionnaires to feed the cloud-based database system CASTOR (CASTOR Electronic Data Capture, Version 1.4, Netherlands).

Blood collection process. A minimum of 3 mL of whole blood for transcriptomic analysis and 5 mL for the Interferon- γ release assays were drawn from each participant. For transcriptomic analysis, specimens were directly collected in Tempus Blood RNA Tubes (Applied Biosystems, 4342792), vigorously shaken, and stored at -80°C . Of note, in Madagascar and Bangladesh, blood samples were first collected in lithium heparin tubes and then transferred in Tempus Blood RNA Tubes for transcriptomic analysis.

RNA extraction process and complementary DNA (cDNA) synthesis. Frozen Tempus Blood RNA tubes were thawed and RNA was manually extracted using the MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (Applied Biosystems by Thermo Fisher Scientific, 4451893) following the manufacturer's instructions. RNA elution was performed by adding 30 μL of Elution Buffer. The purified RNA was transferred to a nuclease-free tube, assessed for quantity and quality (Nanodrop spectrophotometer), and stored at -80°C until needed. The cDNA was synthesized using the Applied High Capacity RNA to cDNA kit (Applied Biosystems by Thermo Fisher Scientific, 4387406). The RT reaction mix was prepared as follows: 10 μL of 2xRT buffer mix, 1 μL of 20x RT Enzyme, and 3 μL of nuclease-free water. Then 6 μL of purified RNA/negative control samples were added and proceeded using random hexamer primers (1 h 37°C , 5 min 95°C and hold 4°C). cDNA was then 1:5 diluted (nuclease-free water) and stored at -20°C for long-term conservation.

Pre-amplification PCR. Prepared cDNA was pre-amplified using specific sequences of TaqMan primer-probes as previously described by Penn-Nicholson et al.²⁶. 5 μL of 2x PCR mix (TaqMan Universal PCR Master Mix 2x) (Applied Biosystems by Thermo Fisher Scientific, 4304437) with 2.5 μL of the specific primers-probes mix (PPM 0.6x), composed of primers of the 6 genes (listed in Supplementary Table 1) (Applied Biosystems by Thermo Fisher Scientific) was mixed. Then 2.5 μL of the diluted cDNA/negative control samples were added and the mixture was incubated 10 min at 95°C followed by 16 cycles of amplification at 95°C for 15 s, 60°C for

4 min, and hold at 4 °C. The pre-amplified PCR products were diluted 1:25 with nuclease-free water and stored at – 20 °C for long-term conservation.

Quantitative Real-Time PCR (qRT-PCR) assay and gene expression analysis. For every target to amplify, 4 µL of pre-amplified DNA was subjected to a real time nucleic acid amplification using 10 µL of TaqMan Universal PCR Master Mix (Applied Biosystems by Thermo Fisher Scientific), 1 µL of primers-probe mix (20×) and 4 µL of nuclease-free water using the following conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 95 °C for 15 s and 60 °C for 1 min for 40 cycles. For analytical reasons, all the PCR reactions were performed in duplicate.

RISK6 score generation. Polymerase chain reaction signals were analyzed using CFX Manager Software version 3.1 (BioRad) in regression mode and expressed as cycle threshold (Ct) values. The step-by-step procedure for computing the 6-gene signature (RISK6) scores was performed as described by Penn-Nicholson et al.²⁶. Briefly, the mean of Ct values was calculated for every targeted genes and combined to generate a score. The score was computed with R script available on <https://bitbucket.org/satvi/risk6/src/master/>.

QuantiFERON-TB Gold Plus and IGRAs-rmsHBHA assays. 1 mL of whole blood was collected directly into each of the QFT-P tubes (Qiagen, Hilden, Germany, 622526) (Nil: Negative Control, TB-Antigens (TB1/TB2) and Mitogen: Positive control) and an extra 1 mL of blood was collected in a heparin tube and stimulated with 10 µg/mL of rmsHBHA (UNICATT, Rome, Italy¹⁰¹⁰¹⁰¹⁰). After 16–24 h incubation at 37 °C, plasma samples were harvested and stored at – 80 °C prior subjected to QFT-P ELISA (Qiagen, Hilden, Germany, 622120), following the manufacturer instructions. Briefly, 50 µL of plasma samples were tested, optical density results were compared to log-normalized values from freshly reconstituted IFN-γ kit standards. To account for potential immunomodulation phenomena unrelated with TB treatment, baseline IFN-γ level values (Nil tubes) were subtracted from antigen-stimulated IFN-γ values (TB1, TB2, Mitogen and rmsHBHA). According to the kit's sensitivity range, the maximum for IFN-γ level values was set at 10 IU/mL and negative values were rescaled to 0.

Statistical analysis. All statistical analyses were performed with R studio (version 4.0.3) software⁵⁴. Graphs were created using the ggplot2 packages⁵⁵. Statistical evaluation of the performance of RISK6 was done by calculating the Area Under the receiver operating characteristic curve (ROC AUC) and associated 95% confidence intervals (CI) using the pROC in R⁵⁶. Discrete variables were analyzed using Fisher's Exact test with Bonferroni's post-hoc test. Normality was assessed using the Shapiro–Wilk Normality Test. Normal, continuous variables were analyzed with Student's t-test. Non-normal, continuous variables were analyzed with the Mann–Whitney test or the Kruskal–Wallis rank-sum test with Dunn's Kruskal–Wallis Multiple Comparisons post-hoc test. Repeated measures of non-independent continuous variables were analyzed using the Friedman rank-sum test, with Wilcoxon–Nemenyi–McDonald–Thompson's post-hoc test. Non-parametric data were presented as median ± IQR and the statistical significance cut-off was considered as a p value of < 0.05. For logistic regression analyses, variables were first evaluated in univariate analyses, then multivariate analyses were performed. Adjustment variables were selected as follows: sociodemographic variables of known clinical importance (e.g., sex, country of origin), TB risk factors (e.g., smoking), and additional sociodemographic variables that were at least moderately associated (p < 0.10) with the outcome in univariate analyses (e.g., prison). Irrelevant adjustment variables were then removed by backward model selection. The combination of variables that minimized the Akaike Information Criterion (AIC) for most tested predictors, while including important adjustment variables, was selected.

Data availability

The RISK6 scores and associated clinical data for all cohorts can be found in Supplementary Tables 2–7.

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Author contributions

J.H. conceived and designed the study, and initiated this project in partnership with N.R., M.H., N.T., S.Ba. and D.G. Patient recruitment, follow-up and data collection was done by E.K., M.U., S.Bi., R.N., P.R., C.R., P.H., J.R., A.R. and R.B. The experiments were performed by R.B., M.N. and S.P. and J.H. performed statistical analysis with contribution from C.C. Data analysis and interpretation of results was implemented by R.B., M.N., J.H. and S.P. The manuscript was written by R.B., and all authors have read, reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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3. Publication 2

Association of baseline white blood cell counts with tuberculosis treatment outcome: a prospective multicentered cohort study

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Article summary

There is a crucial need for alternative non-sputum-based TB tests to assess the efficacy of TB treatment response. In this context, we used a simple immunological tool based on blood cell counts (WBC) in order to monitor the changes of hematological parameters such as WBC, lymphocytes and monocytes proportion during the course of anti-TB treatment in order to characterize their association with treatment outcomes (e.g. cure or treatment failure). For this purpose, we recruited 198 adult, non-immunocompromised, DS-TB and DR-TB culture positive pulmonary TB patients in five different countries including, Bangladesh, Georgia, Lebanon, Madagascar and Paraguay. Patients were followed-up during the course of treatment; at baseline (T0), at two months after treatment initiation (T1), and at the end of treatment (T2). 90.8% of treated patients achieved clinical cure at the end of treatment.

A decrease in white blood cell (WBC) count and an increase in the proportions of lymphocytes has noted in patients throughout the course of TB treatment. Moreover, using a multi-variate analysis, we found that the risk of treatment failure was associated with a high WBC counts in addition to a low proportions of lymphocytes detected in active TB patients at baseline (WBC > 11,450 cells/mm³: p = 0.048; lymphocytes <16.0%: p = 0.039; WBC > 11,450 cells/mm³ and lymphocytes <16.0%: p = 0.024; all thresholds were obtained using Receiver Operating Characteristic (ROC) curve cutoffs).

Overall, using a simple and inexpensive immunological tool, predictive biomarkers that changed significantly in early stages of TB treatment and that would be helpful in predicting early treatment failure were identified. Remarkably, such biomarkers requires the collection of a small volume of blood and relies on the use of a cheap, simple and rapid test. These features facilitate the use of such a tool into clinical poor settings and may support the efforts to incorporate it into a POC test ensuring rapid and accurate prediction of TB treatment response.



Association of baseline white blood cell counts with tuberculosis treatment outcome: a prospective multicentered cohort study



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ABSTRACT

Objectives: Tuberculosis (TB) is the leading infectious cause of death in the world. Cheaper and more accessible TB treatment monitoring methods are needed. Here, we evaluated white blood cell (WBC) absolute counts, lymphocyte, and monocyte proportions during TB treatment, and characterized their association with treatment failure.

Methods: This multicentered prospective cohort study was based in Bangladesh, Georgia, Lebanon, Madagascar, and Paraguay. Adult, non-immunocompromised patients with culture-confirmed pulmonary TB were included and followed up after two months of treatment and at the end of therapy. Blood counts were compared to treatment outcome using descriptive statistics, logistic regression, and Receiver Operating Characteristic (ROC) analyses.

Results: Between December 2017 and August 2020, 198 participants were enrolled, and 152 completed treatment, including 28 (18.5%) drug-resistant patients. The rate of cure at the end of treatment was 90.8% (138/152). WBC absolute counts decreased, and lymphocyte proportions increased throughout treatment. In multivariate analyses, baseline high WBC counts and low lymphocyte proportions were associated with positive sputum culture results at the end of treatment (WBC > 11,450 cells/mm³: $p = 0.048$; lymphocytes < 16.0%: $p = 0.039$; WBC > 11,450 cells/mm³ and lymphocytes < 16.0%: $p = 0.024$). **Conclusion:** High WBC counts and low lymphocyte proportions at baseline are significantly associated with the risk of TB treatment failure.

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Introduction

Tuberculosis (TB) is the leading cause of death by an infectious disease globally, responsible for 1.5 million deaths in 2018 (World Health Organization Geneva, 2019a). The treatment of active pulmonary TB lasts at least 6-months and can cause major side effects (World Health Organization Geneva, 2017, 2019a). Consequently, treatment adherence is poor, particularly in primary care settings (Woimo et al., 2017).

Mycobacterium tuberculosis culture from sputum samples is the gold standard for TB treatment monitoring (Wallis et al., 2016). However, it is time-consuming and requires high-level biosafety laboratories (Horne et al., 2010). Sputum smear microscopy is quicker for treatment monitoring, but it has limited accuracy (Ngabonziza et al., 2016). Hence, blood-based tests are a promising alternative. A variety of blood-based immunoassays, from interferon (IFN)- γ release assays to T cell activation tests, are being evaluated for treatment monitoring (Cingolani et al., 2012; Sali et al., 2018). However, in low-resource countries, these tests need to be adapted to limited laboratory equipment (MacLean et al., 2017) and a simple output (Goletti et al., 2018).

Hematological abnormalities—such as leukocytosis, neutrophilia, or lymphopenia—during pulmonary TB disease have been described in the literature since the eighties (Morris et al., 1989). Low monocyte proportions have been associated with higher rates of progression towards active TB (Rakotosamimanana et al., 2015). Neutrophilia and lymphopenia have been associated with higher mortality risks during TB treatment (Lowe et al., 2013). However, many these studies were either small case-control studies (Abay et al., 2018), focused on the risk of developing active TB disease (Kurup et al., 2016), or assessed kinetics during treatment and not association with treatment failure (Morris et al., 1989; Rakotosamimanana et al., 2015). Most available comprehensive studies were in low-burden, high-healthcare standards areas (Ritchie et al., 2016).

Here, we describe a prospective multicentered cohort study conducted in five low- and middle-income countries across four continents: Bangladesh, Georgia, Lebanon, Madagascar, and Paraguay. Four of these countries are on the 2019 WHO high incidence TB country list (≥ 20 cases per 100,000 population), with the exception of Lebanon (eleven cases per 100,000 population) (World Health Organization Geneva, 2019a). The target population was adult, HIV-uninfected, drug-susceptible (DS-TB), and drug-resistant (DR-TB) pulmonary TB patients.

This study's primary objective was to monitor the evolution of simple hematological parameters during pulmonary TB treatment in higher TB incidence settings: absolute WBC counts, lymphocyte, and monocyte proportions. These markers of inflammation were chosen because they are routinely monitored in primary care settings. The focus was kept on cellular parameters.

A secondary objective was to characterize their association with treatment failure, defined by sputum culture positivity (the gold standard) at the end of treatment, in relation to clinical and sociodemographic factors.

Materials and methods

Study design and sample population

Study design

This descriptive study was nested within a multicentered prospective cohort study evaluating the prognostic value of blood-based immunological markers for TB treatment monitoring, based in five partner institutions from the Mérieux Foundation GABRIEL network (Komurian-Pradel et al., 2013) with the approval of national TB programs: the international center for diarrheal

diseases and research, Bangladesh (icddr,b) in Dhaka, Bangladesh; the National Center for Tuberculosis and Lung Diseases (NTCLD) in Tbilisi, Georgia; the *Laboratoire Microbiologie, Santé et Environnement* (LMSE, Université Libanaise), in Tripoli, Lebanon; the *Institut Pasteur de Madagascar* in Antananarivo, Madagascar; and the *Instituto de Investigaciones en Ciencias de la Salud* (Universidad Nacional de Asunción; IICS-UNA) in Asunción, Paraguay.

Ethical considerations

This study was conducted with the approval of the ethical boards in Bangladesh, the Research Review Committee and the Ethical Review Committee of icddr,b; in Georgia, the Institutional Review Board of the NTCLD (IORG0009467); in Lebanon, the institutional review board of NINI hospital (IRB-F-01); in Madagascar, the Ministry of Public Health and the Ethical Committee for Biomedical Research (reference number: n°099-MSANP/CERBM); in Paraguay, the Research Ethics Committee and the Scientific Committee of the IICS-UNA (IRB number: IRB00011984; Federal Wide Assurance number: FWA00029097). All recruited patients provided written informed consent.

Cohort recruitment, TB diagnosis, and patient follow-up

We calculated that a sample size of 156 was required to reach a level of significance of 95% and a power of 80%, assuming an average rate of treatment failure of 10% (World Health Organization Geneva, 2019a), a baseline prevalence of lymphopenia ($<15\%$ of total WBC) of 30%, and that treatment failure was three times more likely in patients with lymphopenia (Lowe et al., 2013). We aimed to include 200 participants to account for lower prevalence values, failure rates, or missing data.

Patients were recruited if diagnosed with microbiologically confirmed pulmonary TB (positive culture and/or sputum smear and/or GeneXpert; see "Microbiological diagnosis" section for further detail). Patients with HIV or diabetes mellitus (defined by HBA1C levels $>6.5\%$; Cobas (Roche) automated analyzer) and children under 15 years were excluded. In downstream analyses, patients under immunocompromising treatment (corticosteroids, calcineurin inhibitors, biologics, or other chemotherapeutic agents), patients with negative cultures at inclusion, and patients who were lost-to-follow-up were excluded.

Patients were followed up: at inclusion (T0), after two months of treatment (T1), at the end of therapy (T2; 6 months for drug-susceptible (DS-TB) patients, 9–24 months for drug-resistant (DR-TB) patients), and two months after the end of therapy when possible (T3). All patients were on Directly Observed Treatment, Short Course (DOTS), and received treatment according to standard protocols (Aung et al., 2014; World Health Organization Geneva, 2017, 2019a). Detail of therapeutic regimens can be found in Supplementary Table 1.

Microbiological diagnosis and definition of treatment outcome

At each visit, at least one sputum sample per patient was collected for culture (solid and/or liquid media) and smear microscopy (Ziehl-Neelsen and/or Auramine O staining). Molecular amplification of *M. tuberculosis* DNA was conducted at inclusion (GeneXpert MTB/RIF, Cepheid, Sunnyvale, CA). Drug susceptibility testing (DST) and multi-drug resistance diagnosis were performed according to standard protocols (World Health Organization Geneva, 2019b). Microbiological methods and DST protocols are detailed in Supplementary Table 2.

Treatment outcomes were defined as follows: failed (positive sputum culture at T2); cured (negative sputum culture at T2 and no evidence of positive culture at T3); relapse or reinfection (culture conversion at T2 but positive culture recorded at T3); completed (patient not lost to follow up, but no available culture data at T2 or T3) (World Health Organization (WHO), 2013).

On-site whole blood collection and cell count

At each visit, 10 mL of whole blood were drawn, 1 mL of which was collected in EDTA tubes used for the present study. Complete blood counts were performed on automated analyzers (details of manufacturing references per study site are in Supplementary Table 2).

Clinical data collection and standardization

Data collection forms were created with clinicians from partner sites and standardized to ensure dataset homogeneity. Follow-up sheets were used to track sample handling and time flow from the collection in health centers to receipt in laboratories. All forms were translated into the local official language upon request from staff. Data were entered into the cloud-based database system CASTOR (CASTOR Electronic Data Capture, Version 1.4, Netherlands).

Descriptive statistical analysis

Data were cleaned and analyzed in R (version 3.6.2). As the sample size was small, discrete variables were analyzed using Fisher's Exact test with Bonferroni's post-hoc test (Kim, 2017). Normality was assessed using the Shapiro–Wilk Normality Test (test statistics available in Supplementary Table 3). Normal, continuous variables were analyzed with Student's *t*-test. Non-normal, continuous variables were analyzed with the Mann–Whitney test or the Kruskal–Wallis rank-sum test with Dunn's Kruskal–Wallis Multiple Comparisons post-hoc test (Dunn, 1964). Repeated measures of non-independent continuous variables were analyzed using the Friedman rank-sum test, with Wilcoxon–Nemenyi–McDonald–Thompson's post-hoc test (Paro Pedro et al., 2015).

Logistic regression and receiver operating characteristic (ROC) analyses

For these analyses, the assessed outcome was a recorded positive *M. tuberculosis* culture result at T2. Patients with unavailable culture data at T2 and patients with relapse were excluded from the analyses. The evaluated variables were leukocyte counts, lymphocyte proportions, and monocyte proportions at T0 or T1. If missing data exceeded 10% of the sample

size, the variable was not considered. Otherwise, missing data were replaced by the most frequent group (categorical variables) or the mean (continuous variables). Highly skewed, non-normal continuous variables were log-transformed prior to analyses. Variables were first evaluated in univariate logistic regression analyses, then multivariate analyses were performed. Adjustment variables were selected as follows: sociodemographic variables of known clinical importance (e.g., sex, country of origin), TB risk factors (e.g., smoking), and additional sociodemographic variables that were at least moderately associated ($p < 0.10$) with the outcome in univariate analyses (e.g., prison). Irrelevant adjustment variables were then removed by backward model selection. The combination of variables that minimized the Akaike Information Criterion (AIC) for most tested predictors, while including important adjustment variables, was selected (age, sex, smoking habit, drug sensitivity, country of origin). For lymphocyte proportions, odds ratios (OR) were calculated for each increase of 5.0% because the total range of measured values was 5.0–70.0%. Similarly, for absolute WBC counts, OR were calculated for each increase of 1000 cells per mm³.

For both ROC analyses and logistic regression, since the sample size was small, model performance metrics (respectively, the Area Under the Curve (AUC) and the C-statistic) were corrected for optimism using bootstrap to assess model validity as described elsewhere (Smith et al., 2014).

Results

Demographic and clinical characteristics of the cohort

Between December 2017 and February 2020, 198 eligible patients with culture-confirmed active pulmonary TB were recruited in Dhaka (Bangladesh), Tbilisi (Georgia), Tripoli and Akkar (Lebanon), Antananarivo (Madagascar), and Asunción (Paraguay). As of July 2020, 152 of them were followed at least until the end of treatment and had available blood count data (Figure 1). As the T3 time point was later than the local TB program visits, the dropout rate was highest between T2 and T3 (26%).

Among patients followed until T2 at least, 18.4% (28/152) were diagnosed with DR-TB. The sociodemographic and clinical characteristics of DS-TB and DR-TB patients were comparable at inclusion (Table 1). The characteristics of patients at inclusion were compared between study sites (Supplementary Table 4). There was no significant difference in baseline absolute WBC counts and

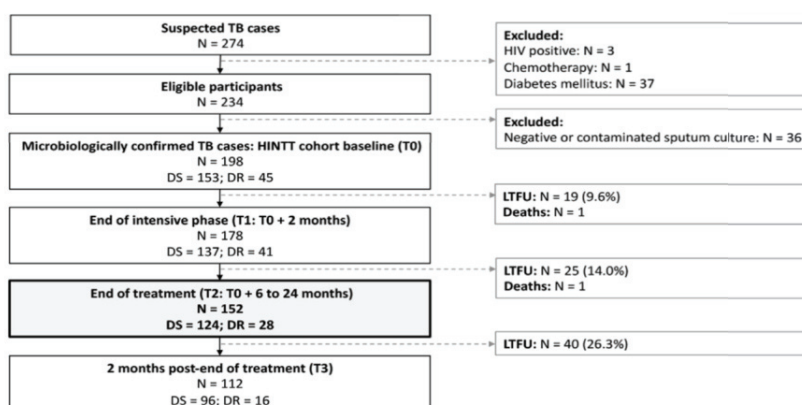


Figure 1. Inclusion and data collection process between December 2017 and July 2020.

DR: drug-resistant. DS: drug-susceptible. LTFU: lost to follow-up. TB: tuberculosis. HIV: human immunodeficiency virus. Treatment for DS-TB patients lasted 6 months. Treatment for DR-TB patients lasted 9–24 months.

Table 1

Sociodemographic and clinical characteristics of drug-susceptible and drug-resistant patients at inclusion.

N	ALL 152	DS-TB 124	DR-TB 28	<i>p</i> DS-TB vs DR-TB
Patient demographics				
Age (years)	28 (22–37.25)	28 (22–39)	27.5 (19.75–33.25)	0.22
Sex (male)	63.2% (96/152)	64.5% (80/124)	57.1% (16/28)	0.52
Country of origin				
Bangladesh	25% (38/152)	16.9% (21/124)	60.7% (17/28)	>0.001
Georgia	21.7% (33/152)	18.5% (23/124)	35.7% (10/28)	0.072
Lebanon	11.8% (18/152)	14.5% (18/124)	0	0.045
Madagascar	23.7% (36/152)	29% (36/124)	0	>0.001
Paraguay	17.8% (27/152)	21% (26/124)	3.6% (1/28)	0.028
BMI at inclusion	18.8 (17.1–21.4)	18.9 (16.9–21.4)	18.8 (17.5–21.0)	0.87
Sputum smear microscopy at inclusion				
High grade (2+ or 3+)	51.1% (76/149)	50.4% (61/121)	53.6% (15/28)	0.83
Low grade (1+ or scanty)	18.8% (43/149)	29.8% (36/121)	25% (7/28)	0.81
Negative	20.1% (30/149)	19.8% (24/121)	21.4% (6/28)	0.79
WBC absolute count at inclusion (/mm ³)	9590 (7468–11770)	9500 (7365–11640)	10,150 (7725–11850)	0.54
Lymphocytes at inclusion (% of WBC)	18 (14–24)	18 (14–23.2)	17 (13.8–24.5)	0.65
Monocytes at inclusion (% of WBC)	5 (2–7.48)	5 (2–8.23)	4 (3–5.25)	0.35
Number of household contacts	4 (3–6)	4 (3–6)	4 (3.75–6.25)	0.91
BCG vaccination	81.1% (99/122)	80.2% (81/101)	85.7% (18/21)	0.64
Risk factors and comorbidities				
Smoking	47.7% (72/151)	48% (59/123)	46.4% (13/28)	1
Alcohol abuse	21.9% (33/151)	23.6% (29/123)	14.3% (4/28)	0.55
Injectable drug use	3.3% (5/150)	2.4% (3/123)	7.4% (2/27)	0.36
Jail detention history	10.1% (15/149)	11.6% (14/121)	3.6% (1/28)	0.44
Chronic HCV infection	2.1% (3/146)	2.5% (3/118)	0	1
Other disease ^a	5.5% (7/128)	6.7% (7/105)	0	0.35
History of TB				
Previous TB	18.9% (28/148)	16.4% (20/122)	30.8% (8/26)	0.1
Of which are documented	78.5% (22/28)	70.0% (14/20)	88.9% (8/9)	0.05
Prior exposure to active TB patients	30.9% (46/149)	31.4% (38/121)	28.6% (8/28)	0.22
Previous TB treatment outcome				
Cured and completed	57.1% (12/21)	56.2% (9/16)	60% (3/5)	1
Completed	14.3% (3/21)	12.5% (2/16)	20% (1/5)	1
Treatment failure	14.3% (3/21)	18.8% (3/16)	0	–
Outcome not evaluated or unknown	14.3% (3/21)	12.5% (2/16)	20% (1/5)	1

DS-TB: drug-susceptible tuberculosis. DR-TB: drug-resistant tuberculosis. BMI: body mass index. IQR: interquartile range. WBC: white blood cells.

^a Asthma, hypertension, inflammation. No patients presented with either pregnancy, renal disease, solid tumors or other cancers, HIV infection, chronic pulmonary disease, or chronic HBV infection. Data were given as % (N) or median (IQR).

lymphocyte proportions between cohorts, but several other parameters differed. Monocyte counts were significantly higher in the Lebanon and Madagascar cohorts than in the other sites. Patients in Bangladesh were younger than those recruited in Georgia and Paraguay. The body mass index (BMI) at inclusion of patients from Bangladesh and Madagascar was significantly lower than in the other study sites. Finally, BCG vaccination rates were significantly lower in the Georgia and Lebanon cohorts.

Microbiological characterization of treatment response over time and assessment of treatment outcome

All enrolled patients were positive for sputum culture at inclusion. Most patients were also positive for sputum smear microscopy (78.2%, 119/152) and/or GeneXpert (98.6%, 150/152). Culture positivity rates decreased significantly upon treatment (Table 2). No significant difference was detected between DR-TB and DS-TB patients (data not shown). At the end of treatment, 90.8% (138/152) of patients were cured, and eight had unavailable culture data and were classified as having completed treatment with no evidence of cure (5.3%, 8/152). One patient

Table 2

Microbiological characterization of treatment response and outcome definition.

Sputum culture results	Positive	Negative	Unavailable
Timepoint			
T0	100% (152/152)	0	0
T1	13.8% (21/152)	70.4% (107/152)	15.8% (24/152)
T2	3.3% (5/152)	94.8% (144/152)	1.9% (3/152)
T3	0.9% (1/112)	43.8% (49/112)	55.4% (62/112)
All patients			
Treatment outcome		DS-TB	DR-TB
Cured and completed ^a	90.8% (138/152)	88.7% (110/124)	100% (28/28)
Completed ^b	5.3% (8/152)	6.5% (8/124)	0
Failed ^c	3.3% (5/152)	4% (5/124)	0
Relapse or reinfection ^d	0.7% (1/152)	0.8% (1/124)	0

T0: baseline. T1: baseline + 2 months. T2: end of treatment. T3: end of treatment + 2 months. DS-TB: drug-susceptible tuberculosis. DR-TB: drug-resistant tuberculosis.

^a Positive culture at T2.^b Negative culture at T2 but positive culture at T3.^c Negative culture at T2 and no evidence of positive culture at T3.^d Patients not lost to follow-up, but with no available culture data at T2 or T3.

Unavailable culture data were caused by contaminations or failure to expectorate sputum. Unavailable sputum smear microscopy results were caused either by technical issues or failure to expectorate enough sputum for both culture and smear.

(0.7%, 1/152) was culture-negative at T2 but had a positive culture result at T3, which was classified as relapse or reinfection. Treatment failure was observed in five DS-TB patients (3.3%, 5/152; Supplementary Table 5 for demographic and microbiological characteristics). Notably, 80% (4/5) of treatment failure patients had high sputum smear microscopy grades at baseline (2+ or 3+). At T1, two of them were culture positive, and one was microscopy positive.

Absolute WBC counts and lymphocyte proportions vary significantly throughout therapy

In all cohorts ($n = 152$), we observed a significant decrease in median absolute WBC counts during treatment (Figure 2A), and a significant increase in median lymphocyte proportions (Figure 2B). Monocyte proportions were heterogeneous between study sites and increased significantly only in the Bangladesh and Georgia cohorts (Figure 2C). At all time points, they were significantly

higher in the Lebanon and Madagascar cohorts. Neutrophil responses were assessed in a subset of the cohort ($n = 129$) and decreased significantly during treatment (Supplementary Figure 1).

Early absolute WBC counts and lymphocyte proportions were significantly different between successfully and unsuccessfully treated TB patients

Absolute WBC counts, lymphocyte, and monocyte proportions were then compared between successfully and unsuccessfully treated patients with available culture data at T2 ($n = 143$; Figure 3). At baseline and after two months, unsuccessfully treated patients had significantly higher absolute WBC counts (Figure 3A) and significantly lower lymphocyte proportions than successfully treated patients (Figure 3B). No significant difference was observed for monocyte proportions (Figure 3C).

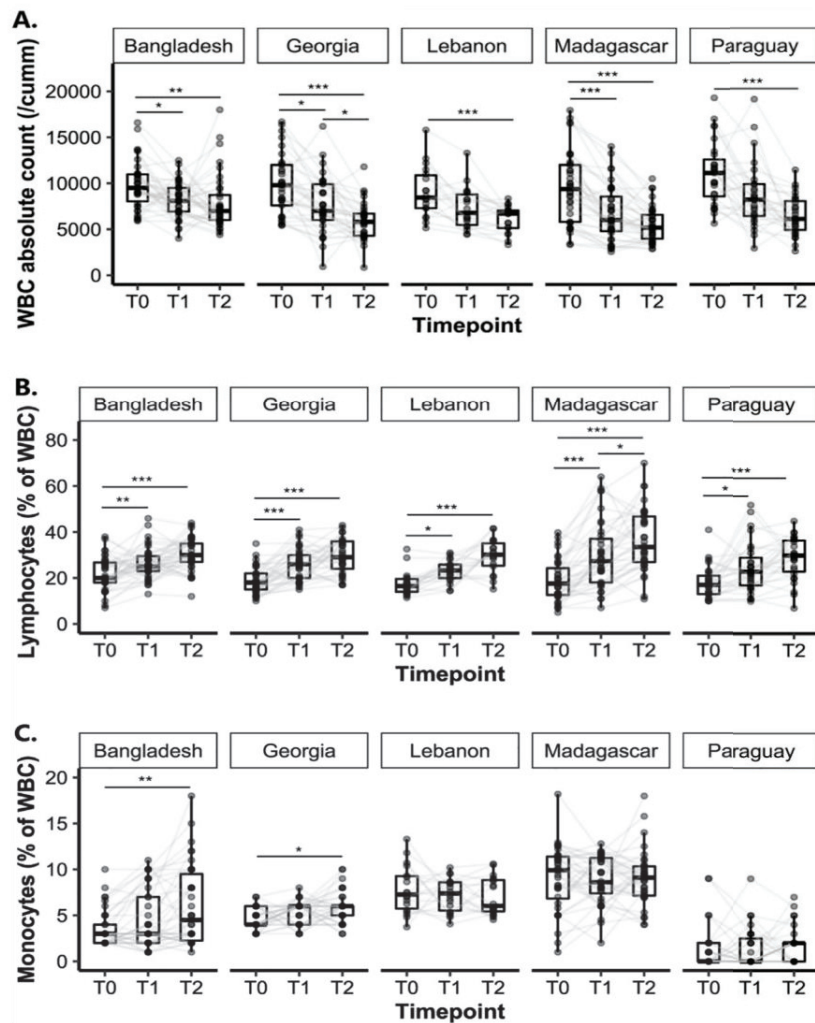


Figure 2. Dynamics of main white blood cell types throughout TB treatment.

(A) White blood cell (WBC) absolute count, cumm: cubic millimeter of whole blood. (B) Lymphocyte percentage of WBC. (C) Monocyte percent of WBC. Data are given as median + interquartile range. Each dot represents one patient at one timepoint. Grey lines connect data points from the same patient. T0: baseline. T1: baseline + 2 months. T2: end of treatment. Data were analyzed using Friedman's test, with the Wilcoxon-Nemenyi-McDonald-Thompson test as a post-hoc correction for multiple pairwise comparisons. *: $p < 0.05$. **: $p < 0.01$. ***: $p < 0.001$.

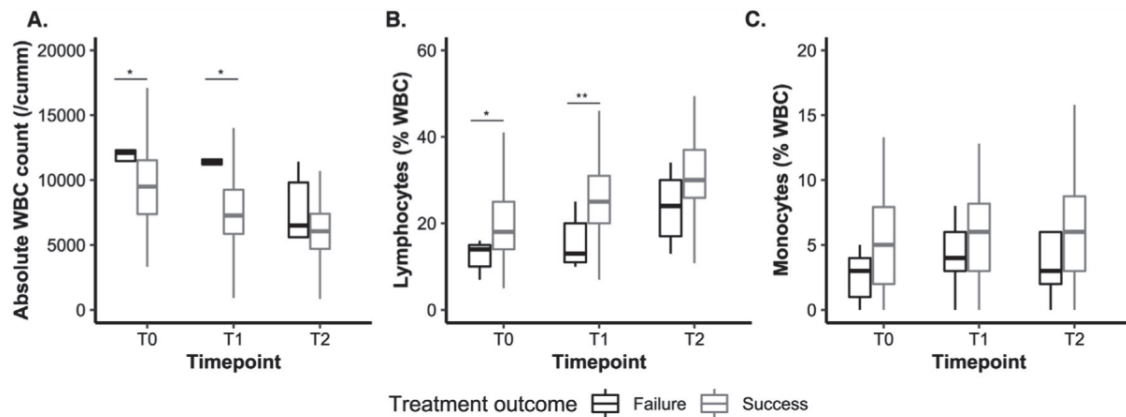


Figure 3. Main white blood cell types over time stratified by sputum smear microscopy and treatment outcome.

White blood cell (WBC) absolute counts (A), lymphocyte percent of WBC (B), and monocyte percent of WBC (C) were assessed over time in cured ($n = 138$) and treatment failure patients ($n = 5$). Data are given as median + interquartile range. T0: baseline. T1: baseline + 2 months. T2: end of treatment. T3: end of treatment + 2 months. cummm: cubic millimeter of whole blood. Treatment failure was defined as a positive sputum culture result at T2. Treatment success was defined as a negative sputum culture result at T2, and no evidence of positive culture at T3. Data were analyzed using the Mann–Whitney U test at each time point. *: $p < 0.05$. **: $p < 0.01$.

As most treatment failure patients had high AFB at baseline (2+ or 3+), we compared WBC absolute counts and lymphocyte proportions according to AFB results, in a subset of all patients presenting with high AFB at baseline (51.7%, 74/143). In this subset, median WBC and lymphocyte counts were similar at baseline, but at T1, WBC counts were higher, and lymphocyte proportions were

lower in unsuccessfully than in successfully treated patients (Supplementary Figure 2).

Table 3
Association of selected baseline sociodemographic and clinical factors with treatment outcome.

Sociodemographic factors and clinical parameters at baseline and at 2 months	Descriptive analysis ($n = 143$)			Logistic regression - association with treatment failure					
	Cured ($n = 138$)	Failed ($n = 5$)	p	Univariate analysis		Multivariate analysis ^a			
				OR (95% CI)	p	aOR	p	AIC	Corrected C-statistic
Age (years)	27.5 (21–37)	31 (29–45)	0.22	1.02 (0.96–1.08)	0.38	–	–	–	–
Sex (male)	62.3% (86/138)	100% (5/5)	0.15	–	–	–	–	–	–
Drug resistance	20.3% (28/138)	0	0.58	–	–	–	–	–	–
Smoking habit	45.3% (62/137)	100% (5/5)	0.021	–	–	–	–	–	–
BMI at inclusion	18.8 (17.1–21.4)	16.0 (15.8–20.9)	0.31	0.87 (0.61–1.15)	0.39	0.74 (0.46–1.05)	0.14	44.68	0.862
Alcohol consumption	21.9% (30/137)	40% (2/5)	0.32	2.38 (0.3–14.9)	0.35	0.52 (0.02–8.55)	0.66	47.23	0.861
History of prison	8.9% (12/135)	40% (2/5)	0.075	6.94 (0.85–46.1)	0.047	1.96 (0.14–25.81)	0.59	47.16	0.856
Previous TB episode	19.4% (26/134)	20% (1/5)	1	1.07 (0.05–7.59)	0.95	1.72 (0.06–29.24)	0.71	47.30	0.860
Absolute WBC count at T0 (cells/mm ³) ^b	9500 (7403–11575)	12,100 (11470–12300)	0.032	1.26 (0.97–1.64)	0.080	1.40 (0.99–2.23)	0.092	43.87	0.862
Lymphocytes at T0 (% of WBC) ^c	18 (14–25)	14 (10–15)	0.019	0.32 (0.10–0.80)	0.032	0.09 (0.0040–0.52)	0.042	37.85	0.866
Monocytes at T0 (% of WBC)	4.9 (2–7.8)	3 (1–4)	0.12	0.75 (0.49–1.03)	0.13	0.21 (0.01–0.78)	0.12	40.57	0.861
Absolute WBC count at T1 (cells/mm ³) ^b	7385 (5907–9382.5)	11,440 (11200–11600)	0.014	1.04 (0.94–1.11)	0.23	4.75 (1.45–55.6)	0.081	35.03	0.860
Lymphocytes at T1 (% of WBC) ^c	25 (20.25–31)	13 (11–20)	0.0079	0.32 (0.11–0.68)	0.0091	0.18 (0.02–0.68)	0.063	39.25	0.864
Monocytes at T1 (% of WBC)	6 (3–8.4)	4 (3–6)	0.41	0.89 (0.66–1.16)	0.40	0.9 (0.4–1.95)	0.79	47.36	0.866
Baseline WBC > 11,450/mm ³	26.8% (37/138)	80% (4/5)	0.024	11.2 (1.60–223.5)	0.031	16.4 (1.52–592)	0.048	41.94	0.867
Baseline lymphocytes <16%	31.9% (44/138)	80% (4/5)	0.044	8.45 (1.21–168.0)	0.061	12.1 (1.44–264)	0.039	42.06	0.858
Baseline WBC > 11,450/mm ³ and lymphocytes <16%	14.5% (20/138)	60% (3/5)	0.029	8.77 (1.37–69.9)	0.019	29.9 (2.19–1088)	0.024	40.73	0.864

Data are given as % (n) or median (interquartile range). Treatment failure was defined as a positive sputum culture result at T2 or T3. BMI: body mass index. OR: odds ratio. aOR: adjusted odds ratio. AIC: Akaike Information Criterion. C-statistic: concordance statistic (corrected for optimism). WBC: white blood cells. T0: baseline. T1: baseline + 2 months. T2: end of treatment. T3: end of treatment + 2 months.

^a Adjusted for age, sex, country of origin, drug resistance strain, and smoking habit.

^b OR or aOR given for each increase in 1000 units.

^c OR or aOR given for each increase in 5 units.

Low baseline lymphocyte proportions are significantly associated with a positive sputum culture at the end of treatment

Sociodemographic characteristics and blood counts at T0 and T1 were compared between cured ($n = 138$) and failed ($n = 5$) patients, and logistic regression was performed (Table 3). In univariate analyses, significant associations with treatment failure were detected for lymphocyte proportions at baseline (OR 0.32, 95% CI 0.10–0.80, $p = 0.032$) and at two months (OR 0.32, 95% CI 0.11–0.68, $p = 0.0091$). No significant association was detected for absolute WBC counts or monocytes proportions at T0 and T1. In multivariate analyses, low lymphocyte proportions at T0 remained moderately associated with treatment failure (adjusted OR 0.090, 95% CI 0.0040–0.52, $p = 0.042$).

ROC analyses were then performed to identify a threshold discriminating successfully and unsuccessfully treated patients based on baseline WBC counts and lymphocyte proportions (Supplementary Table 6). Baseline WBC counts predicted treatment outcome performed with an AUC of 0.788 (95% CI: 0.664–0.912; optimal cutoff $>11,435$ cells/mm³). Baseline lymphocyte proportions performed with an AUC of 0.807 (95% CI: 0.671–0.943; optimal cutoff $<16\%$ of total WBC). The AUC of both variables combined into a new parameter performed better (AUC 0.841 95% CI 0.722–0.959).

Data was then stratified according to the ROC obtained cutoffs. Significant associations were observed in univariate analyses for patients absolute WBC $> 11,450$ cells/mm³ (OR 8.45, 95% CI 1.21–168, $p = 0.031$), and for patients with both WBC $> 11,450$ cells/mm³ and lymphocytes $<16.0\%$ at baseline (OR 8.77, 95% CI 1.37–69.8, $p = 0.019$), but not for lymphocytes $<16.0\%$ alone ($p = 0.061$). These variables remained moderately associated in multivariate analyses (WBC $> 11,450$ cells/mm³: $p = 0.048$; lymphocytes $<16.0\%$: $p = 0.039$; WBC $> 11,450$ cells/mm³ and lymphocytes $<16.0\%$: $p = 0.024$).

Discussion

In this multicentered prospective study, we monitored the evolution of absolute WBC counts, lymphocyte and monocyte proportions during pulmonary TB treatment, and described their association with treatment failure in five cohorts (Bangladesh, Georgia, Lebanon, Madagascar, and Paraguay), four of which were in high-incidence TB countries. We included 152 HIV-negative, culture-confirmed pulmonary TB patients, including 28 drug-resistant TB cases.

We showed that the lymphocyte proportions increased significantly during treatment, whereas the absolute WBC counts decreased significantly. These trends are indicative of general clinical improvement in response to treatment and are consistent with the characteristics of successful therapy. WBC counts are usually higher in TB patients than in healthy individuals, with distinct lymphocyte rates before and after TB treatment (Morris et al., 1989; Rakotosamimanana et al., 2015). No significant evolution of monocyte proportions during treatment was detected in our cohort, consistently with previous work (Rakotosamimanana et al., 2015).

Five patients had positive culture results at the end of treatment and were classified as treatment failure. Two of them had negative smears or cultures at T1, likely due to poor sputum quality. The main finding is that at baseline and after two months of treatment, unsuccessfully treated patients had significantly higher WBC counts and lower lymphocyte proportions than those successfully treated. In adjusted logistic regression analyses, treatment failure was significantly associated with high WBC counts and low lymphocyte proportions at baseline. This result is significant because WBC counts are usually evaluated in TB patients at

baseline and along the therapy course. They are cheap, quick tests that are used routinely in health care centers worldwide. They require a small volume of blood, their output is easy to interpret, and they can be performed without automated equipment. Therefore, they may be a helpful point-of-care tool to help clinicians identify which patients might be less responsive to treatment. Larger studies on this topic are needed to assess whether early WBC or lymphocyte counts should be taken into account when considering changes to treatment composition or duration.

We hypothesize that patients with high baseline WBC counts and low lymphocyte proportions had highly inflammatory clinical patterns. In the context of mycobacterial infection, WBC counts are mostly reflective of neutrophil counts as markers of persisting inflammation or failure to clear the bacteria (Srivastava et al., 2014). Sustained inflammation in chronic TB infection has been described as an impairment of the TB-specific immune response and a marker of active disease (Sia and Rengarajan, 2019). Previous studies have highlighted a correlation between elevated neutrophils, lymphopenia, and TB disease severity, based on chest X-rays (Naghavi et al., 2017; Panteleev et al., 2017). Therefore, a hypothesis is that in this cohort, patients with high baseline WBC and low lymphocytes also had more severe TB manifestations and were less likely to achieve a cure.

We observed that in the subset of our cohort with high smear microscopy grades ($n = 74$), WBC counts and lymphocyte proportions at baseline were not associated with the treatment outcome; however, significant associations were detected at T1. This suggests that in patients who possibly had a higher bacterial burden, cellular inflammatory markers did not stratify risks of failure at baseline, but persistent cellular differences between failed and cured patients were noticeable after the intensive phase of treatment. A limitation is that TB disease severity at baseline could not be assessed in our cohort other than with microscopy (through chest X-rays, Erythrocyte Sedimentation Rate (ESR), or hemoglobin measurements). Further work is needed to assess the relevance of WBC and lymphocytes' association with treatment failure in cohorts with better-defined disease severity.

Moreover, a feature of TB infection is a delayed initiation of an adaptive response after infection, and lymphocyte anergy during chronic infection (Urdahl et al., 2011). This could indicate that *M. tuberculosis*-specific adaptive immunity might be hindered during treatment in patients with high WBC or low lymphocyte proportions. Alternatively, as blood counts are measured in the periphery, this may suggest that *M. tuberculosis*-specific lymphocytes are localized in the lungs for infection clearance: their decreased frequency in peripheral blood would indicate a persisting infection. We observed that the majority of unsuccessfully treated patients (80%, 4/5) presented either high WBC counts or low lymphocyte proportions at baseline, and 60% (3/5) with both, suggesting that the mechanisms behind a persistently activated innate immune response and an anergic adaptive immune response during anti-TB therapy might be linked. Consequently, whether systemic inflammation hinders TB treatment needs to be further investigated.

Our cohort included patients from developing countries, in diverse geographical areas, with varying sociodemographic backgrounds and living standards. We have also enrolled both DS-TB and DR-TB patients, which implies different clinical presentations of the disease. This design suggests similar trends might be occurring in heterogeneous settings and clinical contexts. However, it creates significant limitations and analytical challenges as it hinders the control of some patient- and pathogen-dependent factors such as antibiotic regimens, malnutrition levels, untested infections (e.g., parasites), mycobacterial strains, and genetic background. Moreover, the sample size was small. To account

for these limitations, adjustment with the available patient-dependent factors and optimism corrections with a method adapted to small sample sizes (Smith et al., 2014) was performed. However, the clinical interpretation of these results should remain cautious, given the cohort's heterogeneity and size. Another limitation was the significant dropout rate, particularly between T2 and T3 (26%, 40/152), as T3 was not a timepoint that fell within the time frame of national TB programs. As a consequence, it is likely that some occurrences of TB relapse could not be recorded in our study.

In conclusion, this study confirms that WBC counts and lymphocyte proportions are simple biomarkers for TB treatment monitoring. In this cohort, early measurements of these parameters were associated with treatment failure; these results are encouraging but need to be confirmed on larger cohorts.

Author contributions

JH is the principal investigator and initiated the project together with DG, NT, SBA, GR, NR, and MH. Data was collected by EK, MU, SBI, CA, PR, CR, PH, and RB. CC performed all statistical analyses and wrote the manuscript. All authors have contributed to the content and have given their final approval.

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Conflicts of interest

MH, BMI, and RB had logistic support from the National TB program in Lebanon. DG reports personal fees from Quidel, Qiagen, Janssen, BioMérieux, and Celgene, outside the submitted work. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.ijid.2020.09.017>.

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4. Publication 3

Distinct Cellular Signatures Identified by Spectral Flow Cytometry upon Tuberculosis Diagnosis and at Treatment Completion

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Article summary

Currently, there is a crucial need for optimized TB treatment monitoring methods. Several peripheral blood-based host candidates based on *Mtb*-specific T-cell phenotypes and marker expression profiles have been found as promising surrogates for TB diagnosis and treatment monitoring. Yet, further characterization of immune subpopulations is necessary to establish a full chronological mapping of dynamic host cellular response to TB. The use of high-dimensional single-cell technologies have sought to address this need. Recent studies have highlighted the potential of spectral flow cytometry as a high dimensional single cell analysis technique. So far, no study has characterized, using this technology, the kinetics of host peripheral cell subsets over time in patients treated for pulmonary TB. In this work, a 30-color full spectrum flow cytometry panel was used to perform in-depth phenotypic analysis of T and NK cells upon stimulation with *Mtb* antigens during TB treatment. For this purpose, adults, non-immunocompromised patients with culture confirmed DS-TB were recruited Lebanon and Georgia and were followed during treatment (at T0, T1 and T2). Whole blood was stimulated with QFT-P NIL, TB2 antigenic peptide pool or rmsHBHA. Thereafter, samples were analyzed on a spectral flow cytometer.

Following a hierarchical clustering analysis, spectral flow cytometry analysis based on memory status (naïve, terminally differentiated effector re-expressing CD45RA (TEMRA), central memory (CM) and effector memory (EM)) identified several populations of interest among CD4⁺ (naïve, EM, CM and Treg subsets), CD8⁺ T cells (naïve, EM, TEMRA and MAIT) and Tγδ, among which a proportion were TEMRA. Furthermore, two subsets of NK cells were revealed (NK1 and NK2) based on differential expression of CD38 and CD69. Additionally, at the cluster level, a hierarchical and phenotypic analysis was then performed to identify cellular and functional patterns associated with TB cure upon *Mtb*-specific antigen stimulations. When considering the CD8⁺ T cells, the CD7⁺ TEMRA CD8 populations expressing high levels of CCR4 and CXCR3 were detected within the increased clusters in TB2 samples. Besides, CD27⁻ CD7⁻ EM CD4 populations were identified within the decreased clusters in HBHA samples. On the other hand, as previously mentioned, we detected two subsets of NK cells; NK1 and NK2. NK1 cells that significantly increased between T2 and T0, had a phenotype consistent with an

activated populations whereas NK2 cells that significantly decreased between T1 and T2 had an inactivated profile.

Furthermore, to identify if cellular markers are involved in the discrimination between patients with fast culture conversion from those with a slow conversion, the expression of the activation (HLA-DR, and CD38) and the exhaustion marker (PD-1) was assessed on CD8 TEMRA in response to TB2 stimulation, and those of the activation (CD69 and CD38), and the early maturation marker (CD161) on NK cells in NIL samples. In slow converters, the expression of HLA-DR and PD-1 on TEMRA CD8⁺ T cells and those of CD69 on NK cells, at all-time points were lower than in fast converters. However, the expression of CD38 on both TEMRA CD8⁺ T cells and on NK cells was higher in slow converters than in fast converters. Next, when exploring the discriminatory ability of these markers using the ROC analysis, we found that both the phenotype and functional parameters could be used to discriminate between patients with fast culture conversion from those with a slow conversion. Our results showed that CD8 TEMRA CD38⁺ and NK cells CD38⁺ phenotypes showed a good performance with an AUC > 0.7, for the detection of patients with fast culture conversion from those with slow conversion.

Our overall findings have identified four cellular phenotypes differentially detected between baseline and the end of successful treatment. Moreover, these changes in phenotypic pattern as well as the combination of the phenotypic profile and the expression of CD38 on TEMRA CD8⁺ T cells and NK cells could be surrogate immune markers associated with TB control. However, further validation in cohorts of patients with treatment failure is required to better understand how the change in the immune phenotypes tracks with response to treatment.

Distinct Cellular Signatures Identified by Spectral Flow Cytometry upon Tuberculosis Diagnosis and at Treatment Completion

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Keywords: *Mycobacterium tuberculosis*, tuberculosis, spectral flow cytometry, treatment monitoring; immunophenotyping; heparin-binding hemagglutinin; unsupervised data analysis

ABSTRACT

Tuberculosis (TB) is one of the leading infectious causes of death worldwide. Optimized treatment monitoring methods are needed. The objective of this study was to characterize peripheral immune profiles through spectral cytometry over the course of treatment in patients diagnosed with active TB. This prospective cohort study was based in Tripoli (Lebanon) and Tbilisi (Georgia). Adult, non-immunocompromised patients with culture confirmed drug susceptible TB were followed at treatment initiation (T0), after two months of treatment (T1), and at treatment completion (T2). Whole blood was stimulated with QuantiFERON-TB Gold Plus NIL, TB2 antigenic peptide pool or recombinant methylated *Mycobacterium tuberculosis* (*Mtb*) heparin-binding hemagglutinin produced in *Mycobacterium smegmatis* (rmsHBHA). Cryopreserved white blood cells were analyzed using a 30-marker spectral flow cytometry panel. The discriminatory ability of markers evaluated by area under the receiver-operating characteristic curve (ROC AUC). T-cell phenotypical changes from baseline (T0) to the end of treatment (T2) are detectable in *Mtb*-stimulated samples including a significant increase in terminally differentiated (TEMRA) CD8⁺ T cell populations and a decrease in effector memory CD4⁺ T cell populations. Moreover, two subsets of NK cells were identified NK1 and NK2. Following a phenotypic analysis, we show that there is a significant increase in NK1 cells between T0 and T2 which had a phenotype consistent with an activated population whereas a significant decrease in NK2 cells were detected between T1 and T2 and which had an inactivated profile. Besides, expression of CD38 on TEMRA CD8⁺ T and NK cells showed a higher accuracy (AUC > 0.7), for the detection of patients with slow culture conversion at two months. Overall, our findings offer crucial insights into the underlying immune factors associated with TB cure, and identify phenotypic signature that may influence treatment outcomes. In sum, these phenotypic changes of *Mtb*-specific T cells and NK cells may represent potential surrogate markers associated with TB control. Among them, abundance of TEMRA CD8⁺ T cells, and NK cells in addition to their expression of CD38 are the most promising candidates.

INTRODUCTION

Tuberculosis (TB) remains one of the major infectious causes of death worldwide. In 2020, it was responsible for nearly 10 million TB cases, resulting in 1.3 million deaths¹. TB is treated with a four-drug regimen for 6 months³, but treatment failure and relapse can occur, mostly driven by non-compliance leading to multi-drug resistance issues. Thus, it is essential to rely not only on early diagnosis but biomarkers assessing the outcome during treatment monitoring^{1,13}. Currently, the most common methods used for monitoring TB treatment response are sputum-based tests including smear microscopy and culture^{1,3}. Patients' sputum are often difficult to collect, smear microscopy has limited specificity and sensitivity, and culture methods require a long time¹². In this context, efforts have been made by several TB research groups to identify host immune markers from different accessible biological samples¹⁵³. Peripheral blood-based host candidates based on *Mycobacterium tuberculosis* (*Mtb*)-specific T-cell phenotypes and marker expression profiles have been found as promising surrogates for TB diagnosis and treatment monitoring^{146,176 265,266}. Yet, further characterization of immune subpopulations are necessary to establish a full chronological mapping of dynamic host cellular response to TB¹⁷⁶. High-dimensional single-cell technologies have sought to address this need^{267,268}. Recent studies have highlighted the potential of mass cytometry (also called cytometry by time-of-flight or CyTOF) as a high dimensional single cell analysis techniques²⁵⁰ that overcomes major limitations of conventional flow cytometry. Indeed, CyTOF uses metal-conjugated antibodies, limiting spectral overlap issues encountered in flow cytometry^{182,249}, increasing the abilities to assess wide phenotypic changes of several immune populations using more than 40 parameters at once²⁵⁰. Nevertheless, this technology is limited by high costs, cell loss, and high throughput of cells needed^{251,252}. More recently, these limitations have been overcome by full spectrum flow cytometry (SFC), while still sharing a common workflow with conventional flow cytometry²⁵⁴. This technology has been mostly used in the setting of autoimmune diseases, in the monitoring of cancer patients undergoing immuno- or chemotherapy²⁵⁹. So far, no study has characterized the kinetics of host peripheral cell subsets over time in patients treated for pulmonary TB. In this study, a 30-color full spectrum flow cytometry panel was used to perform in-depth phenotypic analysis of NK and T-cells upon stimulation with *Mtb* antigens at different time-points during TB treatment.

MATERIALS AND METHODS

Study Design and Ethical Statement

This study was a nested multicenter prospective cohort study conducted within the GABRIEL Network ²⁶⁴ in different countries including Georgia and Lebanon. Written informed consent was obtained from all included participants or their legal representative, when unable to consent and standard biosecurity and institutional safety procedures were followed in all study sites. The study protocols were reviewed and approved by the human research ethics committees. In Georgia by the Institutional Review Board of the National Center for Tuberculosis and Lung Diseases (NTCLD) (Reference number: IORG0009467) and in Lebanon by the institutional review board of NINI hospital (Reference number: IRB-F-01).

Cohort Recruitment, Patient Follow-Up, and Clinical Data Collection

Patients were recruited at primary healthcare TB clinics in each country: National Center for Tuberculosis and Lung Diseases (NCTLD) in Tbilisi, Georgia; and National Tuberculosis Program (NTP) centers in Tripoli and Akkar, Lebanon. They were aged ≥ 15 years old and newly diagnosed with pulmonary TB. At screening, patients with chronic human immunodeficiency virus infection or under immunosuppressive treatment or with diabetes mellitus were excluded (**Figure 1**).

Enrolled TB patients were followed-up during the treatment course at three different time points: (i) at baseline upon treatment initiation (T0); (ii) at two months of treatment (T1); (iii) at 6 months at the end of the treatment (T2). All patients has a phenotypically-proven drug-susceptible (DS)-TB, received treatment according to standard protocol and were on Directly Observed Treatment (DOT)^{4,122}. At enrollment and at each follow-up visit, medical history, clinical and demographic data were collected using standardized questionnaires to feed the cloud-based database system CASTOR (CASTOR Electronic Data Capture, Version 1.4, Netherlands) and cleaned and analyzed in R (version 4.0.3).

Blood Collection, Stimulation and White Blood Cell Isolation

1 ml of whole blood was collected directly into each of the QuantiFERON-TB Gold Plus (QFT-P) tubes (Qiagen, Hilden, Germany, 622526) (NIL: Negative Control, TB-Antigens (TB1/TB2)

and Mitogen: Positive control). In this study, three stimulation conditions were used: NIL, TB2, and rmsHBHA. NIL tubes contain no antigens and were used as a negative control. TB2 tubes contains the conventional ESAT-6 and CFP-10 antigens, designed to produce cell-mediated immune responses from CD4⁺ T lymphocytes in addition to new peptides that are able to stimulate both CD4⁺ and CD8⁺ T-cells¹⁰¹. An extra 1 ml of blood was collected in a heparin tube and stimulated with 10 µg/ml of rmsHBHA (UNICATT, Rome, Italy⁶⁴). Detailed whole blood collection stimulation and white blood cell isolation processes were described elsewhere^{168,269}. Prior to staining, fixed white blood cells pellets were thawed in a 37°C water bath for one minute, washed twice with 10 mL of warm RPMI medium, and kept at 37°C until ready.

Spectral Flow Cytometry Protocol

Sample Preparation

In each staining batch, nine samples from a single patient were processed (three time points and three stimulation conditions), as well as an internal control included for downstream control of batch effects (aliquoted cells from a single healthy donor). Detailed sample preparation processes were described elsewhere²⁶⁹.

Spectral Flow Cytometry Staining

The 29-marker mass cytometry panel described previously was translated to spectral flow cytometry²⁶⁹ keeping the same clones as those used in the mass cytometry panel, and adding CD19 to improve resolution. Single staining was performed using white blood cells acquired using the same protocol as patient samples. Single staining samples that were not bright or expressed enough were stained on UltraComp eBeads Plus compensation beads (ThermoFisher Scientific) (**Supplementary Table 1**). Briefly, cells were resuspended in 50µL of Brilliant Stain Buffer (BD Bioscience), stained for 30 minutes at room temperature in the dark, and washed with FACS buffer (PBS 0.1% BSA). Then, fixation with 300µL of freshly reconstituted 4% FA (ThermoFisher Scientific) was performed for 10 minutes, cells were washed twice with BD PhosFlow Perm/Wash Buffer I (1X), and intracellular staining was performed for 30 minutes at room temperature in the dark in BD PhosFlow Perm/Wash Buffer I. Then samples were fixed for 20 minutes in 300µL of freshly reconstituted 1% FA, washed once with PBS, resuspended in 150µL of FACS buffer, and kept at 4°C until acquisition.

Spectral Flow Cytometry Data acquisition, Cleaning and Processing

Samples were analyzed on a Cytex Aurora spectral analyzer hosted by the CYLE flow cytometry core facility at the Cancer Research Center of Lyon (CRCL, UMR INSERM 1052 CNRS 5286, Centre Léon Bérard). Cells were acquired at maximal rate of 10,000 events per second. Quality controls and spectral unmixing were performed using SpectroFlo (Cytex) prior to analysis. Unmixing settings were identical for all patient samples and autofluorescence was extracted individually from an unstained aliquot of each patient sample. Biaxial plots were individually checked for staining quality and compensated prior to further analysis²⁵⁹. Unmixed, compensated.fcs files were then imported into R and cleaned using FlowAI version 3.12. Cleaned files were then gated to exclude debris, doublets, and granulocyte populations which were not in the scope of this study.

Data Analysis

Manual Gating Analysis

In all clean data files, CD45⁺ single cells were isolated, and the proportions of the main white blood cell populations were first assessed manually. Positivity thresholds were set based on unstained controls for Aurora data. The main populations that were identified were found within CD3⁺ T cells. They included CD4⁺ or CD8⁺ T cells and their subsets, TCRγδ⁺ cells, and Mucosal-Associated Invariant T (MAIT) cells (CD8⁺ CD26⁺ CD161⁺ TCRα7.2⁺ cells). The non-T cells studied in this panel were CD3⁻ CD19⁺ B cells, CD3⁻ CD16⁺ CD56⁺ NK cells, CD3⁻ CD14⁺CD16⁻ Monocytes. The workflow for the unsupervised and phenotypic analysis is detailed in supplementary materials.

Statistical Analysis

Statistical analysis and graph representation were performed using the free software R (version 4.0.3) and GraphPad Prism version 6.00 for Windows (GraphPad Software; CA, USA). The proportion (percent of CD45⁺) of each FlowSOM cluster was calculated. For all statistical analyses, exact p-values, test statistics and/or estimates of effect size are provided either in the figure legend. Normality was assessed using the Shapiro Wilk test. The evolution of cluster proportions over time corresponded to repeated measures of non-normal, non-independent

continuous variables, and was analyzed in matched samples using the two-sided Friedman rank sum test with the Wilcoxon–Nemenyi–McDonald Thompson post-hoc test²⁷⁰. Independent, non-normal continuous variables were analyzed with the two-sided Mann–Whitney U test or the Kruskal–Wallis test with Dunn’s Kruskal–Wallis Multiple Comparisons post-hoc test²⁷¹ when more than two categories were compared. Instead, all p-values were computed for each time point, and the p-value corresponding to the null hypothesis being rejected in 5% of all comparisons was used as the significance threshold instead of 0.05²⁷². This novel significance threshold enabled to control type I error while maintaining an exploratory approach; its value was always inferior to 0.05 and is reported in the corresponding figure captions. The Receiver Operating Characteristic (ROC) curve analysis was used to evaluate the performances of functional markers on T cell subsets and on NK cells to differentiate between slow and fast converter groups. Markers with an area under the curve (AUC) > 0.7 were considered as candidate biomarkers.

RESULTS

Patients’ Demographic and Clinical Characteristics

Thirteen individuals (Georgian, n = 8 and Lebanese, n = 5) with culture-confirmed pulmonary TB were included and sampled for spectral flow cytometry analysis (**Figure 1**). Overall, 117 cell samples were analyzed between November 2020 and April 2021. All patients had successful clinical response to standard therapy, and were classified into two response groups according to their culture status at T1 (after two months of treatment): fast converters (n=10; negative culture at T1 and T2) and slow converters (n=3; positive culture at T1 and negative culture at T2). The median age was 41-year-old [interquartile range, IQR 34 - 49], the sex ratio male/female was 3.33. Detailed description of demographic, clinical and microbiological characteristics of patients are given in **Supplementary Table 2**.

Characterization of Immune Cell Phenotypes During TB Management

Seventeen meta-clusters within CD45⁺ immune cells were identified (**Figure 2A**) and were defined by cell-surface lineage markers (**Figure 2B**). A reference map of all automatically detected CD45⁺ cell subsets was then performed (**Figure 2C**). Briefly, spectral flow cytometry

analysis based on memory status (naïve, terminally differentiated effector re-expressing CD45RA (TEMRA), central memory (CM) and effector memory (EM)) identified several populations of interest among CD4⁺ and CD8⁺ T cells: CD4⁺ subsets mostly included naïve, EM, CM and Treg subsets whereas CD8⁺ likely included naïve, EM, two TEMRA phenotypes (1 and 2), MAIT and T $\gamma\delta$, among which a proportion were TEMRA. Furthermore, two subsets of NK cells were revealed (NK1 and NK2) based on differential expression of CD38 and CD69.

Temporal Changes of T-Cell Meta-Cluster Abundances Throughout TB Treatment

Among the identified meta-clusters of T cells, the NIL condition was associated with three significant changes in abundance over time: decrease in CD4⁺ CM (from T1 to T2, $p = 0.03$) and increase in Treg and $\gamma\delta$ TEMRA T cells (from T0 to T2; $p = 0.049$ and $p = 0.028$, respectively). The TB2 stimulation induced an increase in Treg and CD8⁺ TEMRA cells (from T0 to T2; $p = 0.03$ and $p = 0.024$, respectively). The HBHA stimulation resulted in an increase in Treg cells (from T1 to T2; $p = 0.009$) (**Figure 3**).

Cluster Variations According to Antigen Stimulation Throughout TB Treatment

At the cluster level, a hierarchical analysis was conducted to detect significant abundance changes throughout TB treatment according to significant decreases or increases. Among the decreased clusters, were clusters belonging to NK2 and to CD4⁺ subsets (naïve, EM and Treg) in response to HBHA (**Figure 4C**) and a NK2 cluster in response to TB2 (**Figure 4B**). When considering the increased clusters, a phenotypic signature consisting of 11 clusters was evidenced in response to TB2, including immunodominant CD8⁺ subsets ($n = 4$) identified as TEMRA ($n = 3$) and MAIT cells ($n = 1$) in addition to other clusters such as B cells, NK1 ($n = 2$ of each), and CD4⁺ subsets ($n = 3$) identified as naïve, EM and CM cells ($n = 1$ of each) (**Figure 4E**). Among the HBHA-stimulated group, NK1 cell subset was the only increased cell population over time (**Figure 4F**).

Memory CD4⁺ And CD8⁺ T Cell Subsets in Response to *Mtb*-Specific Antigens

Next, we aimed to dissect the CD3⁺ T cell populations specific for each stimulation by characterizing their memory phenotypes, as differences in the memory phenotypes of CD4⁺ and

CD8⁺ T cells were previously found to be associated with different stages of TB infection (active TB versus latent TB infection (LTBI))¹⁶⁷ (**Figure 4H**). In unstimulated samples, CD4⁺ T cell memory phenotypes were parted in CM (67%) and EM (33%). In the TB2-stimulated samples, proportion of TEMRA CD8⁺ was dominant (37.5%) and a group including naïve, EM, CM CD4⁺, EM CD8⁺ and MAIT cells represented 38.5% of T cells . In HBHA-stimulated samples, the EM CD4⁺ phenotype was dominant (40%) and a lower proportion of naïve CD4⁺, Treg and EM CD8⁺ phenotypes was found (20%) (**Figure 4H**).

Terminally Differentiated Effector Memory CD8⁺ T Cell Subsets Are Increased, and Effector Memory CD4⁺ T Cells Are Decreased in Successfully TB-Treated patients

Taking advantage of the phenotypic analysis performed from start to end of TB treatment, we could identify differentially expressed cellular and functional signatures associated with completion of anti-TB treatment upon *Mtb*-specific antigen stimulations (**Figure 4I and Table 1**). When considering the CD8⁺ T cells, the CD7⁺ TEMRA CD8 populations expressing high levels of CCR4 and CXCR3 were detected within the increased clusters in TB2-stimulated samples. Moreover, CM CD4 populations were also detected within the increased clusters in NIL and TB2-stimulated samples characterized by a CD27⁺ ILR7a⁺ CD26⁺ phenotype, which correspond to an inactivated phenotype. Besides, CD27⁻ CD7⁻ EM CD4 populations were identified within the decreased clusters in HBHA-stimulated samples characterized by the absence of expression of activation markers (**Figure 4I and Table 1**).

Activated Natural Killer Subsets increase during TB treatment

As previously mentioned, we detected two subsets of NK cells (NK1 and NK2) that differed in their levels of expression of CD69 and CD38 (**Figure 2A**). From baseline to T2, these two subsets had opposite trend in an antigen-independent manner, as NK1 increased over time, whereas NK2 decreased (**Figure 5B**). As seen in **Figure 4** several clusters were related to NK1 and NK2. In phenotypic analysis, NK1 and NK2 cells, were both characterized by CD16 and CD56 co-expression. NK1 cells had a CD16⁺ CD56⁺ CD38⁺ CD69⁺ CD161⁺ phenotype consistent with an activated populations whereas NK2 cells had an inactivated profile (CD16⁺

CD56⁺ CD38⁻ CD69⁻ CD161⁻) (**Figure 5C**). Overall, we conclude that the NK signature associated with TB control is an enrichment in activated NK cells.

Functional Profile of CD8⁺ TEMRA T Cells and NK Cells Correlates with Culture Conversion at Two-Months of Treatment

Furthermore, to identify if cellular markers are involved in the discrimination between patients with fast culture conversion from those with a slow conversion, the expression of the activation (HLA-DR, and CD38) and the exhaustion marker (PD-1) was assessed on CD8 TEMRA in response to TB2 stimulation, and those of the activation (CD69 and CD38), and the early maturation marker (CD161) on NK cells in NIL samples (**Figure 6**). In slow converters, the expression of HLA-DR and PD-1 on TEMRA CD8⁺ T cells and those of CD69 on NK cells, at all-time points were lower than in fast converters. However, the expression of CD38 on both TEMRA CD8⁺ T cells and on NK cells was higher in slow converters than in fast converters. Next, when exploring the discriminatory ability of these markers using the ROC analysis, we found that both the phenotype and functional parameters could be used to discriminate between patients with fast culture conversion from those with a slow conversion (**Figure 6G**). Our results showed that CD8 TEMRA CD38⁺ and NK cells CD38⁺ phenotypes showed a good performance with an AUC > 0.7, for the detection of patients with fast culture conversion from those with slow conversion.

DISCUSSION

In this study, we used high-dimensional spectral flow cytometry to study peripheral immune cell populations in patients treated for an active TB. Our results have identified four cellular phenotypes differentially detected between baseline and treatment completion. TEMRA CD8⁺ T cells and activated NK cell subset increased over time, whereas EM CD4⁺ T cells and inactivated NK cell subset decreased over time. Moreover, we showed that measuring the expression of CD38 on TB2-specific TEMRA CD8 T cell and on NK cells could be a useful parameter for the detection of patients with fast culture conversion from those with slow conversion. Altogether, these changes in phenotypic pattern as well as the combination of the phenotypic profile and the expression of CD38⁺ on TEMRA CD8⁺ T cells and on NK cells could be surrogate immune markers associated with TB control.

In unsupervised clustering, TB2 antigen has revealed a large panel of immune cell phenotypes (B cells, NK cells, CD4⁺, CD8⁺ and $\gamma\delta$ T cell subsets), which suggests its formulation is a ligand of multiple immune cells that operate together during TB disease and in successfully treated TB. In particular, the TB2-stimulated group revealed a higher proportion of CD8⁺ T cells at the end of TB treatment, consistently with previous works^{273,274}. Among these CD8⁺ T cells, finding a predominance of TEMRA phenotype was also in line with a prior observation indicating that CD8⁺ T cells are the largest responders to TB2 stimulation²⁷⁵. In contrast, HBHA antigen likely revealed EM CD4⁺ T cells in agreement with previous work in which memory phenotypes were associated with HBHA-specific CD4⁺ T cells from TB patients¹⁶⁷.

In the present study, successfully treated TB individuals had significantly higher frequencies of TEMRA CD7⁺ CD8⁺ T cells and significantly lower frequencies of EM CD7⁻ CD27⁻ CD8⁺ T cells compared to patients at baseline. Such peripheral CD8⁺ T cell phenotypes have been previously reported in TB disease patients compared to latent TB infected individuals^{182,276}, to healthy controls²⁷⁷ and throughout the course of anti-TB treatment^{172,275}. Our group has already highlighted the shift in CD8⁺ T cell abundance upon TB2 stimulation using mass cytometry in a prospective cohort study of adult pulmonary TB patients²⁶⁹. Regarding the decrease in differentiated EM CD27⁻ CD7⁻ CD4⁺ T cells population at the end of treatment, it has been previously reported that TB was characterized by a higher frequency of highly differentiated memory phenotype of EM CD4⁺ T cells (CD45RA⁻ CCR7⁻ CD27⁻)²⁷⁸.

Few studies have investigated the implication of the innate immunity in the protective mechanism against *Mtb*, hence, we were interested in studying NK cells, one of the critical components of the early innate immunity. This is the first spectral flow cytometry study assessing the contribution of different NK cell subsets in TB patients at baseline and throughout TB treatment. A recent work has suggested a link between the abundance of NK cells and TB outcome as it showed a significant increase in the abundance of NK cells in cured TB patients when compared to baseline levels¹⁸². Our data showed a greater participation of mature NK cell subsets (CD16⁺ CD56⁺) in TB patients, consistent with data previously reported^{279,280}. The increase in abundance of activated NK cells and the decline of inactivated NK phenotypes at the end TB treatment compared to baseline suggest that NK cells may play an important role in *Mtb* clearance. A previous work has reported that NK cell frequency was restored following successful anti-TB treatment compared to baseline^{182,281}. Another work studying LTBI

individuals (including 12 progressors and 20 non-progressors to TB disease) has demonstrated that NK cell frequencies decreased during the progression to TB disease, whereas no significant change in the NK cell frequency was detected among the non-progressor group¹⁸². It emphasizes the need to further study NK cell dynamics during *Mtb* infection and decipher the phenotypic patterns associated with different stages of infection.

The T cell subsets described above could be considered as potential phenotypic markers for monitoring TB treatment response. In our work, we also identified a different functional profile within the CD8 TEMRA T cell subsets characterized by a decreased expression of activation markers such as CD38 and HLA-DR and an increase in the expression of exhaustion marker; PD-1 over time. Our findings are consistent with previous studies showing a reduction in the expression of T cell activation markers throughout treatment^{266,282}. On the contrary, data from previous study showed that no significant differences were found in PD-1 expression on IFN- γ ⁺ CD8 T cells in active TB patients after treatment completion²⁸³. On the other hand, ROC analysis showed that the expression of the activations markers contributed to the detection of patients with slow culture conversion at two months. CD38 expression on TEMRA CD8⁺ T cells was among the markers which best discriminated fast from slow conversion. Our group has already highlighted using mass cytometry that HLA-DR and CD38 also differentiated slow from fast converters during treatment²⁶⁹. Moreover, the expression of such marker on CD4⁺ T cells was previously found to be significantly higher in patients with slow conversion when compared to those with a fast conversion²⁸⁴. Thus, our results suggested that the expression of CD38 on CD8⁺ TEMRA T cells could provide a useful indicator to detect patients with slow culture conversion at two months. Altogether, these changes in phenotypic pattern as well as the combination of the phenotypic profile and the expression of CD38⁺ in TEMRA CD8⁺ T cells and in NK seems to be promising for use in TB treatment monitoring.

Our study has some limitations. Indeed, the sample size was relatively small and we did not include healthy controls, so we could be able to determine which of each identified immune subsets were specific to TB disease. Similarly, including individuals with LTBI, will also be valuable to determine different stages of TB infection using immune markers identified by spectral flow cytometry. Moreover, further validation in cohorts of patients with treatment failure

is required to better understand how the change in the immune phenotypes tracks with response to treatment.

In summary, our data showed the potential use of a novel immune assay; spectral flow cytometry demonstrating its utility to characterize, on a single cell level, the changes in *Mtb*-specific immune cells in TB patients at baseline and throughout the course of anti-TB treatment. We have identified candidate blood-based host biomarkers for monitoring TB treatment response, based on several *Mtb*-specific CD4⁺ and CD8⁺ T cell memory phenotypes including an increased TEMRA CD8⁺ T cells and a decreased EM CD4⁺ T cells. Besides, we have identified specific changes in NK cell phenotypes and functions during *Mtb* infection and throughout treatment, including an activated NK cell subset that was restored after successful TB treatment and a non-activated one that diminished at the end of treatment, thus highlighting the protective role of innate immunity against TB disease. Our findings also showed that measuring. Moreover, we showed that measuring the expression of CD38 on TB2-specific TEMRA CD8 T cell and on NK cells could be a useful parameter for the detection of patients with slow culture conversion at two months. Overall, these markers seems to be promising for use in TB treatment monitoring. However, further studies are needed to validate our findings in a larger cohort in order to define its usefulness in clinical settings and thus providing suggestions that could lead to new intervention strategies in TB vaccine and drug trials.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved in Georgia by the Institutional Review Board of the NTCLD (IORG0009467) and in Lebanon by the Institutional Review Board of NINI hospital (IRBF-01). The patients/participants provided their written informed consent to participate in this study.

CONFLICT OF INTEREST

None of the authors have a conflict of interest.

AUTHOR CONTRIBUTIONS

FA and JH are the principal investigators of the study, and initiated this project in partnership with NT, and DG. Patient recruitment, follow-up and data collection was done by EK and RB. CC designed and optimized the spectral flow cytometry protocol. The experiments, data analysis and interpretation of results were performed by RB and CC. The manuscript was written by RB and FA. All authors have read, reviewed and approved the final manuscript.

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FIGURES

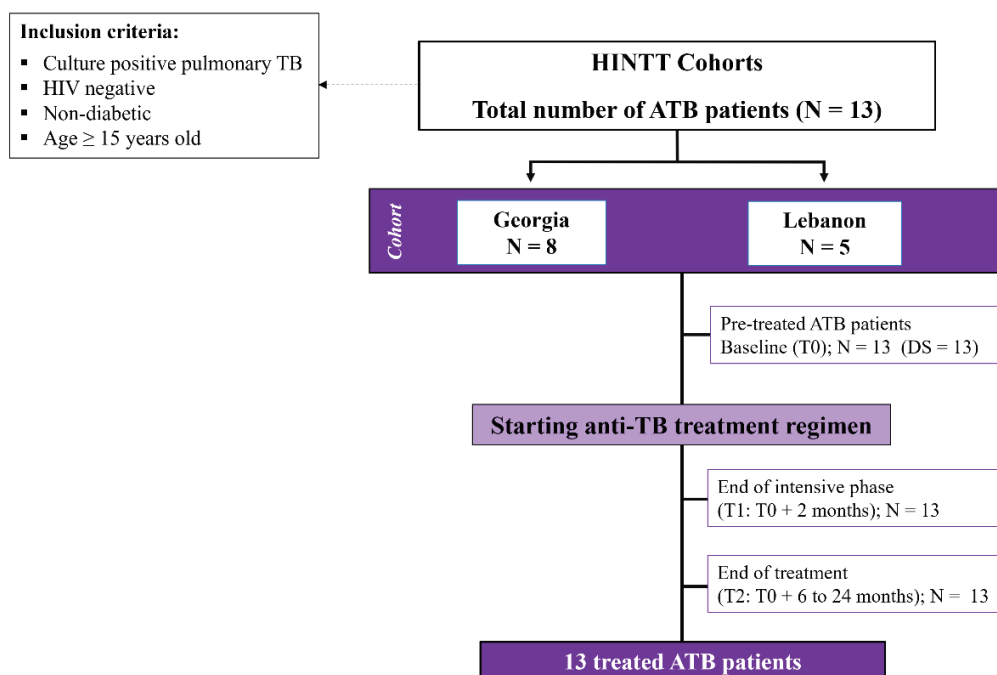


Figure 1 | Flow diagram describing the enrollment and exclusion of participants with ATB disease from the different cohorts. ATB patients were followed-up at three different time points: at baseline (T0), ATB patients who didn't their TB treatment and followed throughout antibiotic therapy: at month 2 (T1), and at the end of treatment (T2). TB: Tuberculosis. ATB: active TB. HIV: Human Immunodeficiency Virus. DS: Drug-susceptible.

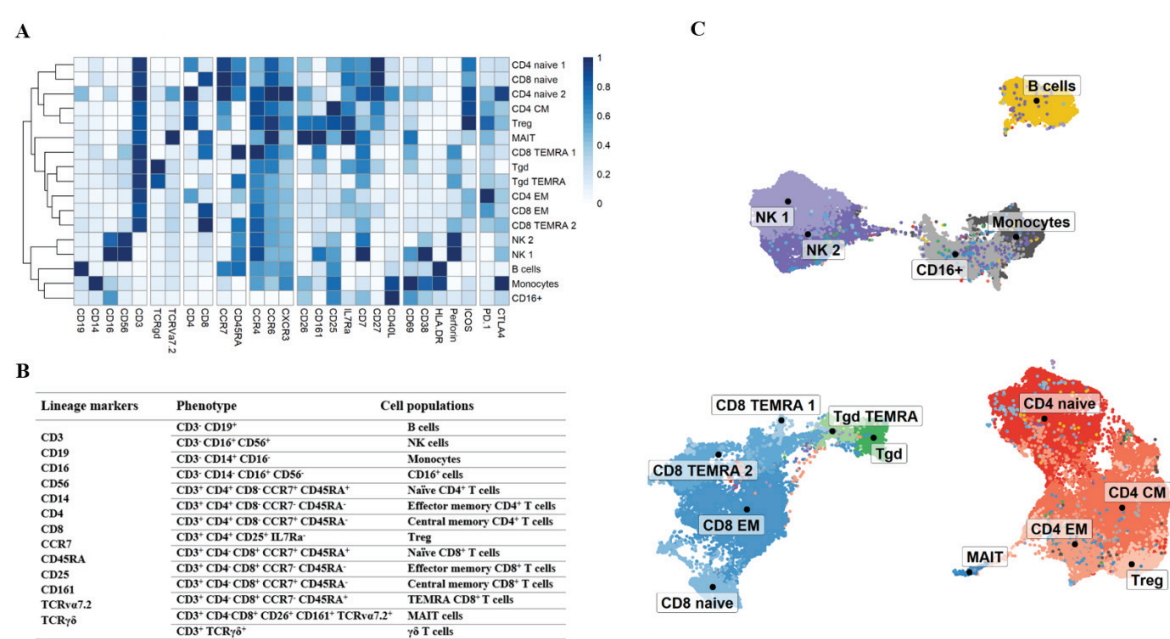


Figure 2 | Characterization of the main immune cells associated with pulmonary TB disease. Whole blood samples were collected from 13 patients newly diagnosed with ATB disease and followed-up at three different time points throughout the anti-TB treatment. These samples were stimulated with *Mtb*-specific antigens (TB2/HBHA) or with negative control (NIL). Hierarchical clustering analysis was performed on the 86,000 CD45⁺ cells identified based on spectral flow cytometry analysis using a 30-marker panel (antibody panel, **Supplementary Table 1**). **(A)** Heatmap showing expression of markers for each of the 17 meta-cluster identified within the CD45⁺ cells. To identify the distinct cell population, each meta-cluster was annotated based on the expression intensity of each marker. **(B)** Lineage-defining markers expression and the phenotype of cell populations in each of the cell meta-clusters identified by FlowSOM analysis.

(c) UMAP visualization of the determined clusters and meta-clusters. NK: Natural killer. TEMRA: Terminally differentiated effector re-expressing CD45RA. CM: Central memory. EM: Effector memory. Treg: Regulatory T cells. MAIT: Mucosal-associated invariant T cells. Tgd: $\gamma\delta$ T cell.

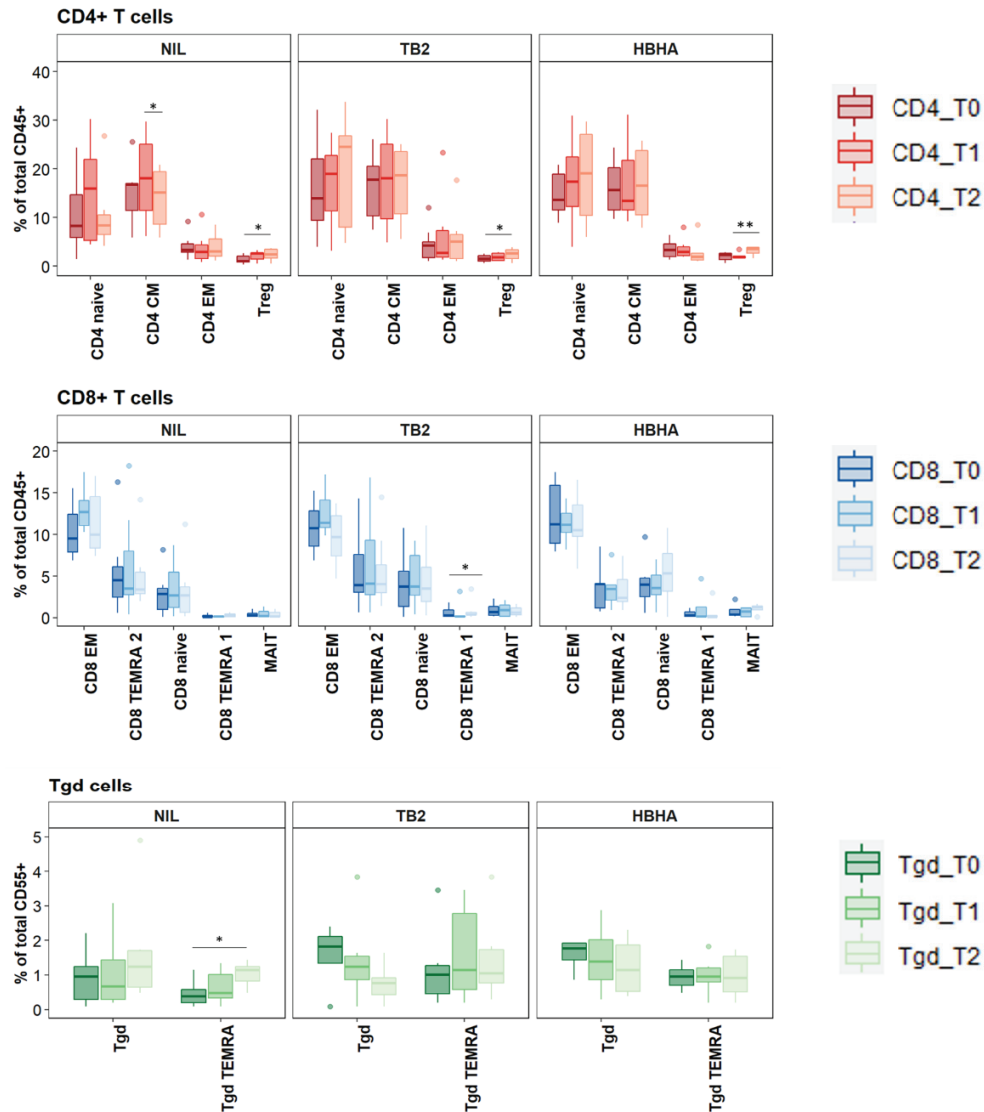


Figure 3 | Changes in the frequencies of T cell phenotypes throughout the treatment. Boxplots showing the variation of the different T cell phenotypes (T cell phenotype frequencies are % of total CD45⁺ cells) in ATB patients during the course of treatment (baseline; T0, two months after treatment initiation; T1 and at the end of treatment; T2) within the CD4⁺, CD8⁺ and $\gamma\delta$ T cell response to *Mtb*-specific antigens (TB2, n = 9 and HBHA, n = 6) and in unstimulated samples

NIL, n = 7. Horizontal lines designate medians, boxes represent the inter-quartile ranges (IQR) and the ranges are represented by whiskers. Statistical significance was calculated using Mann–Whitney U test. * indicates a p-value < 0.05 and ** indicates a p-value < 0.01. CM: Central memory. EM: Effector memory. TEMRA: Terminally differentiated effector re-expressing CD45RA. Treg: Regulatory T cells. MAIT: Mucosal-associated invariant T cells. Tgd: $\gamma\delta$ T cell.

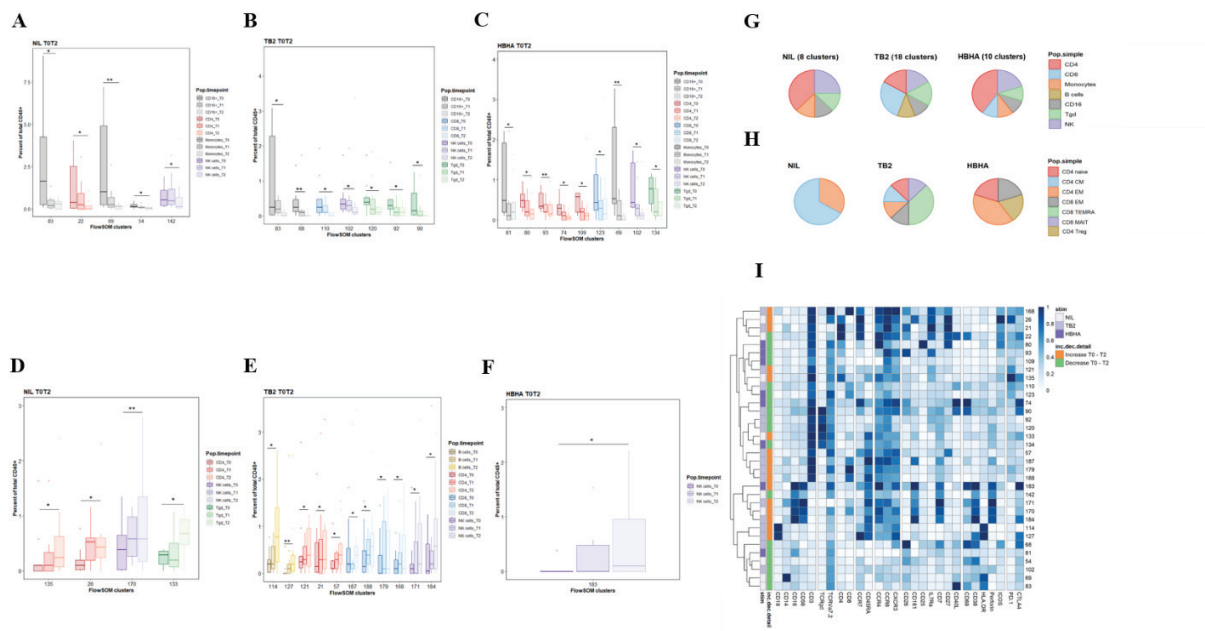


Figure 4 | Significantly different CD45⁺ immune subsets in successfully treated TB patients. The frequencies of each identified FlowSOM cluster were measured throughout the course of anti-TB treatment and in each stimulation condition; the number of matched data points per time point for all panels: NIL, n = 7; TB2, n = 9; HBHA, n = 6. The frequencies of the different immune subsets are % of total CD45⁺. Only significantly different clusters at the end of treatment (T2) relative at baseline (T0) are shown for clarity. **(A to C)** Significantly decreased clusters at the end of treatment (T2) relative to baseline (T0), in unstimulated **(A)**, TB2-stimulated **(B)** and HBHA-stimulated samples **(C)**. **(D to F)** Significantly increased clusters at the end of treatment (T2) relative to baseline (T0), in unstimulated **(D)**, TB2-stimulated **(E)** and HBHA-stimulated samples **(F)**. B cells clusters were presented in yellow, Monocytes and CD16⁺ clusters in grey, NK cell clusters in purple, CD4⁺ clusters in red, CD8⁺ clusters in blue and $\gamma\delta$ T cell clusters in green. Statistical significance was calculated using Friedman rank sum test and Wilcoxon-

Nemenyi-Thompson post-hoc for pairwise comparisons between non-independent observations at T0 and T2. * indicates a p-value < 0.05 and **: p < 0.01. Each line represent one cluster. Scales indicate normalized mass signal intensity. **(G)** Pie charts visualizing the proportion of the identified significant clusters that changes between T0 and T2 in the different groups analyzed in response to different stimulation conditions (NIL, TB2 and HBHA). The arcs indicate the proportion CD4⁺ T cells (red), CD8⁺ T cells (blue), Monocytes (orange), B cells (yellow), CD16⁺ (grey), $\gamma\delta$ T cells (green) and NK cells (purple). The stimulation condition is indicated above each pie chart. **(H)** Pie charts that represent the memory status of CD4⁺ and CD8⁺ T cells in response to different stimulation condition. The arcs represent the proportion of naïve (red), CM (blue), EM (orange) CD4⁺ T cells, EM (grey), TEMRA (green) CD8⁺ T cells, MAIT (purple) and Treg (yellow) in the different groups analyzed in response to different stimulation conditions (NIL, TB2 and HBHA). The stimulation condition is indicated above each pie chart. **(I)** Phenotypic analysis using marker expression heatmap showing the mean expression levels of markers related to the increased (orange color) and decreased (green color) CD45⁺ cell clusters that are significantly different at the end of treatment (T2) compared to baseline (T0).

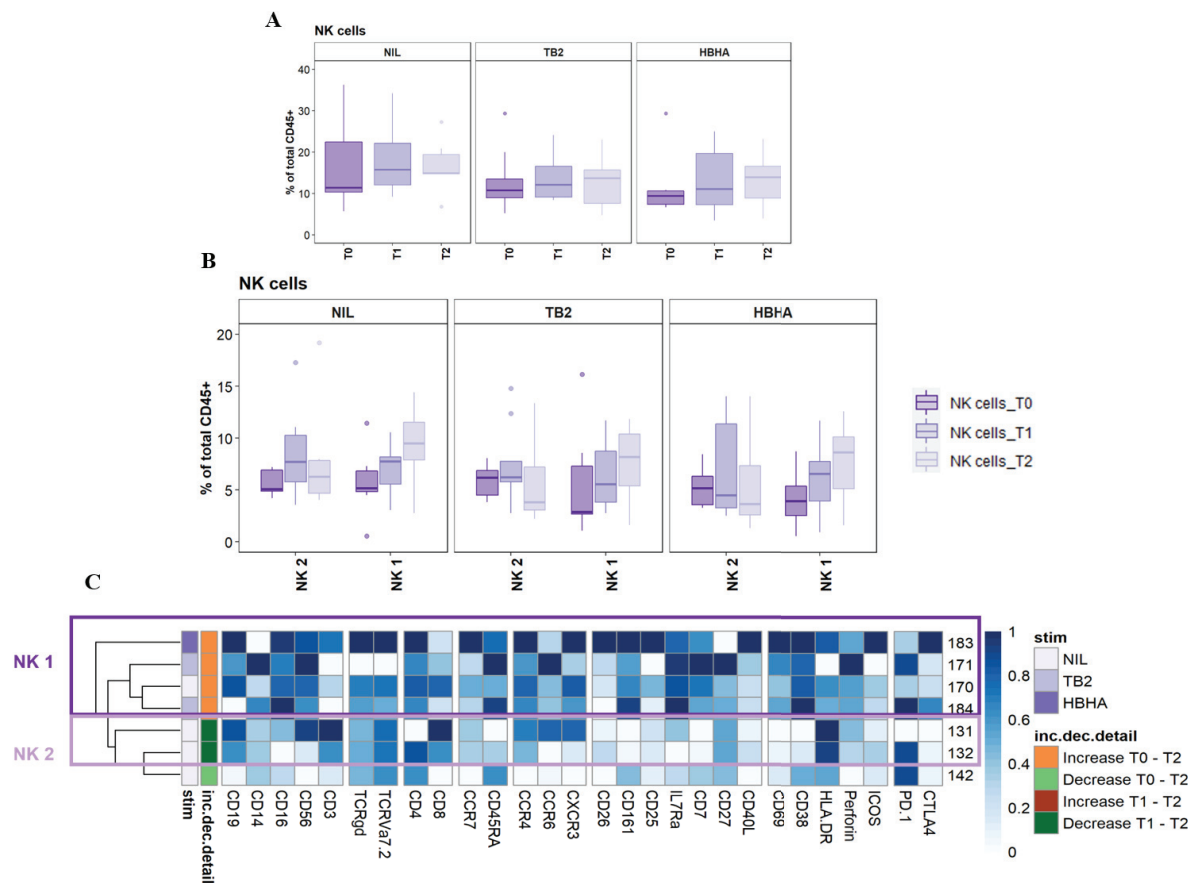


Figure 5 | Analysis of total NK cells (top panel) and NK cell subsets (middle panels) during the course of anti-TB treatment. Boxplots evaluating the changes in the frequencies (that are % of total CD45⁺ cells) of (A) NK cells and (B) NK cell subsets among three different groups including the response induced by: unstimulated (NIL, n = 7) samples and Mtb-specific antigens (TB2, n = 9) and (HBHA, n = 6) in patients with ATB at baseline (T0), followed at two months after treatment initiation (T1) and who had successfully completed their TB treatment at the end of treatment (T2). Horizontal lines designate medians, boxes represent the inter-quartile ranges (IQR) and the ranges are represented by whiskers. Statistical significance was calculated using Mann–Whitney U test. (C) Phenotypic analysis using marker expression heatmap showing the mean expression levels of markers related to the increased (orange color) and decreased (green color) NK cell clusters throughout anti-TB treatment. Each line represent one cluster. Scales indicate normalized mass signal intensity.

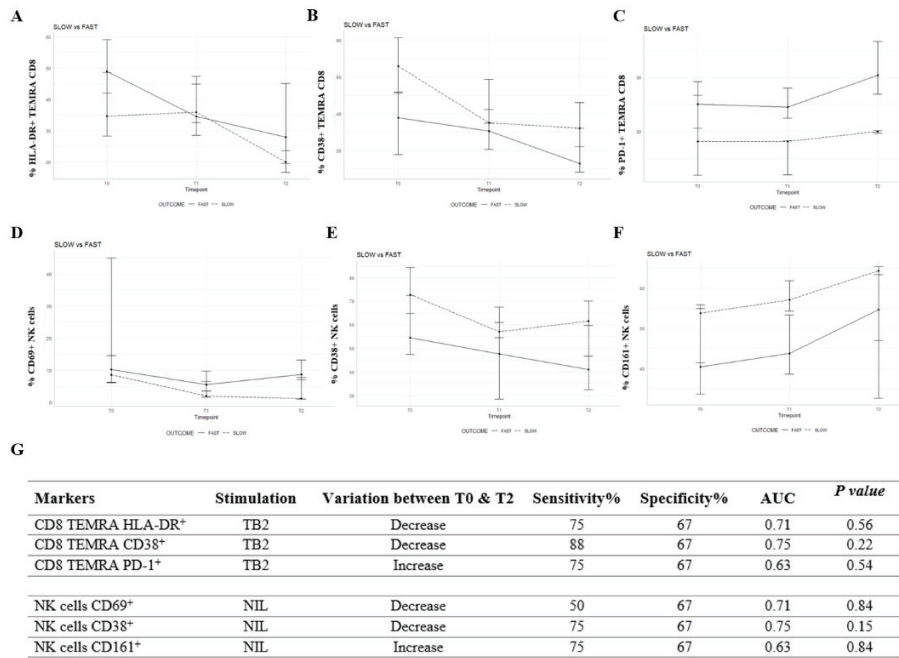


Figure 6 | Functional profile of TEMRA CD8⁺ and NK cells correlating with culture conversion at two-months. Longitudinal Kinetics showing the percentage of activation and exhaustion markers on (A to C) TEMRA CD8⁺ T cells at baseline (T0), 2 months after treatment initiation (T1), and at the end of treatment (T2) in patients with fast and those with a slow culture conversion. (A) HLA-DR⁺, (B) CD38⁺, and (C) PD-1⁺. Longitudinal Kinetics showing the percentage of activation and early maturation markers on (D to F) CD56⁺ NK cells over time. (D) CD69⁺, (E) CD38⁺, and (F) CD161⁺. Dots depict medians and error bars represent the IQR. (G) Receiver operating characteristic (ROC) curve analysis and the respective areas under the curve (AUC) with 95% confidence intervals (CI) showing the ability of functional markers to discriminate between patients with fast and slow culture conversion at baseline (T0). AUC: area under the curve.

TABLE

Table 1 | T cell clusters that significantly changed between baseline and the end of treatment.

Increased clusters from T0 to T2			
	CD8 ⁺ T cells	CD4 ⁺ T cells	NK cells
NIL		- CD27⁺ ILR7a⁺ CD26⁺ CD69⁻ CD40L⁻ CD4⁺ CM - CD27⁻ CD7⁻ CTLA-4⁺ CD4⁺ EM	CD16 ⁺ CD56 ⁺ CD38 ⁺ CD69 ⁺ CD161 ⁺ NK1
TB2	CD7 ⁺ CCR4 ⁺ CXCR3 ⁺ CD8 ⁺ TEMRA	- CD27⁺ ILR7a⁺ CD26⁺ CD69⁻ CD40L⁻ CD4⁺ CM - CD27⁻ CD7⁻ CTLA-4⁺ CD4⁺ EM - CD27⁺ CD69⁻ CD40L⁻ CD26⁻ CTLA-4⁻ CD4⁺ naïve	CD16 ⁺ CD56 ⁺ CD38 ⁺ CD69 ⁺ CD161 ⁺ NK1
HBHA			CD16 ⁺ CD56 ⁺ CD38 ⁺ CD69 ⁺ CD161 ⁺ NK1
Decreased clusters from T0 to T2			
	CD8 ⁺ T cells	CD4 ⁺ T cells	NK cells
NIL		CD27 ⁺ ILR7a ⁺ CD26 ⁺ CD69 ⁺ CD40L ⁺ CD4 ⁺ CM	
TB2	CD7 ⁻ CD27 ⁻ CD8 ⁺ EM		
HBHA	CD7 ⁻ CD27 ⁻ CD8 ⁺ EM	- ICOS⁺ CD25⁺ CD4⁺ Treg - CD27⁻ CD7⁻ CD4⁺ EM - CD27⁻ CD69⁺ CD40L⁺ CD26⁺ CTLA-4⁺ CD4⁺ naïve	

These data were obtained from Figure 4 and Figure 5.

5. Publication 4

New evidence on the role of Natural Killer cells in the control of *Mycobacterium tuberculosis* infection

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Article summary

In the previous publication, we identified two subsets of NK cells which differed by their phenotypic immune profile. These findings suggest that changes in the frequency and phenotypic profiles of NK cell subsets could be associated with TB cure. Such data support the potential contribution of NK cells in the protective immunity against *Mtb* infection in humans.

In this review, we revised novel evidences on the role of NK cells in the context of TB disease. We focused on data generated during the last decade concerning NK cell development, effector functions, and contribution to the innate immune response against *Mtb* infection. In addition, we presented new perceptions gained from *in-vitro* and *in-vivo* studies of NK cells revealing a correlation between these cells and the stages of *Mtb* infection (active TB vs LTBI). We also reported the ability to predict progression from LTBI to active TB disease and to monitor treatment response by evaluating the levels of NK cells. Moreover, we highlighted the current priority for TB research including the understanding of NK-mediated mechanisms of early *Mtb* clearance that may provide novel therapeutic and vaccine targets and open new fields in TB prevention. Finally, we present findings of several clinical studies that have investigated the association between the abundance, biomarkers, functionality of peripheral blood NK cells and human TB.

Overall, we highlighted that NK cells may represent important innate targets that could confer protection against TB. Besides, we underlined that differential subsets, frequency, markers and functions of these cells may be used to discriminate between the diverse stages of TB as well as to monitor the TB treatment response. These data are holding promise and must be validated in larger cohort studies using blood and other samples (e.g. broncho-alveolar lavage). NK cells may therefore represent a new innate immune target with potential clinical applications. These include the improvement of vaccine design and efficacy as well as TB treatment, especially in immunocompromised individuals (e.g. those living with HIV) by conferring them with a T-cell independent protective immunity.

New evidence on the role of Natural Killer cells in the control of *Mycobacterium tuberculosis* infection

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Abstract

Tuberculosis (TB) remains one of the leading causes of death worldwide. The interaction between the host immune system and the pathogen *Mycobacterium tuberculosis* (*Mtb*) can induce three clinical outcomes, which range from the early clearance of *Mtb* to the establishment of latent TB infection (LTBI), and the progression to active TB disease. In recent years, the evidence demonstrated that innate immune cells may highly affect the infection outcomes resulting from the interaction between the host and *Mtb*. Among these are natural killer (NK) cells that have recently attracted an increased interest due to their functional role in controlling *Mtb* infection in humans. Therefore, in this review, we report relevant advances on the contribution of NK cells in the host immune response against *Mtb* infection and propose them as novel innate targets that could confer protection against TB.

Keywords: Tuberculosis, *Mycobacterium tuberculosis*, innate immune cells, Natural Killer cells.

Introduction

Tuberculosis (TB) is one of the leading causes of death from an infectious disease worldwide. According to the world health organization (WHO), one-fourth of the world population is estimated to be infected with *Mycobacterium tuberculosis* (*Mtb*). In 2020, approximately 10 million cases of TB were reported, of whom 1.3 million died from the disease¹. Unfortunately, TB control efforts are threatened by the emergence and increase of multidrug-resistant TB (MDR-TB), the convergence of TB and human immunodeficiency virus (HIV), as well as the lack of vaccines able to confer significant protection against TB. Bacille Calmette-Guerin (BCG) vaccine remains the only authorized vaccine that prevents the development of TB disease⁵. It provides significant protection, especially from disseminated forms of TB in children⁶, however, there is currently no effective vaccine that prevents pulmonary and other forms of TB in adults^{6,7}. Five to 10% of infected individuals develop the contagious, active form of TB disease, while most of them (90%), due to the host's innate and adaptive immune response, control the infection and develop asymptomatic latent TB infection (LTBI). However, a small proportion (10%) of LTBI individuals will develop active TB during their lifetime². Moreover, several studies have reported that some household contacts (HHCs) who are extensively exposed to *Mtb* do not acquire infection, suggesting that these individuals are naturally resistant to *Mtb* infection and are defined as 'resisters'²⁸⁵. Moreover, it was suggested that resisters may have been able to clear *Mtb* infection early with an effective innate immune response before the development of acquired immunity. This early clearance of *Mtb* is attributed to innate immune cells such as monocytes, macrophages, DCs, neutrophils, and natural killer (NK) cells in addition to innate like-immune cells such as gamma-delta T cells, mucosal-associated invariant T cells (MAIT)²⁸⁶, and invariant NK T cells²⁸⁷. Among these cell populations likely to play a role in host immune response against *Mtb*, NK cells have recently been the focus of increasing interest in TB physiopathology²⁸⁸. NK cells appear to play a predominant role against *Mtb* infection through several mechanisms including cytotoxic activity^{289,290}, the release of cytokines^{291,292} addition to their ability to develop immunological memory that induces enhanced effector functions during secondary responses^{293,294}. Thus, the increasing evidence of the role of NK cell function during TB suggests that such cell-mediated innate immunity could be a new target against *Mtb* infection. Therefore, in this review, we focus on the evidence generated during the last decade

about NK cells related to their development, effector functions, and their contribution to the innate immune response against *Mtb* infection.

Biology of human NK cells

Natural killer (NK) cells are large granular lymphocytes that belong to the innate immune system. Those cells represent 10 to 15% of human lymphocytes in the peripheral blood²⁹⁵. They are distributed in the blood, spleen, thymus, liver, lungs as well as in uterus^{296,297}. NK cells are distinguished by the expression of cell surface markers; they are phenotypically identified by the expression of the neural cell adhesion molecule; NCAM (CD56) and the lack of the CD3 expression^{295,298}. Based on the surface density of CD56, two distinct NK cell subsets were identified, the CD56^{bright} and the CD56^{dim}, which exhibit major phenotypical and functional properties. Around 10% of peripheral blood NK cells express high-density levels of CD56 (CD56^{bright}), and lack or express low levels of Fc-γ receptor III (Fc-GR III, CD16), those cells can produce large amounts of cytokines, however, the majority of peripheral NK cells (approximately 90%) express a low level of CD56 (CD56^{dim}), those cells express high levels of CD16, in addition, express more killer immunoglobulin (Ig)-like receptors (KIR), moreover, they are more cytotoxic, even though, it was also demonstrated that the CD56^{dim} are also able to secrete cytokines rapidly^{291,299}, and can release large amounts of IFN-γ and other cytokines. Thus, CD56^{dim} has two functional abilities, which are relevant for rapid immune defenses mediated by the same NK cell subset without however the need for the intervention of another subset present in different tissues. As a result, better outcomes in terms of quality responses and time can be generated^{291,300}. It was demonstrated that NK cells are similar to a group of lymphocytes known as innate lymphoid cells (ILCs). ILCs are currently emerging as essential effectors during the early stages of the immune responses against various bacterial species and were classified into three different groups: ILC1s, ILC2s, and ILC3s. NK cells were recently classified as a member of group 1 ILCs, as both can produce tumor necrosis factor (TNF-α) and interferon-gamma (IFN-γ), however, unlike ILC1s, NK cells have cytotoxic functions³⁰¹.

Effector and regulatory function of NK cells

The functional properties of NK cells are under the control of signals elicited by ligands binding to different activating and inhibitory receptors²⁹⁹. The balance between inhibitory and activating signals determines how the NK cells will distinguish between healthy and target cells (transformed or infected cells). Healthy cells express major histocompatibility complex I (MHC-I) molecules, which act as ligands for inhibitory receptors on NK cells, thus contributing to a ‘self-tolerance’ and are not killed by NK cells³⁰². However, target cells (transformed or infected cells) downregulated or lose the MHC-I molecule, thus lowering the inhibitory signals in NK cells, and are recognized by NK cells as a ‘missing self’. Moreover, upregulation of ligands for activating receptors on NK cells is induced on tumor or infected cells. Consequently, a shift in the balance towards activated NK cells leads to the elimination of target cells directly through NK cell-mediated cytotoxicity or indirectly through the release of pro-inflammatory cytokines³⁰³.

Inhibitory receptors on NK cells

An inhibitory signal is induced from the specific MHC-I-specific receptor in healthy cells, thus, avoiding their destruction by NK cells. However, NK cells can kill target cells (transformed or infected cells) with an altered expression of HLA class I, followed by overexpression of ligands for activating NK cell receptors^{304,305}. The main types of human leucocyte antigen (HLA) class I-specific inhibitory receptors form three groups of NK cell receptors: KIRs (killer Ig-like receptors), NKG2A (natural killer group 2A), and LIRs (leukocyte Ig-like receptors)^{297,306}. KIRs, are type I transmembrane molecules, characterized by two or three Ig-like domains and by either a short or a long cytoplasmic tail. Inhibitory KIRs including KIR2DL1–3, KIR2DL5, and KIR3DL1–3, bind to HLA-A, -B, and -C^{307,308}. CD94/NKG2A belonging to the C-type lectin family is another set of inhibitory receptors expressed as a heterodimer. This receptor recognizes as its ligand the HLA-E, a non-classical HLA class I molecule. LIRs are also inhibitory receptors that belong to the same Ig superfamily as KIRs, it is known as ILTs (Ig-like transcripts) and binds to the non-classical HLA-G molecules³⁰⁹.

Activating receptors on NK cells

Full NK cell activation requires in addition to the lack of MHC-I, a specific recognition of stress-induced molecules by NK cell surface activating receptors. The major NK cell-activating receptors include the natural cytotoxicity receptors (NCRs): NKp46 (NCR1 or CD335), NKp44 (NCR2 or CD336), and NKp30 (NCR3 or CD337). That NCRs bind to a wide variety of ligands expressed on target cells such as viruses, bacteria, and tumor cells³¹⁰. NKp46 is a 46-kDa transmembrane protein that belongs to the Ig superfamily, and it is expressed by all CD56^{bright} CD16⁻ and CD56^{dim} CD16⁺. Both NKp46 and NKp30, a 30-kDa protein, are expressed on mature resting and activated NK cells^{311,312}, however, NKp44 is only expressed on activated NK cells³¹⁰. CD16, another activating receptor that is expressed mainly by the CD56^{dim}, plays also an essential role in antibody-dependent cytotoxicity (ADCC) against IgG-coated target cells^{295,313}. Other important activating receptors include DNAM-1 and natural-killer group 2, member D (NKG2D). NKG2D is a C-type lectin-like type II transmembrane glycoprotein that is expressed as a homodimer on the NK cells³¹⁴. This receptor binds to stress-induced ligands such as MIC-A, MIC-B, and UL16-binding proteins (ULBPs) which are structural homologs to MHC-I molecules³¹⁵. Two additional members of the NKG2 family, such as NKG2C and NKG2E have expressed a heterodimer with CD94^{316,317}, and act as activating receptors that bind to the class Ib molecule Qa-1^{b 318}.

Mechanisms employed by NK cells to control *Mtb* infection

Two effector functions are used by NK cells as primary mechanisms to mediate their protective immunomodulatory effects to control *Mtb* infection. First, NK cells as cytotoxic lymphocytes can directly induce the lysis of *Mtb*-infected cells²⁸⁸. Such cytotoxic function can be initiated through several mechanisms including death receptor ligation and degranulation^{289,290}. Secondly, NK cells can produce diverse inflammatory cytokines in response to the stimulation of activating receptors, in addition to activation signaling induced by inflammatory cytokines^{291,292}.

The role of NK cells in the protective immunity against *Mtb* infection

Recognition of pathogen-associated molecules by specific NK cell receptors represents an essential component of the effector functions of NK cells to mediate protective immunity against *Mtb* infection^{306,319}. It was reported that NK cells can lyse mononuclear phagocytes infected with *Mtb*, through the recognition of up-regulated stress-induced cellular ligands expressed on the

surface of *Mtb*-infected cells, such as UL-16-binding protein (ULBP1) and vimentin (a type-III cytoskeletal protein), by NK cell-activating receptors (NKG2D and NKp46, respectively)^{320,321}. Moreover, it was demonstrated that NKp30 was not involved in the lysis of mononuclear phagocytes, however, such activating receptor was reported to play a role in the lysis of DCs infected with *Mtb* (iDCs), through the recognition of stress-induced cellular ligands, the BAT3³²².

By targeting the infected cells, NK cells can mediate the control of *Mtb* infection through several direct mechanisms such as cytolysis, apoptosis, and phagocytosis. NK cell cytotoxic functions can be mediated through the release of granules comprising perforin, granzymes, and granulysin. Perforin is inserted into the membrane of the target cell and functions as a pore, which facilitates the transport of granzyme and granulysin into the cytoplasm of the target cell³²³. Granzyme B, the major component of NK cells³²⁴, can induce the apoptosis of the target cell, and in the presence of perforin can also lead to nuclear damage²⁹⁰. NK cell cytotoxicity can also be mediated by apoptosis through the recognition of FasL, a ligand expressed on the surface of NK cells, by a death receptor responsible for the lysis of cells, the Fas or CD95^{325,326}. The viability of *Mtb* is limited through the Fas pathway which induces the apoptosis of *Mtb*-infected macrophages³²⁷.

In the lung, during the early stages of TB infection, pro-inflammatory cytokines such as TNF- α , IL-12, and IL-18, released by infected macrophages or dendritic cells (DCs) induce the activation of NK cells, which produce pro-inflammatory cytokines such as INF- γ , TNF- α , IL-22, and other macrophage-activating soluble factors. The release of IFN- γ by NK cells can lead to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS)^{328,329}, followed by the generation of several reactive species that lead to anti-microbial oxidative destruction to membrane lipids and proteins, enzymes and DNA³³⁰. Notably, it was reported that human NK cells can produce the IL-22, a member of the IL-10 cytokine family produced by CD4⁺ and CD8⁺ T cells. It was found that this cytokine plays an important role in the mycobacterial defense through the inhibition of *Mtb* intracellular growth by enhancing the phagolysosomal fusion³³¹.

The bacteria released by the infected cells can directly interact with NK cells by binding the peptidoglycan and mycobacterial cell wall components to TLR2 (Toll-Like Receptor 2), thus

inducing the activation of NK cells and the induction of NKp44. When NK cells are activated, a second interaction is established between NKp44 and its mycobacterial ligands, thus leading to a two-step process that maintains and prolongs the activation of NK cells³³². This direct targeting of *Mtb* bacteria by NK cells can induce the killing of the pathogen through antibody-dependent cellular cytotoxicity (ADCC). It has been previously shown that the cytotoxicity function of NK cells was enhanced by antibodies, thus leading to the limitation of intracellular growth of *Mtb*³³³. Later on, IFN- γ and other cytokines produced by NK cells may lead to the T cell priming and recruitment to the site of infection. Following antigen recognition, CD4⁺ T cells are activated and produce IL-2 which is implicated in the activation of NK cells³³², at the same time NK cells also play an immune-regulatory role on other T cell activities such as CD8⁺ T cells³³⁴ and regulatory T cells (Treg)³³⁵.

Role of glutathione in the control of *Mtb* infection by NK cells

Synthesis of glutathione (GSH), is simultaneously done, for the protection against the toxic effects of ROS and RNI produced by activated macrophages for the control of intracellular *Mtb* infection³³⁶. GSH is a non-protein thiol, not synthesized by mycobacteria, and it is responsible for oxidative balance and cellular homeostasis³³⁷. It has been previously shown that GSH plays an important role in inhibiting the intracellular growth of *Mtb*, through direct anti-mycobacterial function as well as by activating NK cells^{338,339}. It was demonstrated that NK cell cytotoxicity functions were decreased under low GSH levels³⁴⁰. A decreased level of GSH was previously reported in peripheral blood mononuclear cells (PBMCs) isolated from patients with active TB disease when compared to healthy individuals³⁴¹. Thereafter, it was reported that NK cells' cytotoxicity activity was increased following their treatment with N-acetyl cysteine (NAC) and further increased with the addition of cytokines such as IL-2 and IL-12³⁴⁰. Thus, the treatment of NK cells with NAC + IL-2 + IL-12 leads to an increased expression in NK cell ligands such as FasL and CD40L, thus regulating the NK cell induction of apoptosis in target cells and inhibiting the growth of *Mtb*³³⁸.

Role of IL-15 in the control of *Mtb* infection by NK cells

During TB infection, macrophages and monocytes overexpressed the IL-15, a cytokine produced by several cells and acting as a growth factor³⁴². It serves various functions such as survival and

differentiation of NK cells^{343,344}. Moreover, it was demonstrated that IL-15 induces the activation of NK cells and the release of IL-22, an essential cytokine for limiting *Mtb* growth³³¹. Currently, targeting IL-15 modulating NK cell development and activities is a key research focus for obtaining effective human-IL-15 immunotherapy³⁴⁵, therefore such therapy may be promising for TB disease³⁴⁶.

Regulation of NK cell responses by IL-21 during *Mtb* infection

IL-21 is a pleiotropic cytokine that belongs to the γc family of cytokines such as IL-2 and IL-15 that are essential for NK cell activation^{347,348}. During TB infection, IL-21 is produced by activated CD4⁺ T cells and it serves several biological functions including the activation, proliferation, and differentiation of T cells and NK cells³⁴⁹. Recently, a novel role for IL-21 was elucidated in the protection against *Mtb* infection through the regulation of the NK cell responses. It was demonstrated that CD4⁺ T cells of LTBI produced high levels of IL-21 than that produced in active TB patients. Moreover, the IL-2 produced by *Mtb*-activated CD4⁺ T cells activated NK cells and enhances their ability to produce IFN- γ and anti-microbial peptides, thus inducing the lyse of *Mtb*-infected monocytes and inhibiting *Mtb* growth in monocytes³⁵⁰.

Therapeutic effects of recombinant human IL-2 as adjunctive immunotherapy against tuberculosis

IL-2 is a cytokine produced by activated T cells and promotes the activation of NK cells during *Mtb* infection³⁵¹. Moreover, this cytokine was found to increase NK cells' cytotoxicity³⁵². Interestingly, the use of recombinant human interleukin 2 (rhuIL-2) as adjunctive immunotherapy to treat TB disease was previously demonstrated and seems to be promising³⁵³³⁵⁴. More recently, it was shown that such rhuIL-2 treatment increased the NK cell numbers in TB patients, thus improving the host immunity response³⁵⁵.

IL-22 inhibits intracellular growth of *Mtb* by enhancing calgranulin A expression

IL-22 is a cytokine produced by activated NK cells exposed to *Mtb*-infected macrophages, which play a role in limiting the intracellular growth of *Mtb* by enhancing phagolysosomal fusion³³¹. It was found that IL-22 induced in *Mtb*-infected macrophages an increased expression of the gene

encoding the calcium-binding protein (calgranulin A), which in turn mediated the phagosomal maturation. Thus, calgranulin A plays as a central molecule through which IL-22 inhibits the growth of *Mtb* in infected macrophages³⁵⁶.

Possible role of NK cells in the early clearance of *Mtb* infection

Three clinical outcomes are possible upon aerosol exposure to *Mtb*, 90% of infected individuals develop asymptomatic or latent TB infection (LTBI), which is revealed in the cellular immune response to *Mtb*-specific antigens measured by tuberculin skin test (TST) or interferon-gamma release assays (IGRAs). Whilst, 5 to 10% of infected individuals develop symptomatic active TB disease¹. However, several studies have reported that some household contacts (HHCs) who are extensively exposed to *Mtb* do not acquire infection, suggesting that these individuals are naturally resistant to *Mtb* infection and are defined as ‘resisters’²⁸⁵. This resister phenotype is defined as a negative result for both the TST and the IGRAs tests^{285,357}. Moreover, the resisters may have been able to clear *Mtb* infection early with an effective innate immune response before the development of acquired immunity. This phenomenon is called early clearance (EC), which has been the focus of researchers over the past years³⁵⁷. The best indicators of early clearance come from a longitudinal analysis of HHCs of TB patients or those living in high TB burden countries³⁵⁸. In 1941, an evaluation of nursing students showed persistence of negative TST, despite high exposure to patients with TB³⁵⁹. Other studies, including a longitudinal observation of HHCs conversion for two years, also showed persistent negative TST or IGRAs results. The frequency of EC in these studies ranged from 3.4% to 26.8% both in Uganda^{93,360}. Remarkably, it was found that the resister phenotype wasn’t attributable to known clinical, demographic, or risk factors³⁶¹. The EC is most likely attributed to innate immunity, as it occurred before an adaptive immune response can develop^{285,357}. This immune response involves innate immune cells of EC of *Mtb* infection (innate resisters), such as monocytes, macrophages, DCs, neutrophils, and NK cells in addition to innate like-immune cells such as gamma-delta T cells, mucosal-associated invariant T cells (MAIT)²⁸⁶, and invariant NK T cells²⁸⁷. Recently, innate immune cells (NK cells) have received increasing attention in the host defense against *Mtb* infection²⁸⁸. The appearance of NK cells during the early stages of *Mtb* infection supports its possible role in the EC of *Mtb* using direct and indirect mechanisms^{306,319}. Moreover, unlike the other innate immune cells such as macrophages, DCs, and neutrophils, NK cells don’t serve as a

niche for *Mtb* and don't disseminate the bacteria. Thus, NK cells represent critical components of the early immune response against *Mtb* infection. Subsequently, studies investigating immune pathways in NK cells should provide new insights into the mechanisms of EC of *Mtb*, thus providing efficient targets for vaccine development than adaptive immune components.

Evidence of the existence of memory with NK cells against *Mtb* infection

Recently, several discoveries have supported the idea that a small population of NK cells may acquire immunological memory, which can develop long-lived and specific memory cells to a variety of antigens that are capable of rapid recall responses^{362,363}. This phenomenon is termed “innate memory”. Several studies have reported that NK cells have enhanced effector functions induced during the secondary responses to different antigens such as haptens (molecules stimulating the production of antibodies), viruses, and bacteria^{364,365}. This memory-like function can be induced through antigenic and non-antigenic stimuli (activation by cytokines)^{364,366}. Particularly, human studies have supported the evidence of the existence of memory responses mediated by NK cells in the immunity against *Mtb* infection. Molecules such as CD45RO (a marker used to identify memory T cell subsets) and CD27 have been identified in subjects infected with *Mtb*, as markers of “memory-like” NK cells. Previous data showed that NK cells expressing CD45RO⁺ were detected in the pleural fluids of patients with pleural effusion associated with active TB disease. Moreover, it was reported that these memory-like CD45RO⁺ NK cells have increased cytotoxic functions and produced higher levels of cytokines such as IFN- γ and IL-22 in response to stimulation with ILs such as IL-12 and IL-15, respectively, and to BCG when compared to naïve CD45RO⁻ NK cells^{293,294}. Interestingly, it was also demonstrated that memory-like NK cells can be induced following BCG vaccination in both mice and humans, protecting against *Mtb* infection. Mice vaccinated with BCG showed an IL-21-dependent expansion of NKp46⁺ CD27⁺ KLRG1⁺ NK cells existing in the spleen and lymph nodes. These memory-like NK cells were shown to release higher levels of IFN- γ when compared to NKp46⁺ CD27⁻ NK cell subsets. In addition, following their adoptive transfer to unvaccinated animals, they were able to enhance the control of *Mtb* infection in both isolated macrophages and mouse TB-infected lungs. Additionally, individuals with an LTBI showed CD56⁺ CD27⁺ NK cell subsets that we're able to reduce intracellular *Mtb* growth within macrophages more efficiently when compared to the other CD56⁺ CD27⁻ NK cell subset³⁶⁷. Another study has also shown that

memory NK cells directed against *Mtb* infection were modulated by the BCG vaccine. It was reported that BCG vaccination of healthy individuals induced the release of pro-inflammatory cytokines by NK cells in response to *Mtb* infection three months after the first exposure³⁶⁸. Besides, adults with LTBI re-vaccinated with BCG showed an enhancement in IFN- γ response induced by NK cells, and such responses were long-lasting and persistent for up to one year after re-vaccination³⁶⁹. Moreover, BCG vaccination of neonates at birth also induced increased IFN- γ NK cell responses when compared to unvaccinated 5-week and 9-week-old neonates³⁷⁰. Thus the existence of a subset of NK cells with memory-like properties in *Mtb* infection supports the evidence of targeting such cells to improve the efficacy of the TB vaccine, in addition to its potential usefulness in patients with impaired adaptive immunity such as human immunodeficiency virus (HIV).

Clinical studies evaluating the role of NK cells in human TB

Several clinical studies have investigated the association between the abundance and the functionality of NK cells in the peripheral blood and human TB²⁸¹. In some reports, it was demonstrated that NK cells were more abundant in individuals with LTBI compared to healthy controls¹⁸². However, patients with active TB disease were found to have lower frequencies of NK cells when compared to healthy controls and LTBI individuals^{182,281}. Moreover, it was reported that NK cells from individuals with LTBI display higher levels of cytotoxicity when compared to healthy controls¹⁸², however, an impaired cytotoxic function of NK cells was detected in TB patients^{371,372}. Recently, it was established that Broncho-alveolar lavage (BAL) NK cells didn't differ between individuals who resist the development of LTBI and those with LTBI³⁷³. Interestingly, a recent study evaluating the role of NK cells in pulmonary cavity formation found elevated levels of cytotoxicity (abundance of granzyme A and granzyme B) and increased abundance of NK cells in active TB patients with cavity compared to those with non-cavity, suggesting that NK cells may participate in cavity formation in pulmonary TB patients³⁷⁴. Additionally, a research group tested if the abundance of NK cells could inform the progression to active TB disease, subsequently, when analyzing LTBI individuals (including 12 progressors and 20 non-progressors to ATB), they found that NK cell frequencies decreased during the progression to active TB disease, while, no significant changes in the NK cell frequencies were detected within the non-progressors¹⁸². In a few reports in which the NK cell abundance and

activity were monitored throughout anti-TB treatment, the NK cell cytotoxic functions, the frequencies of NK cells in addition to the proportion of CD16⁺ CD56^{bright} NK cell subsets, were restored following successful treatment, reaching levels detected in healthy controls^{182,281}. Moreover, it was also found that cured TB patients had higher levels of NK cells at the end of treatment when compared to pre-treatment, however, patients who didn't respond to treatment showed no significant change in NK cell abundances between pre-and the end of treatment¹⁸². Besides, the expression of NK cell receptors was analyzed by several groups, demonstrating potential markers for distinguishing between different TB infection stages and for treatment monitoring. The expression of inhibitory receptors such as NKG2A on CD16⁺ NK cell subsets was observed to be reduced in active TB patients when compared to healthy controls³⁷⁵, in addition, the expression of TIGIT was decreased on NK cells from LTBI individuals with positive Quantiferon (QFT) when compared to others with QFT-negative²⁸⁰. The evaluation of the proportion of KLRG1, another inhibitory receptor expressed on NK cells, was found to be increased in LTBI individuals when compared to patients with active TB disease, whereas, IFN- γ ⁺ CD56^{bright} CD16^{dim} NK cell subsets expressed higher levels of KLRG1 in active TB patients compared to LTBI individuals. Besides, within the CD56^{bright} CD16^{dim} NK cell subsets, the proportion of this receptor was elevated in active TB patients when compared to healthy controls. Based on these results, they suggested that the expression of KLRG1 on NK cells may be associated with the modulation of the progression from LTBI to active TB disease³⁷⁶. On the other hand, expression of activating receptors such as NKG2C was found to be able to discriminate between different stages of TB infection, it was higher in LTBI individuals (TST-positive) when compared to individuals who resist development of LTBI (TST-negative) and to patients with active TB¹⁸⁸. In addition, the expression of the natural cytotoxicity receptors NKp46 was decreased on NK cells from LTBI individuals with QFT-positive when compared to others with QFT-negative²⁸⁰. In comparison to healthy controls, the frequency of NKp46 on CD16⁺ NK cell and CD56⁺ NK cell subsets was also found to be reduced in active TB patients³⁷⁵. Notably, a persistent decrease in the expression of NKp46 and NKp30 was observed at the end of treatment relative to levels observed in pre-treatment TB patients²⁸¹. Moreover, an assessment of NK cell function was also made. The expression of CD57 on CD56^{dim} NK cells, a marker of differentiation was found to be higher in LTBI individuals with QFT-positive when compared to others with QFT-negative²⁸⁰, in addition, the expression of this marker was

decreased at the end of treatment compared to pre-treatment TB patients¹⁸⁸. Besides, based on the evaluation of the activation marker such as CD69 it was found that the proportion of CD69⁺ NK cells was elevated in active TB patients when compared to LTBI individuals and healthy controls³⁷⁷. Overall, these studies, summarized in Table 1, highlighted a potential role of NK cells in the immune response against pulmonary TB that could inform the stages of TB infection, disease progression in addition to treatment response.

Table 10: Findings from clinical studies evaluating the role of NK cells in human TB

NK cell markers	Intended application	References
NK cell frequencies	Higher in LTBI vs HC	182
	Lower in ATB vs LTBI Lower in ATB vs HC	182,281
	Higher in ATB with cavity vs non-cavity ATB	374
	Decreased in progressor from LTBI to ATB	182
	In cured TB patients: higher at post-treatment vs pre-treatment In uncured TB patients: no significant change at post-treatment vs pre-treatment	182,281
NK cell cytotoxicity		
GZMB⁺PRF⁺ NK cells	Higher in LTBI vs HC	182
granzyme A and granzyme B	Higher in ATB with cavity vs non-cavity ATB	374
Inhibitory receptors on NK cells		
NKG2A on CD16⁺ NK cell	Reduced in ATB vs HC	375
TIGIT	Decreased in LTBI (QFT+) vs QFT-	280
KLRG1⁺ on NK cells KLRG1⁺ on IFN-γ⁺ CD56^{bright} CD16^{dim} NK cells	Increased in LTBI vs ATB Higher in ATB vs LTBI	376
Activating receptors on NK cells		
NKG2C on NK cells	Higher in LTBI (TST+) vs resisters (TST)	188

	Higher in LTBI vs ATB	
NKG2D on CD16 ⁺ NK cells	Lower in ATB vs HC	375
NKp46 on NK cells	Decreased in LTBI (QFT+) vs QFT-	280 375
NKp46 on CD16 ⁺ NK cells and on CD56 ⁺ NK cells	Reduced in ATB vs HC	
Functional markers		
Differentiation marker		
CD57 on CD56 ^{dim} NK cells	Higher in LTBI (QFT+) vs QFT-	280
CD57 on NK cells	Decreased at post-treatment vs pre-treatment	188
Activation marker		
CD69 on NK cells	Elevated in ATB vs LTBI Elevated in ATB vs HC	377

LTBI: latent TB infection. ATB: active tuberculosis. HC: healthy controls. GZB: granzyme B. PRF: perforin. QFT+: positive Quantiferon test. QFT-: negative Quantiferon test. TST+: positive tuberculin skin test. TST-: negative tuberculin skin test.

Conclusions and future directions

Studies of human TB have focused for years on T-cell biology. However, innate immunity, a critical component of the immune system was increasingly recognized as playing a crucial role in the defense against *Mtb* infection. NK cells that belong to the innate immunity, have received attention in studies focusing on TB research, due to their involvement in the control of *Mtb* infection through several mechanisms including cytotoxic activity, the release of cytokines addition to their ability to develop immunological memory that induces enhanced effector functions during secondary responses. Thus, we must look for novel immune cells beyond T cells, and redirect the focus on these lymphocytes which may represent a new protective target against TB. A current priority for TB research is to understand the mechanisms of early clearance of *Mtb*, fortunately, there is a suggestion from studies that NK cells are involved in early *Mtb* clearance. Thus, human studies investigating immune response induced via NK cells between resisters to LTBI and others with LTBI may provide additional insights into the early

clearance of *Mtb* infection, thus providing new targets for drugs and vaccines and importantly opening new fields in TB prevention. In addition, new perceptions gained from *in-vitro* and *in-vivo* studies of NK cells found that there is a correlation between NK cells and the stages of *Mtb* infection (active TB vs LTBI), and reported the ability to predict progression from LTBI to active TB disease and to monitor treatment response by evaluating the levels of NK cells. These findings holding promise must be validated in larger cohort studies using blood and other samples such as bronchoalveolar lavage, in addition, it should be translated into clinical use, which could support NK cells used as a new innate target to improve vaccine design and efficacy for TB patients in addition to immunocompromised individuals suffering from HIV, by conferring them with a T-cell independent protective immunity.

CONFLICT OF INTEREST

None of the authors have a conflict of interest.

AUTHOR CONTRIBUTIONS

The manuscript was written by RB and FA. All authors have read, reviewed, and approved the final manuscript.

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CHAPTER 3: DISCUSSION & FUTURE PERSPECTIVES

1. Non-sputum-based TB tests: an urgent need for the END TB strategy

The WHO's END TB Strategy includes an ambitious target to end the global TB epidemic by 2035³⁷⁸. An essential component to achieve this goal is conducting research leading to drug discovery, improving TB diagnosis, and developing an effective vaccine. As highlighted in my thesis, there is a crucial need for alternative non-sputum-based TB tests. This urgent need is a result of the limitations associated with the current WHO's recommended sputum-based TB tests⁹, in terms of time of results, accuracy, and suitability for use as a POC test. In this context, the WHO and the Foundation for Innovative New Diagnostics (FIND) have determined the following sections as TB research priority needs: (a) Tests for TB screening and triage, (b) Point-of-care biomarker-based tests that do not require sputum, (c) Tests to replace smear microscopy as the initial TB diagnostic test, (d) Next-generation drug-susceptibility tests to inform TB treatment, (e) Next-generation sequencing for detection of drug resistance, (f) Tests for detecting incipient TB and for treatment monitoring, and (g) Tests for TB infection.

Importantly, a series of target product profiles (TPPs) which detailed the different criteria that should be met in those tests were set^{14,379}. Besides, for each of the TPPs, detailed minimal and optimal criteria such as sensitivity, specificity, time to results, and cost were identified. The general aim of this work was to evaluate new TB diagnostic tools according to the recommendations of the WHO.

In this chapter, I will discuss how the studies reported in my thesis have contributed to TB research within those priority needs. Indeed, my work focused on the evaluation of novel non-sputum-based TB tests in the framework of TB diagnosis and the identification of simple and rapid biomarkers for monitoring TB treatment response in adults. First, we evaluated the performance of a human-blood transcriptomic signature; RISK6, in the context of TB diagnosis. Thereafter, we focused on the identification of blood-based immune biomarker tests for TB treatment monitoring. For this purpose, we evaluated simple hematological parameters using an inexpensive technique, followed by the performance of an in-depth phenotypic analysis using a high-dimensional single-cell technology to characterize and identify specific immune cell subsets that could be used as a potential markers for monitoring TB treatment response.

2. Conducting multicenter research: a step toward a well-designed study

My work was carried out in the framework of the HINTT project which represents a multicenter prospective cohort study evaluating the prognostic value of blood-based biomarkers for TB diagnosis and for monitoring TB treatment response. It was conducted within the GABRIEL Network²⁶⁴ in five different countries including Bangladesh, Georgia, Lebanon, Madagascar, and Paraguay, considered high TB burden countries except for Lebanon. Notably, the strength of this study was the choice of countries located in three different continents, which is of particular interest as so much work on TB research has been performed in other countries such as South Africa^{222,233} and China³⁸⁰. Therefore, testing the performance of such tests or biomarkers in diverse cohorts from distinct patient populations and different geographical regions will be an important validation step of the significance and the reproducibility of results. It will allow comparing data and effects between the study sites and, most importantly, validating the generalizability among patient populations from different ethnic groups and across institutions. Remarkably, this multicenter study has promoted capacity building, knowledge sharing, and technology transfer between the different institutions involved in this work.

3. Performance of a six-gene blood transcriptomic signature for TB diagnosis in adults

Transcriptomic studies have attracted the attention of the scientific community within the last years and, as previously mentioned, human blood transcriptomic signatures have shown promising in the field of TB disease. Accordingly, in publication 1, we assessed the performance of RISK6; a PCR-based six-gene blood transcriptomic signature, as a screening and triage test for TB, and also evaluated its utility as a biomarker for monitoring TB treatment response that could be an alternative to the current sputum-based TB tests. Adult non-immunocompromised patients with bacteriologically confirmed active pulmonary TB (ATB), latent TB infection (LTBI), and healthy donors (HD) were enrolled. Moreover, the ATB patients were followed-up during and after treatment completion.

First, we demonstrated that the performance of the RISK6 signature to discriminate between ATB patients and HD displayed a comparable performance in the four cohorts and reached excellent AUC values of 0.94 (95% CI 0.89 to 0.99), with 90.9% sensitivity and 87.8% specificity. Thus, RISK6 signature achieves the minimum criteria set by the WHO for a non-sputum-based screening test¹⁴. It should be noted that RISK6 signature has been previously evaluated in other seven independent cohorts³⁸¹. Importantly, when we compared our results to the previously reported ones, similar data in terms of score range and score changes over time were obtained, despite the heterogeneity of our studies, study design, and marked technical differences. Collectively, the evaluation of the performance of RISK6 within different cohorts from different geographical locations is considered as an important next validation step for the reproducibility of this signature and most importantly to increase its generalizability.

On the other hand, an additional application of RISK6 was found: this signature was able to stratify TB patients according to the infection status. Indeed, this assay has yielded an AUC of 0.93 (95% CI 0.85-1) with 90.9% sensitivity and 88.5% specificity for discriminating the active form of TB disease from the LTBI. Thus, our results are promising, but further validation in larger cohorts with a larger number of latently infected individuals will allow testing such performance. These results are comparable with those of the previously identified 3-genes²¹⁹ and 20-genes¹² transcriptomic studies that were also able to distinguish ATB patients from those with LTBI. However, while several transcriptomic signatures have been reported to have a powerful ability to discriminate between ATB patients LTBI and HD^{219, 12,207,208, 155, 214}, some of them were not generalizable^{219,382}, and others require the measurement of a high number of genes thus limiting their applicability in laboratories located in resource-poor settings. Indeed, among the different published transcriptomic signatures, only two (Sweeney³²¹⁹ and Sambarey¹⁰²¹⁴) were found to achieve the minimal WHO TPPs for a TB triage test³⁸³.

Importantly, we also showed that RISK6 signature scores and mycobacterial loads were correlated. Indeed, consistent with previous studies evaluating either the Xpert MTB/RIF test or the CRP concentration measurements^{384,385}, our results revealed that RISK6 directly correlated with sputum smear grades, suggesting its possible application as a potential tool to identify patients with high transmission risk.

As blood samples were also collected from ATB patients at diagnosis, throughout their anti-TB treatment, and after treatment completion, we were interested to determine if RISK6 could be used as a clinically relevant biomarker for TB treatment response. Subsequently, we demonstrated that RISK6 scores were significantly higher in ATB patients at baseline when compared to the levels observed after two months of treatment. Moreover, the RISK6 scores continued to decrease progressively until the end of treatment reaching scores observed in HD, which confirmed the previous data³⁸¹. These findings were consistent with previous data showing a significant drop in RISK6 scores³⁸¹ and other transcriptomic signature^{215,386 237,387 207,221} throughout TB treatment. Taken together, our results suggested that RISK6, as a blood transcriptomic signature, is modulated during anti-TB treatment, and may thus represent a useful tool to monitor the response to TB treatment.

Notably, many tools are currently in the pipeline set by the WHO and FIND for non-sputum-based TB tests. However, Sweeney³²¹⁹ and RISK6³⁸¹ signatures are the most advanced in the pipeline⁴. Fortunately, our findings reported in publication 1 are considered a step forward to validate the potential use of RISK6 as a non-sputum-based TB diagnostic test as it was evaluated in four additional independent cohorts from different countries and ethnicities.

Finally, the overall findings in my present work and other reported study³⁸¹ provide strong proof that RISK6 can be applied as a non-sputum-based screening and triage test that met the WHO TPP benchmarks. These data support the efforts to incorporate this test into a point-of-care test to reduce its complexity and costs thus facilitating its implementation in resource-poor settings. Indeed, such a novel, simple, and inexpensive tool is highly needed to improve TB diagnosis. It may help reducing TB spread and transmission, especially among populations with higher TB risk, and who are not able to produce sputum samples (e.g. children and HIV co-infected patients). Unfortunately, such populations lived often in areas with high TB burden that are usually disturbed with poverty.

4. Blood-based immune biomarkers predict early TB treatment response

In the second publication, using simple and inexpensive blood cell counts, we were able to identify simple biomarkers such as the decrease in WBC count and the increase in the proportion of lymphocytes occurring throughout TB treatment. Such findings were consistent with previous studies showing that ATB disease is associated with high WBC counts when compared to healthy donors^{388,389}. Most importantly, in this study, we also highlighted that the risk of treatment failure was associated with high WBC counts in addition to low proportions of lymphocytes detected in active TB patients at baseline. Such findings are important given that WBC counts are usually evaluated in TB patients at diagnosis and throughout treatment³⁹⁰.

Overall, in this publication, we identify predictive biomarkers that changed significantly in the early stages of TB treatment and that would help predict early treatment failure. Remarkably, identification of such biomarkers requires the collection of a small volume of blood and relies on the use of a cheap, simple, and rapid test, thus facilitating its use in clinical poor settings in contrast to other complex methods. Subsequently, such characteristics will support the efforts to incorporate this tool into a POC test ensuring rapid and accurate prediction of TB treatment response.

5. Characterization of Mtb-specific immune response using a novel high dimensional single-cell analysis technology

Advances in the field of immunology have identified promising candidate immune markers for monitoring TB treatment response. Fortunately, recent advances such as the emergence of immune profiling tools have allowed an enhanced understanding of the complexity of the immune response, thus a greater identification of new targetable biomarkers based on *Mtb*-specific T-cell phenotypes and marker expression profiles^{146,176}. However, such comprehensive studies of the complex human immune system require in-depth analysis and characterization of immune subpopulations using high-throughput single-cell analysis technologies¹⁷⁶. The emergence of novel cytometry-based assays has sought to address this need. Recent studies have highlighted the potential of spectral flow cytometry as an important technology that allows

combining the phenotypic and functional analysis of a wide variety of immune cell subsets, using more than 40 parameters in a single panel^{259,260}, in the context of different domains such as cancer²⁵⁸ and COVID-19 research²⁶³. Therefore, we were interested to benefit from the high dimensional analysis accessible using spectral flow cytometry to deeply characterize the specific immune response against tuberculosis within our multi-centric clinical cohort study. We have identified a series of immune cell subsets related to T and NK cell subsets that were potentially useful to monitor TB treatment response. Importantly, phenotypic and functional profiles have been attributed to each of the identified T and NK cell subsets. Therefore, the detected changes in our study are affected by the antigenic stimulation condition and are likely to reflect a true modification of the host immune response with anti-TB therapy.

5.1. Candidate T-cell biomarkers for monitoring TB treatment response

In successfully treated TB individuals, significantly higher frequencies of TEMRA CD7⁺ CD8⁺ T cells and significantly lower frequencies of EM CD7⁻ CD27⁻ CD8⁺ T cells were detected when compared to patients at baseline. Such peripheral CD8⁺ T cell phenotypes have been previously reported in TB disease patients compared to latent TB infected individuals^{182,276}, healthy controls²⁷⁷, and throughout anti-TB treatment^{172,275}. Using mass cytometry in a prospective cohort study of adult pulmonary TB patients, our group has previously highlighted the shift in CD8⁺ T cell abundance upon stimulation with antigens of the TB2 tube of the QFT-P assay²⁶⁹. On the other hand, a decrease in the differentiated EM CD27⁻ CD7⁻ CD4⁺ T cells population was detected at the end of treatment. Previously, it has been reported that TB was characterized by a higher frequency of highly differentiated EM CD4⁺ T cells (CD45RA⁻ CCR7⁻ CD27⁻)²⁷⁸.

5.2. NK cell-based biomarkers for monitoring TB treatment response

Few studies have investigated the implication of innate immunity in the protective mechanism against *Mtb*. Hence, we were interested in studying NK cells, one of the critical components of early innate immunity in the context of TB disease. Notably, we performed the first spectral flow cytometry study assessing the contribution of different NK cell subsets in TB patients at baseline and throughout TB treatment. Recent work has suggested a link between the abundance of NK cells and TB outcome as it showed a significant increase in the abundance of these cells in cured

TB patients when compared to baseline levels¹⁸². Our results showed an increase in the abundance of activated NK cells and a decline of inactivated NK phenotypes at the end of TB treatment compared to baseline, suggesting that NK cells may play an important role in *Mtb* clearance. Previously, it was reported that NK cell frequency was restored following successful anti-TB treatment compared to baseline^{182,281}. Another work studying LTBI individuals (including 12 progressors and 20 non-progressors to TB disease) has demonstrated that NK cell frequencies decreased during the progression to TB disease, whereas no significant change in the NK cell frequency was detected among the non-progressors¹⁸². These data emphasize the need to further study NK cell dynamics during *Mtb* infection and decipher the phenotypic patterns associated with different stages of infection.

The use of a novel immune assay, the spectral flow cytometry, to explore the immune response in TB was evaluated for the first within our multi-cohort study. Thereafter, we succeeded to highlight novel immune-based markers using an accessible sample such as blood that could be useful to monitor TB treatment response. This blood-based test may be able to improve the diagnosis of TB allowing faster treatment and thus reduction of TB transmission.

In sum, our data support the concept that studying the immunological profile of ATB patients throughout treatment in the context of *Mtb*-specific T cell and NK cell subsets will generate information that may be useful in biomarker discovery. Our results have identified four cellular phenotypes differentially detected between baseline and treatment completion. TEMRA CD8⁺ T cells and activated NK cell subset increased over time, whereas EM CD4⁺ T cells and inactivated NK cell subset decreased over time. Moreover, changes in phenotypic pattern as well as the combination of the phenotypic profile and the expression of CD38 on TEMRA CD8⁺ T cells and on NK cells could be surrogate immune markers associated with TB control. To use these T cells and NK cell subsets as a tool to monitor response to treatment, there is a need to validate such findings in a larger cohort and evaluate if such subsets are associated with treatment failure. To achieve this, future studies would have to follow up a cohort of successfully treated TB patients and others with treatment failure. The aim will be to determine the usefulness in evaluating treatment outcomes and the utility in clinical settings, thus providing suggestions that could lead to new intervention strategies in TB vaccine and drug developments.

6. A crucial need for validation studies

Potential TB diagnosis/treatment biomarkers identified within our multicenter prospective cohort project should be confirmed by additional independent validation studies. Indeed, in our work evaluating RISK6 in the context of TB diagnosis, a relatively small number of LTBI individuals was recruited. Hereafter, to better estimate specificities and sensitivities for a triage test to discriminate the active form of TB disease from the LTBI, it is required to validate our findings in cohorts with a larger number of LTBI individuals from different study sites. Besides, the use of RISK6 as a diagnostic test for TB relies on its ability to distinguish between ATB and other diseases. Thus, it would be relevant to evaluate the specificity of the RISK6 scores in comparison to respiratory diseases other than TB, which are considered as most difficult to distinguish with. On the other hand, we identified potential immune-based biomarkers for monitoring treatment. However, an ideal treatment monitoring biomarker study would include a large number of participants with treatment failure or relapse to determine how such identified biomarker tracks with treatment response. Additionally, we excluded diabetic and HIV-positive patients and immunosuppressed individuals in general, and our studies were restricted to adults. Therefore, additional validation studies are needed in immunocompromised, HIV-positive patients, individuals with co-morbidities and across different age groups including children and adolescents.

7. Conclusions and perspectives

The studies reported in this thesis contribute to the goal of improving diagnostic and treatment monitoring tests for TB disease according to the TB research priority needs defined by the WHO. A key advantage of such transcriptomic and immunological tools evaluated in this work is that they rely on rapid and simple non-sputum-based test using whole blood which is an easily accessible sample. The first section of the thesis provides strong proof that a six-gene blood transcriptomic signature: RISK6, can be applied as a screening and triage test that met the WHO TPP benchmarks. This host response-based gene signature may be also used for stratifying patients according to their TB infection status, as well as for monitoring patients throughout treatment. The second section identified simple and rapid biomarkers, based on white blood cell counts and lymphocytes proportions, for monitoring TB treatment response. Interestingly, we

demonstrated that measurement of such identified biomarkers would help predict early treatment response such as treatment failure. In the last section, we identified four cellular phenotypes differentially detected between baseline and treatment completion. TEMRA CD8⁺ T cells and activated NK cell subset increased, whereas EM CD4⁺ T cells and inactivated NK cell subset decreased over time. Moreover, our results also suggested that the measure of the expression of CD38⁺ on TEMRA CD8 T cell and on NK cells could be a promising marker associated with TB control. Finally, data from this work provide strong evidence that blood-based tests are promising surrogates for improving TB diagnosis and treatment monitoring.

7.1. Future direction in TB research

Several recent advances have been done in TB research. New approaches for TB diagnosis have been evaluated and succeeded in adults. However, children must be included in this research to ensure that such neglected populations benefit from advances in TB research in tandem with adults. In the last decade, several calls to act on pediatric TB have attracted the attention of researchers to the neglected issue of pediatric TB and have led to important advances³⁹¹. However, despite the TB diagnostic research advances, they remains an urgent priority for pediatric TB research. In, this context, the WHO are now collecting data on pediatric TB and boosting national TB programs to address pediatric TB as a high priority research³⁹². The findings of this thesis highlighted several promising findings in the field of TB diagnosis and treatment monitoring in adults, and reinforce the need for further validation studies to improve the performance of such identified TB diagnostic and treatment monitoring tests to be confirmed in children.

On the other hand, predicting the risk of progression from LTBI to ATB disease is a key area that was not addressed in our studies. Identifying patients at risk of progression would enable resources to be directed to treat patients at higher risk of developing the active form of the disease. To date, several transcriptomic signatures are currently in the pipeline set by the WHO and FIND for non-sputum-based TB tests for detecting incipient TB and for treatment monitoring¹⁴. While all of these tests are in the early stages of development, Sweeney: a three genes signature, and RISK6; a six genes signature that was evaluated and validated in this thesis, are the most advanced in the pipeline to predict the risk of progression to ATB disease and for

treatment monitoring¹⁴. Notably, the findings generated in our studies will be translatable to other associated priorities in TB research. This includes the management of LTBI and is currently done through an ongoing study carried out by the Mérieux Foundation and named APRECIT (Amélioration de la PRise En Charge de l'Infection Tuberculeuse latente en milieu communautaire). Improving screening, overall management of LTBI, and predicting the risk of progression from LTBI to ATB disease are the main objectives of this study. Analysis of T cell subset changes using workflows similar to those shown in this thesis could be performed in LTBI and uninfected household contacts, to further characterize immune factors associated with a higher risk of progression to ATB disease.

7.2. *Translation to clinical applications*

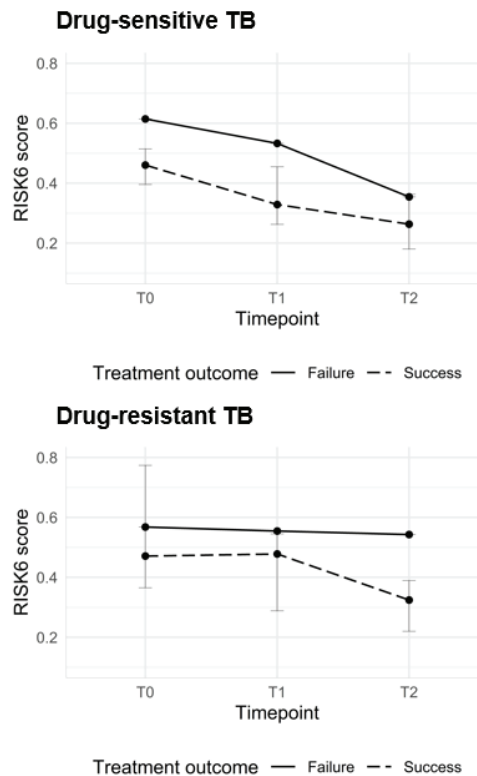
A major issue in TB research is the lack of translation from clinical research to commercially available clinical applications³⁹³. Another major issue is the unavailability of simple, rapid, and inexpensive point of care tests that can be implemented in laboratories located in resource-poor settings³⁹⁴. Indeed, many TB diagnostic tests are currently not suitable to be implemented in low income areas^{394,395}. Our present data provide strong proof that the RISK6 signature can be applied as a non-sputum-based screening and triage test that met the TPPs set by the WHO and FIND. This signature can be applied using a simple qRT-PCR and require the measurement of a small number of genes which facilitates its implementation in clinical laboratories in contrast to other complex methods. Interestingly, our group plan currently to validate the practical utility of the RISK6 signature as a diagnostic TB test using dried blood spots (DBS). Subsequently, this supports the efforts to incorporate RISK6 assay into a point-of-care test ensuring rapid and accurate diagnosis of ATB cases. On the other hand, we also identified potential biomarkers for monitoring treatment response using two immune-based assays. In publication 2, we identified a predictive biomarker that would be helpful in the early prediction of treatment failure, using a routinely, rapid and inexpensive tool. These features offer an advantage to translate it into a point of care application to help clinicians predicting early treatment response of TB patients. Besides, our data on a high-dimensional single-cell technology, based on treatment monitoring using phenotypic profiles of *Mtb*-specific T and NK cells as surrogate markers, warrants further research to translate such findings into clinical application leading to their use in clinical settings. Importantly, such identified immune markers may be highly beneficial to design studies

evaluating novel TB immunotherapy strategies. Finally, in this work, we reported promising findings and advances within TB research, which represent a step toward being able to offer a new, simple, and promising tool for TB diagnosis and treatment monitoring.

Annexes

1. Supplementary data from original publications

1.1. Annex 1 – Supplementary data from publication 1



Supplementary Figure 1: Ability of RISK6 signature to stratify TB patients according to ultimate treatment outcome. Longitudinal Kinetics of RISK6 signature scores across TB treatment time (T0: baseline; T2: 2 months after initiation and T2: at the end of treatment) in patients with cure and those with a treatment failure, stratified into drug-sensitive (n= 95) and drug-resistant TB cases (n= 11). Dots depict medians and error bars represent the IQR.

Supplementary Table 1. TaqMan PCR primer-probes in the RISK6 signature

Primer-Probes
FCGR1B FAM (60X), Hs02341825_m1
GBP2 FAM (60X), Hs00694846_g1
SERPING1 FAM (60X), Hs00934329_m1
SDR39U1 FAM (60X), Hs01016970_g1
TRMT2A FAM (60X), Hs01000041_g1
TUBGCP6 FAM (60X), Hs00363509_g1

Supplementary Table 6: Performance of RISK6 for monitoring TB treatment response (Baseline vs T1, T2 & T3)

Time points	T1	T2	T3
AUC	0.697 (0.571-0.796)	0.871 (0.776-0.943)	0.904 (0.826-0.966)
Optimal threshold	0.497 (0.413-0.508)	0.411 (0.344-0.457)	0.397 (0.363-0.456)
Sensitivity	0.75 (0.531-0.906)	0.857 (0.667-1)	0.929 (0.786-1)
Specificity	0.62 (0.46-0.8)	0.78 (0.64-0.94)	0.84 (0.68-0.94)
Positive predictive value	0.556 (0.468-0.677)	0.631 (0.5-0.867)	0.619 (0.448-0.812)
Negative predictive value	0.798 (0.694-0.912)	0.932 (0.864-1)	0.975 (0.923-1)
p value	<0.0001	<0.0001	<0.0001

Supplementary Table 7: Receiver operating characteristic curve analysis of the performance of RISK6 signature to distinguish active from vs Treated TB patients

ATB vs TREATED TB patients							
Cohort	Cut-off	Sensitivity%	Specificity%	Cases, n	Controls, n	AUC	AUC 95%CI
Georgia	> 0.2447	93.8	65.4	32	26	87.60%	78.2 - 97.1%
Madagascar	> 0.3720	90.9	93.9	44	33	96.10%	91.7 to 100%
Lebanon	> 0.3009	90.5	73.3	21	15	90.20%	80.3 - 100%
Bangladesh	> 0.3625	90.9	65.2	44	23	91.80%	85.5 - 98.1%
All	> 0.3221	90.1	72.7	141	97	90.90%	87.2 - 94.5%

Supplementary Table 8: Association between baseline demographic and clinical parameters of active TB patients with treatment outcome

	Descriptive analysis (n=102)				Logistic regression - association with treatment failure				
	Cured (n=98)	Failure (n=4)	p		Univariate analysis		Multivariate analysis ^a		
					OR_95%CI	p	aOR_95%CI	p	aic
Age (years)	26.5 (21.25-35)	36 (28.75-42)	0.343		0.982 (0.926 - 1.061)	0.587	-	-	-
Gender (male)	59.2% (58/98)	75% (3/4)	0.647		-	-	-	-	-
Drug resistance	15.3% (15/98)	50% (2/4)	0.128		-	-	-	-	-
Smoking habit	45.9% (45/98)	75% (3/4)	0.34		-	-	-	-	-
BMI at baseline	18.63 (17.12-20.86)	15.92 (15.53-16.06)	0.008		1.743 (1.103 - 3.194)	0.036	2.338 (1.256 - 6.412)	0.029	32.9
Alcohol consumption	18.4% (18/98)	25% (1/4)	0.568		1.525 (0.103 - 12.355)	0.713	2.044 (0.117 - 35.52)	0.601	41.3
Jail detention history	5.2% (5/96)	25% (1/4)	0.222		6.067 (0.273 - 58.765)	0.147	7.765 (0.255 - 206.394)	0.185	39.9
Absolute WBC count at T0 (cells/ mm3)	9500 (7650-11345)	12200 (11450-12650)	0.069		1 (0.999 - 1)	0.163	1 (0.999 - 1)	0.129	39
Lymphocytes at T0 (% of WBC)	18 (14-23.5)	15.5 (13-19.5)	0.443		1.043 (0.917 - 1.205)	0.55	1.068 (0.925 - 1.278)	0.407	40.8
Monocytes at T0 (% of WBC)	5 (3-8.28)	3.5 (2.5-4.25)	0.128		1.41 (0.966 - 2.535)	0.156	1.375 (0.88 - 2.784)	0.272	39.8
Score RISK6 at T0	0.46 (0.38-0.52)	0.56 (0.54-0.58)	0.046		0.016 (0 - 15.048)	0.191	0.069 (0 - 48.572)	0.424	40.9
IFN-γ [HBHA] at T0	0.09 (0.01-0.4)	0.27 (0.11-1.4)	0.343		0.968 (0.722 - 1.747)	0.864	0.951 (0.658 - 1.768)	0.816	41.5
Baseline lymphocytes <16%	31.6% (31/98)	50% (2/4)	0.593		0.58 (0.126 - 2.665)	0.451	0.592 (0.121 - 2.934)	0.493	41.1
Baseline WBC >11,450/mm3	23.5% (23/98)	75% (3/4)	0.05		5.016 (1.13 - 42.827)	0.053	9.277 (1.563 - 137.339)	0.034	35.4
Baseline WBC>11,450/mm3 and lymphocytes <16%	14.3% (14/98)	25% (1/4)	0.476		134.318 (0 - NA)	0.992	290.051 (0 - NA)	0.994	37.1

Data are given as % (n) or as a median levels (interquartile ranges). Failure was defined as a patients with positive results of sputum culture at T2 and/or T3. BMI: body mass index. WBC: white blood cells. OR: odds ratio. aOR: adjusted odds ratio. CI: confidence interval. AIC: Akaike Information Criterion. a. Adjusted for age, sex, country of origin, drug resistance strain, and smoking habit.

1.2. Annex 2 – Supplementary data from publication 2

Drug resistant patients were recruited in Bangladesh (17/28, 60.7%), Georgia (10/28, 35.7%), and Paraguay (1/28, 3.6%). Among them, 2/28 (7.1%) were rifampicin-resistant (RR-TB), 4/28 (14.2%) were poly-resistant (PR-TB), 17/28 (60.7%) were MDR-TB, 4/28 (14.2%) were pre-extensively drug resistant (pre-XDR-TB), and 1/28 (3.7%) was XDR-TB. All pre-XDR-TB or XDR-TB patients were recruited in Georgia.

Supplementary Table 1. Therapeutic regimens used in each study site.

	Bangladesh (n = 38)	Georgia (n = 33)	Lebanon (n = 18)	Madagascar (n = 36)	Paraguay (n = 27)
DS-TB patients	New cases (n = 16) 2HRZE/4HR ¹	New cases (n = 15) Re-treated (n = 2) 2HRZE/4HR ^{1,4}	New cases (n = 16) Re-treated (n = 2) 2HRZE/4HR ^{1,4}	New cases (n = 31) Re-treated (n = 5) 2HRZE/4HR ^{1,4}	New cases (n = 20) Re-treated (n = 6) 2HRZE/4HR ^{1,4}
	Re-treated (n = 5) 2SHRZE/1HRZE/5RHE ²	New cases (n = 5) 2HRZE/4HRE ⁵			
		Poly-DR (isoniazid-resistant, n = 1) RZE ⁶			
DR-TB patients	New cases (n = 11) Re-treated (n = 6) 4-6 Km-Mfx-Pto-Cfz-Z- HHigh dose-E/5-6 Mfx-Cfz- Z-E ³	New cases (n = 9) and re-treated (n = 1) RR-TB: H+E+Z+Km+Mfx+Pto+Cfz / Mfx+E+Z+cfz; MDR-TB: H+Z+Km+Lfx+cfz+Bdq / Z+Lfx+cfz+Bdq; Cm+Lfx+Pto+Cs+Cfz+H / Cm+Lfx+Cs+Bdq+Lzd / Lfx+Cs+Bdq+Lzd; Z)+Cm+Mfx+Cs+Pto+Cfz / Lfx+Cfz+Bdq+Lzd Pre-XDR-TB: (Z)+Lfx+Pto+Cfz+Bdq+Lzd / Cs+Cfz+Bdq+Lzd; Bdq+Cfz+Lfx+H+Pto+E+Z / Bdq+Cfz+Lzd+H+E / Bdq+Cfz+Lfx+H+Z / Bdq+Cfz+Lfx+E+Z; H+Z+Km+Lfx+Cfz+Bdq / Z+Lfx+Cfz+Bdq; Cm+Lfx+Cs+Cfz+Bdq+Lzd / Lfx+Cs+Cfz+Bdq+Lzd XDR-TB: Bdq+Lzd+Cfz+Cs	No cases	No cases	Re-treated (n = 1) 4 Km-Hhigh dose- 12E-Z-Mfx-Pto- Cfz

Footnotes: DS-TB: drug-sensitive. DR-TB: drug-resistant. RR-TB: rifampicin-resistant. MDR-TB: multi-drug resistant. XDR: extensively drug resistant. Numbers refer to the total duration in months for each drug. Drug regimens were prescribed according to each study site's National TB program and could not be altered.

Supplementary Table 2. Methods for microbiological diagnosis of tuberculosis, drug susceptibility testing, and cell count in each study site.

Country	Sputum culture			Smear microscopy		Drug susceptibility testing (DST)				Reference of cell counting machine
	MGIT	LJ	OK	ZN	Auramine O	GeneXpert	LPA	LJ-DST	MGIT-DST	
Bangladesh		X		X		X		X		Callatac ES Automated Hematology Analyzer, MEK-7300 (Nihon Kohden) XT-20001 (Sysmex) CELL-DYN Ruby (Abbott) XT2100i and XN1000 (Sysmex) XT-20001 (Sysmex)
Georgia	X	X		X	X	X			X	
Lebanon	X	X		X		X			X	
Madagascar	X				X	X	X	X		
Paraguay			X	X		X				

Footnotes: Crosses indicate the method(s) performed in each study site.

MGIT: Mycobacteria Growth Indicator Tube (liquid medium). LJ: Löwenstein-Jensen (solid medium). OK: Ogawa Kudoh (solid medium). ZN: Ziehl-Neelsen staining. LPA: line probe assay. Smear microscopy was performed after NALC-NaOH decontamination.

Critical drug concentrations used for DST:

MGIT: Streptomycin 4.0 µg/mL, Rifampicin 40.0 µg/mL, Isoniazid 0.2 µg/mL, and Ethambutol 2.0 µg/mL.

L-J: Streptomycin 1.0 µg/mL, Rifampicin 1.0 µg/mL, Isoniazid 0.1 µg/mL, and Ethambutol 5.0 µg/mL.

Supplementary Table 3. Normality assessment of evaluated continuous variables.

Timepoint	T0		T1		T2	
	W value	p-value	W value	p-value	W value	p-value
Body mass index	0.947	>0.001	0.975	0.011	0.977	0.021
Leucocytes (/mm ³)	0.971	0.003	0.444	>0.001	0.575	>0.001
Monocytes (%)	0.945	>0.001	0.964	0.001	0.966	0.001
Lymphocytes (%)	0.957	>0.001	0.924	>0.001	0.976	0.009
Age	0.881	>0.001	-	-	-	-

Footnotes: normality was assessed using the Shapiro-Wilk test. The H0 hypothesis of normality was rejected for p<0.05.

Supplementary Table 4. Sociodemographic characteristics of the cohort, stratified by country.

N	Bangladesh 38	Georgia 33	Lebanon 18	Madagascar 36	Paraguay 27	p
Patient demographics						
Age (years), median	22 (18.2-28) ^G	35 (28-44) ^{B,M,P}	29.5 (22.2-36.5)	26 (19.7-36.7)	29 (23-39)	>0.001
Sex (male)	65.8% (25/38)	78.8% (26/33)	55.6% (10/18)	55.6% (20/36)	55.6% (15/27)	0.22
Drug-resistant strain	44.7% (17/38) ^{L,M,P}	30.3% (10/33) ^M	0 ^{B,G}	0 ^{B,G}	3.7% (1/27) ^B	>0.001
BMI at inclusion, median	17.6 (16.3-20.7) ^{G,L,P}	20.3 (18.8-23.6) ^{B,M}	20.7 (19.4-21.4) ^{B,M}	17.1 (16.3-18.2) ^{G,L,P}	20.2 (17.8-22.4) ^{B,M}	>0.001
White blood cell absolute count at inclusion (/mm ³)	9500 (8050-10975)	9800 (7600-12000)	8455 (7297.5-10875)	9370 (5802.5-12002.5)	11110 (8590-12585)	0.29
Lymphocyte proportion at inclusion (% of WBC)	20 (18-26.7)	18 (15-22)	16.5 (14.3-19.5)	17.6 (12.6-24.2)	17 (13-21)	0.10
Monocyte proportion at inclusion (% of WBC)	3 (2-4) ^{G,L,M,P}	4 (4-6) ^{B,L,M,P}	7.26 (5.7-9.2) ^{B,G,P}	9.9 (6.8-11.3) ^{B,G,P}	0 (0-2) ^{B,G,L,M}	>0.001
Number of household contacts, median	4.5 (3-7)	4 (2.75-4.25)	5 (4-6)	5 (4-6)	5 (4-7)	0.056
BCG	84.2% (32/38) ^{G,L}	36.6% (12/33) ^{B,M,P}	26.7% (4/15) ^{B,M,P}	94.3% (33/35) ^{G,L}	85.7% (18/21) ^{G,L}	>0.001
Risk factors and comorbidities						
Smoking	44.7% (17/38)	57.6% (19/33)	50% (9/18)	38.9% (14/36)	50% (13/26)	0.62
Alcohol abuse	13.2% (5/38)	6.1% (2/33) ^{M,P}	5.6% (1/18)	41.7% (15/36) ^G	38.5% (10/26) ^G	>0.001
Injectable drug use	10.5% (4/38)	0	0	0	3.8% (1/26)	0.052
Jail detention history	5.3% (2/38)	6.1% (2/33)	16.7% (3/18)	2.9% (1/34) ^P	26.9% (7/26) ^M	0.013
Chronic HCV infection	0	6.1% (2/33)	0	2.8% (1/36)	0	-
Other disease ¹	0	0	5.6% (1/18)	8.3% (3/36)	14.3% (3/21)	-
History of TB						
Previous TB	29.7% (11/37)	9.7% (3/31)	11.1% (2/18)	13.9% (5/36)	26.9% (7/26)	0.15
<i>Of which are documented</i>	100% (11/11)	66.7% (2/3)	50% (1/2)	40% (2/5)	85.7% (6/7)	0.12
Prior exposure to active TB patients	28.9% (11/38)	6.2% (2/32)	44.4% (8/18)	36.1% (13/36)	48% (12/25)	0.17
Previous TB outcome						
Cured and completed	45.5% (5/11)	0	50% (1/2)	40% (2/5)	57.1% (4/7)	-
Treatment completed	18.1% (2/11)	0	0	0	14.3% (1/7)	-
Treatment failure	0	33.3% (1/3)	0	20% (1/5)	14.3% (1/7)	-
Outcome not evaluated or unknown	36.4% (4/11)	66.7% (2/3)	50% (1/2)	40% (2/5)	14.3% (1/7)	-
Current TB outcome						
Cured and completed	100% (38/38)	90.9% (30/33)	61.1% (11/18)	97.2% (35/36)	88.9% (24/27)	0.39
Completed	0	3% (1/33)	38.9% (7/18)	0	0	-
Treatment failure	0	6.1% (2/33)	0	2.8% (1/36)	7.4% (2/27)	-
Relapse or reinfection	0	0	0	0	3.7% (1/27)	-

Footnotes: BMI: body mass index. TB: tuberculosis. WBC: white blood cells. Data are given as % (N) or median (interquartile range).

1: asthma, hypertension, inflammation. ^{B, G, L, M, P}: initial of study sites that are different from each other (p<0.05). *: different from all other sites.

Data were compared with Kruskal-Wallis' test with Dunn's post-hoc, or Fisher's test with Bonferroni's post-hoc when significant.

Supplementary Table 5. Characteristics of patients with treatment failure.

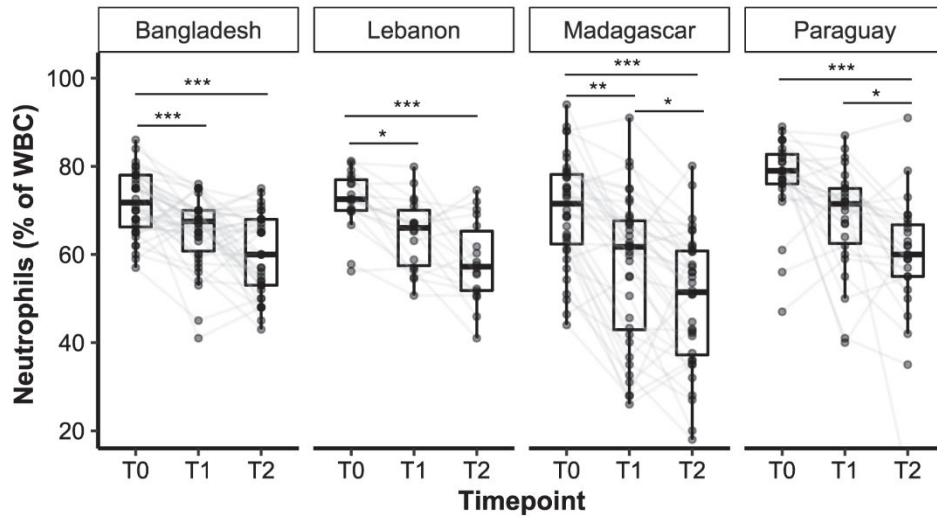
Demographics			T0		T1		T2	
Drug susceptibility	Country	Age (years)	Culture	Smear	Culture	Smear	Culture	Smear
S	Georgia	31	+	2+	+	Scanty	+	Scanty
S	Georgia	58	+	Scanty	<i>Unavailable*</i>	-	+	-
S	Madagascar	45	+	3+	-	1+	+	-
S	Paraguay	24	+	3+	-	-	+	<i>Unavailable**</i>
S	Paraguay	29	+	3+	+	-	+	<i>Unavailable**</i>

Footnotes: T0: baseline. T1: T0 + 2 months. T2 : end of treatment. S: drug-susceptible. LTFU: lost to follow-up. + : positive. - : negative. *contamination during culture. **, not enough sputum.

Supplementary Table 6. Receiver Operating Curve (ROC) analysis.

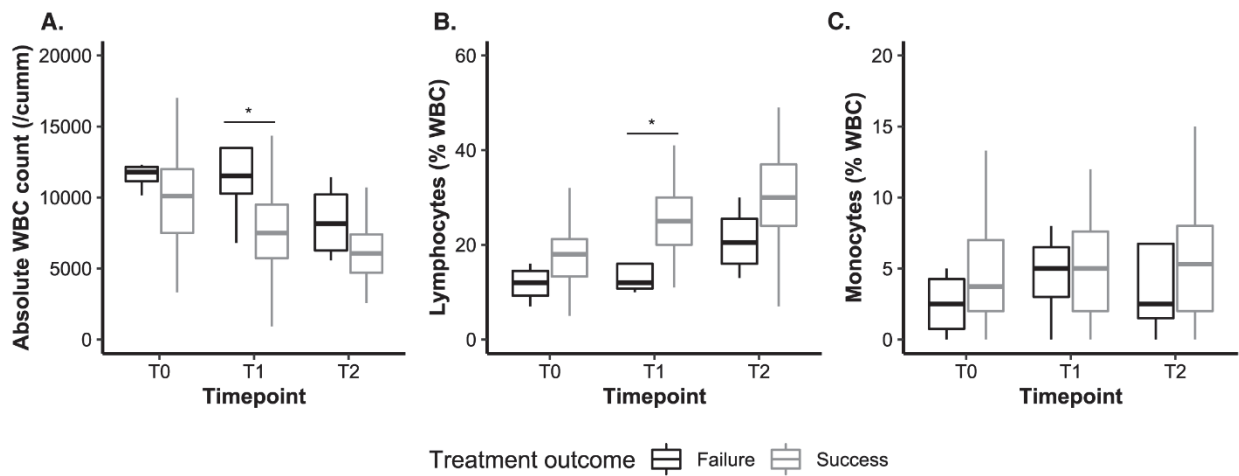
Clinical parameters at baseline	AUC	Sensitivity	Specificity	Optimal threshold
Absolute WBC counts (cells/mm ³)	0.788 (0.664-0.912)	1 (0.8-1)	0.74 (0.57-0.84)	11435 (10105-12072.5)
Lymphocyte proportions (% of WBC)	0.807 (0.671-0.943)	1 (0.8-1)	0.68 (0.58-0.93)	16.0 (10.5-16.5)
Absolute WBC counts (cells/mm ³) + lymphocyte proportions (%)	0.841 (0.723-0.959)	1 (0.8-1)	0.74 (0.62-0.94)	-

Footnotes: WBC: white blood cells. AUC: Area Under the Curve. AUC are given with the 95% confidence interval.



Supplementary Figure 1. Neutrophil proportions of total white blood cells during treatment.

Data are given as median + interquartile range (total n = 129). Each dot represents one patient at one timepoint. Grey lines connect data points from a same patient. T0: baseline. T1: baseline + 2 months. T2: end of treatment. Data were analyzed using Friedman's test, with the Wilcoxon-Nemenyi-McDonald-Thompson test as a post-hoc correction for pairwise multiple comparisons. *: p<0.05. **: p<0.01. ***: p<0.001.



Supplementary Figure 2. Evolution of the main white blood cell types during treatment in patients with high baseline sputum smear microscopy grades.

White blood cell (WBC) absolute counts (A.), lymphocyte percent of WBC (B.); and monocyte percent of WBC (C.) were assessed over time in 74 patients with sputum smear microscopy grades of 2+ or 3+ (success, n = 70; failure, n = 4). Data are given as median + interquartile range. T0: baseline. T1: baseline + 2 months. T2: end of treatment. Data were analyzed using the Mann-Whitney U test. *: p<0.05.

1.3. Annex 3 – Supplementary data from publication 3

SUPPLEMENTARY MATERIALS AND METHODS

Workflow for Unsupervised Analysis

An equal number of events was down sampled from each clean file to enable comparison between patients and time points, and converted to a .csv file for upload into R. For each given file, if there were less than 1,000 CD3⁺ events, the file was removed from analyses. All available panel markers were defined as either lineage or functional markers and were used differently in the analyses. Lineage-defining markers include canonical surface markers such as CD4 or TCR $\gamma\delta$ and display a theoretically stable expression. Functional markers include markers of activation (*i.e.* CD26, CD69), proliferation (*i.e.* CD38), inflammation (*i.e.* CD27, perforin), or migration (*i.e.* CCR7, CCR4) (**Supplementary Table 1**). All data were transformed prior to use in unsupervised analyses (biexponential transformation for Aurora data).

Visualization and Dimension Reduction

Dimension reduction was first performed to visualize heterogeneity phenotype changes between time points and stimulation conditions. UMAP (Uniform Manifold Approximation and Projection; version 3.1)^{396,397} was applied to CD45⁺ single events. Nineteen lineage-defining markers (CD19, CD16, CD56, CD14, CD3, CD4, CD8, CD45RA, CCR7, CCR4, CCR6, TCR $\gamma\delta$, CD56, CD26, CD161, TCR α 7.2, CD152, IL7Ra, and CD25) were used as clustering channels. UMAPs were then performed on the main identified subpopulations (B cells, NK cells, Monocytes, CD4⁺ and CD8⁺) using all functional markers in the panel. UMAP was created in R using the package Spectre³⁹⁸.

FlowSOM Automated Clustering

Unsupervised clustering was performed using the self-organizing map algorithm FlowSOM³⁹⁹ (version 2.7). The same clustering channels were used. To find the optimal number of metaclusters, the expected number of cell subpopulation was estimated as a starting point, then adjustment steps were performed: FlowSOM metaclusters were plotted on UMAPs, and the optimal number was adjusted to provide the best metacluster overlap with manually identified UMAP population islands.

Phenotype Analysis of Automatically Defined Clusters

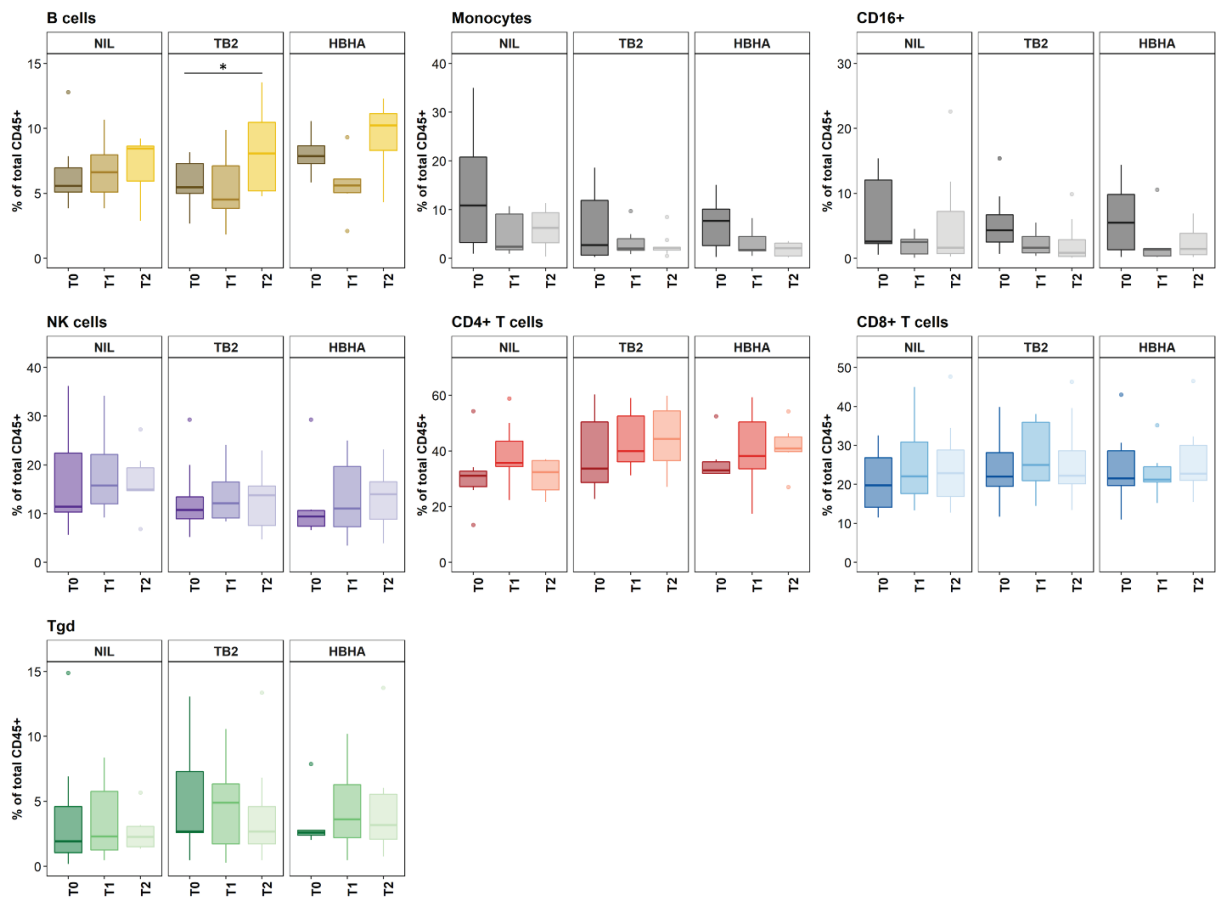
The abundance of each identified cluster as well as the expression levels of functional markers within clusters were studied throughout treatment. Cell type proportions were compared using automatically generated cluster gates. Expression levels were visualized with heatmaps, using the package pheatmap in R ⁴⁰⁰.

SUPPLEMENTARY RESULTS

Higher Levels of B Cell Frequencies in Successfully Treated Individuals for Pulmonary TB Disease

We next investigated whether and how these immune cell frequencies in the population of total leucocytes (% of total CD45⁺ cells) change among patients who proceeded from ATB disease to anti-TB treatment completion. In general, the frequencies of total B cells were significantly higher ($p = 0.03$) in successfully treated individuals (T2) relative to patients with ATB at baseline in TB2-stimulated samples. Notably, no significant change in total B cells frequencies was observed among the groups in response to NIL and HBHA stimulation. Besides, regardless of stimulations no significant differences in total monocytes and CD16⁺ frequencies were detected between treated (T1 and T2) and untreated ATB patients at baseline (**Supplementary Figure 1**).

SUPPLEMENTARY FIGURES



Supplementary Figure1 | Changes in the frequencies of differentially represented immune cells in ATB patients before and throughout anti-TB treatment. Boxplots evaluating the changes in the frequencies (that are % of total CD45⁺ cells) of B cells, Monocytes, CD16⁺, NK cells and T cell subsets (CD4⁺, CD8⁺ and $\gamma\delta$ T cells) in patients with ATB who had successfully completed their TB treatment until the end of treatment (n = 13). Evaluation was done on patients with matched time points (>1000 cells/sample) within three different stimulation conditions including: unstimulated samples (NIL, n = 7 patients), and stimulated samples (TB2; n = 9 patients and HBHA, n = 6 patients), collected from patients at baseline (T0), two months after treatment initiation (T1), and at the end of treatment (T2). Horizontal lines designate medians, boxes represent the inter-quartile ranges (IQR) and the ranges are represented by whiskers. Statistical significance was calculated using Mann–Whitney U test. * indicates a p-value < 0.05. NK: Natural killer. Tgd: $\gamma\delta$ T cell.

SUPPLEMENTARY TABLES

Supplementary Table 1 | Spectral flow cytometry antibody panel components.

ANTIBODIES		AURORA		
Cellular target	Clone	Fluorochrome	Supplier	Single staining ¹
CD45RA	HI100	BUV395	BD	Cells
CD8	RPA-T8	BUV496	BD	Cells
CD14	RMO52	BUV563	BD	Cells
CD27	L128	BUV661	BD	Cells
CD56	NCAM16.2	BUV737	BD	Beads
CD4	RPA-T4	BUV805	BD	Cells
CD197/CCR7	G043H7	BV421	Biolegend	Cells
CD19	HB19	SB436	Thermofisher	Cells
HLA-DR	L243	e450	Thermofisher	Beads
TCRgd	11F2	BV480	BD	Beads
TCRVa 7.2	3C10	BV510	Biolegend	Beads
CD16	3G8	BV570	Biolegend	Cells
CD194/CCR4	L291H4	BV605	Biolegend	Cells
CD183/CXCR3	G025H7	BV650	Biolegend	Cells
CD196/CCR6	11A9	BV711	BD	Beads
CD185/CXCR5	RF8B2	BV750	BD	Beads
CD279/PD-1	EH12.2H7	BV785	Biolegend	Cells
CD26	BA5b	FITC	Biolegend	Cells
CD3	UCHT1	A532	Thermofisher	Cells
CD45	HI30	PerCP	BD	Cells
Perforin	B-D48	PerCP-Cy5.5	Biolegend	Beads
CD69	FN50	PerCP-eFluor710	Thermofisher	Cells
CD25	2A3	PE	BD	Beads
CD278/ICOS	C398.4A	PE Dazzle594	Biolegend	Beads
CD154/CD40L	24-31	PE-Cy5	Biolegend	Cells
CD152/CTLA-4	14D3	PE-Cy7	Thermofisher	Cells
CD161	HP-3G10	APC	Biolegend	Cells
CD127/IL-7Ra	A019D5	AF647	Biolegend	Cells
CD7	CD7-6B	AF700	BD	Cells
CD38	HIT2	APC e780	Thermofisher	Cells

¹ This column indicates which single stainings for unmixing reference controls were bright enough to be performed on fixed white blood cell samples, or had to be performed on UltraComp eBeads Plus compensation beads (ThermoFisher Scientific).

Supplementary Table 2 | Sociodemographic and clinical characteristics of TB patients

SOCIODEMOGRAPHIC CHARACTERISTICS				MICROBIOLOGICAL RESULTS EVOLUTION							TREATMENT OUTCOME
Patient ID	Country	Gender	Age	Smear microscopy T0	Smear microscopy T1	Smear microscopy T2	Culture T0	Culture T1	Culture T2	Culture profile	Treatment outcome
01DSL	LB	F	46	3+	NEG	NEG	POS	NEG	NEG	Fast	Success
02DSL	LB	M	17	Scanty	NEG	NEG	POS	NEG	NEG	Fast	Success
12DSGE	GEO	M	33	NEG	Scanty	NEG	POS	NEG	NEG	Fast	Success
12DSL	LB	M	49	2+	NEG	NEG	POS	POS	NEG	Slow	Success
14DSGE	GEO	M	35	2+	NEG	NEG	POS	NEG	NEG	Fast	Success
15DSGE	GEO	M	41	3+	NEG	NA	POS	NA	NA	NA	NA
16DSGE	GEO	M	49	Scanty	Scanty	NEG	POS	POS	NA	NA	NA
17DSL	LB	F	57	NA	NEG	NEG	POS	NEG	NEG	Fast	Success
20DSL	LB	F	34	2+	NEG	NEG	POS	POS	NEG	Slow	Success
21DSGE	GEO	M	44	Scanty	3+	NEG	POS	NEG	NEG	Fast	Success
26DSGE	GEO	M	34	Scanty	1+	NEG	POS	NEG	NEG	Fast	Success
27DSGE	GEO	M	39	NEG	NEG	NEG	POS	NEG	NEG	Fast	Success
28DSGE	GEO	M	54	Scanty	2+	NEG	POS	NEG	NEG	Fast	Success

LB: Lebanon. GEO: Georgia. F: Female. M: Male. POS: Positive. NEG: Negative.

2. Other original publications

2.1. Annex 4 – Relevance of QuantiFERON-TB Gold Plus and Heparin-Binding Hemagglutinin Interferon- γ Release Assays for Monitoring of Pulmonary Tuberculosis Clearance: A Multicentered Study

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Relevance of QuantiFERON-TB Gold Plus and Heparin-Binding Hemagglutinin Interferon- γ Release Assays for Monitoring of Pulmonary Tuberculosis Clearance: A Multicentered Study

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Background: Tuberculosis (TB) is a leading infectious cause of death. To improve treatment efficacy, quicker monitoring methods are needed. The objective of this study was to monitor the response to a heparin-binding hemagglutinin (HBHA) interferon- γ (IFN- γ) release assay (IGRA) and QuantiFERON-TB Gold Plus (QFT-P) and to analyze plasma IFN- γ levels according to sputum culture conversion and immune cell counts during treatment.

Methods: This multicentered cohort study was based in Bangladesh, Georgia, Lebanon, Madagascar, and Paraguay. Adult, non-immunocompromised patients with culture-confirmed pulmonary TB were included. Patients were followed up at baseline (T0), after two months of treatment (T1), and at the end of therapy (T2). Clinical data and blood samples were collected at each timepoint. Whole blood samples were stimulated with QFT-P antigens or recombinant methylated *Mycobacterium tuberculosis* HBHA

(produced in *Mycobacterium smegmatis*; rmsHBHA). Plasma IFN- γ levels were then assessed by ELISA.

Findings: Between December 2017 and September 2020, 132 participants completed treatment, including 28 (21.2%) drug-resistant patients. rmsHBHA IFN- γ increased significantly throughout treatment (0.086 IU/ml at T0 vs. 1.03 IU/ml at T2, $p < 0.001$) while QFT-P IFN- γ remained constant (TB1: 0.53 IU/ml at T0 vs. 0.63 IU/ml at T2, $p = 0.13$). Patients with low lymphocyte percentages (<14%) or high neutrophil percentages (>79%) at baseline had significantly lower IFN- γ responses to QFT-P and rmsHBHA at T0 and T1. In a small group of slow converters (patients with positive cultures at T1; $n = 16$), we observed a consistent clinical pattern at baseline (high neutrophil percentages, low lymphocyte percentages and BMI, low TB1, TB2, and MIT IFN- γ responses) and low rmsHBHA IFN- γ at T1 and T2. However, the accuracy of the QFT-P and rmsHBHA IGRAs compared to culture throughout treatment was low (40 and 65% respectively). Combining both tests improved their sensitivity and accuracy (70–80%) but not their specificity (<30%).

Conclusion: We showed that QFT-P and rmsHBHA IFN- γ responses were associated with rates of sputum culture conversion. Our results support a growing body of evidence suggesting that rmsHBHA IFN- γ discriminates between the different stages of TB, from active disease to controlled infection. However, further work is needed to confirm the specificity of QFT-P and rmsHBHA IGRAs for treatment monitoring.

Keywords: tuberculosis, immunomonitoring, interferon-gamma release assays, heparin-binding haemagglutinin adhesin, QuantiFERON, treatment monitoring, inflammatory markers

INTRODUCTION

Tuberculosis (TB) is one of the leading causes of death by infectious disease in the world, causing 1.5 million deaths in 2019 (1). The treatment of pulmonary TB requires antibiotic multitherapies that last at least six months (2, 3) and can have toxic side effects. Consequently, treatment adherence is not optimal, especially in primary care settings (4, 5). Currently, anti-TB treatment monitoring relies on *Mycobacterium tuberculosis* (*M. tuberculosis*) detection by sputum smear microscopy and culture when possible (6). Sputum culture is the gold standard, but it is slow and requires high biosafety laboratory environments (7), while smear microscopy is highly sample- and operator-dependent and has poor sensitivity (8, 9). There is a clinical need for quicker anti-TB treatment monitoring tests adapted to primary care settings (10), that require accessible samples (blood, urine, feces) and limited laboratory equipment (11).

QuantiFERON-TB Plus (QFT-P; Qiagen) is an ELISA-based IFN- γ release assay (IGRA) that tests for exposure to *M. tuberculosis*. While it is useful for the triage of suspected TB patients, it cannot discriminate between active and latent TB (12) and has shown little clinical relevance for TB treatment monitoring so far (10). Previous works highlighted a general decrease in IFN- γ levels across TB treatment (13–18), and a study on QuantiFERON Gold In-Tube highlighted the presence of downregulated non-TB specific IFN- γ responses (Mitogen tube) were associated with poor treatment outcomes (19). However, persistently high quantitative results as well as heterogeneous

QFT-P conversion rates make the test unlikely to be adapted for individual treatment monitoring (20–22).

Recently, the use of QFT-P in combination with the detection of IFN- γ responses to recombinant *Mycobacterium smegmatis* heparin-binding hemagglutinin (hereafter called “rmsHBHA IGRA”) as an additional stimulation antigen has shown promise to stratify TB cases by stage of infection and progression to disease (23–27), and to monitor TB treatment outcomes (28). In particular, negative or low levels of IFN- γ production in response to rmsHBHA stimulation have been associated with active TB disease as opposed to latent infection. However, this assay has been explored only in studies in non-TB endemic settings, or with no drug-resistant TB patients.

The primary objective of this prospective multicentered cohort study was to monitor the plasma IFN- γ response to rmsHBHA and QFT-P antigens during anti-TB treatment. Moreover, recent data collected in the same cohort highlighted an association between baseline circulating white blood cells (WBC) and TB treatment outcome (29); hence, a secondary objective was to describe rmsHBHA and QFT-P IFN- γ values in subsets of patients stratified according to demographics, immune cell counts, and culture conversion during treatment. For that purpose, we conducted a cohort study in five countries with low- or middle income status and high- or middle TB incidence rates (30) (Bangladesh, Georgia, Lebanon, Madagascar, and Paraguay), focusing on adult, HIV-uninfected, culture confirmed drug-susceptible or drug-resistant pulmonary TB patients.

MATERIALS AND METHODS

Study Design and Sample Population

This descriptive study was nested within a multicenter prospective cohort study assessing the prognostic value of blood-based immunological markers for TB treatment monitoring. The study was based in five institutions from the Mérieux Foundation GABRIEL network (31), with the approval of national TB programs and the following ethical committees: the international center for diarrheal diseases and research, Bangladesh (icddr,b) in Dhaka, Bangladesh; the National Center for Tuberculosis and Lung Diseases (NTCLD) in Tbilisi, Georgia; the *Laboratoire Microbiologie, Santé et Environnement (LMSE, Université Libanaise)*, in Tripoli, Lebanon; the *Institut Pasteur de Madagascar* in Antananarivo, Madagascar; and the *Instituto de Investigaciones en Ciencias de la Salud (Universidad Nacional de Asunción; IICS-UNA)* in Asunción, Paraguay. All recruited patients provided written informed consent and standard biosecurity and institutional safety procedures were followed in all study sites.

Cohort Recruitment, TB Diagnosis, and Patient Follow-Up

The sample size was evaluated to detect a difference in rmsHBHA IFN- γ between baseline and end of treatment, with the following assumptions: we aimed for a level of significance of 95% and a power of 80%, assuming a minimum average expected difference of 1.6 IU/ml in rmsHBHA IFN- γ levels throughout treatment based on reported estimates (32), with an expected standard deviation of 3 IU/ml at each repeated measurement. We calculated (33) that a sample size of 117 was required to reach significance. As this study was nested in a cohort study with a sample size of 200, we aimed to enroll more patients to account for missing data. Patients were recruited if diagnosed with microbiologically confirmed pulmonary TB (positive culture and/or sputum smear and/or GeneXpert). Patients with HIV or diabetes mellitus and children under 15 years were excluded. In downstream analyses, patients under immunocompromising treatment, patients with negative cultures at inclusion, and patients who were lost-to-follow-up were excluded. Detailed procedures for microbiological diagnosis, drug sensitivity testing, and therapeutic regimen composition were described previously (29).

Patients were followed up: at inclusion (T0), after two months of treatment (T1), at the end of therapy [T2; 6 months for drug-susceptible (DS-TB) patients, nine to 24 months for drug-resistant (DR-TB) patients]. Data were presented for all patients followed up until T2 at least. Patients were on Directly Observed Treatment (DOT) and received treatment according to standard protocols (2, 3, 34). In this study, culture conversion at T1 was used to define three patient subsets: fast converters (definitive culture conversion between T0 and T1), slow converters (definitive culture conversion between T1 and T2), and patients with poor treatment outcome (positive culture results at T2: treatment failure; or positive culture at T3: relapse).

On-Site Whole Blood Collection and Cell Count

At every follow-up visit, 10 ml of whole blood were drawn: 4 ml was used for other downstream analyses, 1 ml was collected in

EDTA tubes and used to measure whole blood cell counts by standardized automated systems available in the study sites as listed previously (29), and 5 ml was used for *in vitro* blood stimulation. For the QFT-P assay, 1 ml of whole blood was seeded directly into each of four QuantiFERON-TB Gold Plus (QFT-P, Qiagen) tubes as per the manufacturer's instructions. The NIL tube contained no antigens and was used as a negative control. The TB1 and TB2 QFT-P tubes are coated with commercial *M. tuberculosis*-specific antigenic peptide pools. TB1 tubes contain two mycobacterial peptides, ESAT-6 (>15aa) and CFP-10 (8–13aa), which elicit specific immune responses from CD4⁺ T lymphocytes (35). TB2 tubes contain an additional commercial peptide pool designed to induce CD8⁺ T lymphocyte stimulation. MIT tubes are coated with commercial phytohemagglutinin-like bacterial antigens and were used as a positive control (35). For the rmsHBHA assay, 1 ml of blood was seeded into a NIL tube which was complemented with rmsHBHA (provided by the Delogu laboratory, UNICATT, Rome, Italy (23)), at a final concentration of 5 μ g/ml. Within 2 h of blood collection, samples were placed at 37°C in a 5% CO₂ atmosphere and incubated for 24 h. After incubation, plasmas were separated from the cell fraction by decantation, and stored at –80°C until further use.

Interferon- γ Release Assay

IFN- γ secretion was quantified using the QFT-P ELISA kit (Qiagen) according to the manufacturer's instructions. Briefly, plasma samples were thawed at room temperature, and 50 μ l of plasma was tested. Optical density results were compared to log-normalized values from freshly reconstituted IFN- γ kit standards. To account for potential immunomodulation phenomena unrelated with TB treatment, baseline IFN- γ level values (NIL tubes) were subtracted from antigen-stimulated IFN- γ values (MIT, TB1, TB2, rmsHBHA). According to the kit's sensitivity range, the maximum for IFN- γ level values was set at 10 IU/ml and negative values were rescaled to 0.

Assay Comparability Between Study Sites

To optimize comparability, a sample handling and processing protocol common to all study sites was developed, and on-site trainings were performed to standardize experimental processes such as instrument settings and timings. A tracking sheet was developed and used to follow sample shipment and standardize storage conditions in all sites. As the models of measurement instruments used in the different sites could not be homogenized, instrument readings were tested with QuantiFERON Control Panel (Qiagen) prior to launching the project. Finally, internal controls were added to each ELISA run to control for experimental variation and verify storage quality. Briefly, whole blood from a healthy donor was collected and stimulated with QFT-P antigens following the same protocol as described previously. Plasma was then separated and aliquoted and added to each ELISA run.

Clinical Data Collection and Statistical Analysis

Standardized clinical report and data collection forms were implemented to ensure dataset homogeneity as described previously (29). Data were entered into the CASTOR database

system (Version 1.4, Netherlands) (36), and cleaned and analyzed in R (version 3.6.2). Discrete variables were analyzed using Fisher's Exact test with Bonferroni's *post-hoc* test (37). Normal, continuous variables were analyzed with Student's *t*-test. Non-normal, continuous variables were analyzed with the Mann–Whitney test, or the Kruskal–Wallis rank sum test with Dunn's Kruskal–Wallis Multiple Comparisons *post-hoc* test (38). Repeated measures of non-independent continuous variables were analyzed using the Friedman rank sum test, with the Wilcoxon–Nemenyi–McDonald–Thompson *post-hoc* test (39). As the HBHA IGRA was not commercialized and QFT-P was designed to screen latent rather than active TB patients, we used Receiver Operating Curve (ROC) analyses to identify optimal IFN- γ thresholds adapted for this cohort, discriminating culture positive from culture negative patients. The overall QFT-P test was considered positive if either TB1 or TB2 was above their respective thresholds. ROC analyses and logistic regression were performed as described previously (29). In particular, multivariate logistic regression analyses were adjusted with the combination of variables that minimized the Akaike Information Criterion (AIC) for most tested predictors, while including important adjustment variables (age, sex, drug sensitivity, country).

RESULTS

Sociodemographic, Clinical, and Microbiological Characteristics of the Cohorts

Between December 2017 and September 2020, 199 eligible patients with culture confirmed active pulmonary TB were

recruited in Dhaka (Bangladesh), Tbilisi (Georgia), Tripoli and Akkar (Lebanon), Antananarivo (Madagascar), and Asunción (Paraguay). As of September 2020, 132 of them were followed at least until the end of treatment and had available IGRA data (Figure 1). Among these patients, 21.2% (28/132) were diagnosed with DR-TB. The sociodemographic characteristics of DS-TB and DR-TB patients were similar at inclusion (Table 1). Sociodemographic characteristics were compared between study sites, and significant differences were observed concerning age, BMI at inclusion, and BCG vaccination rates (Supplementary Table 1). All enrolled patients were sputum culture positive at inclusion. Most patients were also positive for sputum smear microscopy (sensitivity: 78.0%, 103/132) and/or GeneXpert (98.4%, 125/132). Three (3.9%) cases of treatment failure and one (0.7%) case of relapse were recorded (Table 1).

Dynamics of Interferon- γ Levels During Treatment and Influence of Sociodemographic Factors

Plasma IFN- γ levels in response to TB1, TB2, or HBHA stimulations were measured during anti-TB treatment (Figure 2). The median IFN- γ response to TB1 and TB2 remained constant over time, while the median response to rmsHBHA increased significantly (Figure 2A). Individual IFN- γ levels were heterogeneous in all three stimulation conditions (Figure 2B). To account for individual variations, rmsHBHA/TB1 and rmsHBHA/TB2 IFN- γ ratios were evaluated, and a significant increase in both ratios was still observed overall (Supplementary Figures 1A–C). We also measured the TB2–TB1 IFN- γ response, as a proxy for the QFT-P CD8⁺ T-cell response (Supplementary Figure 1D). No significant difference was detected over time.

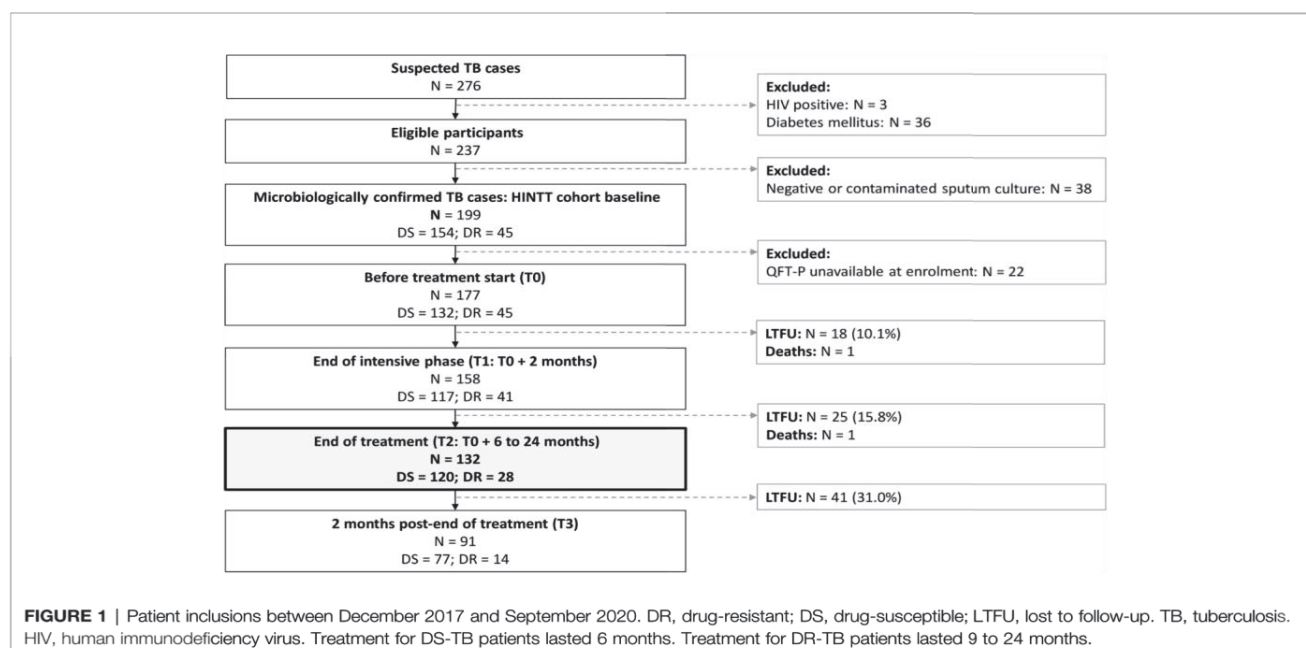


TABLE 1 | Sociodemographic and clinical characteristics of drug-susceptible and drug-resistant patients at inclusion.

N	ALL 132	DS-TB 104	DR-TB 28	P
Patient demographics				
Age (years)	27 (21–36.2)	27 (21–37.2)	27.5 (19.7–33.2)	0.41
Sex (male)	62.9% (83/132)	64.4% (67/104)	57.1% (16/28)	0.51
Treatment outcome				
Cured and completed	95.5% (126/132)	94.2% (98/104)	100% (28/28)	0.34
Completed	1.5% (2/132)	1.9% (2/104)	0	-
Failure	2.3% (3/132)	2.9% (3/104)	0	-
Relapse	0.8% (1/132)	1% (1/104)	0	-
Country of origin				
Bangladesh	28.8% (38/132)	20.2% (21/104)	60.7% (17/28)	>0.001
Georgia	23.5% (31/132)	20.2% (21/104)	35.7% (10/28)	0.13
Lebanon	5.3% (7/132)	6.7% (7/104)	0	0.34
Madagascar	27.3% (36/132)	34.6% (36/104)	0	>0.001
Paraguay	15.2% (20/132)	18.3% (19/104)	3.6% (1/28)	0.073
BMI at inclusion	18.7 (16.9–21.3)	18.83 (16.9–21.4)	18.7 (17.5–21.0)	0.79
WBC absolute count at inclusion (cells/mm ³)	9745 (7365–12032)	9695 (7350–12055)	10150 (7725–11850)	0.65
Neutrophils at inclusion (% of WBC)	75 (68–79.1)	75 (68.3–79)	78 (66.7–80.2)	0.34
Lymphocytes at inclusion (% of WBC)	18 (14–25)	18 (14–25)	17 (13.7–24.5)	0.52
Monocytes at inclusion (% of WBC)	4.4 (2–7)	5 (2–8.0)	4 (3–5.2)	0.42
Number of household contacts	4 (3–6)	4 (3–6)	4 (3.75–6.2)	0.86
BCG vaccination	86.2% (94/109)	86.4% (76/88)	85.7% (18/21)	1
Risk factors and comorbidities				
Smoking	46.2% (61/132)	46.2% (48/104)	46.4% (13/28)	1
Alcohol abuse	22% (29/132)	24% (25/104)	14.3% (4/28)	0.57
Injectable drug use	3.8% (5/131)	2.9% (3/104)	7.4% (2/27)	0.27
Jail detention history	8.5% (11/130)	9.8% (10/102)	3.6% (1/28)	0.57
Chronic HCV infection	1.6% (2/129)	2% (2/101)	0	0.75
Other disease ¹	6.2% (7/113)	7.8% (7/90)	0	0.34
History of TB				
Prior exposure to active TB patients	29% (38/131)	29.1% (30/103)	28.6% (8/28)	0.23
Documented previous TB episode	15.1% (20/132)	11.5% (12/104)	28.5% (8/28)	0.048
Previous TB outcome				
Cured and completed	61.1% (11/18)	61.5% (8/13)	60% (3/5)	1
Treatment completed	11.1% (2/18)	7.7% (1/13)	20% (1/5)	0.49
Outcome not evaluated or unknown	16.7% (3/18)	15.4% (2/13)	20% (1/5)	1
Treatment failure	11.1% (2/18)	15.4% (2/13)	0	-

Data are given as % (N) or median (interquartile range). DS-TB, drug-susceptible tuberculosis; DR-TB, drug-resistant tuberculosis; BMI, body mass index; WBC, white blood cells; 1: asthma, hypertension, inflammation. P-values are given for DS-TB vs. DR-TB.

No patients had HIV, non-TB chronic pulmonary diseases, renal diseases, solid tumors, chronic HBV infection, were pregnant, or under immunosuppressive therapies (corticosteroids, calcineurin inhibitors, biologics).

The impact of sociodemographic parameters on IFN- γ levels was assessed but no significant association was detected (data not shown).

Overall, QFT-P positivity rates remained constant during treatment (T0 vs. T2: 52 vs. 55%, $p = 0.71$), whereas rmsHBHA positivity rates increased significantly (T0 vs. T2: 31 vs. 67%, $p < 0.001$ (Table 2). We also calculated the slopes of rmsHBHA and QFT-P IFN- γ variations during treatment (Table 2). An increased INF- γ response to TB1, TB2, and rmsHBHA was observed in 55.3% (73/132), 56.8% (75/132), and 77.3% (102/132) of patients respectively.

IFN- γ levels over time were then stratified per study site (Figures 2C–E). Similar trends were observed in all cohorts for TB1 and TB2 IFN- γ levels, except in the Madagascar site in which an increase in TB1 IFN- γ was recorded between T0 and T2. Variation in IFN- γ levels produced by rmsHBHA-stimulated samples was different between study sites: similar in increase and order of magnitude in the Bangladesh and Georgia cohorts on the one hand, as well as in Paraguay and Lebanon on the other

hand; however, no increase was observed in the Madagascar cohort, as well as lower IFN- γ values (Supplementary Table 2). Mitogen IFN- γ levels were also significantly lower in the Madagascar cohort than in the Georgia cohort at all timepoints (Supplementary Table 2).

Effect of Neutrophil and Lymphocyte Percentages on Interferon- γ Release Assay Interferon- γ Response During Treatment

We analyzed the distribution of neutrophil percentages, and stratified IFN- γ results according to three groups: low neutrophils (less than the first quartile), intermediate neutrophils (between first and third quartiles), and high neutrophils (Figure 3A; threshold values are available in Supplementary Table 3). Similar analyses were performed with lymphocyte percentages (Figure 3C). We also evaluated the proportion of QFT-P and rmsHBHA positivity at each timepoint, stratified by neutrophil (Figure 3B) and

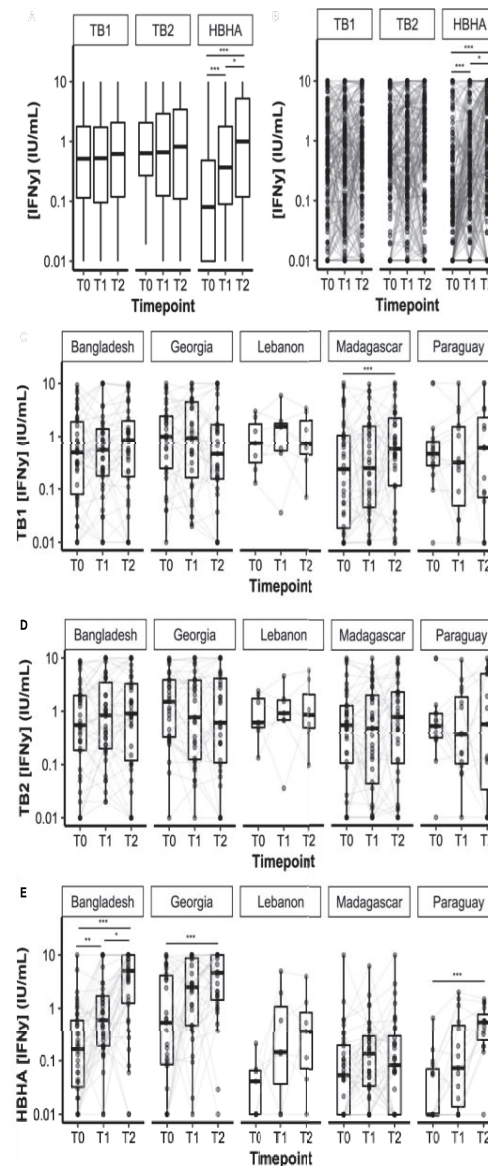


FIGURE 2 | Dynamics of plasma IFN- γ response to QFT-P and HBHA stimulations over the course of TB therapy. Data are given as median + interquartile range. Each black dot represents one patient at one timepoint. Grey lines connect data points from the same patient. **(A)** Median IFN- γ responses in the complete cohort ($n = 132$ per timepoint). **(B)** Individual IFN- γ responses in the complete cohort. **(C-E)** Stratification per study site. Bangladesh ($n = 38$), Georgia ($n = 31$), Lebanon ($n = 7$), Madagascar ($n = 36$), Paraguay ($n = 20$). T0: baseline. T1: baseline + 2 months. T2: end of treatment. Data were compared using Friedman's Exact Test with the Wilcoxon-Nemenyi-McDonald-Thompson post-hoc, or the Mann-Whitney U test. **(B)** * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

TABLE 2 | Qualitative evolution of QFT-P and HBHA IFN- γ levels during treatment.

Positivity rate at each timepoint	HBHA	TB1 only	TB2 only	QFT-P (TB1 and/or TB2)
T0	31.1% (41/132)	4.5% (6/132)	34.8% (46/132)	52.3% (69/132)
T1	56.1% (74/132)	2.3% (3/132)	42.4% (56/132)	50.8% (67/132)
T2	67.4% (89/132)	2.3% (3/132)	43.9% (58/132)	55.3% (73/132)
Trend between T0 and T1				
Increase	66.7% (88/132)	56.1% (74/132)	47.7% (63/132)	—
Decrease	24.2% (32/132)	41.7% (55/132)	47.7% (63/132)	—
Constant	9.1% (12/132)	2.3% (3/132)	4.5% (6/132)	—
Trend between T0 and T2				
Increase	77.3% (102/132)	55.3% (73/132)	56.8% (75/132)	—
Decrease	15.2% (20/132)	40.9% (54/132)	41.7% (55/132)	—
Constant	7.6% (10/132)	3.8% (5/132)	1.5% (2/132)	—

Data are given as % (N). T0, baseline; T2, end of treatment; QFT-P, QuantiFERON-TB Gold Plus; Constant, no difference in IFN- γ levels between T0 and T2, regardless of variations during treatment. Positivity was set at 0.75 IU/ml for QFT-P and at 0.22 IU/ml for HBHA based on ROC analyses.

lymphocyte percentages (Figure 3D). As HBHA stimulation was not performed using a commercial kit, Receiver Operating Curve (ROC) analyses were performed to identify the optimal rmsHBHA IFN- γ threshold value differentiating culture-positive patients from culture-negative patients at any timepoint. The resulting Area Under the Curve (AUC) was maximized for an IFN- γ cutoff value of 0.24 IU/ml (AUC 0.725, 95% CI 0.674–0.777). Overall, neutrophil and lymphocyte percentages directly impacted IFN- γ responsiveness to TB-specific antigens: QFT-P and rmsHBHA IFN- γ levels and positivity rates were significantly higher in patients with low neutrophil (Figures 3A, B) or with high lymphocyte proportions (Figures 3C, D). This statistically significant trend was also observed when comparing the

subgroup of patients with both low neutrophil and high lymphocyte percentages to the rest of the cohort (data not shown).

Effect of the Culture Conversion Status at 2 Months on the Interferon- γ Release Assay Interferon- γ Response Throughout Treatment

Overall, 112 patients had available culture data at T0, T1, and T2. Most patients were fast converters (definitive culture conversion between T0 and T1; 82.1%, 92/112) or slow converters (definitive culture conversion between T1 and T2; 14.2%, 16/112). Poor treatment outcomes were recorded in four patients (treatment failure, 2.7%, 3/112; relapse, 0.9%, 1/112; data not shown).

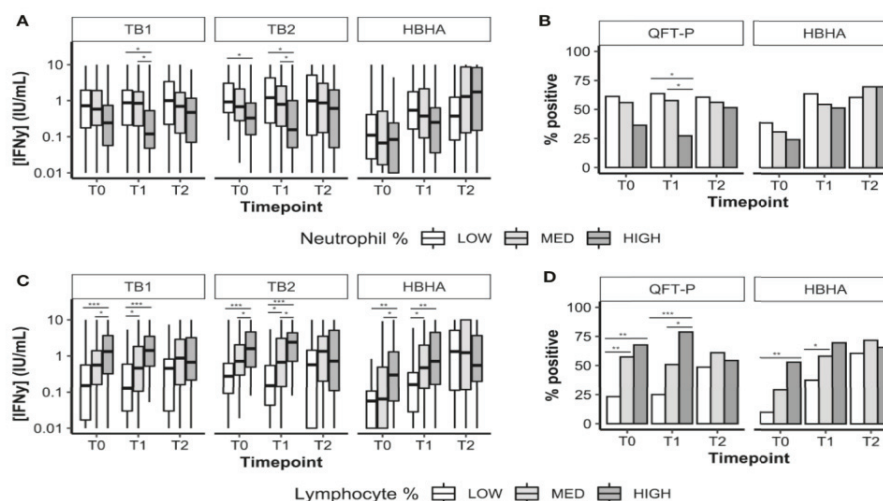


FIGURE 3 | Plasma IFN- γ response to TB-specific QFT-P antigens or HBHA stimulation in patients stratified by WBC counts over the course of TB therapy. (A, C) Quantitative IFN- γ response. Data are given as median + interquartile range and were compared using Kruskal–Wallis' test with Dunn's *post-hoc* when necessary. (B, D) Data were given as a percentage of each group and were compared using Fisher's Exact Test with Bonferroni's correction when necessary. Positivity was set at 0.75 IU/ml for QFT-P TB1, 0.71 IU/ml for QFT-P TB2 (the overall QFT-P result was positive if TB1 and/or TB2 were positive) and at 0.22 IU/ml for HBHA, based on ROC analyses. Low, medium, and high WBC or lymphocyte groups were defined as follows: low: <1st quartile; medium: 1st–3rd quartiles; high: >3rd quartile. WBC, white blood cells; T0, baseline; T1, baseline + 2 months; T2, end of treatment; **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

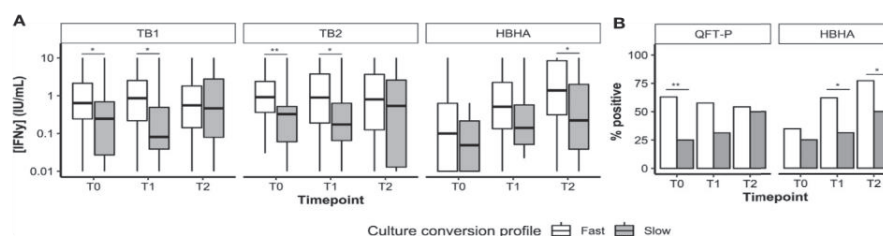


FIGURE 4 | Culture conversion profiles and plasma IFN- γ dynamics. Plasma IFN- γ levels (A, represented as medians + interquartile range) and IGRA positivity rates (B). T0, enrolment; T1, T0 + 2months; T2, end of treatment; Fast, conversion between T0 and T1 ($n = 92$). Slow, conversion between T1 and T2 ($n = 16$). * $p < 0.05$; ** $p < 0.01$ (Mann-Whitney U Test or Fisher's Exact Test).

Among successfully cured patients ($n = 108$), median IFN- γ levels (Figure 4A) as well as QFT-P and rmsHBHA IGRA positivity rates (Figure 4B) were stratified according to the culture conversion profiles. In slow converters, TB1 and TB2 IFN- γ levels at T0 and T1 and rmsHBHA IFN- γ levels at T2 were significantly lower than in fast converters. Similarly, QFT-P positivity rates at T1 and rmsHBHA positivity rates at T1 and T2 were significantly lower in slow converters.

Then, we calculated the sensitivity, specificity, and accuracy of the QFT-P and rmsHBHA IGRAs for TB treatment monitoring at T1 and T2, using culture as a reference standard (Supplementary Table 4). At T1 and T2 respectively, the accuracy of the QFT-P IGRA was of 44 and 46%, and the accuracy of TB2-TB1 was of 52 and 55%. For the rmsHBHA IGRA, we evaluated the test performances of negative rmsHBHA results (*i.e.* rmsHBHA IFN- $\gamma \leq 0.22$ IU/mL), since lower rmsHBHA IFN- γ values were observed before treatment. The accuracy of the rmsHBHA IGRA was of 64 and 65% at T1 and T2, respectively. Finally, we generated a score which was positive when the QFT-P result was positive and the rmsHBHA result was negative. The sensitivity of this combined score was of 86% at T1 and 82% at T2, and its accuracy reached 77% at T1 and 81% at T2, but its specificity remained inferior to 30% at both timepoints. Similar results were observed with a score combining rmsHBHA and TB2-TB1.

Association Between White Blood Cell Counts, Culture Conversion, and Interferon- γ Release Assay Interferon- γ Response During Treatment

We compared the immune cell counts (Supplementary Table 5) and the baseline sociodemographic characteristics (Supplementary Table 6) of patients according to their culture conversion profiles. No difference was detected between slow and fast converters for immune cell counts, but at T0 and T1, patients with treatment failure or relapse had significantly higher neutrophil percentages (at T0, median 84%, interquartile range (IQR) 81.5–86.5; at T1, 79%, IQR 75–81.75), and lower lymphocyte percentages (at T0, 12.5%, IQR 9.2–15.2; at T1, 15.5%, IQR 11–21.2) than successfully treated patients. The BMI at inclusion was significantly lower in slow than in fast converters, and slower conversion rates were observed in the Madagascar cohort.

Then, logistic regression analyses were performed to identify associations between slow culture conversion and immune cell counts or IGRA results (Table 3). In univariate analyses, significant associations were detected between slow conversion and MIT IFN- γ at T0 (odds ratio (OR) 0.78, $p = 0.001$) and T1 (OR 0.84, $p = 0.021$), with QFT-P IGRA positivity at T0 (OR 0.19, $p = 0.008$), and with rmsHBHA IGRA positivity at T1 (OR 0.24, $p = 0.015$) and T2 (OR 0.29, $p = 0.029$). The BMI at inclusion was also associated (OR 0.791, $p = 0.025$).

In multivariate analyses, significant associations were maintained for MIT IFN- γ at T0 (adjusted OR 0.65, $p = 0.009$), QFT-P IGRA positivity at T0 (aOR 0.045, $p = 0.013$), and HBHA IGRA positivity at T1 (aOR 0.076, $p = 0.045$). No significant association was found otherwise (Supplementary Table 7). Adjusting the models with neutrophil and monocyte proportions at baseline yielded similar results, but with higher AIC values (Supplementary Table 8).

Overall, we observed a slow converter profile including consistent clinical patterns at baseline (low BMI, high neutrophil percentages, low lymphocyte percentages, low TB1 and TB2 IFN- γ responses), as well as a downregulated rmsHBHA response at the end of treatment.

DISCUSSION

In this multicentered prospective study, we assessed the value of QFT-P or rmsHBHA-based IGRAs for pulmonary TB sputum culture conversion monitoring in five cohorts (Bangladesh, Georgia, Lebanon, Madagascar, and Paraguay). We recruited 132 HIV-uninfected culture confirmed pulmonary TB patients, including 28 DR-TB cases. To our knowledge, this is the first time that QFT-P and HBHA IGRAs are prospectively evaluated for treatment monitoring in DS-TB and DR-TB cohorts from high-TB incidence countries.

Consistently with previous works (20, 21), we found that individual monitoring of TB1 and TB2 IFN- γ levels during treatment showed little relevancy; we observed important inter-patient heterogeneity, and no significant changes in median values over time. On the contrary, median rmsHBHA

TABLE 3 | Associations between time to culture conversion, IFN- γ response, and selected clinical parameters.

Parameter	Timepoint	Descriptive analysis (n = 108)			Univariate analysis		Multivariate analysis ¹			
		Slow responders (n = 16; reference)	Fast responders (n = 92)	P	OR (95%CI)	p	aOR (95%CI)	p	C	AIC
MIT IFN- γ (IU/ml)	T0	4.9 (1.8–10)	10 (9.82–10)	0.0028	0.78 (0.67–0.91)	0.001	0.65 (0.44–0.86)	0.009	0.75	57.5
	T1	8.3 (3.9–10)	10 (9.93–10)	0.0039	0.84 (0.72–0.98)	0.021	0.77 (0.56–1.01)	0.076	0.68	63.9
	T2	9.4 (4.8–10)	10 (9.94–10)	0.023	0.88 (0.76–1.04)	0.11	0.78 (0.57–1.05)	0.10	0.66	64.7
Positive QFT-P IGRA	T0	25% (4/16)	63% (58/92)	0.006	0.19 (0.051–0.61)	0.008	0.045 (0.002–0.35)	0.013	0.77	57.6
	T1	31.2% (5/16)	56.5% (52/92)	0.10	0.33 (0.099–0.99)	0.059	0.39 (0.064–1.97)	0.27	0.64	66.2
	T2	50% (8/16)	53.3% (49/92)	0.98	0.88 (0.29–2.57)	0.81	3.27 (0.59–24.1)	0.20	0.65	65.7
Positive HBHA IGRA	T0	25% (4/16)	35.9% (33/92)	0.57	0.62 (0.16–1.96)	0.44	0.39 (0.075–1.71)	0.24	0.64	66.7
	T1	31.2% (5/16)	65.2% (60/92)	0.013	0.24 (0.071–0.73)	0.015	0.076 (0.003–0.67)	0.045	0.74	61.9
	T2	50% (8/16)	77.2% (71/92)	0.033	0.29 (0.097–0.89)	0.029	0.79 (0.17–4.13)	0.77	0.61	67.1
Lymphocyte % of WBC	T0	17.5 (12.8–19.5)	19.0 (15–26)	0.099	0.93 (0.84–1.00)	0.086	0.94 (0.83–1.05)	0.33	0.64	66.4
	T1	23.0 (16.2–28.0)	25.0 (20.7–31)	0.099	0.93 (0.86–0.99)	0.052	0.92 (0.83–0.99)	0.078	0.68	63.3
	T2	29.5 (23.5–36.2)	30.0 (25.9–36)	0.93	1.01 (0.95–1.06)	0.76	1.01 (0.94–1.08)	0.89	0.62	67.5
Body mass index	T0	17.0 (16.3–18.6)	19.7 (17.4–21.5)	0.0088	0.79 (0.63–0.93)	0.025	0.78 (0.56–1.02)	0.098	0.65	65.5

T0, inclusion. T1, T0 + 2 months. T2, end of treatment. OR, odds ratio; aOR, adjusted odds ratio; CI, confidence interval; WBC, white blood cells; C, model C statistic; AIC, Akaike Information Criterion. Only parameters with significant association to the outcome were shown; other tested parameters are available in **Supplementary Table 7**. Slow culture conversion was defined as a persistently positive culture result at T1 followed by a culture conversion at T2. For MIT IFN- γ , associations were calculated for each unit increase. For lymphocyte proportions, associations were calculated for each increase of 5%. ¹models were adjusted for age, sex, country of origin, drug resistance strain, body mass index at inclusion, and BCG vaccination rate.

IFN- γ levels increased significantly throughout treatment, and an increase was observed in most patients. This is consistent with studies associating high rmsHBHA IFN- γ levels to latency and controlled infection (23, 25–27), as well as in children (28) and in adults (32) receiving anti-TB treatment. The differences observed between the QFT-P and rmsHBHA IFN- γ responses during treatment can be explained by distinct antigen compositions. TB1 and TB2 are peptide pools obtained from secreted antigens, whereas rmsHBHA is a native protein found in mycobacterial cell walls *in vivo*; hence, antigen processing and presentation may differ. Bacterial pathogenesis mechanisms (40) as well as the bactericidal effect of anti-TB treatment could also affect the release of QFT-P and HBHA antigens. In addition, mycobacterial immune escape mechanisms involving HBHA (41, 42) could explain the downregulated *in vitro* IFN- γ responses to rmsHBHA during active disease.

Characterization of the association between QFT-P, rmsHBHA IFN- γ , and mycobacterial clearance has led us to identify two subsets of conversion rates. In particular, slower culture conversion was associated with QFT-P negativity at T0, consistently with a prior study linking negative or indeterminate QFT-P results with poor treatment outcomes (43), and with HBHA IGRA negativity at T1. More generally, both a general immunosuppression with low non-specific IFN- γ (44), and low *M. tuberculosis*-specific IFN- γ (45) have been demonstrated during active TB. Thus, an anergic early T-cell-driven response might be involved in slower mycobacterial clearance (43). At the other end of the spectrum, lower levels of IFN- γ in slow converters at T2 suggest a link between magnitude of the rmsHBHA-mediated response and mycobacterial clearance.

Our data indicate that rmsHBHA and/or QFT-P IFN- γ had low specificity and accuracy compared to the gold standard culture conversion. Because of the small cohort size, this result must be interpreted with caution; but if confirmed, it could suggest that the increase in rmsHBHA IFN- γ might be

representative of general immune recovery during treatment rather than a specific response to *M. tuberculosis*. Here, this hypothesis is supported by the fact that a low IFN- γ response to non-TB specific stimulation (Mitogen tubes) at T0 was also significantly associated with slow culture conversion in multivariate analysis. In addition, immune cross-reactions with HBHA homologs present in environmental mycobacteria have been previously reported (23).

Finally, our study had several limitations. The sample size was relatively small, and patients were included in diverse geographical areas and had different antibiotic regimens. As a consequence, malnutrition levels, untested co-infections (besides HIV and virus B and C hepatitis), different genetic and epigenetic backgrounds, or potential differences in bacterial loads during sputum collection could not be controlled. We were intrigued by differences in IFN- γ response to HBHA in the different study sites, which could be linked to ethnic-specific influences over *M. tuberculosis* responses (46). Although adjustment with sociodemographic factors and optimism corrections with a method adapted to small sample sizes (47) were performed, our results need to be confirmed in larger cohorts.

In conclusion, this study described the associations between mycobacterial clearance and immune responses to QFT-P and rmsHBHA IGRAs throughout anti-TB treatment. Lower QFT-P and rmsHBHA IFN- γ levels were associated with slower mycobacterial clearance. Our results support a growing body of evidence suggesting that rmsHBHA IFN- γ discriminates between the different stages of TB. However, the specificity of both IGRAs was insufficient for treatment monitoring. Further research is needed to clarify how the rmsHBHA response is regulated at the cellular level during treatment, and whether there is any specific interaction with mycobacterial clearance. In particular, evaluating how long rmsHBHA IFN- γ values remain stable after treatment would help assess whether it could be a relevant biomarker for relapse prediction.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by: in Bangladesh, the Research Review Committee and the Ethical Review Committee of icddr, b; in Georgia, the Institutional Review Board of the NTCLD (IORG0009467); in Lebanon, the Institutional Review Board of NINI hospital (IRB-F-01); In Madagascar, the Ministry of Public Health and the Ethical Committee for Biomedical Research (reference number: n°099-MSANP/CERBM); in Paraguay, the Research Ethics Committee and the Scientific Committee of the IICS-UNA (IRB number: IRB00011984; Federalwide Assurance number: FWA00029097). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JH is the principal investigator and initiated the project together with DG, NT, SBa, GR, NR, and MH. Data were collected by EK, MU, SBi, CA, PR, CR, PH, JR, AR, and RB. CC performed all the analyses and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.616450/full#supplementary-material>

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Supplementary data

Supplementary Tables.

Supplementary Table 1. Sociodemographic characteristics of the cohort, stratified by country.

N	Bangladesh 38	Georgia 31	Lebanon 7	Madagascar 36	Paraguay 20	p
Patient demographics						
Age (years), median (IQR)	22 (18.25-28) ^{G,P}	34 (28-43) ^{B,M}	23 (20.5-28.5)	26 (19.75-36.75)	28.5 (22.5-38.25) ^B	>0.001
Sex (male), % (N)	65.8% (25/38)	77.4% (24/31)	42.9% (3/7)	55.6% (20/36)	55% (11/20)	0.23
Drug resistance, % (N)	44.7% (17/38) ^{M,P}	32.3% (10/31) ^M	0	0 ^B	5% (1/20) ^B	>0.001
Treatment failure, % (N)	2.6% (1/38)	6.5% (2/31)	0	2.8% (1/36)	0	0.72
BMI at inclusion, median (IQR)	17.6 (16.3-20.7) ^{G,L,P}	20.3 (18.8-23.8) ^{B,M}	20.9 (20.2-21.2) ^{B,M}	17.1 (16.3-18.2) ^{G,L,P}	20.8 (18.5-22.7) ^{B,M}	>0.001
White blood cell absolute count at inclusion (/cumm)	9500 (8050-10975)	9800 (7000-12050)	8610 (6780-12450)	9370 (5802.5-12002.5)	11180 (8690-13567.5)	0.52
Lymphocyte proportion at inclusion (% of WBC)	20 (18-26.75) ^L	18 (15-21.5)	13.4 (12.55-15.6) ^B	17.6 (12.6-24.28)	18.5 (15.5-23)	0.041
Number of household contacts, median (IQR)	4.5 (3-7)	4 (3-4.75)	5 (4-5.5)	5 (4-6)	4 (4-6)	0.18
BCG vaccination, % (N)	84.2% (32/38)	38.7% (12/31)	14.3% (1/7)*	91.7% (33/36)	94.1% (16/17)	>0.001
Risk factors and comorbidities						
Smoking, % (N)	84.2% (32/38)	38.7% (12/31)	14.3% (1/7)	91.7% (33/36)	50% (10/20)	0.49
Alcohol abuse, % (N)	44.7% (17/38) ^M	58.1% (18/31) ^{M,P}	28.6% (2/7)	38.9% (14/36) ^{B,G}	35% (7/20) ^G	0.0010
Injectable drug use, % (N)	13.2% (5/38)	6.5% (2/31)	0	41.7% (15/36)	5% (1/20)	0.10
Jail detention history, % (N)	10.5% (4/38)	0	0	0	25% (5/20)	0.064
Chronic HCV infection, % (N)	5.3% (2/38)	6.5% (2/31)	14.3% (1/7)	2.9% (1/34)	0	0.69
Other disease ¹ , % (N)	0	3.2% (1/31)	0	2.8% (1/36)	16.7% (3/18)	0.060
History of TB						
Previous TB, % (N)	29.7% (11/37)	10.3% (3/29)	14.3% (1/7)	13.9% (5/36)	20% (4/20)	0.10
Prior exposure to active TB patients, % (N)	28.9% (11/38)	6.7% (2/30)	42.9% (3/7)	36.1% (13/36)	45% (9/20)	0.36
Previous TB outcome						
Cured and completed, % (N)	42.9% (3/7)	0	0	0	75% (3/4)	1
Treatment completed, % (N)	28.6% (2/7)	0	0	0	0	1
Outcome not evaluated or unknown, % (N)	0	66.7% (2/3)	0	0	25% (1/4)	1
Treatment failure, % (N)	0	33.3% (1/3)	0	33.3% (1/3)	0	1

Footnotes: BMI: body mass index. IQR: interquartile range. TB: tuberculosis. WBC: white blood cells.

1: asthma, hypertension, inflammation.

Data were compared with Kruskal-Wallis' test with Dunn's post-hoc, or Fisher's test with Bonferroni's post-hoc when significant.

B, G, L, M, P: initial of study sites that are different from each other (p<0.05). *: different from all other sites.

Supplementary Table 2. QFT-P or HBHA IFN- γ levels in the different study sites.

Parameter	Timepoint	All N = 132	Bangladesh N = 38	Georgia N = 31	Lebanon N = 7	Madagascar N = 36	Paraguay N = 20	<i>p</i> (countries)
TB1 [IFN- γ] (IU/mL)	T0	0.52 (0.11-1.78)	0.49 (0.08-1.9)	1 (0.24-2.42)	0.74 (0.33-1.8)	0.25 (0.02-1.04)	0.36 (0.24-0.74)	0.12
	T1	0.53 (0.1-1.73)	0.55 (0.18-1.4)	0.94 (0.17-4.49)	1.53 (0.53-1.78)	0.26 (0.05-1.54)	0.35 (0.11-1.47)	0.23
	T2	0.62 (0.12-2.07)	0.85 (0.17-2)	0.46 (0.16-1.68)	0.72 (0.47-2.15)	0.6 (0.12-2.21)	0.53 (0.08-1.99)	0.96
TB2 [IFN- γ] (IU/mL)	T0	0.64 (0.27-2.06)	0.55 (0.18-1.98)	1.51 (0.33-3.88)	0.62 (0.5-1.76)	0.56 (0.1-1.29)	0.53 (0.3-0.91)	0.24
	T1	0.66 (0.12-2.91)	0.84 (0.2-3.6)	0.77 (0.12-3.83)	0.92 (0.68-1.62)	0.48 (0.04-2.08)	0.37 (0.12-1.7)	0.47
	T2	0.82 (0.11-3.43)	0.9 (0.12-3.29)	0.61 (0.11-4.17)	0.87 (0.5-2.55)	0.8 (0.1-2.33)	0.59 (0.08-3.75)	0.92
MIT [IFN- γ] (IU/mL)	T0	10 (6.47-10)	10 (7.43-10)	10 (10-10) ^M	7.91 (5.12-10)	9.44 (1.98-10) ^G	10 (5.41-10)	0.0025
	T1	10 (8.07-10)	10 (6.53-10)	10 (10-10) ^M	10 (10-10)	9.54 (5.35-10) ^G	10 (7.72-10)	0.0075
	T2	10 (8.59-10)	10 (7.72-10) ^G	10 (10-10) ^{B,M}	10 (9.75-10)	9.87 (7.42-10) ^G	10 (7.09-10)	0.0095
HBHA [IFN- γ] (IU/mL)	T0	0.08 (0.01-0.48)	0.16 (0.03-0.6) ^P	0.54 (0.08-4.14) ^{M,P}	0.04 (0.01-0.07)	0.05 (0.01-0.2) ^G	0.05 (0.01-0.07) ^B	>0.001
	T1	0.37 (0.09-1.78)	0.6 (0.19-1.72) ^{M,P}	2.5 (0.48-8.71) ^{L,M,P}	0.14 (0.07-1.28) ^G	0.14 (0.03-0.31) ^{B,G}	0.09 (0.02-0.45) ^B	>0.001
	T2	1 (0.12-5.24)	5.05 (1.25-10) *	4.65 (1.46-10) *	0.36 (0.08-0.92)	0.08 (0.01-0.31)	0.5 (0.18-1.09)	>0.001

Footnotes: T0: baseline. T1: baseline + 2 months. T2: end of treatment. All values are given after subtraction of NIL [IFN- γ]. ^{B, G, L, M, P}: initial of study sites that are different from each other ($p < 0.05$). *: different from all other sites. Data were compared using Kruskal-Wallis's non-parametric test with Dunn's post-hoc when significant.

Supplementary Table 3. Blood count thresholds for stratified IFN- γ analysis.

	T0		T1		T2	
	Q1	Q3	Q1	Q3	Q1	Q3
WBC (cells/mm³)	7300	12000	5900	9500	4900	7400
Neutrophils (% of WBC)	70	80	60	70	50	70
Lymphocytes (% of WBC)	14	25	20	31	26	37

Footnotes: T0: baseline. T1: baseline + 2 months. T2: end of treatment. Q1: first quartile. Q3: third quartile. WBC: total white blood cells.

Supplementary Table 4. Assay performances of the QFT-P and rmsHBHA IGRAs compared to sputum culture.

Test	Thresholds	Timepoint	Sensitivity	Specificity	Accuracy
Smear microscopy	-	<i>T1</i>	81.1	50	76.6
		<i>T2</i>	92.9	33.3	91.5
QFT-P IGRA	TB1 $\geq$ 0.75 IU/mL and TB2 $\geq$ 0.71 IU/MI	<i>T1</i>	45.3	35.3	43.8
		<i>T2</i>	45.7	66.7	46.2
QFT-P TB2-TB1	$\geq$ 0.03 IU/MI	<i>T1</i>	51.6	52.9	51.8
		<i>T2</i>	54.3	66.7	54.6
HBHA IGRA	$\leq$ 0.22 IU/mL	<i>T1</i>	64.2	64.7	64.3
		<i>T2</i>	66.9	0	65.4
HBHA and QFT-P IGRA	-	<i>T1</i>	86.3	23.5	76.8
		<i>T2</i>	82.7	0	80.8
HBHA and TB2-TB1	-	<i>T1</i>	80	35.3	73.2
		<i>T2</i>	85	0	83.1

Footnotes: T0: baseline. T1: baseline + 2 months. T2: end of treatment.

For all IGRA variables, cutoffs adapted for this study on active TB patients were calculated using AUC analyses. Respective cutoffs are indicated in the “Threshold” column. The overall QFT-P test was considered positive if either TB1 or TB2 were above the indicated thresholds. The “HBHA and QFT-P IGRA” variable was defined as follows: positive when HBHA-IGRA results are negative and QFT-P results are positive; negative when HBHA-IGRA results are positive and/or QFT-P results are negative or indeterminate. The “HBHA and TB2-TB1” variable was defined as follows: positive when HBHA-IGRA results are negative and TB2-TB1 is strictly greater than the indicated threshold; negative when HBHA-IGRA results are positive and/or TB2-TB1 is equal to or lesser than the indicated threshold.

Supplementary Table 5. Associations between time to culture conversion and WBC counts.

N	Timepoint	Successfully treated patients with available T1 culture results (n = 112)			
		Fast converters 92	Slow converters 16	Failure or relapse 4	<i>p</i>
Absolute WBC count (per mm ³)	<i>T0</i>	9500 (7302.5-11425)	9385 (7822.5-14325)	12200 (12082.5-13400)	<i>0.077</i>
	<i>T1</i>	7535 (6247.5-9367.5)	8500 (6220-10900)	10780 (9470-11300)	<i>0.14</i>
	<i>T2</i>	6190 (4742.5-7812.5)	6750 (4387.5-7687.5)	6050 (5595-7247.5)	<i>0.97</i>
Neutrophil % of WBC	<i>T0</i>	75 (68-79)	75 (71.97-78.25)	84 (81.5-86.5)*	<i>0.022</i>
	<i>T1</i>	67.55 (60-72.17)	67.5 (61.1-75)	79 (75-81.75)*	<i>0.043</i>
	<i>T2</i>	60.15 (54.75-68)	58.5 (51.75-66.97)	64.5 (59-71.75)	<i>0.49</i>
Lymphocyte % of WBC	<i>T0</i>	19 (15-26)	17.5 (12.8-19.5)	12.5 (9.2-15.2)*	<i>0.017</i>
	<i>T1</i>	25 (20.7-31)	23 (16.2-28.0)	15.5 (11-21.2)*	<i>0.027</i>
	<i>T2</i>	30 (25.9-36)	29.5 (23.5-36.2)	21 (17.7-26.5)	<i>0.21</i>
High absolute WBC count (>3rd quartile)	<i>T0</i>	21.7% (20/92)	31.2% (5/16)	75% (3/4)	<i>0.050</i>
	<i>T1</i>	20.7% (19/92)	43.8% (7/16)	75% (3/4)*	<i>0.014</i>
	<i>T2</i>	29.3% (27/92)	25% (4/16)	25% (1/4)	<i>1</i>
High neutrophil % (>3rd quartile)	<i>T0</i>	22.8% (21/92)	18.8% (3/16)	100% (4/4)*	<i>0.0053</i>
	<i>T1</i>	23.9% (22/92)	31.2% (5/16)	75% (3/4)	<i>0.081</i>
	<i>T2</i>	28.3% (26/92)	31.2% (5/16)	50% (2/4)	<i>0.62</i>
Low lymphocyte % (<1st quartile)	<i>T0</i>	14.1% (13/92)	37.5% (6/16)	50% (2/4)*	<i>0.020</i>
	<i>T1</i>	19.6% (18/92)	37.5% (6/16)	50% (2/4)	<i>0.12</i>
	<i>T2</i>	22.8% (21/92)	31.2% (5/16)	75% (3/4)	<i>0.06</i>

Footnotes: Data are given as median (interquartile range) or % (N). WBC: white blood cells. T0: baseline. T1: baseline + 2 months. T2: end of treatment. Fast converters: culture conversion between T0 and T1. Slow converters: culture conversion between T1 and T2. Treatment failure: positive culture at T2 or T3 (end of treatment + 2 months). *: significantly different from both other groups (Kruskal-Wallis test + Dunn's post-hoc test).

Supplementary Table 6. Sociodemographic characteristics and culture conversion profile.

N	Fast converters 92	Slow converters 16	Failure or relapse 4	<i>p</i>
Patient demographics				
Age (years), median (IQR)	26.5 (21-36.25)	33.5 (25.75-47.75)	38 (30.5-48.25)	0.099
Sex (male), % (N)	63% (58/92)	62.5% (10/16)	100% (4/4)	1
Drug resistance, % (N)	26.1% (24/92)	18.8% (3/16)	0	0.76
Country of origin, % (N)				
Bangladesh	38% (35/92)	18.8% (3/16)	0	0.14
Georgia	26.1% (24/92)	25% (4/16)	50% (2/4)	0.93
Lebanon	5.4% (5/92)	0	0	0.34
Madagascar	10.9% (10/92)*	56.2% (9/16)*	25% (1/4)	>0.001
Paraguay	19.6% (18/92)	0	25% (1/4)	0.053
BMI at inclusion, median (IQR)	19.7 (17.3-21.4)*	17.0 (16.2-18.6)*	17.5 (15.9-20.2)	0.0088
White blood cell absolute count at inclusion (/mm ³)	9500 (7302.5-11425)	9385 (7822.5-14325)	12200 (12082.5-13400)	0.75
Lymphocyte proportion at inclusion (% of WBC)	19 (15-26)	17.5 (12.8-19.55)	12.5 (9.25-15.25)	0.099
Number of household contacts, median (IQR)	4 (3-6)	3.5 (3-5.25)	5.5 (4.75-8)	0.44
BCG vaccination, % (N)	83.8% (62/74)	100% (13/13)	100% (2/2)	0.19
Risk factors and comorbidities				
Smoking, % (N)	42.4% (39/92)	43.8% (7/16)	100% (4/4)	1
Alcohol abuse, % (N)	17.4% (16/92)	12.5% (2/16)	50% (2/4)	0.32
Injectable drug use, % (N)	4.4% (4/91)	0	0	1
Jail detention history, % (N)	7.7% (7/91)	6.2% (1/16)	50% (2/4)	1
Chronic HCV infection, % (N)	1.4% (1/71)	8.3% (1/12)	0	0.27
Other disease ¹ , % (N)	5% (4/80)	8.3% (1/12)	0	0.51
History of TB				
Previous TB, % (N)	17.3% (16/92)	12.5% (2/16)	25% (1/4)	0.73
Prior exposure to active TB patients, % (N)	26.4% (24/91)	12.5% (2/16)	75% (3/4)	1

Footnotes: BMI: body mass index. IQR: interquartile range. TB: tuberculosis. WBC: white blood cells.

1: asthma, hypertension, inflammation. Data were compared with the Kruskal-Wallis test and Dunn's post hoc, or Fisher's test. *: groups significantly different from each other.

Supplementary Table 7. Associations between time to culture conversion and IFN- γ response.

Parameter	Timepoint	Univariate analysis		Multivariate analysis ¹			
		OR (95%CI)	<i>p</i>	aOR (95%CI)	<i>p</i>	C	AIC
TB1 IFN- γ	<i>T0</i>	0.87 (0.611 - 1.09)	0.32	0.914 (0.652 - 1.213)	0.55	0.62	67.1
	<i>T1</i>	0.879 (0.646 - 1.08)	0.30	1.051 (0.74 - 1.392)	0.74	0.61	67.4
	<i>T2</i>	1.01 (0.831 - 1.18)	0.91	1.365 (1.002 - 1.943)	0.058	0.66	63.6
TB2 IFN- γ	<i>T0</i>	0.81 (0.543 - 1.03)	0.17	0.856 (0.592 - 1.143)	0.33	0.65	66.4
	<i>T1</i>	0.874 (0.659 - 1.07)	0.25	0.992 (0.715 - 1.289)	0.96	0.62	67.5
	<i>T2</i>	0.99 (0.818 - 1.15)	0.90	1.162 (0.911 - 1.493)	0.22	0.62	66.0
HBHA IFN- γ	<i>T0</i>	0.336 (0.023 - 0.916)	0.25	0.241 (0.004 - 1.068)	0.36	0.68	64.8

	<i>T1</i>	0.989 (0.814 - 1.15)	<i>0.89</i>	1.068 (0.746 - 1.515)	<i>0.69</i>	0.61	67.3
	<i>T2</i>	0.843 (0.674 - 0.993)	<i>0.072</i>	0.983 (0.712 - 1.333)	<i>0.91</i>	0.64	67.5

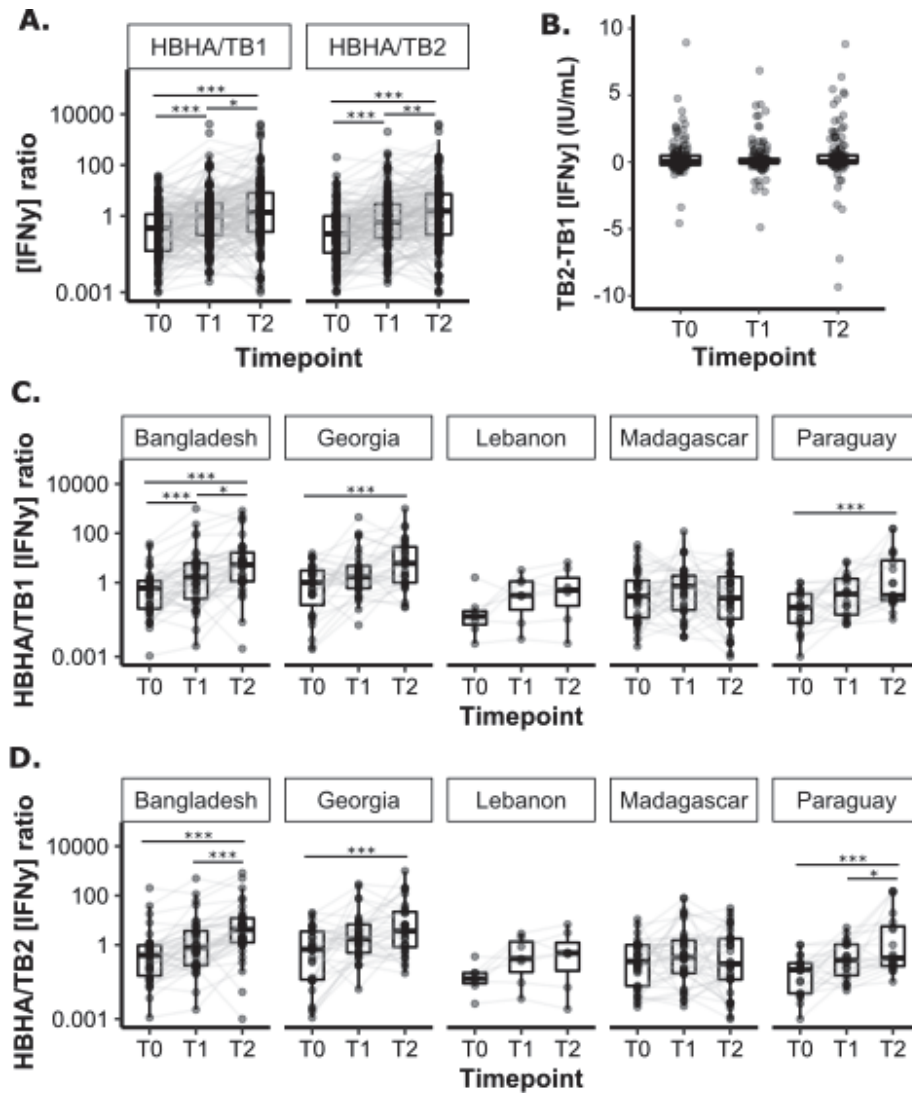
Footnotes: T0: inclusion. T1: T0 + 2 months. T2: end of treatment. OR: odds ratio. aOR: adjusted odds ratio. CI: confidence interval. WBC: white blood cells. C: model C statistic. AIC: Akaike Information Criterion. Slow culture conversion was defined as a persistently positive culture result at T1 followed by a culture conversion at T2. TB1, TB2 and HBHA IFN- γ levels were measured in IU/mL. For continuous independent variables, associations were calculated for each unit increase. 1: models were adjusted for age, sex, country of origin, drug resistance strain, body mass index at inclusion, and BCG vaccination rate.

Supplementary Table 8. Associations between time to culture conversion and IFN- γ response, adjusted for neutrophil and monocyte proportions at baseline.

Parameter	Timepoint	Multivariate analysis ¹			
		aOR (95% CI)	<i>p</i>	C	AIC
TB1 IFN- γ	<i>T0</i>	0.939 (0.642 - 1.34)	<i>0.73</i>	0.62	68.0
	<i>T1</i>	0.957 (0.636 - 1.32)	<i>0.80</i>	0.61	68.0
	<i>T2</i>	1.42 (1.027 - 2.08)	<i>0.054</i>	0.68	63.6
TB2 IFN- γ	<i>T0</i>	0.844 (0.563 - 1.206)	<i>0.36</i>	0.64	67.2
	<i>T1</i>	0.928 (0.64 - 1.247)	<i>0.64</i>	0.62	67.8
	<i>T2</i>	1.19 (0.927 - 1.57)	<i>0.16</i>	0.67	66.1
HBHA IFN- γ	<i>T0</i>	0.341 (0.006 - 1.185)	<i>0.45</i>	0.66	66.4
	<i>T1</i>	1.133 (0.779 - 1.661)	<i>0.49</i>	0.62	67.6
	<i>T2</i>	1.004 (0.723 - 1.39)	<i>0.98</i>	0.62	68.1
MIT IFN- γ	<i>T0</i>	0.62 (0.391 - 0.857)	<i>0.013</i>	0.74	58.7
	<i>T1</i>	0.711 (0.498 - 0.954)	<i>0.034</i>	0.70	62.8
	<i>T2</i>	0.799 (0.555 - 1.122)	<i>0.19</i>	0.63	66.3
Positive QFT-P IGRA	<i>T0</i>	0.045 (0.002 - 0.404)	<i>0.022</i>	0.75	59.5
	<i>T1</i>	0.279 (0.036 - 1.631)	<i>0.17</i>	0.66	66.1
	<i>T2</i>	2.69 (0.462 - 21.2)	<i>0.29</i>	0.66	66.9
Positive HBHA IGRA	<i>T0</i>	0.551 (0.057 - 4.366)	<i>0.57</i>	0.61	67.8
	<i>T1</i>	0.075 (0.003 - 0.689)	<i>0.045</i>	0.72	62.7
	<i>T2</i>	0.623 (0.098 - 4.45)	<i>0.62</i>	0.61	67.8
Lymphocyte % of WBC	<i>T0</i>	0.89 (0.482 - 1.685)	<i>0.71</i>	0.61	67.9
	<i>T1</i>	0.898 (0.788 - 0.988)	<i>0.055</i>	0.66	63.0
	<i>T2</i>	1.00 (0.915 - 1.09)	<i>0.98</i>	0.61	68.1
Body mass index	<i>T0</i>	0.912 (0.578 - 1.35)	<i>0.66</i>	0.64	66.1

Footnotes: T0: inclusion. T1: T0 + 2 months. T2: end of treatment. aOR: adjusted odds ratio. CI: confidence interval. WBC: white blood cells. C: model C statistic. AIC: Akaike Information Criterion. Slow culture conversion was defined as a persistently positive culture result at T1 followed by a culture conversion at T2. TB1, TB2 and HBHA IFN- γ levels were measured in IU/mL. 1: models were adjusted for age, sex, country of origin, drug resistance strain, body mass index at inclusion, BCG vaccination rate, and neutrophil and monocyte proportion at baseline.

Supplementary Figure



Supplementary Figure 1. Dynamics of plasmatic IFN- γ response to QFT-P and HBHA stimulations over the course of TB therapy. Data are given as median + interquartile range. **A.** Evolution of the HBHA/TB1 and HBHA/TB2 IFN- γ ratios throughout treatment. **B.** Evolution of the TB2-TB1 IFN- γ response (QFT-P CD8+ T cell response) throughout treatment. Stratification per study site of the HBHA/TB1 (**C.**) and HBHA/TB2 (**D.**) ratios. Bangladesh (n = 38), Georgia (n = 31), Lebanon (n = 7), Madagascar (n = 36), Paraguay (n = 20). Each black dot represents one patient at one timepoint. Grey lines connect data points from a same patient. T0: baseline. T1: baseline + 2 months. T2: end of treatment. Data were compared using Friedman's Exact Test with the Wilcoxon-Nemenyi-McDonald-Thompson post-hoc, or the Mann-Whitney U test (panel B). *: p<0.05; **: p<0.01; ***: p<0.001.

3. Oral communications

- JUNE 20 - 22, 2022

MYCODAYS congress that took place in LYON - FRANCE

- NOVEMBER 7 - 10, 2021

11th GABRIEL network meeting: The first prize winner of the "GABRIEL's Young Scientist Award 2021" for my oral presentation titled "Multi-country evaluation of RISK6, a 6-gene blood transcriptomic signature, for tuberculosis diagnosis and treatment monitoring". VEYRIER-DU-LAC, FRANCE

- JULY 9 – 12, 2021

31st ECCMID, European Congress of Clinical Microbiology & Infectious Diseases that took place online.

- MAY 20, 2021

DécripThèse – PhD day of the E2M2 doctoral school. LYON, FRANCE.

- DECEMBER 7 – 9, 2020

1st GABRIEL webinar, Hot Topics in Infectious Diseases that took place online.

- JUNE 17 - 20, 2019

10th GABRIEL network meeting. Hot Topics in Infectious Diseases. BEIRUT, LEBANON.

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