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Identification of human and pathogen molecular variants associated to tuberculosis heterogeneity

Identificação de variantes moleculares do humano e patogénio associados à heterogeneidade da tuberculose

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I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

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ABSTRACT

Tuberculosis (TB) imposes high human and economic tolls. One of the most striking features of TB is the variability of infection outcomes, which has been classically attributed to environmental and host determinants. More recently, studies uncovering *Mycobacterium tuberculosis* complex (MTBC) genomic diversity have shown the potential importance of pathogen-related factors to the disease pathogenesis. We approached this question from different angles, by combining the study of the pathogen properties, the host immune response and the clinical features of TB, within a cohort of 681 culture-confirmed pulmonary TB (PTB) cases diagnosed at the Hospital de São João, a major healthcare center in Porto, Portugal, between 2007 and 2013.

We started by developing a severity assessment tool for stratifying mortality risk in PTB patients. Five risk features were selected for the prediction model: hypoxemic respiratory failure (OR 4.7, 95% CI 2.8-7.9), age ≥50 years (OR 2.9, 95% CI 1.7-4.8), bilateral lung involvement (OR 2.5, 95% CI 1.4-4.4), ≥1 significant comorbidity – HIV infection, diabetes *mellitus*, liver failure or cirrhosis, congestive heart failure and chronic respiratory disease – (OR 2.3, 95% CI 1.3-3.8), and hemoglobin <12 g/dL (OR 1.8, 95% CI 1.1-3.1). A TB risk assessment tool (TReAT) was developed, stratifying patients with low (score ≤2), moderate (score 3-5) and high (score ≥6) mortality risk. The mortality associated with each group was 2.9%, 22.9% and 53.9%, respectively. The model performed equally well in the validation cohort.

After focusing on the host clinical prognostic predictors, in the second part of the project we assessed the impact of *M. tuberculosis* diversity on the disease clinical severity. We started by developing a clinical decision tree to classify the severity of the disease and by applying it to a selected group of 133 individuals that in our cohort did not present known predictor or precipitator TB factors. We found that, for this group of patients, no association existed between the severity of disease and the phylogeny of the infecting bacteria. We also found that *M.*

tuberculosis isolates from patients with mild disease grew significantly slower, while strains associated to moderate outcome had a longer lag phase and reached the highest plateau, after a steep exponential phase. To gain in-depth knowledge of the genetic basis for differential mycobacterial growth, we performed whole genome sequencing analysis. We detected several single nucleotide polymorphisms (SNPs) in genes that were previously associated with growth suppression and identified novel gene candidates involved in membrane transport and biosynthetic pathways.

Finally, in the third part of this work, we studied the architecture of the immune response triggered by the different isolates of *M. tuberculosis*. Sixteen clinical isolates associated with different clinical severity of TB were selected and used to infect peripheral blood mononuclear cells (PBMCs) from non-treated/non-recent latent TB infected (LTBI) donors or past/cured TB patients. Independently of the host genetics, we identified two distinct groups of *M. tuberculosis* isolates: high *versus* low inflammatory triggers. Furthermore, we report that PBMCs from past TB patients produced less IL-1β than those from LTBI participants in response to a variety of isolates, whereas the opposite was observed for IL-1RA. LTBI subjects elicited responses with significantly higher IL-1β/IL-1RA ratios than those from TB patients, thus suggesting this ratio as a discriminator of risk for latent to active TB progression.

Overall, we provide a new clinical prediction rule for the risk of death in TB patients and propose a new classification tree for TB severity. On the pathogen side, we unveiled the differential growth of clinical isolates associated with moderate outcomes of TB as a distinctive feature. On the host side, we suggest the ratio IL-1β/IL-1RA as a possible biomarker of disease resistance *versus* susceptibility to TB. Our findings present new platforms for active and latent TB management and open new avenues for basic research, to unveil host and pathogen determinants of TB outcomes.

RESUMO

A tuberculose (TB) continua a impor elevados custos económicos e humanos. Uma das características mais notáveis da TB é a variabilidade de resultados da infeção, que tem sido classicamente atribuída a determinantes ambientais e do hospedeiro. Trabalhos mais recentes estudaram a diversidade genómica do *Mycobacterium tuberculosis* complex (MTBC) e revelaram o potencial impacto dos fatores da bactéria na patogénese da TB. Esta questão foi abordada por diferentes ângulos, combinando o estudo das propriedades do patogénio, a resposta imune do hospedeiro e as características clínicas de TB, a partir de uma coorte de 681 casos de TB pulmonar confirmados por cultura, diagnosticados no Hospital de São João, um centro clínico de excelência do Porto, Portugal, entre 2007 e 2013.

Começámos por desenvolver uma ferramenta de avaliação de gravidade clínica para estratificar o risco de mortalidade de doentes com TB pulmonar. Cinco fatores de risco foram selecionados para o modelo de predição: insuficiência respiratória hipoxémica (OR 4.7, 95% CI 2.8-7.9), idade ≥50 anos (OR 2.9, 95% CI 1.7-4.8), envolvimento pulmonar bilateral (OR 2.5, 95% CI 1.4-4.4), ≥1 comorbilidade significativa – infeção HIV, diabetes *mellitus*, insuficiência hepática ou cirrose, insuficiência cardíaca congestiva e doença respiratória crónica – (OR 2.3, 95% CI 1.3-3.8), e hemoglobina <12 g/dL (OR 1.8, 95% CI 1.1-3.1). Desenvolveu-se a *tuberculosis risk assessment tool* (TReAT), estratificando doentes com baixo (pontuação ≤2), moderado (pontuação 3-5) e alto (pontuação ≥6) risco de mortalidade. A mortalidade em cada grupo foi de 2.9%, 22.9% e 53.9%, respetivamente. O modelo manteve um bom desempenho na coorte de validação.

Após nos termos focado nos preditores clínicos de prognóstico do hospedeiro, na segunda parte do projeto quisemos analisar o impacto da diversidade de *M. tuberculosis* na gravidade da doença. Desenvolvemos uma árvore de decisão clínica para classificar a gravidade da doença e aplicámo-la a um grupo selecionado de 133 doentes que na nossa coorte não apresentavam fatores preditores ou precipitantes conhecidos de TB. Neste grupo não se verificou uma associação entre a gravidade clínica e a filogenia da bactéria infetante. Porém, mostrámos que os isolados de *M. tuberculosis* de indivíduos com doença ligeira crescem de forma significativamente

mais lenta, enquanto que as estirpes associadas com TB moderada apresentam uma fase *lag* mais longa e atingem um patamar mais elevado, após uma fase uma íngreme fase exponencial. Para conhecer em detalhe as bases genéticas do crescimento micobacteriano, realizámos uma análise de sequenciação genómica. Detetaram-se diversos *single nucleotide polymorphisms* (SNPs) em genes que previamente tinham sido associados com a supressão de crescimento e identificámos novos genes candidatos envolvidos no transporte de membrana e em vias biossintéticas.

Finalmente, na terceira parte do trabalho, estudámos a arquitetura da resposta imune desencadeada por diferentes estirpes de *M. tuberculosis*. Dezasseis isolados clínicos associados a diferentes gravidades clínicas de TB foram selecionados e usados para infetar células mononucleares do sangue periférico (PBMCs) de dadores com infeção latente não recente e não tratada ou de doentes com TB passada/curada. Independentemente da genética do hospedeiro, identificámos dois grupos distintos de isolados de *M. tuberculosis* muito e pouco inflamatórias. Além disso, mostrámos que PBMCs de doentes com TB passada produzem menos IL-1β em resposta a uma variedade de isolados, enquanto o oposto se verificou para IL-1RA. Dadores com infeção latente apresentaram respostas com razão IL-1β/IL-1RA significativamente mais elevada.

Em suma, propomos uma nova regra de predição clínica para o risco de mortalidade por TB e uma nova árvore de classificação de gravidade da doença. Na vertente do patogénio, desvendámos um perfil de crescimento distinto dos isolados clínicos associados com TB moderada. Na vertente do hospedeiro, os nossos resultados sugerem que a razão IL-1β/IL-1RA poderá ser um biomarcador de resistência *versus* suscetibilidade para TB. Estes dados fornecem novas plataformas para a investigação básica dos determinantes do hospedeiro e patogénio na heterogeneidade da TB.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AFB	Acid fast bacilli
AIDS	Acquired immune deficiency syndrome
AIM2	Absent in melanoma 2
ALOX	Arachidonate lypoxigenase
ANOVA	Analysis of variance
APC	Antigen-presenting cell
AUC	Area under the curve
BALF	Bronchoalveolar lavage fluid
bp	Base pair(s)
BCG	Bacillus Calmette-Guérin
BDL	Below detection level
BWA	Burrows wheeler aligner
CAMP	Cathelicidin antimicrobial peptide
CDC	Chest disease centre
CFP-10	Culture filtrate protein 10
CFU	Colony-forming unit
CHARMS	Checklist for critical appraisal and data extraction for systematic reviews of prediction modelling studies
CHF	Congestive heart failure
CI	Confidence intervals
COPD	Chronic obstructive pulmonary disease
CPR	Clinical prediction rule
CRD	Chronic respiratory disease
CRP	C-reactive protein
CT	Computed tomography
CTL	Cytotoxic T cells
DAF	Derived allele frequencies
DCs	Dendritic cells

DNA	Deoxyribonucleic acid
DOTS	Directly observed treatment, short course
DST	Drug sensitivity testing
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
ESAT-6	Early secretory antigenic target 6
FDG-PET	Fluorodeoxyglucose positron emission tomography
GO	Gene ontology
GU	Growth units
GWAS	Genome-wide association studies
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HDT	Host-directed therapies
HIV	Human immunodeficiency virus
HSJ	Hospital de São João
IFN	Interferon
IGRA	IFN-γ release assay
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Interferon-inducible protein
IQR	Interquartile range
LAM	Latin-American-Mediterranean
LED	Light emitting diode
LPA	Line probe assays
LSP	Large sequence polymorphisms
LTB4	Leukotriene B4
LTBI	Latent tuberculosis infection
LXA4	Lipoxin A4
Mb	Megabase
MDR	Multidrug resistant
MFS	Major facilitator superfamily
MGB	Minor groove binding

MHC	Major histocompatibility complex
MIRU-VNTR	Variable-number tandem repeats of mycobacterial interspersed repetitive units
MOI	Multiplicity of infection
mRNA	Messenger RNA
MSMD	Mendelian susceptibility to mycobacterial disease
MTBC	*Mycobacterium tuberculosis* complex
NFQ	Non-fluorescent quencher
NGS	Next generation sequencing
NK	Natural killer T cells
NLR	NOD-like receptors
NLRP3	NLR pyrin domain containing 3
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOS2	Nitric oxide synthase 2
OADC	Oleic acid/albumin/dextrose/catalase
OR	Odds ratio
PAMP	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDIM	Phthiocerol dimycocerosate
PGE2	Prostaglandin E2
PIM	Phosphatidylinositolmannoside
PPD	Purified protein derivative
PRR	Pattern recognition receptors
PTB	Pulmonary tuberculosis
RAG	Recombination activating gene
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROC	Receiving operator characteristic
ROI	Reactive oxygen intermediates

RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SIFT	Sorting intolerant from tolerant
SNP	Single nucleotide polymorphism
STROBE	Strengthening the reporting of observational studies in epidemiology
TB	Tuberculosis
t_d	Doubling time
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TReAT	Tuberculosis risk assessment tool
Treg	Regulatory T cells
TST	Tuberculin skin test
VDR	Vitamin D receptor
WHO	World Health Organization
XDR	Extensively drug resistant

THESIS PLANNING

The present thesis is organized in 4 different chapters.

In **Chapter 1**, a general introduction is presented, launching the fundamentals on the present knowledge and questions on tuberculosis (TB). An historical perspective of the fight against TB and all the major epidemiological breakthroughs till the present time are described. It follows a review of the etiological agent, *Mycobacterium tuberculosis*, and the basis for its genetic diversity and how different genotypes are linked to clinical and immune phenotypes. Then, a summary of the literature on the host immune response and host-pathogen interaction is provided. Chapter 1 ends with an overview of the hypothetical link of inflammation level gradient and TB clinical heterogeneity.

In **Chapter 2**, the aims of the present dissertation are proposed.

Chapter 3 describes the cohort of TB patients diagnosed at the Hospital de São João, Porto, between 2007 and 2013, in which the present thesis was based. After a full demographic, clinical and radiological characterization, mortality risk factors were identified in a derivation set of 681 pulmonary TB (PTB) patients selected. In this longitudinal, retrospective study, a clinical prediction rule was generated, with 6-month mortality as the outcome measure, and patients were stratified according to a severity score into low-, moderate- and high-risk groups. The results were validated in an independent cohort from a non-hospital center. This was the first prognostic scoring system to be proposed in a high-income region of low to intermediate TB incidence for both inpatient and outpatient settings.

The clinical characterization of TB cases, for which matched *M. tuberculosis* infecting strains were available, offered the foundations to the biological studies described in **Chapter 4**. To underpin the molecular mechanisms underlying the heterogeneity of TB clinical manifestations, cases with predictor or precipitator factors (comorbid conditions) were excluded, arriving to a study cohort of 133 otherwise healthy TB patients. Then, the biology of infecting *M. tuberculosis* strains

was dissected through phylogenetic stratification and whole genome analysis, correlating these data with phenotypic features of associated disease severity and bacterial growth kinetics. Next, in **Chapter 5**, we explored the architecture of the immune response triggered by the different isolates of *M. tuberculosis* and propose a possible biomarker of risk of transition from latent to active TB.

The general discussion of the developed work is presented in **Chapter 6**. Future perspectives of the ongoing work are also considered in this section.

CHAPTER 1 – GENERAL INTRODUCTION

Part of this chapter was submitted as a review manuscript

Title: *Modulation of inflammation by host, pathogen and extrinsic factors in tuberculosis: a meaningful troika?*

Authors: Helder Novais Bastos, Nuno S. Osório, Sebastien Gagneux, Iñaki Comas and Margarida Saraiva

1. Tuberculosis: a global health problem

Tuberculosis (TB) is estimated to have caused 20% of the deaths in the Western globe cities during the 19th century. Rates of infection were as high as 79-90% and TB was named "the captain of all these men of death" [1]. These figures reflected the impact of industrialization on the incidence of the disease mainly in urban areas, where malnutrition and poor living conditions allied with overcrowded dwellings and congested factories generated the perfect environment for TB to spread. Two seminal discoveries were decisive in the fight against TB. First, the conclusion of its infectious nature by the French military surgeon Jean-Antoine Villemin, in a report in 1865 where he described TB transmission by inoculation from humans to rabbits [2]. Later, in 1882 the German physician Robert Koch identified the bacterial agent [3]. These discoveries paved the way for the development of diagnostic, preventive and therapeutic tools to fight TB, which together with improved social and living circumstances, led to drastic reductions in the global disease incidence, reaching historical lows in most Western countries.

Mortality rates in Europe and North America began to decline in the mid-19th century. The sanatorium movement took a relevant part in this fight against TB. Therapeutic interventions undertook during this time were largely empiric, ranging from the simplest bed rest in the horizontal position, to invasive procedures, like artificial pneumothorax, thoracoplasty, plombage and lung resection [4]. The later has recently deserved renewed attention in selected patients with TB caused by drug-resistant organisms [5]. The introduction of streptomycin in 1946 made sanatorium and collapse therapy in all its forms obsolete for susceptible bacteria [6], and further developments allowed for shorter, less toxic and more effective treatment regimens.

However, 70 years after introducing antibiotic treatment, we are still far from TB elimination, as defined by <1 case per 1 million population [7]. Instead, TB remains a major cause or morbidity and mortality in the developing world, accounting for approximately 1.8 million deaths every year [8]. With nearly 10.4 million new cases occurring worldwide, *Mycobacterium tuberculosis* continues to

rank among the leading causes of death by an infectious agent [8]. Further aggravating this scenario, one quarter of the world's population is estimated to be latently infected with *M. tuberculosis*, providing a virtually impossible to eliminate reservoir [9, 10]. South Africa has nowadays the highest incidence of TB in the world, which is the leading cause of natural death in this country [8]. Records from Cape Town show a clear decrease of the fatality rate after the introduction of combination chemotherapy [11]. However, childhood TB remained unchanged, suggesting that active transmission remained high, and notification rates returned to pre-chemotherapy levels with the advent of the human immunodeficiency virus (HIV)-epidemic in the 1990s [11]. Since its initial appearance in the 1980s, the HIV-1 infection contributed to large escalations in the incidence of TB, affecting more heavily the sub-Saharan Africa [12]. More or less at the same time, the dissolution of the USSR produced a major socioeconomic crisis and the collapse of the health services, which also resulted in a significant increase of new TB cases in that region [10, 13]. Moreover, recent times have witnessed the appearance of a new force driving the TB epidemic in the former USSR countries: the emergence of multidrug-resistant (MDR) strains [14]. **Figure I.1** shows the trends in TB incidence from 1990 to 2014. The expanding TB problem was declared a "Global Emergency" by the World Health Organization (WHO) in 1993. At the time, it was envisaged that the efficient use of existing tools would result in a progressive regression of disease incidence towards worldwide elimination [15]. The widespread implementation and success of the WHO global control program, subsequently labelled as the directly observed treatment, short course (DOTS) strategy [16], is estimated to have saved up to six million lives [17]. Despite this undeniable success, to meet the TB

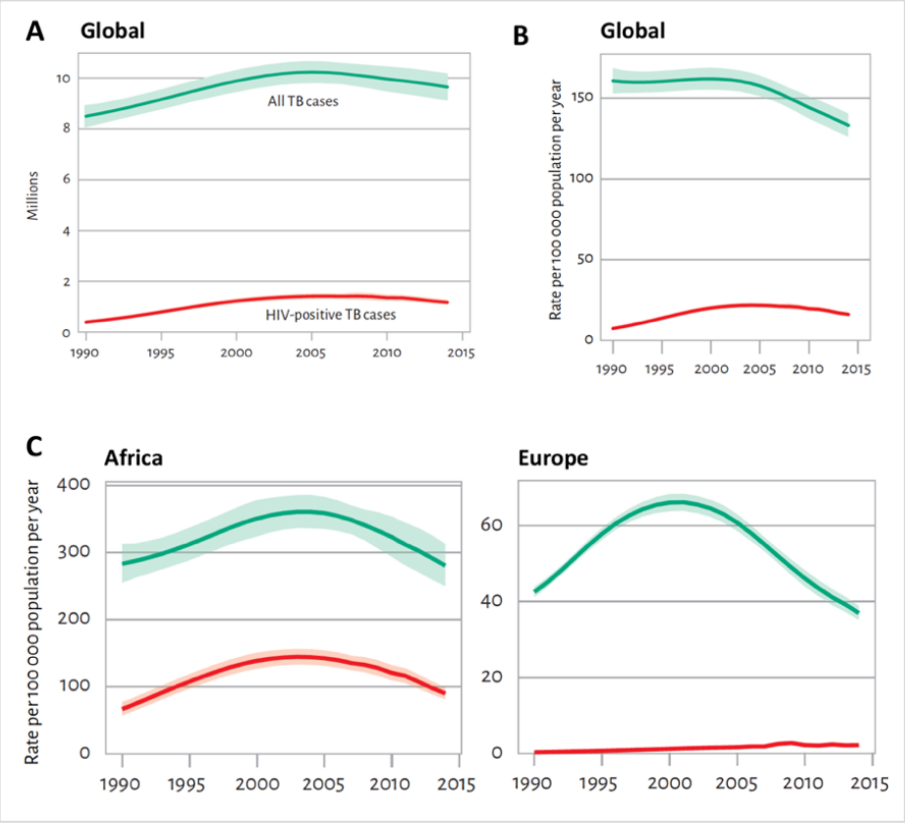

Figure I.1 – Trends in TB incidence from 1990 to 2014. **(A)** Global estimated absolute numbers of TB cases (in millions per year). **(B)** Global estimated rates of TB incidence (per 100 000 population per year) and **(C)** by WHO African and European regions. In Portugal, the estimated incidence was around 25/100 000 inhabitants in 2014. Green line represents all TB cases (including HIV-positive). Red line represents HIV-positive TB. *Adapted from* WHO report [8].

elimination target by 2050, the incidence of TB should decrease at an average rate of 20% annually [10], which will be hardly achievable with the available tools. It is now evident that only the integration of new technological advances in diagnostics, therapeutic strategies and vaccines will accelerate the pace of TB decline [10]. Whereas new drug regimens that can shorten treatment time will have a positive impact on global control, only a vaccine effective at stopping transmission at any of the points of the bacteria infection cycle will be the final punch against the disease.

1.1. Current and future perspectives on prevention, diagnosis and treatment

The vaccine *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG), almost a century old, is the only tool currently in use to prevent TB, but its efficacy against the predominant pulmonary form of TB is inconsistent [18]. BCG is effective against TB meningitis and milliary TB among children, but its protective effect wanes with time, with virtually no effect on adult TB [19]. However, although 15 TB vaccine candidates have been tested in pre-clinical or clinical trials since 2005, none has shown high efficacy so far [20]. It should be noted that TB vaccine development has faced several obstacles. First, animal models in current use only partially resemble the broad spectrum of human outcomes after exposure to *M. tuberculosis*, either due to biological differences of the host or difficulty in mimicking the natural infection conditions in the experimental setting [21]. Second, phase III trials require large cohorts, comprising several tens of thousands of participants, to validate new vaccine's effectiveness. Third, since the time for progression to active disease is largely unpredictable, clinical trials likely need to last for several years for evaluation of efficacy. One option toward this goal is stratification based on biomarkers which would identify the study participants at greatest risk to develop active TB disease within the duration of a standard clinical trial [22, 23]. However, such biomarkers are still unknown.

Despite rapid progress over the last decade, vaccine development and biomarker research have not yet been successfully applied in the area of TB management. Hence the importance of screening latent TB infection (LTBI) in selected risk population groups to prevent progression to active transmissible disease [7]. Latent-infected subjects are clinically asymptomatic and have no radiographic lung lesions of active TB, but present a specific immune response detected by an IFN-γ release assay (IGRA) or the tuberculin skin test (TST) [24]. TST is based on skin infiltration caused by intradermal injection of purified protein derivative (PPD), which is a crude mixture of antigens many of which are shared by *M. tuberculosis*, *M. bovis*, BCG and several species of environmental mycobacteria [25]. The two commercial IGRA kits, QuantiFERON-TB Gold In-Tube (QFT-GIT®) and T-SPOT.TB®, measure *in vitro* IFN-γ production by whole blood

enzyme-linked immunosorbent assay (ELISA) or an enzyme-linked immunospot (ELISPOT) assay by stimulated peripheral blood mononuclear cells (PBMCs) [26, 27]. Blood is stimulated with *M. tuberculosis* antigens culture filtrate protein 10 (CFP-10), early secretory antigenic target 6 (ESAT-6) and, in the case of QFT-GIT®, also with TB7.7 (Rv2654c) [27, 28], providing better sensitivity and specificity to identify LTBI in a single patient visit compared to TST [29]. *In vitro* tests may also discriminate true negative responses from anergy [30]. It should be noted that both TST and IGRA have a low accuracy in immune-compromised patients and they cannot distinguish between LTBI and active disease [31, 32]. Alternative biomarkers have been proposed to distinguish active TB, such as interleukin (IL)-17, interferon-inducible protein (IP)-10 and IL-10 [33], with IP-10 retaining high accuracy in the HIV infected patients in several reports [34, 35]. However, none of these biomarkers accurately identify those subjects with LTBI who are at risk of developing active disease [36, 37]. The most advanced research in this topic has been conveyed by the analysis of whole blood transcriptomic mRNA expression signatures. Recently, a 16-gene RNA signature was discovered in a set of 46 progressors (TB patients) and 107 matched controls that correlated with the risk of developing active TB [38]. Noteworthy, the 16 genes that comprise the transcriptomic signature are all regulated by type I and II IFNs, suggesting that chronic peripheral activation of the IFN response, previously shown to be associated with active TB disease at the time of diagnosis [39-42], may also precede the onset of active TB disease.

While improved TB vaccines or better biomarkers of progression of infection are not available for current use, the central programmatic intervention in the Stop TB Strategy has been focused on the diagnosis and treatment of active disease [10]. The main objective of this program is to reduce transmission by early case detection, by increased diagnostic accuracy and treatment outcomes and by a reduction in case fatality.

Staining for acid fast bacilli (AFB) has been the cornerstone of TB diagnosis, despite having a sensitivity of merely 50-60% [43], leaving a large portion of cases to be diagnosed based only on clinical judgment and radiography evaluation. International guidelines recommend 2 to 3 consecutive sputum

smears, with at least one being an early morning specimen [44, 45], but the incremental yield of a third smear is low (2–3%) [46, 47]. Sensitivity may be improved by fluorescent microscopy, with similar specificity, which is further enhanced by sample concentration [48], and also can reduce reading time to 1 minute for a single smear, compared to 4 minutes for a conventional smear [49]. Recently, the WHO has recommended the replacement of conventional fluorescence microscopes by light emitting diode (LED) fluorescent microscopes, that are more robust and function in a standard light room [50]. Yet, culture is still the gold standard method for diagnosis [51], presenting increased sensitivity over AFB smear, which is crucial in smear negative patients, usually occurring in the context of high rates of HIV coinfection, and allows to distinguish between non-tuberculous and tuberculous mycobacteria [52]. But mycobacterial culture requires a high degree of laboratory capacity, which is often not available in high-TB burden areas. Its utility is also limited because of the positivity delay (4-6 weeks in the case of solid media culture) [53]. Newer liquid media culture systems use fluorometric growth indicators that provide better sensitivity over solid media, with an increased yield of about 10%, and allow faster *M. tuberculosis* identification, usually within 2-3 weeks [54, 55]. They are also useful for drug sensitivity testing (DST), based on a proportional method of comparing growth in a critical concentration of antibiotics *versus* antibiotic-free media. For those reasons, liquid culture systems became the standard of care in developed nations [56].

The demand for faster diagnosis prompted the development of methods based on polymerase chain reaction (PCR) for the detection of pathogen's DNA. Line probe assays (LPA) contain specific oligonucleotide probes immobilized on a strip that hybridize with target PCR products, activating a colorimetric indicator [57]. Their main use has been for the rapid detection of drug resistant TB, with the advantage of reporting results in a matter of hours, much less than the traditional methods. The most exciting advance in TB diagnostics has been the automated PCR using the GeneXpert MTB/RIF platform [58]. This method is able to simultaneously identify the presence of *M. tuberculosis*, with higher sensitivity than smear microscopy (89% if smear-positive, 67% if smear-negative) [59], as

well as the *rpoB* gene mutation status, which encodes for the majority of rifampin resistance and is a strong indicator of concurrent isoniazid resistance (MDR TB) [60]. One study showed that Xpert shortened the detection of rifampin resistance from 106 days with traditional DST to 1 day [43]. However, two recent randomized studies of Xpert *versus* microscopy in real-world settings failed to show a significant impact of the former on morbidity or mortality at 6 months [61, 62]. Furthermore, Xpert does not provide the full drug susceptibility profile and also detects the DNA of nonviable *M. tuberculosis*, thus limiting its use for the identification of extensively drug resistant TB (XDR-TB) and for treatment monitoring [43].

Effective therapy is also central to any strategy for controlling TB. High cure rates are achieved with the standard regimen for drug-susceptible TB, established four decades ago, comprising 6 to 9 months of isoniazid and rifampicin, combined with pyrazinamide and ethambutol for the first 2 months [8, 63]. However, the treatment regimen has two major drawbacks [63]. One is the duration of therapy, raising concerns regarding the adherence and the possibility of emerging drug resistance caused by treatment default. The other limitation is the lack of tolerance in some patients, who may need dose adjustments or even suspension due to drug side effects that are frequently reported. Efforts have been made to shorten treatment without increasing the risk of relapse. One trial reported 4 months of standard treatment (2 months of daily isoniazid, rifampicin, ethambutol and pyrazinamide, followed by 2 months of daily isoniazid and rifampicin) in adults with noncavitary disease and culture conversion after 2 months yielded an acceptable relapse rate of 7% [64]. Moxifloxacin and gatifloxacin have also been tried in treatment-shortening trials [65-69]. However, three large, multicentre phase 3 trials (REMox [70], OFLOTUB [71], and RIFAQUIN [72]) reported a relapse risk increase from less than 5% with 6 month-treatment to more than 10% with 4 months. Similar results were found with high doses of rifampicin and rifapentine [73]. There are currently eight antituberculosis drugs in phase 2–3 trials, either to drug-susceptible or MDR TB [73]. Recently two new drugs for MDR-TB (bedaquiline and delamanid) were approved [74, 75], but they will probably yield a little impact of the global epidemiology of TB, since only a

minority of cases with MDR-TB are actually detected [76]. A growing body of evidence places the development of host-directed therapies (HDTs) as a foremost innovative step for the treatment of TB and at the forefront of precision medicine approaches.

To conclude, while innovative diagnostics, treatment and prevention measures are being fostered, they must be combined with better quality of laboratory services and TB national control policies to effectively hasten epidemiological changes towards elimination of TB in both low- and high-incidence countries.

2. The etiological agent(s) of TB: *Mycobacterium tuberculosis* complex

TB is caused by a group of phylogenetically closely related bacteria, collectively known as the *Mycobacterium tuberculosis complex* (MTBC). Within this complex, *M. tuberculosis* and *Mycobacterium africanum* (in West Africa) are responsible for virtually all human cases of TB [77]. These are obligate human pathogens and have no known animal reservoir [78]. *Mycobacterium canetti* is a rare cause of TB (<100 isolates reported), occurring mainly in patients from the Horn of Africa [77, 79], and presenting a few distinctive features. It produces cordless isolates after Ziehl–Neelsen staining, with rapidly growing, smooth and shiny colonies on solid growth media, referred as “smooth tubercle bacilli” MTBC [79]. The lack of inter-human transmission of *M. canetti* suggests a yet unknown environmental reservoir [77]. Other members of the MTBC include the animal-adapted *Mycobacterium bovis* (cattle), *Mycobacterium caprae* (sheep and goats), *Mycobacterium microti* (voles) and *Mycobacterium pinnipedii* (seals and sea lions) [80].

Mycobacteria are irregular rods of 0.3–0.5 μm in diameter with variable length, non-spore forming aerobic bacteria that by virtue of lacking an outer cell membrane are considered to fit into the Gram positive bacteria category [81, 82]. The typical feature of acid-alcohol staining fastness is due to the high content of

mycolic acids (>50% of its dry weight) within the mycobacterial cell wall [82]. The thickness of the mycolic acids layer impairs the entry of nutrients, being responsible for the slow growth of the bacteria, but it also increases cellular resistance to degradation through lysosomal enzymes [83]. In optimal laboratory conditions, *M. tuberculosis* may achieve a maximum generation time of around 16 hours [84]. This extremely slow growth rate has two consequences of clinical significance: i) the infection develops in an insidious process of several weeks or months before becoming clinically evident, and ii) at 37 °C and under optimal conditions of oxygen and nutrients on solid culture media, identifiable mycobacterial colonies may not appear for 3 to 6 weeks [82]. When they do appear, the colonies are irregular, waxy, and white to light-yellow coloured, with bacteria piled up into clumps or ridges [84].

The *M. tuberculosis* strain H37Rv is the most studied in research laboratories. Its genome has been completely sequenced in 1998 [85], which has opened grounds to identify genes that translate into virulence factors, antigens and also candidate targets for HDTs against TB.

2.1. Global phylogeography of *M. tuberculosis* and the origin of TB

Given the low mutation rate and lack of horizontal gene transfer and of genetic recombination [86-88], the human-adapted members of the MTBC were long assumed to be essentially identical. Over the years, the genotyping of clinical isolates by spoligotyping [89], MIRU-VNTR [90], single and large sequence polymorphisms [91, 92] and more recently by whole genome sequencing [93], revealed the existence of 7 different phylogenetic lineages of human TB-causing bacteria [92, 94]. A long period of coevolution of the pathogen with different populations of modern humans following the out-of-Africa migrations [95] resulted in the fact that different bacterial lineages prevail in different geographical regions of the world (**Figure I.2**). Lineage 1 (*Indo-Oceanic*) predominates in East Africa and South-East Asian countries; Lineage 2 (*East-Asian*) in all Eastern Asia; Lineage 3 (*East-African-Indian*) in Central Asia and East Africa; Lineage 4 (*Euro-American*), the most widespread, is commonly found in Europe, America, Africa and Middle-East; Lineages 5 and 6 (*West-African 1 and 2*)

are restricted to West Africa and finally, Lineage 7 has only been found in Ethiopia [94, 96]. Echoes of this type of interaction are still observed in cosmopolitan settings, where the phylogeny of the isolate correlates with the ethnic origin of the patient [97, 98].

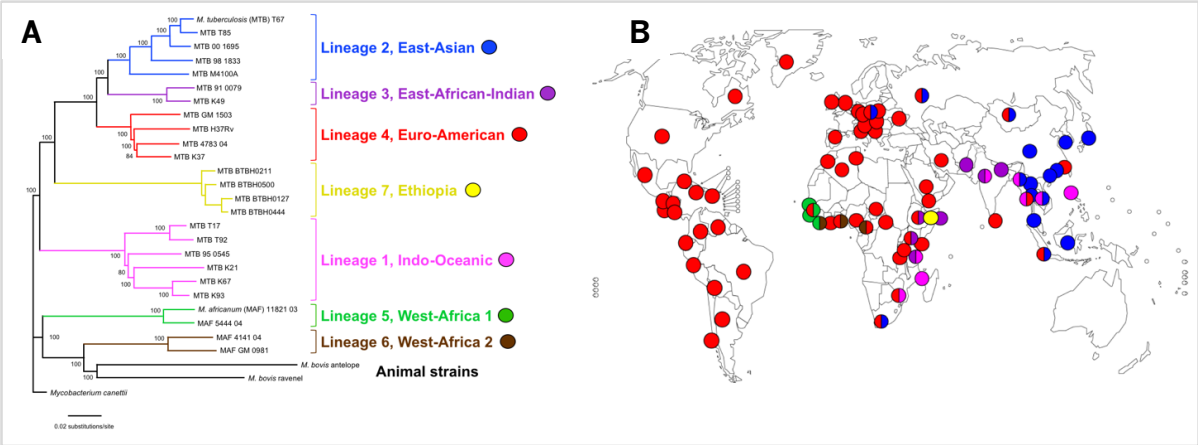

Figure I.2 – Global phylogeography of MTBC (A) Maximum parsimony phylogeny based on molecular typing of 964 specimens from patients in Ethiopia, 2006-2010. *Adapted from* Firdessa et al [94]. Phylogeny shown is based on 13,199 nucleotide positions that were variable in at least 1 of the 28 MTBC strains represented in the tree. Numbers near nodes indicate percentage of bootstrap replicates supporting the topology after 1000 pseudoreplicates. **(B)** Each coloured dot represent the dominant MTBC lineage(s) in each country. *Adapted from* Gagneux [77] and Coscolla et al [96].

The African origin of TB has been supported by several studies [95, 99-101], including by the fact that all 7 MTBC lineages are represented in that continent [94, 95]. Besides large human migrations into Eurasia starting 70 000 years ago, which led to the emergence of the phylogenetically-related lineages 2, 3 and 4, collectively known as the "modern" lineages, a few other major branching events have been proposed. One of the most important seems to be the Neolithic demographic transition 10 000 years ago, when animal domestication and agricultural developments boosted the population expansion and crowding that helped to sustain the infectious cycle [95]. In the last centuries, the shape of the phylogeographic distribution of *M. tuberculosis* strains changed again, to its present form (Figure I.2), as a result of the continuous waves of European migration to the overseas colonial possessions and simultaneous increase of the human population density in a pace that had never been seen before [99, 100].

Importantly, MTBC diversity can be found at very different evolutionary and geographical scales (**Figure I.3**). For example, a phylogeographical structure can also be observed at the sublineage level within Lineage 4 [102], with some globally distributed sublineages, while others are more geographically restricted. Moreover, there is an increasing body of evidence documenting the diversification of the initial infecting bacteria in sub-populations within a single patient [103]. Out of all the diversity observed, from macro- to micro-evolutionary scales, the challenge will be to identify those genetic changes that represent an adaptation of the bacteria to the host immune responses.

2.2. Driving forces of genetic diversity in *M. tuberculosis* and the potential impact on phenotype heterogeneity

Until early 1990s, when the first molecular strain-typing techniques were developed, there was a general belief that genetic diversity within MTBC was too limited to account for any differences in the infection course [104]. However, it is now well established that the genetic variation within the MTBC is higher than originally expected.

Across all lineages, more than 30 000 single nucleotide polymorphisms (SNPs) have been described [95]. The majority of the mutations in coding regions that have accumulated in the different MTBC phylogenetic branches are non-synonymous (i.e. that alter the amino acid sequence in the protein), with a large proportion of them placed inside highly conserved regions in other mycobacteria [93, 95, 99, 105, 106], thus suggesting a functional impact. Interestingly, there is evidence that the immune system may be, in fact, one major driving force in *M. tuberculosis* evolution, as shown by the disruption of adaptation of *M. tuberculosis* to sympatric human populations in the case of HIV patients [107] and by the evidence of positive selection in the *M. tuberculosis* genome driven by non-antibiotic forces [108]. In contrast to most other pathogens, that have evolved to evade host immunity by antigenic variation, *M. tuberculosis* seems to have explored the opposite strategy, since the known human T-cell epitopes (i.e. membrane proteins) are evolutionarily hyperconserved, with more than 95% of

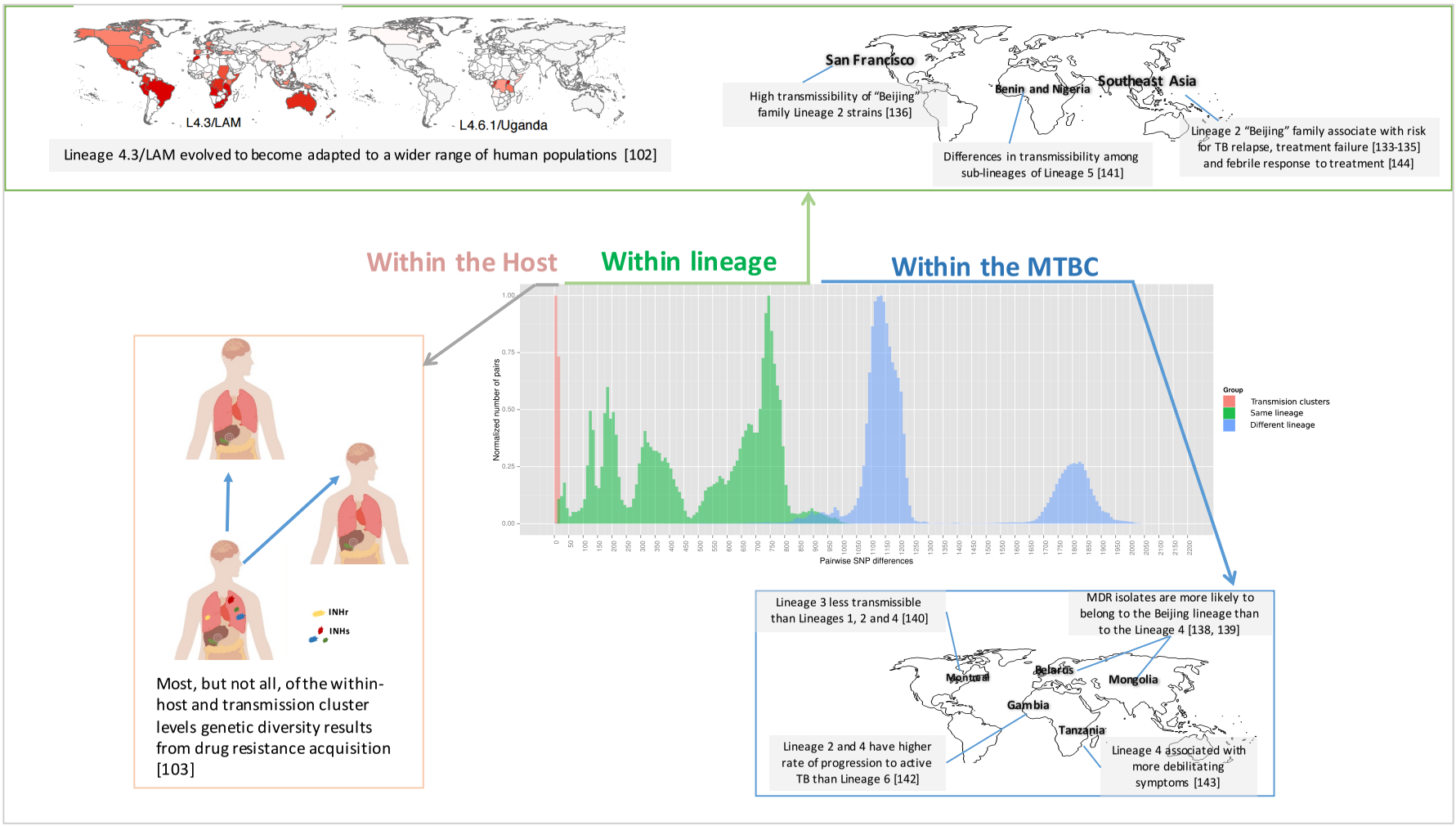

Figure I.3 - The levels of genetic diversity across the MTBC and its epidemiological and clinical impact. The maximum genetic distance between any two strains of the MTBC is around 2,000 SNPs. Then, different sublineages within the same MTBC lineage differ from each other at a maximum of 1,000 SNPs. Some within-host diversity results from drug resistance acquisition, but additional diversity can be observed emerging independently of antibiotic pressure. *From* Bastos HN et al. (submitted).

the 491 individual epitopes analysed in one study having no amino acid change at all [93]. These data suggest that *M. tuberculosis* may have evolved to induce T cell responses to its own advantage, in order to ensue tissue destruction and the development of cavitary disease, contributing to its successful transmission [109]. This aspect is corroborated by the observation that TB/HIV coinfected patients with low CD4+ T-cell counts are less likely to present cavitations [12]. The formation of the granuloma itself, needed to control the infection, may also paradoxically offer a niche for long-term survival of the bacteria, as studies showed that many asymptomatic humans harbour virulent bacteria in their tuberculous granulomas [110, 111]. On the other hand, a subset of predicted T cell epitopes in *M. tuberculosis* that evolved to escape the host immune system have been identified [108]. This is important, as the alteration of a single amino acid in an epitope can have a large impact in its affinity to a specific HLA molecule, thus influencing the T cell synapse and modulating the level and type of immune response elicited [112, 113]. These epitopes could potentially be exploited as vaccine components as envisaged for other diseases.

In 1995, an outbreak of TB in a low-risk rural area of the United States of America was traced to an isolate designated CDC1551, which attracted attention due to an unusually high skin test conversion rate and unusually large PPD reactions [114]. Later, Manca et al showed that CDC1551 isolates were more potent than others in inducing the secretion of inflammatory mediators by infected monocytes [115], providing the evidence for a link between bacterial genotypes and immune response phenotypes. Subsequent works by further supported these observations *in vitro* [116-120] and in experimental infections [121-125]. In turn, the differential stimulation of the immune response may impact the pathogen virulence. Thus, certain isolates of *M. tuberculosis* appear to have acquired immune evasion strategies to imbalance the immune system in their benefit. As an example of this, the hypervirulent isolate HN878 prevents the development of protective T helper (Th) 1 cells and enhances the expression of type I IFNs in infected mice [122, 126]. Virulent strains also appear to be able to escape host immunity by differentially modulating the death of the infected cell. Indeed, some *M. tuberculosis* isolates inhibit apoptosis and trigger necrosis of host

macrophages to delay the initiation of adaptive immunity [127]. Similarly, neutrophil necrotic cell death can be induced, with subsequent spilling of the noxious granule-contents into the surrounding tissue and bacteria spreading to the extracellular space [128].

The clinical relevance of MTBC diversity at the lineage and sublineage levels is also increasingly recognized [96], as highlighted in Figure I.3. Experimental studies exploring the molecular mechanisms underlying differential virulence of MTBC strains found differences in growth rates [118, 119, 129, 130], gene expression [106, 130], metabolic profiles [131] and cell wall composition [118, 132]. It is conceivable that these findings account for the differences on the level of the inflammatory response generated by the host. Lineage 2 (Beijing) strains have been associated with treatment failure and relapse [133-135] and a highly transmissible sublineage of Lineage 2 has been found in San Francisco [136]. Moreover, one *in vitro* study demonstrated that Lineage 2 may acquire drug resistances more rapidly than Lineage 4 [137], which was confirmed in reports from Belarus and Mongolia, where MDR isolates were more likely to belong to the Lineage 2 Beijing family of strains [138, 139]. Lineage 3 was reported to be less transmissible than Lineages 1, 2 and 4 across Montreal, Canada [140], whereas striking differences in transmissibility among sublineages of Lineage 5 in Benin and Nigeria were registered [141]. Lineages 2 and 4 were shown to have a higher rate of progression to active TB comparatively to Lineage 6 in Gambia [142]. In addition, more debilitating symptoms (such as weight loss) have been associated with Lineage 4 strains in Tanzania [143], whereas increased febrile response was seen in patients infected with Beijing genotype strains [144]. Moreover, Lineage 4 was linked mainly to pulmonary TB [145], while lineages 2 [145-147] and 3 [148] were reported to associate more with extrapulmonary disease.

Taken together, these findings suggest that genetic differences within MTBC account, at least partially, for the phenotypic heterogeneity seen in host immune responses and, by consequence, in the clinical presentation of TB.

3. An overview of the transmission and the immune response to *M. tuberculosis*

3.1. Transmission

Infectious droplet nuclei are generated when pulmonary or laryngeal TB patients cough, sneeze, shout, or sing [149]. Depending on the environment, these tiny particles can remain suspended in the air for several hours [150]. Transmission occurs after inhalation by a second individual of airborne particles carrying just a few mycobacteria [151]. Although transmission models of TB have not yet been fully established, it is thought that mainly the smaller droplets, containing fewer bacteria, will make it past the upper respiratory track and into the relatively sterile environment of the distal alveoli [152, 153]. It is thus considered that infection with *M. tuberculosis* results in a low dose infection. Only 10 to 30% of the individuals that contact with *M. tuberculosis* become infected and although not being capable of eliminating the pathogen, the majority develops an immune response that is able to contain infection in its latent form [82].

3.2. Recognition and early events after infection: the innate immune response

Much of what is known on the immune response to *M. tuberculosis* (**Figure I.4**) comes from studies using the mouse model of experimental infection. Research showed early recruitment of phagocytic cells into the airways and alveoli of the infected mice [154], following the inhalation of airborne droplets carrying *M. tuberculosis*. Important cellular players at this stage are macrophages, neutrophils and dendritic cells (DCs), which sense the presence of the bacteria through the activation of various pattern recognition receptors (PRRs) by microbial components known as pathogen-associated molecular patterns (PAMPs) [155]. Some examples of PRRs are toll like receptors (TLRs), nucleotide-binding oligomerization domain (NOD) receptors, DC-SIGN and dectin-1 (C-type lectin receptors) [154, 156, 157] and all of them have been

shown to be involved in the recognition of *M. tuberculosis* [154]. The TLRs have been the most widely studied in the context of TB, among which TLR2, -4 and -9 are the best characterized [157-159]. A vast array of inflammatory mediators are released following mycobacterial recognition by TLRs, shaping the immune response to *M. tuberculosis* [156, 160]. For instance, *in vitro* studies show that mycobacterial triggering of both TLR2 and TLR4 are important for the production of tumor necrosis factor (TNF) [158, 161-163], TLR2 and TLR9 are major drivers of IL-12 production [158, 161, 164] and IL-10 secretion by antigen-presenting cells (APCs) is highly dependent on mycobacterial TLR2 engagement [165, 166].

The activated innate immune cells also take up the bacteria to attempt at destroying it [167]. Resident macrophages provide the first phagocytic response and initiate a number of effector mechanisms, such as phagosome/lysosome fusion [168], generation of reactive nitrogen (RNI) and oxygen intermediates (ROI) [169, 170] and apoptosis [171, 172]. However, there is also evidence suggesting that *M. tuberculosis* can use macrophage intracellular media to replicate and to persist within lung granulomas [171-174]. Neutrophils are heavily recruited after infection and become the most abundant cell type found in the bronchoalveolar lavage of active pulmonary TB patients [175], where they exert important microbicidal activities [176]. Berry et al [39] described a transcriptional signature dominated by a neutrophil-driven IFN-inducible gene profile, consisting of both IFN-γ and type I IFNs (IFN-α and IFN-β) signalling, that was specific to patients with active TB and correlated with radiological severity. Several other reports also emphasize the contribution of neutrophils to pathologic exacerbation in TB [177-180].

DCs are fundamental players in the host immune response to mycobacterial challenges, standing out as the main APCs, crucial for B and T cell activation [181]. They engulf and process *M. tuberculosis* or apoptotic bodies and are directed to the draining mediastinal lymph nodes through the presence of specific cytokine and chemokine gradients to initiate the adaptive immune response [182, 183], which further discussed in the next section.

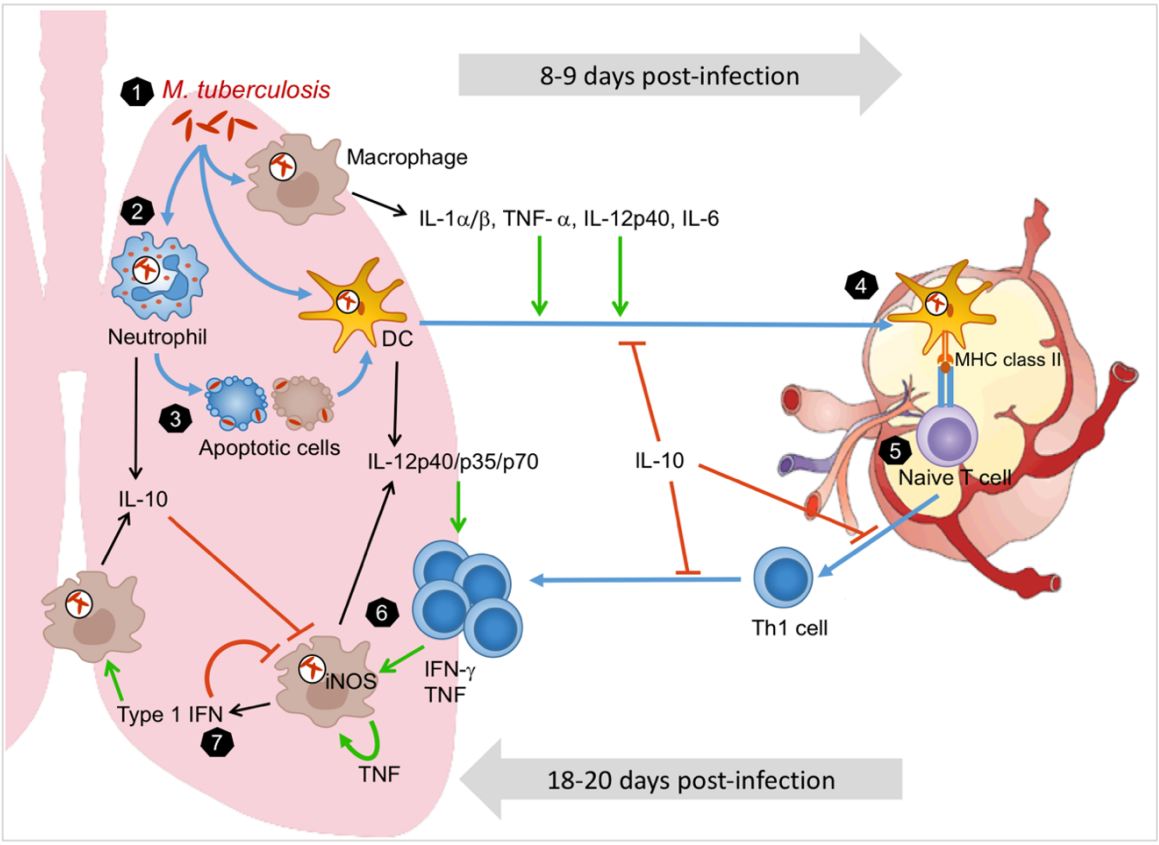

Figure I.4 – The innate and adaptive immune response network after *M. tuberculosis* infection. Following aerosol infection with *M. tuberculosis* **(1)**, the bacteria is taken up by the resident phagocytes **(2)**, including neutrophils, alveolar macrophages and dendritic cells (DCs). Macrophages and neutrophils represent the front line of host defence, locally secreting several antimicrobial peptides and inflammatory mediators, while DCs, the main antigen-presenting cells (APC), migrate to the draining mediastinal lymph nodes to activate and induce proliferation of antigen-specific naïve CD4+ T cells. The outcome of infection is favored when the infected macrophages or neutrophils undergo apoptosis **(3)**, delivering the compartmentalized antigen in apoptotic bodies to DCs, thus increasing their traffic rate to lymph nodes **(4)**. This process is further influenced by innate cytokines and chemokines (green arrows = stimulates; red marks = inhibits), which also promotes naïve T cell differentiation toward a Th1 phenotype **(5)**. Initially, the bacilli prevent phagolysosomal fusion and is able to survive and replicate inside macrophages. Protective antigen-specific Th1 cells migrate back to the lungs and produce IFN-γ, leading to macrophage activation and cytokine production **(6)**. IL-12 (p40/p35) is essential for the initial induction of protective IFN-γ T-cell responses against *M. tuberculosis*, while IL-12p70 is required for expanding and sustaining this process. Bacterial control is enhanced through phagosome maturation and induction of microbicidal factors, including iNOS. TNF promotes intracellular killing and is required for long term maintenance of the tuberculous granuloma. Certain hypervirulent *M. tuberculosis* strains induce the secretion of type I IFN by macrophages **(7)**, which acts in an autocrine way or via IL-10 production to inhibit macrophage effector functions and secretion of cytokines/chemokines. Neutrophils are also dominant producers of IL-10 in the lung, providing an anti-inflammatory feedback loop that prevents granulocyte-mediated exacerbated pathology. IL-10 can also block chemotactic factors that control DCs trafficking to the draining lymph nodes and Th1 cell trafficking back to the lungs. In the lymph nodes,

3.3. The adaptive immune response

Activated DCs arrive to the draining lymph nodes, where they present processed bacterial antigens at the cell surface via major histocompatibility complexes (MHC) I and II, leading to the activation of naïve CD8+ and CD4+ T cells, respectively. This process creates the micro-environment needed for differentiation and proliferation of CD8+ cytotoxic T cells (CTLs) and, under IL-12 and IFN-γ influence, of CD4+ Th1 cells [183]. Then, protective antigen-specific Th1 cells migrate back to the lungs and produce IFN-γ, enhancing macrophage activation and cytokine production [167, 184] (Figure I.4). Bacterial control is promoted through phagosome maturation and induction of microbicidal factors, which in the mouse model of infection include the production of nitric oxide (NO) and its metabolites (RNI) in a reaction catalyzed by the enzyme inducible NO synthase (iNOS or NOS2) [185, 186]. Mice deficient on *NOS2* are highly susceptible to mycobacterial challenges [185-187]. Moreover, mycobacterial growth is enhanced when iNOS is inhibited in infected human alveolar macrophages [188, 189].

With a still relatively unappreciated role, Th17 cell subset also takes part in the immune response of TB. These cells mainly secrete IL-17 which is known to modulate granuloma formation and is required for BCG vaccine-dependent accelerated recruitment of IFN-γ producing Th1 cells to the site of infection [190, 191]. However, it has been shown that repeated BCG exposure of *M. tuberculosis*-infected mice leads to increased Th17 cell responses accompanied by neutrophil/granulocyte recruitment and consequently increased immunopathology [192]. Therefore, Th17 cells need to be tightly regulated during mycobacterial infections to avoid serious tissue damage.

The relevance of adaptive immune responses to control *M. tuberculosis* infection is reiterated on HIV infected patients with low CD4+ T cell counts, rendering these patients more prone to develop disseminated TB [193, 194]. A similar outcome is observed in recombination activating gene (*RAG*) deficient mice, which are unable to produce B and T lymphocytes and succumb to *M. tuberculosis* shortly after infection [195, 196]. Other cell subsets have also been studied under the scope of TB, including CTLs [197, 198], Th2 [199] and B cells

[200-204], however they showed inconsistent results. Foxp3+ regulatory T (Treg) cells, on the other hand, have been pointed as important players in the control of immune responses to infection, to prevent exacerbated immunopathology [154]. Their role in TB is suggested by the increased numbers detected in patients with active disease, when compared to latent infected individuals [205]. In the mouse model, Tregs were also shown to proliferate and accumulate at the mycobacterial infection sites [206], where they suppress the development of IFN-γ-producing CD4+ T cells and overall Th expansion [207, 208].

As already highlighted, to orchestrate the different components of the cellular responses to TB, an extensive array of immune mediators is required. Important examples of cytokines involved will be described in the following sections.

3.4. Inflammatory mediators

Upon recognition and internalization of *M. tuberculosis*, a vast array of inflammatory mediators is released to the vicinity of the infected cell [157, 209]. These mediators include, among others, IL-12, including subunit IL-12p40, IFN-γ, IL-17, IL-6, IL-1β, type I IFN, TNF-α and eicosanoids [157, 209]. Together these molecules shape the immune response to *M. tuberculosis*.

3.4.1. IL-12p40, homodimers and heterodimers

IL-12p40 associates to another IL-12p40 molecule to form the homodimer IL-12p80 [209, 210], which is secreted by phagocytes readily after infection and is essential to induce a migratory phenotype in DCs, allowing these cells to travel to the lymph node where naïve T cells will be activated [154, 184, 209]. This IL-12p80-dependent mechanism is believed to be enhanced through the expression of a splice variant of the IL-12 receptor β1 (IL-12Rβ1, which binds to IL-12p40) by DCs early after infection [211]. Although IL-12p80 is important for the early events leading to the adaptive immune response, this molecule is unable to trigger the production of IFN-γ, a critical cytokine required for the control of *M.*

tuberculosis [209]. This role belongs to another mediator within the IL-12 family of cytokines.

Unlike IL-12p40, IL-12p35 is not significantly expressed in the early stages after infection [209]. Accordingly, the critical role of IL-12p70 (also known as IL-12), a product of dimerization of IL-12p40 with IL-12p35, may not involve the initial triggering of an adaptive immune response. Instead, IL-12p70 is essential for the induction and maintenance of IFN-γ-producing T cells, through Th1 lineage differentiation, which are protective in later stages of infection [212, 213].

IL-12p40 also dimerizes with IL-23p19, forming the bioactive molecule IL-23. This cytokine is produced in response to *M. tuberculosis* by macrophages and DCs in a TLR2-dependent manner [214, 215]. In contrast to IL-12p70, IL-23 is not essential to control *M. tuberculosis* infection, as IL-23p19 deficient mice remain capable of controlling mycobacterial growth and of maintaining stable levels of local IFN-γ mRNA expression [213]. However, IL-23 is required to sustain optimal Th17 cell responses, IL-6 and TNF [213, 216-219], and partially compensates for the generation of IFN-γ-producing CD4+ T cells in the absence of IL-12p70 [213]. Also, the IL-23/IL-17 immune axis is critical to regulate the early recruitment of neutrophils to the infection site [220].

3.4.2. IFN-γ

The primary source of IFN-γ are CD4+ T cells [221]. Antigen specific CD8+ T cells and cells of the innate immune system γδ T cells, natural killer (NK) T cells, NK cells and macrophages, also produce this cytokine during mycobacterial infection [222, 223], although they generally do not compensate for CD4+ T cells insufficiency [154]. However, it has been hypothesized that these innate sources play an important protective role in the case of T cell immunodeficiency as seen in HIV/TB coinfected individuals [223].

The role of IFN-γ in the immune response to *M. tuberculosis* infection is central, as illustrated both in the mouse model [224, 225] and in humans [226]. Indeed, TB control is severely compromised in subjects bearing genetic deficiencies that impair Th1 cell differentiation, IFN-γ production or the cellular

response to this molecule, leading to increased susceptibility even to weakly virulent mycobacteria [227, 228]. *Ifng* deficient mice have defective macrophage activation and low NOS2 expression [225]. As a result, there is unrestricted mycobacterial growth and granulomas become quickly necrotic. However, IFN-γ-induced NO production cannot explain totally the antimycobacterial action of this cytokine [229]. Treatment of cells with IFN-γ stimulate autophagic pathways that promote mycobacterial phagosomes to mature into phagolysosomes and increase *M. tuberculosis* killing [230, 231]. On the other hand, IFN-γ also have anti-inflammatory properties, by negatively regulating IL-17 production and neutrophil recruitment [232-234], both of which might be beneficial in early stages of infection, but have detrimental effects if they persist in chronic phase.

Given its critical role against mycobacterial infection, the detection of TB antigen-specific circulating effector memory T cells through IGRA has emerged as useful immunological marker of *M. tuberculosis* infection [154]. However, IFN-γ production is not a direct correlate of protection and, in fact, patients with more severe TB disease have the highest IFN-γ levels in the serum [235] and bronchoalveolar lavage fluid (BALF) [236], which fall during successful therapy [235, 236].

3.4.3. IL-17

The differentiation from naïve precursor cells to Th17 cells, a subset of IL-17-producing CD4+ T cells [237], depends on the production of IL-23, IL-1β, IL-6, and TNF-α [223, 238] and it is inhibited by IFN-γ or IL-4 [239]. The importance of Th17 cells is illustrated by the fact that defective production of Th17-related cytokines influences the outcome of the infection [240]. Despite the presence of Th17 cells during infection, γδ T cells were shown to be the primary source of IL-17 in response to IL-23 in *M. tuberculosis* infection [241, 242].

Experimental evidence in the mouse model support the role of IL-17 in the formation, maintenance and long-term integrity of granuloma in mycobacterial infection [191, 242]. In mice, the absence of IL-17 provokes a significant reduction in the mononuclear and polymorphonuclear inflammatory infiltrates to

the lung [240, 243]. Conversely, uncontrolled IL-17 signaling drives the accumulation of a large number of granulocytes, leading to increased tissue damage and host susceptibility upon TB infection [190]. In the context of vaccination against TB, IL-23 improves the vaccine efficacy when used as an adjuvant [244], by inducing a protective Th17 memory population [243]. Hence, a fine-tuned Th17 immune response is essential to ensure that infection is resolved without permanent sequelae.

3.4.4. IL-6

IL-6 is produced mainly by monocytes/macrophages [245] and epithelial cells [246] in early phases of the *M. tuberculosis* infection. This cytokine is usually found in elevated levels in the BALF from involved sites with TB [247]. IL-6 is known to exert a series of effects during *M. tuberculosis* infection. For instance, IL-6 signaling (together with transforming growth factor [TGF]-β) plays a critical role during the initial stages of Th17 cell differentiation, while inhibiting the generation of Treg cells induced by TGF-β [248]. Moreover, IL-6 is required for the induction of an optimal T cell response [249], probably through a set of mechanisms that include the regulation of acute phase proteins synthesis [250], regulation of serum iron levels [251], B-cell [252] and T-cell differentiation [248, 253-255]. Though IL-6 deficient mice are able to control *M. tuberculosis* after lower dose aerosol infection, they rapidly succumb when exposed to high doses of bacteria [256].

3.4.5. TNF-α

TNF is a critical cytokine involved in the control of *M. tuberculosis* growth by promoting intracellular killing and participating in the long-term maintenance of the tuberculous granuloma [257]. TNF deficient mice are unable to control the growth of *M. tuberculosis* and quickly succumb to infection [258-260]. In humans, pharmacological disruption of the TNF signaling pathway also increases susceptibility to TB, particularly favoring the transition from latent infection to

active disease [261]. In apparent contrast to these findings, an increase in plasma TNF levels was reported to associate with clinical deterioration observed early in the treatment of severe TB [262]. This implies that a balanced TNF production is needed to ensure favorable TB outcome. Furthermore, anti-TNF therapy was successfully used in patients with severe pulmonary TB [263], presumably by diminishing extreme inflammation.

3.4.6. The type I IFN/IL-1β/eicosanoids circle within the inflammasome network

Recent investigations ascribe great importance to the IL-1 family of cytokines during the immune response in TB. IL-1β is synthesized as an inactive precursor, pro-IL-1β, which requires cleavage by caspase-1 in order to attain its active form. Pro-IL-1β is virtually absent in naïve myeloid cells, but upon stimulation by pro-inflammatory cytokines (IL-1, TNF, TGF-β), stressors and by various TLRs, an inflammatory signaling pathway is activated through TAK1 (TGF-β-activated kinase 1) [264], promoting the nuclear translocation of NF-κB which upregulates pro-IL-1β mRNA transcription [265, 266]. Caspase-1 activation is accomplished within the inflammasome, an intracellular complex of proteins that are assembled after activation of PRRs in innate immune cells, mainly the NOD-like receptors (NLRs) NLRP1, NLRP3, NLRC4, and NAIP [267, 268]. IL-1β is readily secreted after bacterial challenge [269-272] and enhances the monocyte to macrophage transition, with improved phagocytic and antigen-presentation functions [273]. This cytokine also plays a role in the T-cell activation and T-cell-dependent antibody production, and guiding the transmigration of leukocytes into the stress location [274]. Moreover, IL-6 and IL-23 production are both IL-1β-dependent [274, 275], justifying the failure to induce Th17 differentiation and IL-17 production in IL-1 receptor (IL-1R) type I-deficient mice [276]. In addition to local inflammation, IL-1β is also responsible for systemic responses, such as generation of fever and acute-phase proteins production [274]. Given the importance of this molecular pathway against infections, it is not surprising to

find that IL-1R deficient mice are extremely sensitive to low-dose aerosol infection with *M. tuberculosis* [277-280]. On the other hand, IL-1β is highly expressed in the BALF from pulmonary TB patients compared with that from healthy controls [281] and correlated with more advanced disease [282]. These data suggest that initial triggering of a protective IL-1β response is critical for host survival upon infection with *M. tuberculosis* and that keeping the balanced level of cytokine is vital to prevent disease progression. Previous studies have shown that continuous IL-1β expression promotes sustained recruitment of granulocytes to the lung, induces the expression of additional inflammatory mediators such as prostaglandin E2 (PGE2) and stimulates tissue-damaging metalloproteinases [283, 284].

More recently, the function of IL-1β within the context of eicosanoid pathway regulation was further explored. *M. tuberculosis*-infected *Il1r1*–/– mice present increased bacterial load, associated to reduced levels of PGE2 in BALF, while those of leukotriene B4 (LTB4) and lipoxin A4 (LXA4) are higher as compared to wild-type animals [285]. The causal link between these molecules appears to be the modulation of the infected cell death type by the eicosanoid mediators balance, which is known to affect the viability of the infection [173, 286, 287]. PGE2 induces apoptosis of infected macrophages, a mechanism associated with protection against mycobacteria. In contrast, LXA4 induces a necrotic phenotype, which favors bacterial proliferation and infection of bystander cells, thus enabling the formation of new infectious foci [127, 171-173, 288]. In line with this model, arachidonate 5-lypoxigenase (ALOX5) deficient mice, which are unable to produce LTB4 and LXA4, therefore shifting the eicosanoid metabolic pathway towards PGE2 production, are more resistant to *M. tuberculosis* [173, 288]. Human studies also highlight the relevance of eicosanoids in TB, as individuals harboring polymorphisms in *ALOX5* and *LTA4H* (involved in LTA4 synthesis) show increased susceptibility to TB [289, 290].

The extent of the role of eicosanoids in TB is not yet fully understood. There is evidence suggesting that PGE2 is also important for the regulation of the crosstalk between IL-1β and type I IFNs [285]. The latter subvert anti-tuberculous host defenses by inhibiting iNOS and IL-12p40, while inducing the

immunosuppressive mediators IL-10 and IL-1 receptor antagonist (IL-1RA) [154, 279]. Type I IFNs also reduce IL-1β production, either indirectly, through IL-10-mediated pro-IL-1β transcriptional repression, or directly suppressing the NLRP1b and NLRP3 inflammasome-dependent caspase-1 activation [291]. Such inflammasome inhibition by type I IFNs also reduces the maturation of IL-18, an important inducer of IFN-γ [292]. Type I IFNs were found to independently counter-regulate the IL-1 function and the prostaglandin axis in *M. tuberculosis*-infected human and murine cells and, conversely, IL-1α/β and PGE2 inhibit IFN-β expression [285]. These findings highlight the existing crosstalk between these pathways in TB and indicate a protective role mediated by IL-1/PGE2 through suppression of type I IFNs (**Figure I.5**).

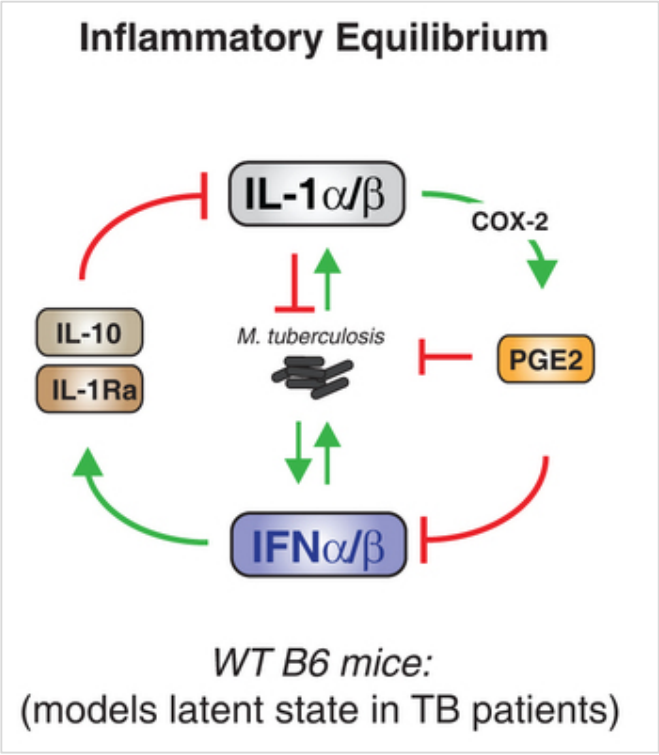

Figure I.5 – Schematic summary of IL-1–type I IFN counter-regulation during *M. tuberculosis* infection. Virulent *M. tuberculosis* directly induces IL-1α and IL-1β as well as type I IFNs in phagocytic myeloid cells. While pro-inflammatory IL-1 is required for bacterial control, type I IFNs subvert anti-tuberculous host defenses by inducing the immunosuppressive mediators IL-10 and IL-1RA. The balance between both pathways determines an inflammatory equilibrium whereby mycobacterial bacilli are contained to produce a latent or subclinical chronic infection. Through COX-2-mediated PGE2 induction, IL-1 inhibits bacterial growth either by promoting apoptosis of infected macrophages, or by opposing the type I IFNs function. *From* Mayer-Barber et al. [285].

3.4.7. IL-10 and IL-1RA balancing the inflammatory response

The initial triggering of a protective immune response is critical for host survival upon infection with *M. tuberculosis*, as described above, and keeping the appropriate level of immunity is vital to prevent disease progression. Once active disease is established, and the bacterial burden increases, the host immunity is further activated leading to a potentially dangerous level of inflammation. In this context, IL-10 and IL-1RA induction may be beneficial to counteract tissue destruction caused by the infection itself, shifting the architecture of the immune response towards a tissue regenerative one [293-295].

IL-10 is an important anti-inflammatory mediator and is found increased in the sputum [296], BALF [297] and serum [235] of patients with pulmonary TB. This cytokine acts in an autocrine/paracrine way, especially on the innate compartment, to inhibit the production of pro-inflammatory cytokines such as IL-1, IL-6, TNF and IL-12 [298-301], which in turn compromises the production of IFN-γ by T cells [302-304], IL-12p40-dependent migration of infected DC to the draining lymph nodes [184, 305] and the lytic activity of T cells [304]. A good clinical example of the importance of IL-10 in the regulation of TB-induced inflammation was brought by the finding that anergic subjects, designated this way because they fail to develop a delayed-type hypersensitivity responses to mycobacterial antigens and, therefore, lack dermal reactivity to tuberculin PPD, have constitutively active IL-10-producing T cells [306]. Some human data suggest the genetic association between IL-10 polymorphisms and susceptibility to TB [307-309]. Yet, these data remain largely inconsistent, with studies in some populations reporting association of IL-10 SNPs with TB susceptibility and others not [310-313].

In the animal model, transgenic mice that overexpress IL-10 have enhanced susceptibility to BCG [314] and *M. tuberculosis* [315], because of an inability to mount a protective immune response. Nevertheless, studies focusing in IL-10 deficient mice show contradictory results. When IL-10 activity is blocked during the chronic stage of infection (day 90 post-infection) in CBA/J mice, that by default are susceptible to *M. tuberculosis*, an increased survival was observed, accompanied by diminished bacterial burdens and enhanced Th1 immunity

[316]. Moreover, IL-10 deficient mice showed enhanced control of the bacterial load in the lung and spleen during *M. tuberculosis* infection independently of the genetic backgrounds studied, BALB/c and C57BL/6, after one month of disease [317]. Conversely, data from other studies show that infected IL-10 deficient mice in C57BL/6 background , were either transiently protected [318], or had similar bacterial burdens as wild type mice [319, 320]. This was despite enhanced levels of IFN-γ, IL-12p40 and iNOS [319]. Furthermore, at late stages of infection (over 60 days) these mice succumb and show increased bacterial burdens, due to an overall lack of control of the inflammatory responses, leading to tissue damage and disease progression [321]. These data underline the key role played by IL-10 in dampening the Th1 response to limit chronic lung inflammation in TB.

Another potentially relevant counter-regulatory mediator is IL-1RA, a natural competitive antagonist of IL-1Rs, exerting its anti-inflammatory effects by blocking the binding of IL-1α and IL-1β to the IL-1R [322, 323]. Similarly to IL-1β [281], studies have shown that IL-1RA is also significantly increased in BALF [282] from TB patients. However, while circulating IL-1β was undetectable, elevated serum concentrations of IL-1RA were found in patients with active TB as compared to control subjects [324], suggesting that IL-1RA could be used as a biomarker of disease activity. The precise impact of IL-1RA on TB pathogenesis remains elusive. Anakinra, a recombinant human IL-1RA, has been used on a large number of patients with different inflammatory diseases, from gout to rheumatoid arthritis [325], and still, records show little or absent risk of *M. tuberculosis* reactivation in latently infected individuals [325, 326].

4. Host immune imbalances in TB: from genetic determinants to environmental factors

The status of the host immune system is a critical determinant of TB outcome. Many studies unequivocally show that immune imbalances resulting from genetic variants, life habits or comorbidities affecting the host determine the course of disease, notably transmission, immediate manifestation of active TB or transition from latent infection to active TB (**Figure I.6**). The outstanding question remains: how can we predict the outcome of infection for a given individual? This question has prompted genetic association studies over the years, but a definite answer is still unavailable.

4.1. Host genetics: role in immunomodulation

Family clustering of TB cases offered the first clues for a putative genetic susceptibility to TB [327, 328]. However, family clustering could also be explained by common environmental factors and closer physical proximity that would increase the chance of *M. tuberculosis* transmission. In this sense, compelling evidence for the role of host genetic factors in TB was provided by twin studies, which showed higher concordance rates for monozygotic than for dizygotic pairs [329, 330]. Further evidence came from the description of ethnic differences associated to TB, namely suggesting that subjects of African ancestry are more prone to become infected than caucasians, possibly due to differential permissiveness of macrophages to bacilli intracellular growth [331, 332]. The search for specific molecular pathways affected by single gene defects predisposing to disease progression has however failed to provide specific answers, except in the case of vulnerability to severe mycobacteriosis observed in otherwise healthy children [333]. This rare inherited condition was named Mendelian susceptibility to mycobacterial disease (MSMD), ranging from localized to disseminated infections with one or more mycobacterial species, including the most virulent *M. tuberculosis* [228]. The first reports occurred with children with impaired IL-12-dependent cellular response due to autosomal

recessive IL-12Rβ1 deficiency [334-336]. Other MSMD-causing mutations have been described in autosomal (*IFNGR1*, *IFNGR2*, *IL12B*, *STAT1*, *IRF8*, *ISG15*) and X-linked (*NEMO*, *CYBB*) genes [337-341], which are all physiologically related, as they all result in an impairment of the CD4 T cell-mediated immunity. MSMD provided decisive evidence on the critical protective role of the IL-12/IL-23/IFN-γ loop, that was further confirmed in the context of secondary immunodeficiencies, such as in HIV infection.

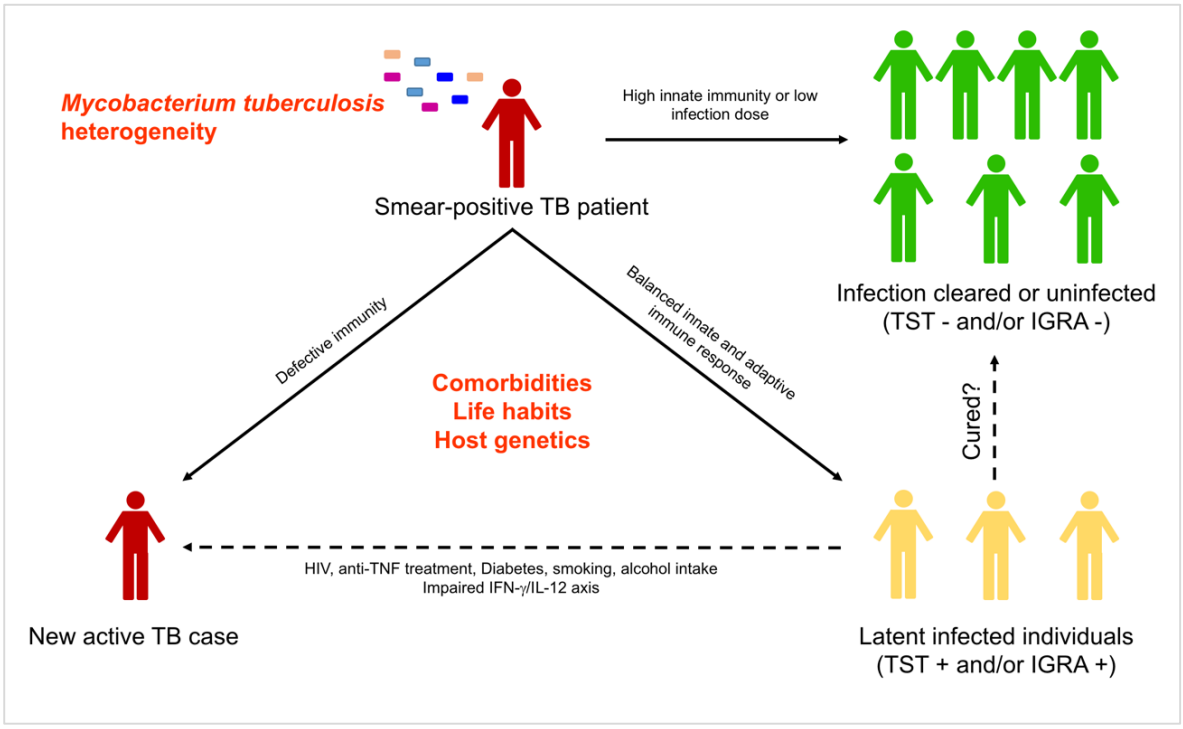

Figure I.6 – Heterogeneity of outcomes after *M. tuberculosis* exposure. Although roughly 10 million new tuberculosis (TB) cases are still reported every year, it is thought to represent only a minor proportion of individuals exposed. As many as 70% of people exposed do not get infected, which can be explained in different ways: (a) the new host presents an highly effective innate immune response capable of rapidly clearing the infection, without developing a detectable adaptive immune response, i.e., negative tuberculin skin test (TST) and/or IFN-γ release assay (IGRA) results; (b) environmental factors that decrease the transmissibility of airborne contaminated droplets; (c) infection dose or time exposed was insufficient to lead to infection. It is estimated that nearly 2 billion people of the world's population contain the infection in a dormant asymptomatic state. The majority of these (around 90%) will keep this latent TB under the control of an effective innate and adaptive immune response. Some of these will clear the infection, but will still demonstrate reactivity to mycobacterial antigens with TST and/or IGRA. However, 5-10% will develop active TB during their lifetime (half during the first 2 years after infection), usually triggered by an immunosuppressive event. Other precipitating factors include metabolic diseases, life habits and host genetics. Increasing evidence suggests bacteria diversity as major player in the TB pathogenesis. *Developed and expanded from the work by* O'Garra et al. [154].

In the case of adult patients, genetic studies did not show any major susceptibility locus [342, 343] and remarkably no variants of genes from the IL-12/IFN-γ axis have been consistently found [310, 343]. Nevertheless, several specific candidate gene-based studies were performed over the years and a number of genetic variants were associated with TB susceptibility. This is the case of NRAMP1, DC-SIGN, TLRs, vitamin D receptor, TNF, IL-1β, IL-10 or some HLA class II molecules [310, 344, 345]. Remarkably, these data remain largely inconsistent, with studies in some populations reporting association with TB susceptibility and others not [310, 311, 346]. As an example, different polymorphisms in the human TLR2 gene were reported to associate with increased susceptibility to TB [347-350], whereas such association was not observed in other studies [351-354]. This discrepancy might be explained on the basis of a dynamic host-pathogen genetic and pathogen phenotypic interplay [145]. This example calls for the need of integrating the pathogen genotype in human genetic association studies. More recently, the rise of genome-wide association studies (GWAS) allowed the unbiased identification of numerous SNPs with impact on pulmonary TB risk [355-363]. However, results did not support previous candidate-based studies and so far have not been validated on different populations [344, 363]. In all, the lack of clear genetic variants associated with TB susceptibility most likely reflects the fact that adult pulmonary TB is associated with complex genetic traits, where gene-gene interactions (epistasis) and extrinsic factors play a far more important role in an individual's susceptibility to develop the disease than single polymorphisms on their own [364, 365].

4.2. The modulation of the immune response by extrinsic factors

Evidence shows that the progression from LTBI to pulmonary TB in adults usually reflects an impairment of host resistance due to non-genetic factors, such as aging, life habits, AIDS, diabetes or other comorbidities [36]. The converging point of these extrinsic factors is the immune imbalance they impose to the host. Recent changes in the human population are, through these factors, changing the

face of the TB epidemics, in many cases contributing to increase the number of new active TB cases [366, 367]. However, for most of these factors much more research is needed to understand the molecular basis of the interaction.

4.2.1. Coinfections

A role for coinfection with other pathogens during infection and transmission of TB has been addressed. A major driver of the current TB epidemics has been the HIV syndemic, as it dramatically decreases the host protective responses to TB in a $CD4^+$ T cell count dependent manner [12]. However, the risk of developing TB is largely increased in HIV infected individuals even before CD4+ T cell count decreases [368, 369]. TB/HIV coinfection is complex and leads to an acceleration of both diseases. The pathogenesis of TB is different in HIV-coinfected individuals [369, 370] resulting in the lack of cavitation [371, 372] and in a higher incidence of disseminated disease [373, 374]. A direct impact of HIV infection in *M. tuberculosis* physiology has been recently drawn. Coinfected individuals seem to have a detrimental alternative activation of macrophages, with less NO expression and poorly-formed granulomas, which in turn down-regulates *M. tuberculosis* DosR regulon [375], a set of genes known to be induced during anaerobic dormancy [376]. Therefore, changes in the host immunity resulting from HIV coinfection may remodel the bacteria functioning, which further rewires the host tissue micro-environment.

Other less studied coinfections with impact on TB include helminths, flu and *Helicobacter pylori*. In TB patients coinfected with helminths a more advanced form of disease was reported [377], possibly related with decreased Th1 and Th17 cell responses and increased secretion of IL-10 [378, 379]. In the case of influenza, the increased susceptibility to *M. tuberculosis* infection is mediated by type I IFN signalling [380]. Contrastingly, infection with the ubiquitous bacteria *H. pylori* may help to avoid progression to active TB in latent individuals due to enhanced IFN-γ and other Th1-like cytokine responses generated in response to *H. pylori* and that restrain *M. tuberculosis* [381]. It is interesting that of these three

infections only influenza is also a lung disease, thus indicating that distant events also shape the lung micro-environment.

4.2.2. The host microbiome

Not only coinfections with other pathogens, but also alterations in the commensal bacterial populations of the host may impact the outcome of TB. This is not surprising, considering that the microbiota composition plays an important role at the immunological level, by shaping the early development of the immune system, so that it can develop balanced responses to infections [382]. Using mouse models of infections depleted of commensal gut microbiota after antibiotic treatment, it has been shown that the risk of colonization by respiratory pathogens, like *Streptococcus pneumoniae* [383], *Staphylococcus aureus* [384] and *Klebsiella pneumoniae*, increased [385]. Limited available data suggest that the gut microbiota of mice infected with *M. tuberculosis* also changes drastically [386]. Furthermore, early studies in this topic reported an increase in the lung bacterial burden in germ-free mice early post *M. tuberculosis* infection [387]. Thus, specific microbiota may distally alter the local environment and eventually the course of disease. How the intestinal microbiota distally affects pulmonary immunity and how the gut-lung axis may impact TB await further research.

While the effects of the microbiota of the gut on health status have been well established [388], we are still very far from understanding the composition and the impact of changes in the oral-nasal cavity and the lung microbiota [389]. When *M. tuberculosis* firstly infects the host it has to find its way to the lower respiratory tract, where it will establish infection and resort to a collection of immune evasion strategies [154]. So, *M. tuberculosis* has to initially evade microbiota-activated macrophages of the upper respiratory tract [390]. It remains to be seen if the presence/absence of certain species in the upper respiratory airway generates a more permissive environment for the establishment of TB infection. Thus, restoring key players of the microbiota may help to fight invasion or even improve immunity, possibilities not yet explored in

TB infection [391]. Compared with the gut microbiome studies, the lung microbiome is still in its infancy and remains an exciting area for future research.

4.2.3. Non-communicable comorbidities

Among the non-communicable comorbidities, diabetes stands out as a major risk factor for TB. Diabetic patients have a three times higher risk of developing TB than healthy individuals [392, 393]. Owing to the dimension of the diabetes epidemics, predicted to affect 552 million individuals in the world by 2030 [394], its predicted impact on the global numbers of TB cases is therefore alarming. This is particularly worrying as the prevalence of diabetes is increasing in TB-endemic areas, thus putting at risk a considerable number of individuals. Not only does diabetes increase the risk for TB, but it also worsens disease severity [395], is a risk factor for death in TB patients [396, 397] and is associated with increased failure of standard TB treatment [395, 398, 399]. Evidence coming from human patients and experimental models of infection suggest that the interaction of *M. tuberculosis* with macrophages and DCs is impaired in the context of diabetes, leading to an initial hypo-inflammatory state [400-403]. Once the infection is established, there is evidence for increased inflammation in diabetic TB patients, as an augmented level of pro-inflammatory cytokines in the peripheral blood is measured, likely due to hyperactive T cell responses [401-403].

Similarly to diabetes, other chronic diseases are expected to increase globally in prevalence over the coming years. This is the case of chronic obstructive pulmonary disease (COPD) [404], specially in developing countries with high TB incidence [405]. Studies also showed that subjects with COPD have greater risk to undergo the latent *M. tuberculosis* infection to active disease transition [406, 407] and they also die more compared to TB patients without this comorbid condition [396, 406]. It is clear that COPD and TB share a few common risk factors, such as smoking and malnutrition [408]. Nevertheless, impaired lung function due to COPD itself has been associated with increased risk of TB, after adjusting for confounding factors, like smoking or corticosteroids use [409]. COPD is characterized by a disruption of defence mechanisms that

increase susceptibility to lower respiratory tract bacterial infections, including *M. tuberculosis*. These include decreased mucocilliary clearance of the airways and impaired macrophage phagocytosis [410, 411], as well as adaptive immune function impairment, as a result of the frail effector T cell response to bacterial antigens and accumulation of suppressive Tregs. At the cytokine level, circulating levels of Treg-generated cytokines IL-10 and TGF-ß are elevated [412].

In populations with high incidence of TB, there have been an increased number of TB cases reported in patients treated with TNF antagonists [413]. TNF is a potent inducer of tissue destruction associated with autoimmune diseases, but it is also a critical cytokine involved in the control of *M. tuberculosis*, as already mentioned above. Consequently, the risk of reactivation of latent TB is 1.6-25.2 times higher in rheumatoid arthritis patients receiving biologic inhibitors of TNF activity, compared to conventional immunosuppressive therapy [261, 413, 414]. For that reason, currently all national guidelines recommend the exclusion of active TB disease and LTBI in patients in whom anti-TNF therapy is considered [414].

4.2.4. Environmental factors

Smoking exposure is an independent risk factor for *M. tuberculosis* infection, progression to active disease and for poor treatment outcomes [415-417]. The immunological mechanisms are just starting to be unveiled and include reduced production of TNF, IL-1β and IFN-γ by *in vitro* infected alveolar macrophages [418], decreased number of DCs [419, 420] and compromised recruitment of CD4+IFN-γ+ T cells to the lung, thus weakening the formation of granuloma [421]. More recently, alveolar macrophages from smokers were found to exhibit lysosomal accumulations of tobacco smoke particulates, which impaired their migration towards *M. tuberculosis*-infected cells [422].

Alcohol consumption has also been pointed as a predisposing factor to *M. tuberculosis* infection and to greater progression from initial infection to active disease [423-425]. The mechanism of susceptibility was partially exposed in a mouse study, where consumption of a liquid ethanol diet for 9 weeks blunted the

CD4+ and CD8+ T-cells responses and resulted in significantly higher bacterial burden in the lung [426]. Other study also identified impaired lung CD4+ and draining lymph node T-cell IFN-γ responses of alcohol-consuming mice compared with control mice [427].

One of the oldest described associations with TB has been the poor nutritional status, although it remains unclear whether malnutrition is a cause or a consequence of the disease [428]. It is known, however, the dismal impact that nutritional deprivation has on immune dysregulation [429]. This association is even worse in the framework of HIV infection [430]. Nutritional status not only can affect the function of T cells and other immune cells, but in addition impacts the pharmacodynamics of the drugs [431]. One particular nutrient, vitamin D, has been the focus of renewed attention by researchers [432, 433]. Historically, both vitamin D and exposure to sunlight, which endogenously promotes the conversion in the skin of 7-dehydrocholesterol into pre-vitamin D_3, were used in the treatment of TB [434]. Insufficiency of this molecule has been linked to higher risk of active TB [435, 436] and increased propensity for extrapulmonary involvement [437]. Therefore, variation in sunlight may be behind some of the differences in TB incidence rate and clinical manifestations across the globe [435]. The immunomodulatory role of vitamin D is well established both during innate and adaptive immune responses leading to several changes in immune responses, including the induction of cathelicidin antimicrobial peptide (CAMP), beta-defensin and the promotion of authophagy and/or bacterial killing [438, 439]. Finally, vitamin D levels have been shown to impact adaptive immune responses by influencing Th cell function and by promoting Tregs [440]. Despite all the evidence, several clinical trials have shown that supplementation of drug regimens with vitamin D does not improve TB outcomes in general population [441], but it has been shown to accelerate sputum conversion in patients with the *tt* genotype of the *TaqI* vitamin D receptor (*VDR*) polymorphism [442]. It remains to be seen if supplementation with vitamin D can play a role in preventing infection or progress to active TB.

5. Concluding remarks

The complexity of TB pathogenesis and natural history of infection, the perfect adaptation of *M. tuberculosis* to the human host and the incredibly high number of factors that impact on the disease, make the understanding of TB one of the greater challenges in research. It remains to be explained why only few individuals exposed develop active disease, whereas the majority will either clear the infection or become latently infected (Figure I.6) [443]. Most interestingly, even among the active TB patients, the clinical manifestation of disease is highly variable, with mild to extensive pulmonary involvement (**Figure I.7**), extrapulmonary and disseminated forms. So, TB is not a unique type of disease, but instead a continuous spectrum of TB likely exists [444]. While the exact mechanisms governing the transitions along the *M. tuberculosis* infection stages are still unknown, many observations indicate a role for the imbalances of the host immune response. The best evidence supporting this thesis is the case of latent to active disease transition due to acquired immunosuppressive conditions, such as HIV coinfection and anti-TNF treatment, as previously described. Nevertheless, the immune condition of an infected subject is probably shaped by many other factors, including host genetics and by extrinsic factors that alter the local micro-environment [36], but also by the heterogeneity of the infecting bacteria [445]. Altogether, this troika determines the threshold of the immune response generated during infection and consequently the disease outcome. Modulating these thresholds and uncovering the links between host, pathogen and micro-environments should allow for the discovery of solid correlates of protection, molecular markers for disease prognosis, and the development of safe and effective HDT to TB [446-448].

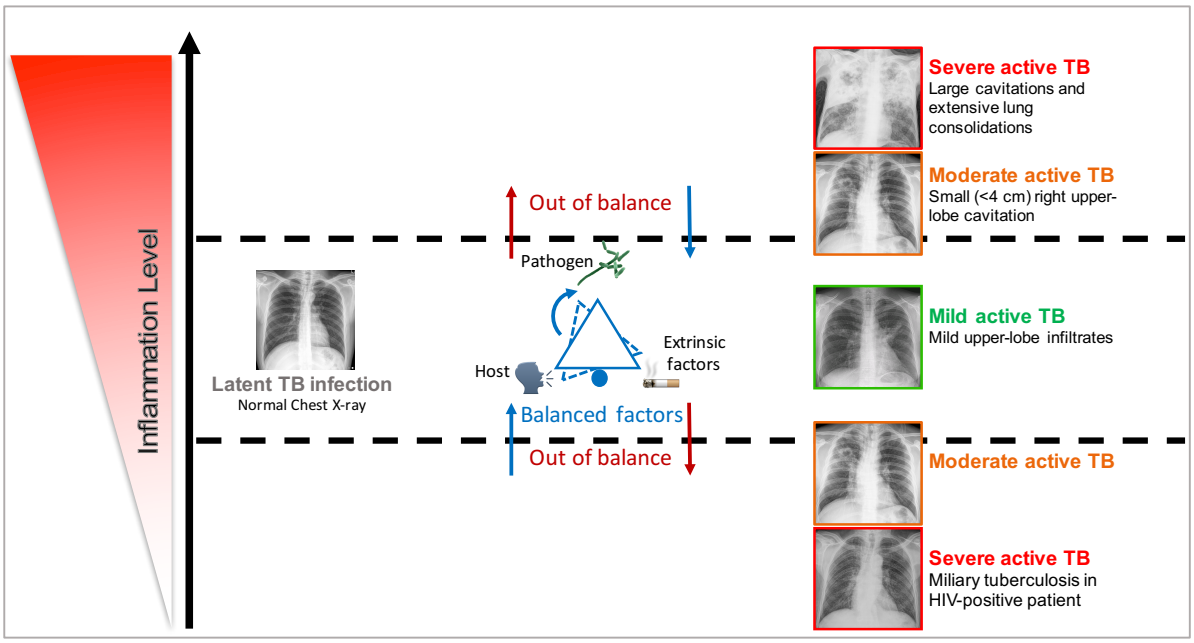

Figure I.7 – Causes of tuberculosis presentation heterogeneity and severity. The clinical manifestations of disease are highly variable. This continuous spectrum of TB is likely shaped by host, bacterial and extrinsic interactions orchestrating the type and intensity of the immune response generated during infection. While high inflammatory responses are detrimental to the host, even causing respiratory failure in the acute phase, very low thresholds of inflammation, as seen with HIV-infected patients, may not be enough to trigger the defence mechanisms needed to control TB. Hence, imbalances on the inflammatory response may lead to different outcomes of disease. Protection results from a fine-tuned balance between pro- and anti-inflammatory mediators, inducing mild to moderate disease that will presumably resolve without permanent sequelae. *From* Bastos HN et al. (submitted).

REFERENCES

1. Rubin SA. Tuberculosis. Captain of all these men of death. Radiol Clin North Am. 1995;33(4):619-39.
2. Villemin JA. Études expérimentales et cliniques sur tuberculose. Paris: Ballière et Fils; 1868.
3. Koch R. Die Aetiologie der Tuberculose. Berl Klin Wochenschr 1882;19:221–30.
4. Murray JF, Schraufnagel DE, Hopewell PC. Treatment of Tuberculosis. A Historical Perspective. Ann Am Thorac Soc. 2015;12(12):1749-59.
5. Fox GJ, Mitnick CD, Benedetti A, Chan ED, Becerra M, Chiang CY, et al. Surgery as an Adjunctive Treatment for Multidrug-Resistant Tuberculosis: An Individual Patient Data Metaanalysis. Clin Infect Dis. 2016;62(7):887-95.
6. Mitchison D, Davies G. The chemotherapy of tuberculosis: past, present and future. Int J Tuberc Lung Dis. 2012;16(6):724-32.
7. Lönnroth K, Migliori GB, Abubakar I, D'Ambrosio L, de Vries G, Diel R, et al. Towards tuberculosis elimination: an action framework for low-incidence countries. Eur Respir J. 2015;45(4):928-52.
8. World Health Organization. Global tuberculosis report 2016. Geneva: World Health Organization; 2016.
9. Houben RM, Dodd PJ. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. PLoS Med. 2016;13(10):e1002152.
10. Dye C, Glaziou P, Floyd K, Raviglione M. Prospects for tuberculosis elimination. Annu Rev Public Health. 2013;34:271-86.
11. Hermans S, Horsburgh CR Jr, Wood R. A century of tuberculosis epidemiology in the northern and southern hemisphere: The differential impact of control interventions. PLoS One. 2015;10(8):e0135179.
12. Kwan CK, Ernst JD. HIV and tuberculosis: a deadly human syndemic. Clin Microbiol Rev. 2011;24(2):351-76.
13. Raviglione MC, Pio A. Evolution of WHO policies for tuberculosis control, 1948-2001. Lancet. 2002;359(9308):775-80.
14. Lange C, Abubakar I, Alffenaar JW, Bothamley G, Caminero JA, Carvalho AC, et al. Management of patients with multidrug-resistant/extensively drug-resistant tuberculosis in Europe: a TBNET consensus statement. Eur Respir J. 2014;44(1):23-63.
15. World Health Organization. Tuberculosis: a global emergency. World Health Forum. 1993;14(4):438.
16. Jassal MS, Bishai WR. Epidemiology and challenges to the elimination of global tuberculosis. Clin Infect Dis. 2010;50(Suppl 3):S156-64.
17. Glaziou P, Floyd K, Korenromp EL, Sismanidis C, Bierrenbach AL, Williams BG, et al. Lives saved by tuberculosis control and prospects for achieving the 2015 global target for reducing tuberculosis mortality. Bull World Health Organ. 2011;89(8):573-82.
18. Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, et al. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA. 1994;271(9):698-702.
19. Wejse C. Tuberculosis elimination in the post Millennium Development Goals era. Int J Infect Dis. 2015;32:152-5.
20. Ahsan MJ. Recent advances in the development of vaccines for tuberculosis. Ther Adv Vaccines. 2015;3(3):66-75.
21. Karp CL, Wilson CB, Stuart LM. Tuberculosis vaccines: barriers and prospects on the quest for a transformative tool. Immunol Rev. 2015;264(1):363-81.
22. Kaufmann SH, Evans TG, Hanekom WA. Tuberculosis vaccines: time for a global strategy. Sci Transl Med. 2015;7(276):276fs8.
23. Weiner J 3rd, Kaufmann SH. Recent advances towards tuberculosis control: vaccines and biomarkers. J Intern Med. 2014;275(5):467-80.
24. Getahun H, Matteelli A, Chaisson RE, Raviglione M. Latent Mycobacterium tuberculosis infection. N Engl J Med. 2015;372(22):2127-35.

25. Pai M, Riley LW, Colford JM Jr. Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review. Lancet Infect Dis. 2004;4(12):761-76.
26. Mori T, Sakatani M, Yamagishi F, Takashima T, Kawabe Y, Nagao K, et al. Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens. Am J Respir Crit Care Med. 2004;170(1):59-64.
27. Grimes CZ, Hwang LY, Williams ML, Austin CM, Graviss EA. Tuberculosis infection in drug users: interferon-gamma release assay performance. Int J Tuberc Lung Dis. 2007;11(11):1183-9.
28. Arlehamn CS, Sidney J, Henderson R, Greenbaum JA, James EA, Moutaftsi M, et al. Dissecting mechanisms of immunodominance to the common tuberculosis antigens ESAT-6, CFP10, Rv2031c (hspX), Rv2654c (TB7.7), and Rv1038c (EsxJ). J Immunol. 2012;188(10):5020-31.
29. Menzies D, Pai M, Comstock G. Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research. Ann Intern Med. 2007;146(5):340-54.
30. Goletti D, Sanduzzi A, Delogu G. Performance of the tuberculin skin test and interferon-γ release assays: an update on the accuracy, cutoff stratification, and new potential immune-based approaches. J Rheumatol Suppl. 2014;91:24-31.
31. Sester M, van Leth F, Bruchfeld J, Bumbacea D, Cirillo DM, Dilektasli AG, et al. Risk assessment of tuberculosis in immunocompromised patients. A TBNET study. Am J Respir Crit Care Med. 2014;190(10):1168-76.
32. Diel R, Loddenkemper R, Nienhaus A. Predictive value of interferon-γ release assays and tuberculin skin testing for progression from latent TB infection to disease state: a meta-analysis. Chest. 2012;142(1):63-75.
33. You E, Kim MH, Lee WI, Kang SY. Evaluation of IL-2, IL-10, IL-17 and IP-10 as potent discriminative markers for active tuberculosis among pulmonary tuberculosis suspects. Tuberculosis. 2016;99:100-8.
34. Vanini V, Petruccioli E, Gioia C, Cuzzi G, Orchi N, Rianda A, et al. IP-10 is an additional marker for tuberculosis (TB) detection in HIV-infected persons in a low-TB endemic country. J Infect. 2012;65(1):49-59.
35. Wergeland I, Pullar N, Assmus J, Ueland T, Tonby K, Feruglio S, et al. IP-10 differentiates between active and latent tuberculosis irrespective of HIV status and declines during therapy. J Infect. 2015;70(4):381-91.
36. Esmail H, Barry CE 3rd, Young DB, Wilkinson RJ. The ongoing challenge of latent tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2014;369(1645):20130437.
37. Petruccioli E, Scriba TJ, Petrone L, Hatherill M, Cirillo DM, Joosten SA, et al. Correlates of tuberculosis risk: predictive biomarkers for progression to active tuberculosis. Eur Respir J. 2016;48(6):1751-63.
38. Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, et al. A blood RNA signature for tuberculosis disease risk: a prospective cohort study. Lancet. 2016;387(10035):2312-22.
39. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature. 2010;466(7309):973-7.
40. Bloom CI, Graham CM, Berry MP, Rozakeas F, Redford PS, Wang Y, et al. Transcriptional blood signatures distinguish pulmonary tuberculosis, pulmonary sarcoidosis, pneumonias and lung cancers. PLoS One. 2013;8(8):e70630.
41. Kaforou M, Wright VJ, Oni T, French N, Anderson ST, Bangani N, et al. Detection of tuberculosis in HIV-infected and -uninfected African adults using whole blood RNA expression signatures: a case-control study. PLoS Med. 2013;10(10):e1001538.
42. Kaforou M, Wright VJ, Levin M. Host RNA signatures for diagnostics: an example from paediatric tuberculosis in Africa. J Infect. 2014;69(Suppl 1):S28-31.
43. Boehme CC, Nicol MP, Nabeta P, Michael JS, Gotuzzo E, Tahirli R, et al. Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. Lancet. 2011;377(9776):1495-505.

44. World Health Organization. Same-day diagnosis of tuberculosis by microscopy: policy statement 2011 [4 Sep 2016]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK131903/.
45. Jensen PA, Lambert LA, Iademarco MF, Centers for Disease Control and Prevention. Guidelines for preventing the transmission of Mycobacterium tuberculosis in health-care settings, 2005. MMWR. 2005;54(RR-17):1–141.
46. Mase SR, Ramsay A, Ng V, Henry M, Hopewell PC, Cunningham J, et al. Yield of serial sputum specimen examinations in the diagnosis of pulmonary tuberculosis: a systematic review. Int J Tuberc Lung Dis. 2007;11(5):485–95.
47. Rieder HL, Chiang CY, Rusen ID. A method to determine the utility of the third diagnostic and the second follow-up sputum smear examinations to diagnose tuberculosis cases and failures. Int J Tuberc Lung Dis. 2005;9(4):384-91.
48. Hooja S, Pal N, Malhotra B, Goyal S, Kumar V, Vyas L. Comparison of Ziehl Neelsen & Auramine O staining methods on direct and concentrated smears in clinical specimens. Indian J Tuberc. 2011;58(2):72-6.
49. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006;6(9):570-81.
50. World Health Organization. Fluorescent Light-Emitting Diode (LED) Microscopy for Diagnosis of Tuberculosis: Policy Statement. Geneva 2011.
51. World Health Organization. Guidelines for treatment of tuberculosis - 4rd edition Geneva: World Health Organization; 2010.
52. Hargreaves NJ, Kadzakumanja O, Whitty CJM, Salaniponi FML, Harries AD, Squire SB. “Smear-negative” pulmonary tuberculosis in a DOTS programme: poor outcomes in an area of high HIV seroprevalence. Int J Tuberc Lung Dis. 2001;5(9):847–54.
53. Toman K, Frieden T, Toman K. World Health Organization. Toman’s tuberculosis: case detection, treatment, and monitoring: questions and answers. Organization WH, editor. Geneva2004.
54. Cruciani M, Scarparo C, Malena M, Bosco O, Serpelloni G, Mengoli C. Meta-analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without solid media, for detection of mycobacteria. J Clin Microbiol. 2004;42(5):2321-5.
55. Williams-Bouyer N, Yorke R, Lee HI, Woods GL. Comparison of the BACTEC MGIT 960 and ESP culture system II for growth and detection of mycobacteria. J Clin Microbiol. 2000;38(11):4167-70.
56. World Health Organization. Expert group meeting on the use of liquid culture media. Use of liquid TB culture and drug susceptibility testing (DST) in low and medium income settings 2007 [4 Sep 2016]. Available from: http://www.who.int/tb/laboratory/use_of_liquid_tb_culture_summary_report.pdf?ua=1.
57. World Health Organization. Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB): Policy Statement. Committee WGAbtGR, editor. Geneva2008.
58. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med. 2010;363(11):1005-15.
59. Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. Xpert® Mtb/Rif assay for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane Database Syst Rev. 2014;1:1–166.
60. Lawn SD, Nicol MP. XpertR MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. Future Microbiol. 2011;6(9):1067–82.
61. Theron G, Zijenah L, Chanda D, Clowes P, Rachow A, Lesosky M, et al. Feasibility, accuracy, and clinical effect of point-of-care Xpert MTB/RIF testing for tuberculosis in primary-care settings in Africa: a multicentre, randomised, controlled trial. Lancet. 2014;383(9915):424–35.
62. Churchyard GJ, Stevens WS, Mametja LD, McCarthy KM, Chihota V, Nicol MP, et al. Xpert MTB/RIF versus sputum microscopy as the initial diagnostic test for tuberculosis: a cluster-randomised trial embedded in South African roll-out of Xpert MTB/RIF. The Lancet Global Health. 2015;3(8):e450–7.
63. Dheda K, Barry CE 3rd, Maartens G. Tuberculosis. Lancet. 2016;387(10024):1211-26.

64. Johnson JL, Hadad DJ, Dietze R, Maciel EL, Sewali B, Gitta P, et al. Shortening treatment in adults with noncavitary tuberculosis and 2-month culture conversion. Am J Respir Crit Care Med. 2009;180(6):558-63.
65. Wang JY, Wang JT, Tsai TH, Hsu CL, Yu CJ, Hsueh PR, et al. Adding moxifloxacin is associated with a shorter time to culture conversion in pulmonary tuberculosis. Int J Tuberc Lung Dis. 2010;14(1):65-71.
66. Dorman SE, Johnson JL, Goldberg S, Muzanye G, Padayatchi N, Bozeman L, et al. Substitution of moxifloxacin for isoniazid during intensive phase treatment of pulmonary tuberculosis. Am J Respir Crit Care Med. 2009;180(3):273-80.
67. Conde MB, Efron A, Loredo C, De Souza GR, Graça NP, Cezar MC, et al. Moxifloxacin versus ethambutol in the initial treatment of tuberculosis: a double-blind, randomised, controlled phase II trial. Lancet. 2009;373(9670):1183-9.
68. Rustomjee R, Lienhardt C, Kanyok T, Davies GR, Levin J, Mthiyane T, et al. A Phase II study of the sterilising activities of ofloxacin, gatifloxacin and moxifloxacin in pulmonary tuberculosis. Int J Tuberc Lung Dis. 2008;12(2):128-38.
69. Burman WJ, Goldberg S, Johnson JL, Muzanye G, Engle M, Mosher AW, et al. Moxifloxacin versus ethambutol in the first 2 months of treatment for pulmonary tuberculosis. Am J Respir Crit Care Med. 2006;174(3):331-8.
70. Gillespie SH, Crook AM, McHugh TD, Mendel CM, Meredith SK, Murray SR, et al. Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. N Engl J Med. 2014;371(17):1577-87.
71. Merle CS, Fielding K, Sow OB, Gninafon M, Lo MB, Mthiyane T, et al. A four-month gatifloxacin-containing regimen for treating tuberculosis. N Engl J Med. 2014;371(17):1588-98.
72. Jindani A, Harrison TS, Nunn AJ, Phillips PP, Churchyard GJ, Charalambous S, et al. High-dose rifapentine with moxifloxacin for pulmonary tuberculosis. N Engl J Med. 2014;371(17):1599-608.
73. Wallis RS, Maeurer M, Mwaba P, Chakaya J, Rustomjee R, Migliori GB, et al. Tuberculosis--advances in development of new drugs, treatment regimens, host-directed therapies, and biomarkers. Lancet Infect Dis. 2016;16(4):e34-46.
74. Kakkar AK, Dahiya N. Bedaquiline for the treatment of resistant tuberculosis: promises and pitfalls. Tuberculosis. 2014;94(4):357-62.
75. Gupta R, Geiter LJ, Wells CD, Gao M, Cirule A, Xiao H. Delamanid for Extensively Drug-Resistant Tuberculosis. N Engl J Med. 2015;373(3):291-2.
76. Zumla AI, Gillespie SH, Hoelscher M, Philips PP, Cole ST, Abubakar I, et al. New antituberculosis drugs, regimens, and adjunct therapies: needs, advances, and future prospects. Lancet Infect Dis. 2014;14(4):327-40.
77. Gagneux S. Host–pathogen coevolution in human tuberculosis. Phil Trans R Soc B. 2012;367(1590):850–9.
78. de Jong BC, Antonio M, Gagneux S. Mycobacterium africanum–review of an important cause of human tuberculosis in West Africa. PLoS Negl Trop Dis. 2010;4:e744.
79. Aboubaker Osman D, Bouzid F, Canaan S, Drancourt M. Smooth Tubercle Bacilli: Neglected Opportunistic Tropical Pathogens. Front Public Health. 2016;3:283.
80. Smith NH, Kremer K, Inwald J, Dale J, Driscoll JR, Gordon SV, et al. Ecotypes of the Mycobacterium tuberculosis complex. J Theor Biol. 2006;239(2):220-5.
81. Wayne LG, Kubica GP. The Mycobacteria. Bergey's manual of systematic bacteriology. 1986;2:1435–57.
82. North RJ, Jung YJ. Immunity to tuberculosis. Annu Rev Immunol. 2004;22:599-623.
83. Brennan PJ, Nikaido H. The envelope of mycobacteria. Annual Review of Biochemistry. 1995;64:29–63.
84. Beste DJ, Espasa M, Bonde B, Kierzek AM, Stewart GR, McFadden J. The genetic requirements for fast and slow growth in mycobacteria. PLoS One. 2009;4(4):e5349.
85. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 1998;393(6685):537-44.
86. Eldholm V, Norheim G, von der Lippe B, Kinander W, Dahle UR, Caugant DA, et al. Evolution of extensively drug-resistant Mycobacterium tuberculosis from a susceptible ancestor in a single patient. Genome Biol. 2014;15(11):490.

87. David HL. Probability distribution of drug-resistant mutants in unselected populations of Mycobacterium tuberculosis. Appl Microbiol. 1970;20(5):810-4.
88. Ford CB, Lin PL, Chase MR, Shah RR, Iartchouk O, Galagan J, et al. Use of whole genome sequencing to estimate the mutation rate of Mycobacterium tuberculosis during latent infection. Nat Genet. 2011;43(5):482-6.
89. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J Clin Microbiol. 1997;35:907–14.
90. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of Mycobacterium tuberculosis. J Clin Microbiol. 2006;44:4498–510.
91. Stucki D, Malla B, Hostettler S, Huna T, Feldmann J, Yeboah-Manu D, et al. Two new rapid SNP-typing methods for classifying Mycobacterium tuberculosis complex into the main phylogenetic lineages. PLoS One. 2012;7:e41253.
92. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, Narayanan S, et al. Variable host-pathogen compatibility in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A. 2006;103(8):2869-73.
93. Comas I, Chakravartti J, Small PM, Galagan J, Niemann S, Kremer K, et al. Human T cell epitopes of Mycobacterium tuberculosis are evolutionarily hyperconserved. Nat Genet. 2010;42:498-503.
94. Firdessa R, Berg S, Hailu E, Schelling E, Gumi B, Erenso G, et al. Mycobacterial lineages causing pulmonary and extrapulmonary tuberculosis, Ethiopia. Emerg Infect Dis. 2013;19(3):460-3.
95. Comas I, Coscolla M, Luo T, Borrell S, Holt KE, Kato-Maeda M, et al. Out-of-Africa migration and Neolithic coexpansion of Mycobacterium tuberculosis with modern humans. Nat Genet. 2013;45(10):1176-82.
96. Coscolla M, Gagneux S. Consequences of genomic diversity in Mycobacterium tuberculosis. Semin Immunol. 2014;26(6):431-44.
97. Hirsh AE, Tsolaki AG, DeRiemer K, Feldman MW, Small PM. Stable association between strains of Mycobacterium tuberculosis and their human host populations. Proc Natl Acad Sci USA. 2004;101(14):4871–6.
98. Reed MB, Pichler VK, McIntosh F, Mattia A, Fallow A, Masala S, et al. Major Mycobacterium tuberculosis Lineages Associate with Patient Country of Origin. J Clin Microbiol. 2009;47(4):1119–28.
99. Hershberg R, Lipatov M, Small PM, Sheffer H, Niemann S, Homolka S, et al. High functional diversity in Mycobacterium tuberculosis driven by genetic drift and human demography. PLoS Biol. 2008;6:e311.
100. Wirth T, Hildebrand F, Allix-Beguec C, Wölbeling F, Kubica T, Kremer K, et al. Origin, spread and demography of the Mycobacterium tuberculosis complex. PLoS Pathog. 2008;4:e1000160.
101. Brisse S Gutierrez MC, Brosch R, et al. Ancient origin and gene mosaicism of the progenitor of Mycobacterium tuberculosis. PLoS Pathog. 2005;1:e5.
102. Stucki D, Brites D, Jeljeli L, Coscolla M, Liu Q, Trauner A, et al. Mycobacterium tuberculosis lineage 4 comprises globally distributed and geographically restricted sublineages. Nat Genet. 2016;48(12):1535-43.
103. Pérez-Lago L, Comas I, Navarro Y, González-Candelas F, Herranz M, Bouza E, et al. Whole genome sequencing analysis of intrapatient microevolution in Mycobacterium tuberculosis: potential impact on the inference of tuberculosis transmission. J Infect Dis. 2014;209(1):98-108.
104. Lin PL, Flynn JL. Understanding latent tuberculosis: a moving target. J Immunol. 2010;185:15-22.
105. Fleischmann RD, Alland D, Eisen JA, Carpenter L, White O, Peterson J, et al. Whole-genome comparison of Mycobacterium tuberculosis clinical and laboratory strains. J Bacteriol. 2002;184(19):5479-90.
106. Rose G, Cortes T, Comas I, Coscolla M, Gagneux S, Young DB. Mapping of genotype-phenotype diversity among clinical isolates of Mycobacterium tuberculosis by sequence-based transcriptional profiling. Genome Biol Evol. 2013;5(10):1849-62.

107. Fenner L, Egger M, Bodmer T, Furrer H, Ballif M, Battegay M, et al. HIV Infection Disrupts the Sympatric Host–Pathogen Relationship in Human Tuberculosis. PLoS Genet. 2013;9(3):e1003318.
108. Osório NS, Rodrigues F, Gagneux S, Pedrosa J, Pinto-Carbó M, Castro AG, et al. Evidence for Diversifying Selection in a Set of Mycobacterium tuberculosis Genes in Response to Antibiotic- and Nonantibiotic-Related Pressure. Mol Biol Evol. 2013;30(6):1326-36.
109. Rodrigo T, Caylà JA, García de Olalla P, Galdós-Tangüis H, Jansà JM, Miranda P, et al. Characteristics of tuberculosis patients who generate secondary cases. Int J Tuberc Lung Dis. 1997;1(4):352-7.
110. Bouley DM, Ghori N, Mercer KL, Falkow S, Ramakrishnan L. Dynamic nature of host-pathogen interactions in Mycobacterium marinum granulomas. Infect Immun. 2001;69(12):7820-31.
111. Tufariello JM, Chan J, Flynn JL. Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. Lancet Infect Dis. 2003;3(9):578-90.
112. da Silva J. The evolutionary adaptation of HIV-1 to specific immunity. Curr HIV Res. 2003;1(3):363-71.
113. Copin R, Coscollá M, Efstathiadis E, Gagneux S, Ernst JD. Impact of in vitro evolution on antigenic diversity of Mycobacterium bovis bacillus Calmette-Guerin (BCG). Vaccine. 2014;32(45):5998-6004.
114. Valway SE, Sanchez MP, Shinnick TF, Orme I, Agerton T, Hoy D, et al. An outbreak involving extensive transmission of a virulent strain of Mycobacterium tuberculosis. N Engl J Med. 1998;338(10):633-9.
115. Manca C, Tsenova L, Barry CE 3rd, Bergtold A, Freeman S, Haslett PA, et al. Mycobacterium tuberculosis CDC1551 induces a more vigorous host response in vivo and in vitro, but is not more virulent than other clinical isolates. J Immunol. 1999;162(11):6740-6.
116. Carmona J, Cruz A, Moreira-Teixeira L, Sousa C, Sousa J, Osorio NS, et al. Mycobacterium tuberculosis strains are differentially recognized by TLRs with an impact on the immune response. PLoS One. 2013;8(6):e67277.
117. Portevin D, Gagneux S, Comas I, Young D. Human macrophage responses to clinical isolates from the Mycobacterium tuberculosis complex discriminate between ancient and modern lineages. PLoS Pathog. 2011;7(3):e1001307.
118. Krishnan N, Malaga W, Constant P, Caws M, Tran TH, Salmons J, et al. Mycobacterium tuberculosis lineage influences innate immune response and virulence and is associated with distinct cell envelope lipid profiles. PLoS One. 2011;6(9):e23870.
119. Sarkar R, Lenders L, Wilkinson KA, Wilkinson RJ, Nicol MP. Modern lineages of Mycobacterium tuberculosis exhibit lineage-specific patterns of growth and cytokine induction in human monocyte-derived macrophages. PLoS One. 2012;7(8):e43170.
120. Wang C, Peyron P, Mestre O, Kaplan G, van Soolingen D, Gao Q, et al. Innate immune response to Mycobacterium tuberculosis Beijing and other genotypes. PLoS One. 2010;5(10):e13594.
121. Dormans J, Burger M, Aguilar D, Hernandez-Pando R, Kremer K, Roholl P, et al. Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different Mycobacterium tuberculosis genotypes in a BALB/c mouse model. Clin Exp Immunol. 2004;137(3):460–8.
122. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, et al. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha/beta. Proc Natl Acad Sci U S A. 2001;98(10):5752-7.
123. López B, Aguilar D, Orozco H, Burger M, Espitia C, Ritacco V, et al. A marked difference in pathogenesis and immune response induced by different Mycobacterium tuberculosis genotypes. Clin Exp Immunol. 2003;133(1):30-7.
124. Dunn PL, North RJ. Virulence ranking of some Mycobacterium tuberculosis and Mycobacterium bovis strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice. Infect Immun. 1995;63(9):3428-37.
125. Aguilar D, Hanekom M, Mata D, Gey van Pittius NC, van Helden PD, Warren RM, et al. Mycobacterium tuberculosis strains with the Beijing genotype demonstrate variability in virulence associated with transmission. Tuberculosis. 2010;90(5):319-25.

126. Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, Murray PJ, et al. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res. 2005;25(11):694-701.
127. Divangahi M, Behar SM, Remold H. Dying to live: how the death modality of the infected macrophage affects immunity to tuberculosis. Adv Exp Med Biol. 2013;783:103-20.
128. Corleis B, Korbel D, Wilson R, Bylund J, Chee R, Schaible UE. Escape of Mycobacterium tuberculosis from oxidative killing by neutrophils. Cell Microbiol. 2012;14(7):1109-21.
129. Gehre F, Otu J, DeRiemer K, de Sessions PF, Hibberd ML, Mulders W, et al. Deciphering the growth behaviour of Mycobacterium africanum. PLoS Negl Trop Dis. 2013;7(5):e2220.
130. Homolka S, Niemann S, Russell DG, Rohde KH. Functional genetic diversity among Mycobacterium tuberculosis complex clinical isolates: delineation of conserved core and lineage-specific transcriptomes during intracellular survival. PLoS Pathog. 2010;6(7):e1000988.
131. Khatri B, Fielder M, Jones G, Newell W, Abu-Oun M, Wheeler PR. High throughput phenotypic analysis of Mycobacterium tuberculosis and Mycobacterium bovis strains' metabolism using biolog phenotype microarrays. PLoS One. 2013;8(1):e52673.
132. Portevin D, Sukumar S, Coscolla M, Shui G, Li B, Guan XL, et al. Lipidomics and genomics of Mycobacterium tuberculosis reveal lineage-specific trends in mycolic acid biosynthesis. Microbiology Open. 2014;3(6):823-35.
133. Parwati I, Alisjahbana B, Apriani L, Soetikno RD, Ottenhoff TH, van der Zanden AG, et al. Mycobacterium tuberculosis Beijing genotype is an independent risk factor for tuberculosis treatment failure in Indonesia. J Infect Dis. 2010;201(4):553-7.
134. Lan NT, Lien HT, Tung le B, Borgdorff MW, Kremer K, van Soolingen D. Mycobacterium tuberculosis Beijing genotype and risk for treatment failure and relapse, Vietnam. Emerg Infect Dis. 2003;9(12):1633-5.
135. Sun YJ, Lee AS, Wong SY, Paton NI. Association of Mycobacterium tuberculosis Beijing genotype with tuberculosis relapse in Singapore. Epidemiol Infect. 2006;134(2):329-32.
136. Kato-Maeda M, Kim EY, Flores L, Jarlsberg LG, Osmond D, Hopewell PC. Differences among sublineages of the East-Asian lineage of Mycobacterium tuberculosis in genotypic clustering. Int J Tuberc Lung Dis. 2010;14(5):538-44.
137. Ford CB, Shah RR, Maeda MK, Gagneux S, Murray MB, Cohen T, et al. Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. Nat Genet. 2013;45(7):784-90.
138. Wollenberg KR, Desjardins CA, Zalutskaya A, Slodovnikova V, Oler AJ, Quiñones M, et al. Whole genome sequencing of Mycobacterium tuberculosis provides insight into the evolution and genetic composition of drug-resistant tuberculosis in Belarus. J Clin Microbiol. 2016. Epub Nov 30. doi: 10.1128/JCM.02116-16.
139. Gurjav U, Erkhembayar B, Burneebaatar B, Narmandakh E, Tumenbayar O, Hill-Cawthorne GA, et al. Transmission of multi-drug resistant tuberculosis in Mongolia is driven by Beijing strains of Mycobacterium tuberculosis resistant to all first-line drugs. Tuberculosis. 2016;101:49-53.
140. Albanna AS, Reed MB, Kotar KV, Fallow A, McIntosh FA, Behr MA, et al. Reduced transmissibility of East African Indian strains of Mycobacterium tuberculosis. PLoS One. 2011;6(9):e25075.
141. Gehre F, Antonio M, Faïhun F, Odoun M, Uwizeye C, de Rijk P, et al. The first phylogeographic population structure and analysis of transmission dynamics of M. africanum West African 1--combining molecular data from Benin, Nigeria and Sierra Leone. PLoS One. 2013;8(10):e77000.
142. de Jong BC, Hill PC, Aiken A, Awine T, Antonio M, Adetifa IM, et al. Progression to active tuberculosis, but not transmission, varies by Mycobacterium tuberculosis lineage in The Gambia. J Infect Dis. 2008;198(7):1037–43.
143. Stavrum R, PrayGod G, Range N, Faurholt-Jepsen D, Jeremiah K, Faurholt-Jepsen M, et al. Increased level of acute phase reactants in patients infected with modern Mycobacterium tuberculosis genotypes in Mwanza, Tanzania. BMC Infect Dis. 2014;14:309.
144. van Crevel R, Nelwan RH, de Lenne W, Veeraragu Y, van der Zanden AG, Amin Z, et al. Mycobacterium tuberculosis Beijing genotype strains associated with febrile response to treatment. Emerg Infect Dis. 2001;7(5):880-3.

145. Caws M, Thwaites G, Dunstan S, Hawn TR, Lan NT, Thuong NT, et al. The influence of host and bacterial genotype on the development of disseminated disease with Mycobacterium tuberculosis. PLoS Pathog. 2008;4(3):e1000034.
146. Kong Y, Cave MD, Zhang L, Foxman B, Marrs CF, Bates JH, et al. Association between Mycobacterium tuberculosis Beijing/W lineage strain infection and extrathoracic tuberculosis: Insights from epidemiologic and clinical characterization of the three principal genetic groups of M. tuberculosis clinical isolates. J Clin Microbiol. 2007;45(2):409-14.
147. Kong Y, Cave MD, Yang D, Zhang L, Marrs CF, Foxman B, et al. Distribution of insertion- and deletion-associated genetic polymorphisms among four Mycobacterium tuberculosis phospholipase C genes and associations with extrathoracic tuberculosis: a population-based study. J Clin Microbiol. 2005;43(12):6048-53.
148. Lari N, Rindi L, Cristofani R, Rastogi N, Tortoli E, Garzelli C. Association of Mycobacterium tuberculosis complex isolates of M. bovis and Central Asian (CAS) genotypic lineages with extrapulmonary disease. Clin Microbiol Infect. 2009;15(6):538-43.
149. American Thoracic Society/CDC. Diagnostic standards and classification of tuberculosis. Am Rev Respir Dis. 1990;142:725-35.
150. Wells WF. Aerodynamics of droplet nuclei. In: Airborne contagion and air hygiene. Cambridge: Harvard University Press; 1995.
151. Fennelly KP, Jones-López EC, Ayakaka I, Kim S, Menyha H, Kirenga B, et al. Variability of infectious aerosols produced during coughing by patients with pulmonary tuberculosis. Am J Respir Crit Care Med. 2012;186(5):450-7.
152. Wells WF, Ratcliffe HL, Grumb C. On the mechanics of droplet nuclei infection; quantitative experimental air-borne tuberculosis in rabbits. Am J Hyg. 1948;47:11–28.
153. Bates J, Potts W, Lewis M. Epidemiology of Primary Tuberculosis in an Industrial School. N Engl J Med. 1965;272:714–7.
154. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. Annu Rev Immunol. 2013;31:475–527.
155. Bhatt K, Salgame P. Host innate immune response to Mycobacterium tuberculosis. J Clin Immunol. 2007;27(4):347-62.
156. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.
157. Kleinnijenhuis J, Oosting M, Joosten LA, Netea MG, Van Crevel R. Innate immune recognition of Mycobacterium tuberculosis. Clin Dev Immunol. 2011;2011:405310.
158. Bafica A, Scanga C, Feng C, Leifer C, Cheever A, Sher A. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. J Exp Med. 2005;202:1715-24.
159. Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, Fenton MJ. Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. J Immunol. 1999;163(7):3920-7.
160. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007;449(7164):819-26.
161. Reiling N, Hölscher C, Fehrenbach A, Kröger S, Kirschning CJ, Goyert S, et al. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. J Immunol. 2002;169(7):3480-4.
162. Underhill DM, Ozinsky A, Smith KD, Aderem A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc Natl Acad Sci U S A. 1999;96(25):14459-63.
163. Means TK, Jones BW, Schromm AB, Shurtleff BA, Smith JA, Keane J, et al. Differential effects of a Toll-like receptor antagonist on Mycobacterium tuberculosis-induced macrophage responses. J Immunol. 2001;166(6):4074-82.
164. Pompei L, Jang S, Zamlynny B, Ravikumar S, McBride A, Hickman SP, et al. Disparity in IL-12 release in dendritic cells and macrophages in response to Mycobacterium tuberculosis is due to use of distinct TLRs. J Immunol. 2007;178(8):5192-9.
165. Jang S, Uematsu S, Akira S, Salgame P. IL-6 and IL-10 induction from dendritic cells in response to Mycobacterium tuberculosis is predominantly dependent on TLR2-mediated recognition. J Immunol. 2004;173(5):3392-7.

166. Nair S, Ramaswamy PA, Ghosh S, Joshi DC, Pathak N, Siddiqui I, et al. The PPE18 of Mycobacterium tuberculosis interacts with TLR2 and activates IL-10 induction in macrophage. J Immunol. 2009;183(10):6269-81.
167. Cooper AM. Cell-mediated immune response in TB. Annu Rev Immunol. 2009;27:393-422.
168. Vergne I, Chua J, Singh SB, Deretic V. Cell biology of mycobacterium tuberculosis phagosome. Annu Rev Cell Dev Biol. 2004;20:367-94.
169. Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci U S A. 2000;97(16):8841-8.
170. Adams LB, Dinauer MC, Morgenstern DE, Krahenbuhl JL. Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to Mycobacterium tuberculosis using transgenic mice. Tuber Lung Dis. 1997;78(5-6):237-46.
171. Divangahi M, Desjardins D, Nunes-Alves C, Remold HG, Behar SM. Eicosanoid pathways regulate adaptive immunity to Mycobacterium tuberculosis. Nat Immunol. 2010;11(8):751-8.
172. Behar SM, Martin CJ, Booty MG, Nishimura T, Zhao X, Gan HX, et al. Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis. Mucosal Immunol. 2011;4(3):279-87.
173. Chen M, Divangahi M, Gan H, Shin DS, Hong S, Lee DM, et al. Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. J Exp Med. 2008;205(12):2791-801.
174. Hossain MM, Norazmi MN. Pattern recognition receptors and cytokines in Mycobacterium tuberculosis infection--the double-edged sword? Biomed Res Int. 2013;2013:179174.
175. Eum SY, Kong JH, Hong MS, Lee YJ, Kim JH, Hwang SH, et al. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. Chest. 2010;137(1):122-8.
176. Eruslanov EB, Lyadova IV, Kondratieva TK, Majorov KB, Scheglov IV, Orlova MO, et al. Neutrophil responses to Mycobacterium tuberculosis infection in genetically susceptible and resistant mice. Infect Immun. 2005;73(3):1744-53.
177. Alemán M, Beigier-Bompadre M, Borghetti C, de la Barrera S, Abbate E, Isturiz M, et al. Activation of peripheral blood neutrophils from patients with active advanced tuberculosis. Clin Immunol. 2001;100(1):87-95.
178. Kisich KO, Higgins M, Diamond G, Heifets L. Tumor necrosis factor alpha stimulates killing of Mycobacterium tuberculosis by human neutrophils. Infect Immun. 2002;70(8):4591-9.
179. Pokkali S, Das SD. Augmented chemokine levels and chemokine receptor expression on immune cells during pulmonary tuberculosis. Hum Immunol. 2009;70(2):110–5.
180. Pedrosa J, Saunders BM, Appelberg R, Orme IM, Silva MT, Cooper AM. Neutrophils play a protective nonphagocytic role in systemic Mycobacterium tuberculosis infection of mice. Infect Immun. 2000;68(2):577-83.
181. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245-52.
182. Robinson RT, Orme IM, Cooper AM. The onset of adaptive immunity in the mouse model of tuberculosis and the factors that compromise its expression. Immunol Rev. 2015;264(1):46-59.
183. Dorhoi A, Reece ST, Kaufmann SH. For better or for worse: the immune response against Mycobacterium tuberculosis balances pathology and protection. Immunol Rev. 2011;240(1):235-51.
184. Khader SA, Partida-Sanchez S, Bell G, Jelley-Gibbs DM, Swain S, Pearl JE, et al. Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. J Exp Med. 2006;203(7):1805-15.
185. Scanga CA, Mohan VP, Tanaka K, Alland D, Flynn JL, Chan J. The inducible nitric oxide synthase locus confers protection against aerogenic challenge of both clinical and laboratory strains of Mycobacterium tuberculosis in mice. Infect Immun. 2001;69(12):7711-7.
186. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc Natl Acad Sci U S A. 1997;94(10):5243-8.

187. Jung YJ, LaCourse R, Ryan L, North RJ. Virulent but not avirulent Mycobacterium tuberculosis can evade the growth inhibitory action of a T helper 1-dependent, nitric oxide Synthase 2-independent defense in mice. J Exp Med. 2002;196(7):991-8.
188. Nicholson S, Bonecini-Almeida Mda G, Lapa e Silva JR, Nathan C, Xie QW, Mumford R, et al. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. J Exp Med. 1996;183(5):2293-302.
189. Nozaki Y, Hasegawa Y, Ichiyama S, Nakashima I, Shimokata K. Mechanism of nitric oxide-dependent killing of Mycobacterium bovis BCG in human alveolar macrophages. Infect Immun. 1997;65(9):3644-7.
190. Torrado E, Cooper AM. IL-17 and Th17 cells in tuberculosis. Cytokine Growth Factor Rev. 2010;21(6):455-62.
191. Okamoto Yoshida Y, Umemura M, Yahagi A, O'Brien RL, Ikuta K, Kishihara K, et al. Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. J Immunol. 2010;184(8):4414-22.
192. Cruz A, Fraga AG, Fountain JJ, Rangel-Moreno J, Torrado E, Saraiva M, et al. Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with Mycobacterium tuberculosis. J Exp Med. 2010;207(8):1609-16.
193. Jones BE, Young SM, Antoniskis D, Davidson PT, Kramer F, Barnes PF. Relationship of the manifestations of tuberculosis to CD4 cell counts in patients with human immunodeficiency virus infection. Am Rev Respir Dis. 1993;148(5):1292-7.
194. Lawn SD, Butera ST, Shinnick TM. Tuberculosis unleashed: the impact of human immunodeficiency virus infection on the host granulomatous response to Mycobacterium tuberculosis. Microbes Infect. 2002;4(6):635-46.
195. Ehlers S, Benini J, Held HD, Roeck C, Alber G, Uhlig S. Alphabeta T cell receptor-positive cells and interferon-gamma, but not inducible nitric oxide synthase, are critical for granuloma necrosis in a mouse model of mycobacteria-induced pulmonary immunopathology. J Exp Med. 2001;194(12):1847-59.
196. Feng CG, Jankovic D, Kullberg M, Cheever A, Scanga CA, Hieny S, et al. Maintenance of pulmonary Th1 effector function in chronic tuberculosis requires persistent IL-12 production. J Immunol. 2005;174(7):4185-92.
197. Mogues T, Goodrich ME, Ryan L, LaCourse R, North RJ. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. J Exp Med. 2001;193(3):271-80.
198. Rolph MS, Raupach B, Köbernick HH, Collins HL, Pérarnau B, Lemonnier FA, et al. MHC class Ia-restricted T cells partially account for beta2-microglobulin-dependent resistance to Mycobacterium tuberculosis. Eur J Immunol. 2001;31(6):1944-9.
199. Rook GA, Hernández-Pando R, Zumla A. Tuberculosis due to high-dose challenge in partially immune individuals: a problem for vaccination? J Infect Dis. 2009;199(5):613-8.
200. Abebe F, Bjune G. The protective role of antibody responses during Mycobacterium tuberculosis infection. Clin Exp Immunol. 2009;157(2):235-43.
201. Vordermeier HM, Venkataprasad N, Harris DP, Ivanyi J. Increase of tuberculous infection in the organs of B cell-deficient mice. Clin Exp Immunol. 1996;106(2):312-6.
202. Maglione PJ, Xu J, Chan J. B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with Mycobacterium tuberculosis. J Immunol. 2007;178(11):7222-34.
203. Turner J, Frank AA, Brooks JV, Gonzalez-Juarrero M, Orme IM. The progression of chronic tuberculosis in the mouse does not require the participation of B lymphocytes or interleukin-4. Exp Gerontol. 2001;36(3):537-45.
204. Johnson CM, Cooper AM, Frank AA, Bonorino CB, Wysoki LJ, Orme IM. Mycobacterium tuberculosis aerogenic rechallenge infections in B cell-deficient mice. Tuber Lung Dis. 1997;78(5-6):257-61.
205. Marin ND, París SC, Vélez VM, Rojas CA, Rojas M, García LF. Regulatory T cell frequency and modulation of IFN-gamma and IL-17 in active and latent tuberculosis. Tuberculosis. 2010;90(4):252-61.
206. Scott-Browne JP, Shafiani S, Tucker-Heard G, Ishida-Tsubota K, Fontenot JD, Rudensky AY, et al. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. J Exp Med. 2007;204(9):2159-69.

207. Kursar M, Koch M, Mittrücker HW, Nouailles G, Bonhagen K, Kamradt T, et al. Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. J Immunol. 2007;178(5):2661-5.
208. Urdahl KB, Shafiani S, Ernst JD. Initiation and regulation of T-cell responses in tuberculosis. Mucosal Immunol. 2011;4(3):288-93.
209. Cooper AM, Mayer-Barber KD, Sher A. Role of innate cytokines in mycobacterial infection. Mucosal Immunol. 2011;4(3):252-60.
210. Watford WT, Moriguchi M, Morinobu A, O'Shea JJ. The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev. 2003;14(5):361-8.
211. Robinson RT, Khader SA, Martino CA, Fountain JJ, Teixeira-Coelho M, Pearl JE, et al. Mycobacterium tuberculosis infection induces il12rb1 splicing to generate a novel IL-12Rbeta1 isoform that enhances DC migration. J Exp Med. 2010;207(3):591-605.
212. Cooper AM, Kipnis A, Turner J, Magram J, Ferrante J, Orme IM. Mice lacking bioactive IL-12 can generate protective, antigen-specific cellular responses to mycobacterial infection only if the IL-12 p40 subunit is present. J Immunol. 2002;168(3):1322-7.
213. Khader SA, Pearl JE, Sakamoto K, Gilmartin L, Bell GK, Jelley-Gibbs DM, et al. IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-gamma responses if IL-12p70 is available. J Immunol. 2005;175(2):788-95.
214. Re F, Strominger JL. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. J Biol Chem. 2001;276(40):37692-9.
215. Teixeira-Coelho M, Cruz A, Carmona J, Sousa C, Ramos-Pereira D, Saraiva AL, et al. TLR2 deficiency by compromising p19 (IL-23) expression limits Th 17 cell responses to Mycobacterium tuberculosis. Int Immunol. 2011;23:89-96.
216. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem. 2003;278(3):1910-4.
217. Ghilardi N, Kljavin N, Chen Q, Lucas S, Gurney AL, De Sauvage FJ. Compromised humoral and delayed-type hypersensitivity responses in IL-23-deficient mice. J Immunol. 2004;172(5):2827-33.
218. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med. 2005;201(2):233-40.
219. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, et al. Divergent pro- and anti-inflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med. 2003;198(12):1951-7.
220. Freches D, Korf H, Denis O, Havaux X, Huygen K, Romano M. Mice genetically inactivated in interleukin-17A receptor are defective in long-term control of Mycobacterium tuberculosis infection. Immunology. 2013;140(2):220-31.
221. Orme IM, Roberts AD, Griffin JP, Abrams JS. Cytokine secretion by CD4 T lymphocytes acquired in response to Mycobacterium tuberculosis infection. J Immunol. 1993;151(1):518-25.
222. Vesosky B, Turner OC, Turner J, Orme IM. Gamma interferon production by bovine gamma delta T cells following stimulation with mycobacterial mycolylarabinogalactan peptidoglycan. Infect Immun. 2004;72(8):4612-8.
223. Zuñiga J, Torres-García D, Santos-Mendoza T, Rodriguez-Reyna TS, Granados J, Yunis EJ. Cellular and humoral mechanisms involved in the control of tuberculosis. Clin Dev Immunol. 2012;2012(2012):193923.
224. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med. 1993;178(6):2243-7.
225. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart T, Bloom BR. An essential role for Interferon-γ in resistance to Mycobacterium tuberculosis infection. J Exp Med. 1993;178:2249–54.
226. Ottenhof TH, Kumararatne D, Casanova JL. Novel human immunodeficiencies reveal the essential role of type-1 cytokines in immunity to intracellular bacteria. Immunol Today. 1998;19:491–4.
227. Levin M, Newport M. Understanding the genetic basis of susceptibility to mycobacterial infection. Proc Assoc Am Physicians. 1999;111(4):308-12.

228. Bustamante J, Boisson-Dupuis S, Abel L, Casanova JL. Mendelian susceptibility to mycobacterial disease: genetic, immunological, and clinical features of inborn errors of IFN-γ immunity. Semin Immunol. 2014;26(6):454-70.
229. MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science. 2003;302(5645):654-9.
230. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell. 2004;119(6):753–66.
231. Colombo MI, Gutierrez MG, Romano PS. The two faces of autophagy: Coxiella and Mycobacterium. Autophagy. 2006;2(3):162–4.
232. Cruz A, Khader SA, Torrado E, Fraga A, Pearl JE, Pedrosa J, et al. Cutting edge: IFN-gamma regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection. J Immunol. 2006;177(3):1416-20.
233. Desvignes L, Ernst JD. Interferon-gamma-responsive nonhematopoietic cells regulate the immune response to Mycobacterium tuberculosis. Immunity. 2009;31(6):974-85.
234. Nandi B, Behar SM. Regulation of neutrophils by interferon-γ limits lung inflammation during tuberculosis infection. J Exp Med. 2011;208(11):2251–62.
235. Verbon A, Juffermans N, Van Deventer SJ, Speelman P, Van Deutekom H, Van Der Poll T. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. Clin Exp Immunol. 1999;115(1):110-3.
236. Tsao TC, Huang CC, Chiou WK, Yang PY, Hsieh MJ, Tsao KC. Levels of interferon-γ and interleukin-2 receptor-α for bronchoalveolar lavage fluid and serum were correlated with clinical grade and treatment of pulmonary tuberculosis. Int J Tuberc Lung Dis. 2002;6(8):720–7.
237. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005;6(11):1123-32.
238. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17-producing human T helper cells. Nature Immunology. 2007;8(9):942–9.
239. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol. 2005;6(11):1133-41.
240. Khader S, Cooper A. IL-23 and IL-17 in tuberculosis. Cytokine. 2008;41(2):79-83.
241. Lockhart E, Green AM, Flynn JL. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol. 2006;177(7):4662-9.
242. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, et al. IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. J Immunol. 2007;178(6):3786-96.
243. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat Immunol. 2007;8(4):369-77.
244. Wozniak TM, Ryan AA, Britton WJ. Interleukin-23 restores immunity to Mycobacterium tuberculosis infection in IL-12p40-deficient mice and is not required for the development of IL-17-secreting T cell responses. J Immunol. 2006;177(12):8684-92.
245. Horii Y, Muraguchi A, Suematsu S, Matsuda T, Yoshizaki K, Hirano T, et al. Regulation of BSF-2/IL-6 production by human mononuclear cells. Macrophage-dependent synthesis of BSF-2/IL-6 by T cells. J Immunol. 1988;141(5):1529-35.
246. Stríz I, Mio T, Adachi Y, Romberger DJ, Rennard SI. Th2-type cytokines modulate IL-6 release by human bronchial epithelial cells. Immunol Lett. 1999;70(2):83-8.
247. Law K, Weiden M, Harkin T, Tchou-Wong K, Rom WN Chi C. Increased release of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha by bronchoalveolar cells lavaged from involved sites in pulmonary tuberculosis. Am J Respir Crit Care Med. 1996;153(2):799-804.
248. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441(7090):235-8.

249. Leal IS, Smedegârd B, Andersen P, Appelberg R. Interleukin-6 and interleukin-12 participate in induction of a type 1 protective T-cell response during vaccination with a tuberculosis subunit vaccine. Infect Immun. 1999;67(11):5747-54.
250. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. Biochem J. 1990;265(3):621-36.
251. Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest. 2004;113(9):1271-6.
252. Kishimoto T. Factors affecting B-cell growth and differentiation. Annu Rev Immunol. 1985;3:133-57.
253. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. Annu Rev Immunol. 2009;27:485-517.
254. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. J Exp Med. 2012;209(7):1241-53.
255. Okada M, Kitahara M, Kishimoto S, Matsuda T, Hirano T, Kishimoto T. IL-6/BSF-2 functions as a killer helper factor in the in vitro induction of cytotoxic T cells. J Immunol. 1988;141(5):1543-9.
256. Ladel CH, Blum C, Dreher A, Reifenberg K, Kopf M, Kaufmann SH. Lethal tuberculosis in interleukin-6-deficient mutant mice. Infect Immun. 1997;65(11):4843-9.
257. Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. J Immunol. 2002;168(9):4620-7.
258. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, et al. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. Immunity. 1995;2(6):561-72.
259. Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD, et al. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. J Immunol. 1999;162(6):3504-11.
260. Kaneko H, Yamada H, Mizuno S, Udagawa T, Kazumi Y, Sekikawa K, et al. Role of tumor necrosis factor-alpha in Mycobacterium-induced granuloma formation in tumor necrosis factor-alpha-deficient mice. Lab Invest. 1999;79(4):379-86.
261. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med. 2001;345(15):1098-104.
262. Bekker LG, Maartens G, Steyn L, Kaplan G. Selective increase in plasma tumor necrosis factor-alpha and concomitant clinical deterioration after initiating therapy in patients with severe tuberculosis. J Infect Dis. 1998;178(2):580-4.
263. Wallis RS, van Vuuren C, Potgieter S. Adalimumab treatment of life-threatening tuberculosis. Clin Infect Dis. 2009;48(10):1429-32.
264. Sakurai H. Targeting of TAK1 in inflammatory disorders and cancer. Trends Pharmacol Sci. 2012;33(10):522-30.
265. Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol. 2009;27:693-733.
266. Roth S, Rottach A, Lotz-Havla AS, Laux V, Muschaweckh A, Gersting SW, et al. Rad50-CARD9 interactions link cytosolic DNA sensing to IL-1β production. Nat Immunol. 2014;15(6):538-45.
267. Dubois H, Wullaert A, Lamkanfi M. General strategies in inflammasome biology. Curr Top Microbiol Immunol. 2016;397:1-22.
268. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. Cell. 2014;157(5):1013-22.
269. Koo IC, Wang C, Raghavan S, Morisaki JH, Cox JS, Brown EJ. ESX-1-dependent cytolysis in lysosome secretion and inflammasome activation during mycobacterial infection. Cell Microbiol. 2008;10(9):1866-78.
270. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, Cheever A, et al. Caspase-1 independent IL-1beta production is critical for host resistance to mycobacterium tuberculosis and does not require TLR signaling in vivo. J Immunol. 2010;184(7):3326-30.

271. McElvania Tekippe E, Allen IC, Hulseberg PD, Sullivan JT, McCann JR, Sandor M, et al. Granuloma formation and host defense in chronic Mycobacterium tuberculosis infection requires PYCARD/ASC but not NLRP3 or caspase-1. PLoS One. 2010;5(8):e12320.
272. Mishra BB, Moura-Alves P, Sonawane A, Hacohen N, Griffiths G, Moita LF, et al. Mycobacterium tuberculosis protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. Cell Microbiol. 2010;12(8):1046-63.
273. Schenk M, Fabri M, Krutzik SR, Lee DJ, Vu DM, Sieling PA, et al. Interleukin-1β triggers the differentiation of macrophages with enhanced capacity to present mycobacterial antigen to T cells. Immunology. 2014;141(2):174-80.
274. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol. 2009;27:519-50.
275. Harris KM, Fasano A, Mann DL. Cutting edge: IL-1 controls the IL-23 response induced by gliadin, the etiologic agent in celiac disease. J Immunol. 2008;181(7):4457-60.
276. Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J Exp Med. 2006;203(7):1685-91.
277. Di Paolo NC, Shafiani S, Day T, Papayannopoulou T, Russell DW, Iwakura Y, et al. Interdependence between Interleukin-1 and Tumor Necrosis Factor regulates TNF-dependent control of Mycobacterium tuberculosis Infection. Immunity. 2015;43(6):1125-36.
278. Fremond CM, Togbe D, Doz E, Rose S, Vasseur V, Maillet I, et al. IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to Mycobacterium tuberculosis infection. J Immunol. 2007;179(2):1178-89.
279. Mayer-Barber KD, Andrade BB, Barber DL, Hieny S, Feng CG, Caspar P, et al. Innate and adaptive interferons suppress IL-1α and IL-1β production by distinct pulmonary myeloid subsets during Mycobacterium tuberculosis infection. Immunity. 2011;35(6):1023-34.
280. Juffermans NP, Florquin S, Camoglio L, Verbon A, Kolk AH, Speelman P, et al. Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. J Infect Dis. 2000;182(3):902-8.
281. Condos R, Rom WN, Liu YM, Schluger NW. Local immune responses correlate with presentation and outcome in tuberculosis. Am J Respir Crit Care Med. 1998;157(3 Pt 1):729-35.
282. Tsao TC, Li L, Hsieh M, Liao S, Chang KS. Soluble TNF-alpha receptor and IL-1 receptor antagonist elevation in BAL in active pulmonary TB. Eur Respir J. 1999;14(3):490-5.
283. Harris JE, Fernandez-Vilaseca M, Elkington PT, Horncastle DE, Graeber MB, Friedland JS. IFNgamma synergizes with IL-1beta to up-regulate MMP-9 secretion in a cellular model of central nervous system tuberculosis. FASEB J. 2007;21(2):356-65.
284. O'Kane CM, Elkington PT, Jones MD, Caviedes L, Tovar M, Gilman RH, et al. STAT3, p38 MAPK, and NF-kappaB drive unopposed monocyte-dependent fibroblast MMP-1 secretion in tuberculosis. Am J Respir Cell Mol Biol. 2010;43(4):465–74.
285. Mayer-Barber KD, Andrade BB, Oland SD, Amaral EP, Barber DL, Gonzales J, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. Nature. 2014;511(7507):99-103.
286. Chen M, Gan H, Remold HG. A mechanism of virulence: virulent Mycobacterium tuberculosis strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. J Immunol. 2006;176(6):3707-16.
287. Keane J, Remold HG, Kornfeld H. Virulent Mycobacterium tuberculosis strains evade apoptosis of infected alveolar macrophages. J Immunol. 2000;164(4):2016-20.
288. Bafica A, Scanga CA, Serhan C, Machado F, White S, Sher A, et al. Host control of Mycobacterium tuberculosis is regulated by 5-lipoxygenase-dependent lipoxin production. J Clin Invest. 2005;115(6):1601-6.
289. Tobin DM, Vary JC Jr, Ray JP, Walsh GS, Dunstan SJ, Bang ND, et al. The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans. Cell. 2010;140(5):717-30.
290. Herb F, Thye T, Niemann S, Browne EN, Chinbuah MA, Gyapong J, et al. ALOX5 variants associated with susceptibility to human pulmonary tuberculosis. Hum Mol Genet. 2008;17(7):1052-60.
291. Guarda G, Braun M, Staehli F, Tardivel A, Mattmann C, Förster I, et al. Type I interferon inhibits interleukin-1 production and inflammasome activation. Immunity. 2011;34(2):213-23.

292. Okamura H, Tsutsi H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature. 1995;378(6552):88-91.
293. Gopal R, Monin L, Torres D, Slight S, Mehra S, McKenna KC, et al. S100A8/A9 proteins mediate neutrophilic inflammation and lung pathology during tuberculosis. Am J Respir Crit Care Med. 2013;188(9):1137-46.
294. Lázár-Molnár E, Chen B, Sweeney KA, Wang EJ, Liu W, Lin J, et al. Programmed death-1 (PD-1)-deficient mice are extraordinarily sensitive to tuberculosis. Proc Natl Acad Sci U S A. 2010;107(30):13402-7.
295. Larson RP, Shafiani S, Urdahl KB. Foxp3(+) regulatory T cells in tuberculosis. Adv Exp Med Biol. 2013;783:165-80.
296. Almeida AS, Lago PM, Boechat N, Huard RC, Lazzarini LC, Santos AR, et al. Tuberculosis is associated with a down-modulatory lung immune response that impairs Th1-type immunity. J Immunol. 2009;183(1):718-31.
297. Bonecini-Almeida MG, Ho JL, Boéchat N, Huard RC, Chitale S, Doo H, et al. Down-modulation of lung immune responses by interleukin-10 and transforming growth factor beta (TGF-beta) and analysis of TGF-beta receptors I and II in active tuberculosis. Infect Immun. 2004;72(5):2628-34.
298. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med. 1991;174(5):1209-20.
299. Boonstra A, Rajsbaum R, Holman M, Marques R, Asselin-Paturel C, Pereira JP, et al. Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. J Immunol 2006;177(11):7551-8.
300. Dillon S, Agrawal A, Van Dyke T, Landreth G, McCauley L, Koh A, et al. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. J Immunol. 2004;172(8):4733-43.
301. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol. 1991;147(11):3815-22.
302. Gong JH, Zhang M, Modlin RL, Linsley PS, Iyer D, Lin Y, et al. Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression. Infect Immun. 1996;64(3):913-8.
303. Rojas RE, Balaji KN, Subramanian A, Boom WH. Regulation of human CD4(+) alphabeta T-cell-receptor-positive (TCR(+)) and gammadelta TCR(+) T-cell responses to Mycobacterium tuberculosis by interleukin-10 and transforming growth factor beta. Infect Immun. 1999;67(12):6461-72.
304. de la Barrera S, Aleman M, Musella R, Schierloh P, Pasquinelli V, Garcia V, et al. IL-10 down-regulates costimulatory molecules on Mycobacterium tuberculosis-pulsed macrophages and impairs the lytic activity of CD4 and CD8 CTL in tuberculosis patients. Clin Exp Immunol. 2004;138(1):128-38.
305. Demangel C, Bertolino P, Britton WJ. Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. Eur J Immunol. 2002;32(4):994-1002.
306. Boussiotis VA, Tsai EY, Yunis EJ, Thim S, Delgado JC, Dascher CC, et al. IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. J Clin Invest. 2000;105(9):1317-25.
307. Awomoyi AA, Marchant A, Howson JM, McAdam KP, Blackwell JM, Newport MJ. Interleukin-10, polymorphism in SLC11A1 (formerly NRAMP1), and susceptibility to tuberculosis. J Infect Dis. 2002;186(12):1808-14.
308. Oh JH, Yang CS, Noh YK, Kweon YM, Jung SS, Son JW, et al. Polymorphisms of interleukin-10 and tumour necrosis factor-alpha genes are associated with newly diagnosed and recurrent pulmonary tuberculosis. Respirology. 2007;12(4):594-8.
309. Oral HB, Budak F, Uzaslan EK, Baştürk B, Bekar A, Akalin H, et al. Interleukin-10 (IL-10) gene polymorphism as a potential host susceptibility factor in tuberculosis. Cytokine. 2006;35(3-4):143-7.
310. Azad AK, Sadee W, Schlesinger LS. Innate immune gene polymorphisms in tuberculosis. Infect Immun. 2012;80:3343–59.

311. Möller M, de Wit E, Hoal EG. Past, present and future directions in human genetic susceptibility to tuberculosis. FEMS Immunol Med Microbiol. 2010;581(1):3-26.
312. Delgado JC, Baena A, Thim S, Goldfeld AE. Ethnic-specific genetic associations with pulmonary tuberculosis. J Infect Dis. 2002;186(10):1463-8.
314. Murray PJ, Wang L, Onufryk C, Tepper RI, Young RA. T cell-derived IL-10 antagonizes macrophage function in mycobacterial infection. J Immunol. 1997;158(1):315-21.
315. Turner J, Gonzalez-Juarrero M, Ellis DL, Basaraba RJ, Kipnis A, Orme IM, et al. In vivo IL-10 production reactivates chronic pulmonary tuberculosis in C57BL/6 mice. J Immunol. 2002;169(11):6343-51.
316. Beamer GL, Flaherty DK, Assogba BD, Stromberg P, Gonzalez-Juarrero M, de Waal Malefyt R, et al. Interleukin-10 promotes Mycobacterium tuberculosis disease progression in CBA/J mice. J Immunol. 2008;181(8):5545-50.
317. Redford PS, Boonstra A, Read S, Pitt J, Graham C, Stavropoulos E, et al. Enhanced protection to Mycobacterium tuberculosis infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. Eur J Immunol. 2010;40(8):2200-10.
318. Roach DR, Martin E, Bean AG, Rennick DM, Briscoe H, Britton WJ. Endogenous inhibition of antimycobacterial immunity by IL-10 varies between mycobacterial species. Scand J Immunol. 2001;54(1-2):163-70.
319. Jung YJ, Ryan L, LaCourse R, North RJ. Increased interleukin-10 expression is not responsible for failure of T helper 1 immunity to resolve airborne Mycobacterium tuberculosis infection in mice. Immunology. 2003;109(2):295-9.
320. North RJ. Mice incapable of making IL-4 or IL-10 display normal resistance to infection with Mycobacterium tuberculosis. Clin Exp Immunol. 1998;113(1):55-8.
321. Higgins DM, Sanchez-Campillo J, Rosas-Taraco AG, Lee EJ, Orme IM, Gonzalez-Juarrero M. Lack of IL-10 alters inflammatory and immune responses during pulmonary Mycobacterium tuberculosis infection. Tuberculosis. 2009;89(2):149-57.
322. Dripps DJ, Brandhuber BJ, Thompson RC, Eisenberg SP. Interleukin-1 (IL-1) receptor antagonist binds to the 80-kDa IL-1 receptor but does not initiate IL-1 signal transduction. J Biol Chem. 1991;266(16):10331-6.
323. Granowitz EV, Clark BD, Mancilla J, Dinarello CA. Interleukin-1 receptor antagonist competitively inhibits the binding of interleukin-1 to the type II interleukin-1 receptor. J Biol Chem. 1991;266(22):14147-50.
324. Juffermans NP, Verbon A, van Deventer SJ, van Deutekom H, Speelman P, van der Poll T. Tumor necrosis factor and interleukin-1 inhibitors as markers of disease activity of tuberculosis. Am J Respir Crit Care Med. 1998;157(4 Pt 1):1328-31.
325. Lopalco G, Rigante D, Giannini M, Galeazzi M, Lapadula G, Iannone F, et al. Safety profile of anakinra in the management of rheumatologic, metabolic and autoinflammatory disorders. Clin Exp Rheumatol. 2016;34(3):531-8.
326. Cantarini L, Lopalco G, Caso F, Costa L, Iannone F, Lapadula G, et al. Effectiveness and tuberculosis-related safety profile of interleukin-1 blocking agents in the management of Behçet's disease. Autoimmun Rev. 2015;14(1):1-9.
327. Dubos RJ, Dubos J. The white plague; tuberculosis, man and society. 1st ed. Boston, MA: Little Brown; 1952.
328. Puffer R. Familial susceptibility to tuberculosis; its importance as a public health problem. Cambridge, MA: Harvard University Press; 1944.
329. Kallmann FJ, Reisner D. Twin studies on the significance of genetic factors in tuberculosis. Am Rev Tuberc. 1943;47:549–74.
330. Comstock GW. Tuberculosis in twins: a re-analysis of the Prophit survey. Am Rev Respir Dis. 1978;117(4):621-4.
331. Stead WW, Senner JW, Reddick WT, Lofgren JP. Racial differences in susceptibility to infection by Mycobacterium tuberculosis. N Engl J Med. 1990;322(7):422–7.
332. Crowle AJ, Elkins N. Relative permissiveness of macrophages from black and white people for virulent tubercle bacilli. Infect Immun. 1990;58(3):632–8.
333. Al-Muhsen S, Casanova JL. The genetic heterogeneity of mendelian susceptibility to mycobacterial diseases. J Allergy Clin Immunol. 2008;122(6):1043-51.

334. Boisson-Dupuis S, El Baghdadi J, Parvaneh N, Bousfiha A, Bustamante J, Feinberg J, et al. IL-12Rβ1 deficiency in two of fifty children with severe tuberculosis from Iran, Morocco, and Turkey. PLoS One. 2011;6(4):e18524.
335. Ozbek N, Fieschi C, Yilmaz BT, de Beaucoudrey L, Demirhan B, Feinberg J, et al. Interleukin-12 receptor beta 1 chain deficiency in a child with disseminated tuberculosis. Clin Infect Dis. 2005;40(6):e55-8.
336. Alcaïs A, Quintana-Murci L, Thaler DS, Schurr E, Abel L, Casanova JL. Life-threatening infectious diseases of childhood: single-gene inborn errors of immunity? Ann N Y Acad Sci. 2010;1214:18-33.
337. Casanova JL, Abel L, 581–620. Annu. Rev. Immunol. 20. Genetic dissection of immunity to mycobacteria: the human model. Annu Rev Immunol. 2002;20:581-620.
338. Filipe-Santos O, Bustamante J, Chapgier A, Vogt G, de Beaucoudrey L, Feinberg J, et al. Inborn errors of IL-12/23- and IFN-gamma-mediated immunity: molecular, cellular, and clinical features. Semin Immunol. 2006;18(6):347-61.
339. Hambleton S, Salem S, Bustamante J, Bigley V, Boisson-Dupuis S, Azevedo J, et al. IRF8 mutations and human dendritic-cell immunodeficiency. N Engl J Med. 2011;365(2):127-38.
340. Bustamante J, Arias AA, Vogt G, Picard C, Galicia LB, Prando C, et al. Germline CYBB mutations that selectively affect macrophages in kindreds with X-linked predisposition to tuberculous mycobacterial disease. Nat Immunol. 2011;12(3):213-21.
341. Bogunovic D, Byun M, Durfee LA, Abhyankar A, Sanal O, Mansouri D, et al. Mycobacterial disease and impaired IFN-γ immunity in humans with inherited ISG15 deficiency. Science. 2012;337(6102):1684-8.
342. Bellamy R, Beyers N, McAdam KP, Ruwende C, Gie R, Samaai P, et al. Genetic susceptibility to tuberculosis in Africans: a genome-wide scan. Proc Natl Acad Sci U S A. 2000;97(14):8005-9.
343. Alcais A, Fieschi C, Abel L, Casanova JL. Tuberculosis in children and adults: two distinct genetic diseases. J Exp Med. 2005;202:1617–21.
344. Qu HQ, Fisher-Hoch SP, McCormick JB. Knowledge gaining by human genetic studies on tuberculosis susceptibility. J Hum Genet. 2011;56(3):177-82.
345. Abel L, El-Baghdadi J, Bousfiha AA, Casanova JL, Schurr E. Human genetics of tuberculosis: a long and winding road. Philos Trans R Soc Lond B Biol Sci. 2014;369(1645):20130428.
346. Schurz H, Daya M, Möller M, Hoal EG, Salie M. TLR1, 2, 4, 6 and 9 Variants Associated with Tuberculosis Susceptibility: A Systematic Review and Meta-Analysis. PLoS One. 2015;10(10):e0139711.
347. Ogus AC, Yoldas B, Ozdemir T, Uguz A, Olcen S, Keser I, et al. The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. Eur Respir J. 2004;23(2):219-23.
348. Thuong NT, Hawn TR, Thwaites GE, Chau TT, Lan NT, Quy HT, et al. A polymorphism in human TLR2 is associated with increased susceptibility to tuberculous meningitis. Genes Immun. 2007;8(5):422-8.
349. Velez DR, Wejse C, Stryjewski ME, Abbate E, Hulme WF, Myers JL, et al. Variants in toll-like receptors 2 and 9 influence susceptibility to pulmonary tuberculosis in Caucasians, African-Americans, and West Africans. Hum Genet. 2010;127:65-73.
350. Yim JJ, Lee HW, Lee HS, Kim YW, Han SK, Shim YS, et al. The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. Genes Immun. 2006;7(2):150-5.
351. Biswas D, Gupta SK, Sindhwani G, Patras A. TLR2 polymorphisms, Arg753Gln and Arg677Trp, are not associated with increased burden of tuberculosis in Indian patients. BMC Res Notes. 2009;2:162.
352. Ma MJ, Xie LP, Wu SC, Tang F, Li H, Zhang ZS, et al. Toll-like receptors, tumor necrosis factor-α, and interleukin-10 gene polymorphisms in risk of pulmonary tuberculosis and disease severity. Hum Immunol. 2010;71(10):1005-10.
353. Selvaraj P, Harishankar M, Singh B, Jawahar MS, Banurekha VV. Toll-like receptor and TIRAP gene polymorphisms in pulmonary tuberculosis patients of South India. Tuberculosis. 2010;90(5):306-10.

354. Xue Y, Zhao ZQ, Wang HJ, Jin L, Liu CP, Wang Y, et al. Toll-like receptors 2 and 4 gene polymorphisms in a southeastern Chinese population with tuberculosis. Int J Immunogenet. 2010;37(2):135-8.
355. Thye T, Vannberg FO, Wong SH, Owusu-Dabo E, Osei I, Gyapong J, et al. Genome-wide association analyses identifies a susceptibility locus for tuberculosis on chromosome 18q11.2. Nat Genet. 2010;42(9):739-41.
356. Thye T, Owusu-Dabo E, Vannberg FO, van Crevel R, Curtis J, Sahiratmadja E, et al. Common variants at 11p13 are associated with susceptibility to tuberculosis. Nat Genet. 2012;44(3):257-9.
357. Chimusa ER, Zaitlen N, Daya M, Moller M, van Helden PD, Mulder NJ, et al. Genome-wide association study of ancestry-specific TB risk in the South African coloured population. Hum Mol Genet. 2013;23(3):796–809.
358. Mahasirimongkol S, Yanai H, Mushiroda T, Promphittayarat W, Wattanapokayakit S, Phromjai J, et al. Genome-wide association studies of tuberculosis in Asians identify distinct at-risk locus for young tuberculosis. J Hum Genet. 2012;57(6):363-7.
359. Dai Y, Zhang X, Pan H, Tang S, Shen H, Wang J. Fine mapping of genetic polymorphisms of pulmonary tuberculosis within chromosome 18q11.2 in the Chinese population: a case-control study. BMC Infect Dis. 2011;11:282.
360. Wang X, Tang NL, Leung CC, Kam KM, Yew WW, Tam CM, et al. Association of polymorphisms in the Chr18q11.2 locus with tuberculosis in Chinese population. Hum Genet. 2013;132(6):691-5.
361. Curtis J, Luo Y, Zenner HL, Cuchet-Lourenço D, Wu C, Lo K, et al. Susceptibility to tuberculosis is associated with variants in the ASAP1 gene encoding a regulator of dendritic cell migration. Nat Genet. 2015;47(5):523-7.
362. Png E, Alisjahbana B, Sahiratmadja E, Marzuki S, Nelwan R, Balabanova Y, et al. A genome wide association study of pulmonary tuberculosis susceptibility in Indonesians. BMC Med Genet. 2012;13:5.
363. Grant AV, Sabri A, Abid A, Abderrahmani Rhorfi I, Benkirane M, Souhi H, et al. A genome-wide association study of pulmonary tuberculosis in Morocco. Hum Genet. 2016;135(3):299-307.
364. de Wit E, van der Merwe L, van Helden PD, Hoal EG. Gene-gene interaction between tuberculosis candidate genes in a South African population. Mamm Genome. 2011;22(1-2):100-10.
365. Daya M, van der Merwe L, van Helden PD, Möller M, Hoal EG. Investigating the Role of Gene-Gene Interactions in TB Susceptibility. PLoS One. 2015;10(4):e0123970.
366. Anyama N, Bracebridge S, Black C, Niggebrugge A, Griffin SJ. What happens to people diagnosed with tuberculosis? A population-based cohort. Epidemiol Infect. 2007;135(7):1069-76.
367. Marais BJ, Lönnroth K, Lawn SD, Migliori GB, Mwaba P, Glaziou P, et al. Tuberculosis comorbidity with communicable and non-communicable diseases: integrating health services and control efforts. Lancet Infect Dis. 2013;13(5):436-48.
368. Sonnenberg P, Glynn JR, Fielding K, Murray J, Godfrey-Faussett P, Shearer S. How soon after infection with HIV does the risk of tuberculosis start to increase? A retrospective cohort study in South African gold miners. J Infect Dis. 2005;191(2):150-8.
369. Deffur A, Mulder NJ, Wilkinson RJ. Co-infection with Mycobacterium tuberculosis and human immunodeficiency virus: an overview and motivation for systems approaches. Pathog Dis. 2013;69(2):101-13.
370. Diedrich CR, Flynn JL. HIV-1/Mycobacterium tuberculosis coinfection immunology: how does HIV-1 exacerbate tuberculosis? Infect Immun. 2011;79(4):1407-17.
371. Raviglione MC, Narain JP, Kochi A. HIV-associated tuberculosis in developing countries: clinical features, diagnosis, and treatment. Bull World Health Organ. 1992;70(4):515-26.
372. Harries AD, Maher D, Nunn P. An approach to the problems of diagnosing and treating adult smear-negative pulmonary tuberculosis in high-HIV-prevalence settings in sub-Saharan Africa. Bull World Health Organ. 1998;76(6):651-62.
373. Crump JA, Ramadhani HO, Morrissey AB, Saganda W, Mwako MS, Yang LY, et al. Bacteremic disseminated tuberculosis in sub-saharan Africa: a prospective cohort study. Clin Infect Dis. 2012;55(2):242-50.
374. Crump JA, Reller LB. Two decades of disseminated tuberculosis at a university medical center: the expanding role of mycobacterial blood culture. Clin Infect Dis. 2003;37:1037–43.

375. Walter ND, de Jong BC, Garcia BJ, Dolganov GM, Worodria W, Byanyima P, et al. Adaptation of Mycobacterium tuberculosis to Impaired Host Immunity in HIV-Infected Patients. J Infect Dis. 2016;214(8):1205-11.
376. Leistikow RL, Morton RA, Bartek IL, Frimpong I, Wagner K, Voskuil MI. The Mycobacterium tuberculosis DosR regulon assists in metabolic homeostasis and enables rapid recovery from nonrespiring dormancy. J Bacteriol. 2010;192(6):1662-70.
377. Resende Co T, Hirsch CS, Toossi Z, Dietze R, Ribeiro-Rodrigues R. Intestinal helminth co-infection has a negative impact on both anti-Mycobacterium tuberculosis immunity and clinical response to tuberculosis therapy. Clin Exp Immunol. 2007;147(1):45-52.
378. Chatterjee S, Nutman TB. Helminth-induced immune regulation: implications for immune responses to tuberculosis. PLoS Pathog. 2015;11(1):e1004582.
379. George PJ, Anuradha R, Kumar NP, Sridhar R, Banurekha VV, Nutman TB, et al. Helminth infections coincident with active pulmonary tuberculosis inhibit mono- and multifunctional CD4+ and CD8+ T cell responses in a process dependent on IL-10. PLoS Pathog. 2014;10(9):e1004375.
380. Redford PS, Mayer-Barber KD, McNab FW, Stavropoulos E, Wack A, Sher A, et al. Influenza A virus impairs control of Mycobacterium tuberculosis coinfection through a type I interferon receptor-dependent pathway. J Infect Dis. 2014;209(2):270-4.
381. Perry S, de Jong BC, Solnick JV, de la Luz Sanchez M, Yang S, Lin PL, et al. Infection with Helicobacter pylori is associated with protection against tuberculosis. PLoS One. 2010;5(1):e8804.
382. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell. 2014;157(1):121-41.
383. Schuijt TJ, Lankelma JM, Scicluna BP, de Sousa e Melo F, Roelofs JJ, de Boer JD, et al. The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. Gut. 2016;65(4):575-83.
384. Gauguet S, D'Ortona S, Ahnger-Pier K, Duan B, Surana NK, Lu R, et al. Intestinal Microbiota of Mice Influences Resistance to Staphylococcus aureus Pneumonia. Infect Immun. 2015;83(10):4003-14.
385. Clarke TB. Early innate immunity to bacterial infection in the lung is regulated systemically by the commensal microbiota via nod-like receptor ligands. Infect Immun. 2014;82(11):4596-606.
386. Winglee K, Eloe-Fadrosh E, Gupta S, Guo H, Fraser C, Bishai W. Aerosol Mycobacterium tuberculosis infection causes rapid loss of diversity in gut microbiota. PLoS One. 2014;9(5):e97048.
387. Huempfner HR, Deuschle KW. Experimental Tuberculosis in Germ-Free and Conventional Mice. Am Rev Respir Dis. 1966;93(3P1):465-7.
388. Forbes JD, Van Domselaar G, Bernstein CN. The Gut Microbiota in Immune-Mediated Inflammatory Diseases. Front Microbiol. 2016;7:1081.
389. Hauptmann M, Schaible UE. Linking microbiota and respiratory disease. FEBS Lett. 2016. Epub Sep 17. doi: 10.1002/1873-3468.12421.
390. Cambier CJ, Takaki KK, Larson RP, Hernandez RE, Tobin DM, Urdahl KB, et al. Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. Nature. 2014;505(7482):218-22.
391. Ubeda C, Bucci V, Caballero S, Djukovic A, Toussaint NC, Equinda M, et al. Intestinal microbiota containing Barnesiella species cures vancomycin-resistant Enterococcus faecium colonization. Infect Immun. 2013;81(3):965-73.
392. Restrepo BI, Schlesinger LS. Impact of diabetes on the natural history of tuberculosis. Diabetes Res Clin Pract. 2014;106(2):191-9.
393. Bates M, Marais BJ, Zumla A. Tuberculosis Comorbidity with Communicable and Noncommunicable Diseases. Cold Spring Harb Perspect Med. 2015;5(11):a017889.
394. Whiting DR, Guariguata L, Weil C, Shaw J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract. 2011;94(3):311-21.
395. Jiménez-Corona ME, Cruz-Hervert LP, García-García L, Ferreyra-Reyes L, Delgado-Sánchez G, Bobadilla-Del-Valle M, et al. Association of diabetes and tuberculosis: impact on treatment and post-treatment outcomes. Thorax. 2013;68(3):214-20.
396. Bastos HN, Osório NS, Castro AG, Ramos A, Carvalho T, Meira L, et al. A prediction rule to stratify mortality risk of patients with pulmonary tuberculosis. PLoS One. 2016;11(9):e0162797.

397. Baker MA, Harries AD, Jeon CY, Hart JE, Kapur A, Lönnroth K, et al. The impact of diabetes on tuberculosis treatment outcomes: a systematic review. BMC Med. 2011;9:81.
398. Alisjahbana B, Sahiratmadja E, Nelwan EJ, Purwa AM, Ahmad Y, Ottenhoff TH, et al. The effect of type 2 diabetes mellitus on the presentation and treatment response of pulmonary tuberculosis. Clin Infect Dis. 2007;45(4):428-35.
399. Viswanathan V, Vigneswari A, Selvan K, Satyavani K, Rajeswari R, Kapur A. Effect of diabetes on treatment outcome of smear-positive pulmonary tuberculosis - a report from South India. J Diabetes Complications. 2014;28(2):162-5.
400. Hodgson K, Morris J, Bridson T, Govan B, Rush C, Ketheesan N. Immunological mechanisms contributing to the double burden of diabetes and intracellular bacterial infections. Immunology. 2015;144(2):171-85.
401. Martinez N, Kornfeld H. Diabetes and immunity to tuberculosis. Eur J Immunol. 2014;44(3):617-26.
402. Restrepo BI, Schlesinger LS. Host-pathogen interactions in tuberculosis patients with type 2 diabetes mellitus. Tuberculosis. 2013;93 Suppl:S10-4.
403. Ronacher K, Joosten SA, van Crevel R, Dockrell HM, Walzl G, Ottenhoff TH. Acquired immunodeficiencies and tuberculosis: focus on HIV/AIDS and diabetes mellitus. Immunol Rev. 2015;264(1):121-37.
404. World Health Organization. Global burden of disease website 2016 [8 December 2016]. Available from: http://www.who.int/topics/global_burden_of_disease/en/.
405. van Zyl Smit RN, Pai M, Yew WW, Leung CC, Zumla A, Bateman ED, et al. Global lung health: the colliding epidemics of tuberculosis, tobacco smoking, HIV and COPD. Eur Respir J. 2010;35(1):27-33.
406. Inghammar M, Ekbom A, Engström G, Ljungberg B, Romanus V, Löfdahl CG, et al. COPD and the risk of tuberculosis--a population-based cohort study. PLoS One. 2010;5(4):e10138.
407. Lee CH, Lee MC, Shu CC, Lim CS, Wang JY, Lee LN, et al. Risk factors for pulmonary tuberculosis in patients with chronic obstructive airway disease in Taiwan: a nationwide cohort study. BMC Infect Dis. 2013;13(194).
408. O'Toole RF, Shukla SD, Walters EH. TB meets COPD: An emerging global co-morbidity in human lung disease. Tuberculosis. 2015;95(6):659-63.
409. Inghammar M, Löfdahl CG, Winqvist N, Ljungberg B, Egesten A, Engström G. Impaired pulmonary function and the risk of tuberculosis: a population-based cohort study. Eur Respir J. 2011;37(5):1285-7.
410. Sethi S, Murphy TF. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. N Engl J Med. 2008;359(22):2355-65.
411. Taylor AE, Finney-Hayward TK, Quint JK, Thomas CM, Tudhope SJ, Wedzicha JA, et al. Defective macrophage phagocytosis of bacteria in COPD. Eur Respir J. 2010;35(5):1039-47.
412. Bhat TA, Panzica L, Kalathil SG, Thanavala Y. Immune Dysfunction in Patients with Chronic Obstructive Pulmonary Disease. Ann Am Thorac Soc. 2015;12(Suppl 2):S169-75.
413. Gómez-Reino JJ, Carmona L, Valverde VR, Mola EM, Montero MD, BIOBADASER Group. Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk: a multicenter active-surveillance report. Arthritis Rheum. 2003;48(8):2122-7.
414. Duarte R, Campainha S, Cotter J, Rosa B, Varela P, Correia A, et al. Position paper on tuberculosis screening in patients with immune mediated inflammatory diseases candidates for biological therapy. Acta Reumatol Port. 2012;37(3):253-9.
415. Slama K, Chiang CY, Enarson DA, Hassmiller K, Fanning A, Gupta P, et al. Tobacco and tuberculosis: a qualitative systematic review and meta-analysis. Int J Tuberc Lung Dis. 2007;11(10):1049-61.
416. Lin HH, Ezzati M, Murray M. Tobacco smoke, indoor air pollution and tuberculosis: a systematic review and meta-analysis. PLoS Med. 2007;4(1):e20.
417. Bates MN, Khalakdina A, Pai M, Chang L, Lessa F, Smith KR. Risk of tuberculosis from exposure to tobacco smoke: a systematic review and meta-analysis. Arch Intern Med. 2007;167(4):335-42.
418. O'Leary SM, Coleman MM, Chew WM, Morrow C, McLaughlin AM, Gleeson LE, et al. Cigarette smoking impairs human pulmonary immunity to Mycobacterium tuberculosis. Am J Respir Crit Care Med. 2014;190(12):1430-6.

419. Robbins CS, Dawe DE, Goncharova SI, Pouladi MA, Drannik AG, Swirski FK, et al. Cigarette smoke decreases pulmonary dendritic cells and impacts antiviral immune responsiveness. Am J Respir Cell Mol Biol. 2004;30(2):202-11.
420. Givi ME, Folkerts G, Wagenaar GT, Redegeld FA, Mortaz E. Cigarette smoke differentially modulates dendritic cell maturation and function in time. Respir Res. 2015;16:131.
421. Shaler CR, Horvath CN, McCormick S, Jeyanathan M, Khera A, Zganiacz A, et al. Continuous and discontinuous cigarette smoke exposure differentially affects protective Th1 immunity against pulmonary tuberculosis. PLoS One. 2013;8(3):e59185.
422. Berg RD, Levitte S, O'Sullivan MP, O'Leary SM, Cambier CJ, Cameron J, et al. Lysosomal disorders drive susceptibility to tuberculosis by compromising macrophage migration. Cell. 2016;165(1):139-52.
423. Feingold AO. Association of tuberculosis with alcoholism. South Med J. 1976;69:1336-7.
424. Olin JS, Grzybowski S. Tuberculosis and alcoholism. Can Med Assoc J. 1966;94:999-1001.
425. Brown KE, Campbell AH. Tobacco, alcohol and tuberculosis. Br J Dis Chest 1961;55:150-8.
426. Mason CM, Dobard E, Zhang P, Nelson S. Alcohol exacerbates murine pulmonary tuberculosis. Infect Immun. 2004;72(5):2556-63.
427. Porretta E, Happel KI, Teng XS, Ramsay A, Mason CM. The impact of alcohol on BCG-induced immunity against Mycobacterium tuberculosis. Alcohol Clin Exp Res. 2012;36(2):310-7.
428. Schaible UE, Kaufmann SH. Malnutrition and infection: complex mechanisms and global impacts. PLoS Med. 2007;4(5):e115.
429. Cegielski JP, McMurray DN. The relationship between malnutrition and tuberculosis: evidence from studies in humans and experimental animals. Int J Tuberc Lung Dis. 2004;8(3):286-98.
430. Scalcini M, Occenac R, Manfreda J, Long R. Pulmonary tuberculosis, human immunodeficiency virus type-1 and malnutrition. Bull Int Union Tuberc Lung Dis. 1991;66(1):37-41.
431. Jeremiah K, Denti P, Chigutsa E, Faurholt-Jepsen D, PrayGod G, Range N, et al. Nutritional supplementation increases rifampin exposure among tuberculosis patients coinfected with HIV. Antimicrob Agents Chemother. 2014;58(6):3468-74.
432. Davies PD. A possible link between vitamin D deficiency and impaired host defence to Mycobacterium tuberculosis. Tubercle. 1985;66(4):301-6.
433. Hewison M. Vitamin D and the immune system: New perspectives on an old theme. Rheum Dis Clin N Am. 2012;38:125–39.
434. Martineau AR, Wilkinson KA, Newton SM, Floto RA, Norman AW, Skolimowska K, et al. IFN-gamma- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37. J Immunol. 2007;178(11):7190-8.
435. Martineau AR, Nhamoyebonde S, Oni T, Rangaka MX, Marais S, Bangani N, et al. Reciprocal seasonal variation in vitamin D status and tuberculosis notifications in Cape Town, South Africa. Proc Natl Acad Sci U S A. 2011;108(47):19013-7.
436. Kim JH, Park JS, Cho YJ, Yoon HI, Song JH, Lee CT, et al. Low serum 25-hydroxyvitamin D level: an independent risk factor for tuberculosis? Clin Nutr. 2014;33(6):1081-6.
437. Pareek M, Innes J, Sridhar S, Grass L, Connell D, Woltmann G, et al. Vitamin D deficiency and TB disease phenotype. Thorax. 2015;70(12):1171-80.
438. Coussens AK, Wilkinson RJ, Hanifa Y, Nikolayevskyy V, Elkington PT, Islam K, et al. Vitamin D accelerates resolution of inflammatory responses during tuberculosis treatment. Proc Natl Acad Sci U S A. 2012;109(38):15449-54.
439. Campbell GR, Spector SA. Vitamin D inhibits human immunodeficiency virus type 1 and Mycobacterium tuberculosis infection in macrophages through the induction of autophagy. PLoS Pathog. 2012;8(5):e1002689.
440. Chambers ES, Hawrylowicz CM. The impact of vitamin D on regulatory T cells. Curr Allergy Asthma Rep. 2011;11(1):29-36.
441. Cegielski P, Vernon A. Tuberculosis and vitamin D: what's the rest of the story? Lancet Infect Dis. 2015;15(5):489-90.
442. Martineau AR, Timms PM, Bothamley GH, Hanifa Y, Islam K, Claxton AP, et al. High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. Lancet. 2011;377(9761):242-50.

443. Young DB, Perkins MD, Duncan K, Barry CE 3rd. Confronting the scientific obstacles to global control of tuberculosis. J Clin Invest. 2008;118(4):1255-65.
444. Barry CE 3rd, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Microbiol. 2009;7(12):845-55.
445. Coscolla M, Gagneux S. Does M. tuberculosis genomic diversity explain disease diversity? Drug Discov Today Dis Mech. 2010;7(1):e43.
446. Zumla A, Maeurer M; Host-Directed Therapies Network, Chakaya J, Hoelscher M, Ntoumi F, Rustomjee R, et al. Towards host-directed therapies for tuberculosis. Nat Rev Drug Discov. 2015;14(8):511-12.
447. Zumla A, Maeurer M, Host-Directed Therapies Network (HDT-NET) Consortium. Host-directed therapies for tackling multi-drug resistant tuberculosis: learning from the Pasteur-Bechamp debates. Clin Infect Dis. 2015;61(9):1432–8.
448. Wallis RS, Hafner R. Advancing host-directed therapy for tuberculosis. Nat Rev Immunol. 2015;15(4):255–63.

CHAPTER 2 – OBJECTIVES OF THE THESIS

Although tuberculosis (TB) is a main global cause of morbidity and mortality, its pathophysiology remains only partly characterized. Upon exposure, some subjects become infected and only a smaller proportion of these undergo transition to active disease during their lifetime. Remarkably, among the active TB patients, the clinical manifestation of disease is also highly variable, with mild or extensive pulmonary involvement, extrapulmonary or disseminated forms of TB. While the precise mechanisms governing the transitions along this spectrum remain largely unknown, immune imbalances are widely accepted as key participants in this process. These imbalances are shaped by the host genetics, the bacterial heterogeneity and by extrinsic factors. Altogether, this troika orchestrates the type and intensity of the immune response generated during infection and consequently its outcome. This project aimed at building and exploring a biological bank of samples from TB-exposed individuals or patients and of *M. tuberculosis* isolates that would provide a platform for genetic and biological studies. In this thesis, we focused on the contribution of host and bacteria factors to the heterogeneity of TB, in the light of inflammation as a central process for pathogenesis. The specific objectives, leading to the results presented in this dissertation are described below.

1. **Clinical characterization of TB patients, identification of risk factors and stratification of severity**

   After identifying cases of culture-confirmed pulmonary TB (PTB) diagnosed at the Hospital de São João, Porto, Portugal, between 2007 and 2013, a clinical characterization was undergone by collecting demographic and standard clinical information. This task led to the development of a TB risk assessment tool (TReAT), with the aim of stratifying the risk of death among PTB patients, thereby possibly helping on the decision for different management options. The study of clinical features of the cohort also provided the foundations for the following tasks, in terms of selecting cases without predictor or

precipitator TB factors for biological studies and to characterize the severity of TB within this group.

2. **Correlating the pathogen genotypic and phenotypic properties with TB clinical features and severity**

   The cohort described above, where the patient's clinical data and the matched *M. tuberculosis* infecting strains were available, provided a unique ground to investigate the complex interplay between the pathogen properties and the disease severity. The purpose of this task was to explore the phylogenetic distribution of *M. tuberculosis* isolates collected from a group of patients without known predictor or precipitator TB factors in Hospital de São João, a major healthcare center in the Porto area, Portugal. Furthermore, we aimed to understand the possible links between the phylogenetic structure and bacterial growth properties of molecularly characterized strains with clinical phenotypes of severity.

3. **Correlating the cytokine expression profile induced in human cells by different *M. tuberculosis* isolates associated with specific clinical features**

   We proposed to investigate the interaction of selected *M. tuberculosis* isolates with human peripheral blood mononuclear cells (PBMCs) to evaluate if a specific pattern of inflammatory responses associated with the outcome of TB. Representative *M. tuberculosis* strains were selected based on their association with the extreme (mild *versus* severe) clinical manifestations of TB, in order to uncover a possible relationship between the profile of the inflammatory response induced by clinical isolates and their association with severity of disease. We also compared the cell responses of non-progressor (non-recent/non-treated latent infected donors) with progressor subjects (past TB patients), to underpin potential immunological correlates of protection/susceptibility for latent to active TB transition.

By achieving the proposed objectives, we will enhance our understanding on the intricate network of events modulating inflammation in TB, which will possibly help to build more effective vaccines and host directed therapies to stop this disease being one of the main killers of our times.

CHAPTER 3 – A PREDICTION RULE TO STRATIFY MORTALITY RISK OF PATIENTS WITH PULMONARY TUBERCULOSIS

The results presented in this chapter were published:

(i) **in an international peer reviewed journal**

Bastos HN, Osório NS, Castro AG, Ramos A, Carvalho T, Meira L, Araújo D, Almeida L, Boaventura R, Fragata P, Chaves C, Costa P, Portela M, Ferreira I, Magalhães SP, Rodrigues F, Sarmento-Castro R, Duarte R, Guimarães JT, Saraiva M. A prediction rule to stratify mortality risk of patients with pulmonary tuberculosis. PLoS One. 2016; 11(9): e0162797

(ii) **in conferences**

European Respiratory Society (ERS) Annual Congress 2016 | London, 3-7 September 2016

A prediction rule to stratify mortality risk of patients with pulmonary tuberculosis (Oral communication) | Authors: Helder Novais e Bastos, Nuno Osório, António Gil Castro, Angélica Ramos, Teresa Carvalho, Leonor Meira, David Araújo, Leonor Almeida, Rita Boaventura, Patrícia Fragata, Catarina Chaves, Patrício Costa, Ivo Ferreira, Sara Magalhães, Fernando Rodrigues, Rui Sarmento Castro, Raquel Duarte, João Tiago Guimarães, Margarida Saraiva

Eur Respir J 2016; 48 (Suppl. 60): OA4825

ABSTRACT

Tuberculosis imposes high human and economic tolls, including in Europe. This study was conducted to develop a severity assessment tool for stratifying mortality risk in pulmonary tuberculosis (PTB) patients. A derivation cohort of 681 PTB cases was retrospectively reviewed to generate a model based on multiple logistic regression analysis of prognostic variables with 6-month mortality as the outcome measure. A clinical scoring system was developed and tested against a validation cohort of 103 patients. Five risk features were selected for the prediction model: hypoxemic respiratory failure (OR 4.7, 95% CI 2.8-7.9), age ≥50 years (OR 2.9, 95% CI 1.7-4.8), bilateral lung involvement (OR 2.5, 95% CI 1.4-4.4), ≥1 significant comorbidity – HIV infection, diabetes *mellitus*, liver failure or cirrhosis, congestive heart failure and chronic respiratory disease – (OR 2.3, 95% CI 1.3-3.8), and hemoglobin <12 g/dL (OR 1.8, 95% CI 1.1-3.1). A tuberculosis risk assessment tool (TReAT) was developed, stratifying patients with low (score ≤2), moderate (score 3-5) and high (score ≥6) mortality risk. The mortality associated with each group was 2.9%, 22.9% and 53.9%, respectively. The model performed equally well in the validation cohort. We provide a new, easy-to-use clinical scoring system to identify PTB patients with high-mortality risk in settings with good healthcare access, helping clinicians to decide which patients are in need of closer medical care during treatment.

INTRODUCTION

Tuberculosis (TB) remains a major global health problem, with an estimated 9.6 million new cases and 1.5 million deaths in 2014 [1]. In Portugal, the incidence was still 25/100.000 inhabitants (intermediate incidence rate) and, contrasting to the majority of other European countries, most of the new TB cases are Portuguese native. A very recent report [2] analysed the social profile of the highest TB incidence areas in Portugal between 2002 and 2012 and concluded that immigrants comprised only 1.6 to 1.8% in the region of Porto, while the highest proportion was seen in the Lisbon area (8.4-8.8%), where larger migration influx has occurred mainly from sub-Saharan African former colonies. In spite of the greater incidence as compared to other countries, treatment success rate in Portugal is high [1] and the case-fatality rate has been below the European Union average [3], which accounts for the efficiency of the national healthcare system.

An increased risk of death from TB has been attributed to drug resistance acquisition and HIV coinfection, especially in developing countries with high incidence [3]. However, following population-based epidemiological studies in regions of low and intermediate TB incidence, other predictors of mortality have been identified. This was the case of increasing age, male gender, the occurrence of extrapulmonary TB and several comorbidities [3-7]. Therefore, objective clinical assessment of risk factors may help lowering the death rate associated with TB by selecting those patients who might be in need of increased clinical supervision or advanced medical treatment.

The use of clinical prediction rules (CPR) gained has relevance in the field of lung diseases in the last decades [8]. Although several prediction scores have been developed in the field of TB, most of them are available for diagnostic purposes [9-13], with only three providing prognosis-centred CPRs [6, 14, 15]. Among these, none is representative of a low to intermediate incidence region, with low rates of drug resistance, in both hospital and ambulatory settings. In this context, we developed a TB risk assessment tool (TReAT) based on readily available clinical features, with the aim of stratifying the risk of death among

pulmonary TB (PTB) patients and possibly helping on the decision for different management options.

MATERIALS AND METHODS

1. Study design and patient population

For the derivation (training) set, the clinical records of patients with *Mycobacterium tuberculosis* positive culture at a University-affiliated hospital (Hospital de São João - HSJ, Porto) during the period of 7 years (2007-2013) were retrospectively analysed. TB cases were defined according to the WHO guidelines and treatment was administered by DOT 5-7 days/week, with the recommended treatment regimens [16]. Exclusion criteria were: i) exclusively extrathoracic TB; ii) age <18 years and iii) lack of information (no registries found). Subjects were categorized according to the disease site as: i) exclusively pulmonary; ii) pleural, with or without proven PTB; or iii) combined extrathoracic and PTB. Extrathoracic involvement was defined as disease in organs other than the lungs or pleura, with either *M. tuberculosis* culture isolation or histologic demonstration of caseating granulomas [16].

The validation set was provided by the Chest Disease Centre (CDC) of Vila Nova de Gaia, an ambulatory referral centre for TB screening and treatment in a large urban area of the north of Portugal. Since mortality of patients diagnosed at the hospital is higher than what is observed in the non-hospital setting, we forced a ratio cases (deaths) to controls (survivors) similar to the derivation cohort, by using an entry-time-matched validation set. Cases that immediately preceded and/or immediately followed each of the fatalities occurring between 2007 and 2014 were defined as time-matched controls (survivors). No particular pairing was performed as the order of the individuals in the dataset was arbitrary.

The reporting of this study conforms to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement [17] and to the CHecklist for critical Appraisal and data extraction for systematic Reviews of prediction Modelling Studies (CHARMS) [18].

2. Data collection

For the derivation set, data was collected from both the HSJ clinical files and the Portuguese regional surveillance system (SVIG-TB) database. Specifically, information was collected for: age, gender and lifestyle factors (smoking status and alcohol intake); HIV infection status; pharmacological immunosuppression; active cancer; diabetes *mellitus*; liver failure or cirrhosis; stages 4 and 5 chronic kidney failure; congestive heart failure (CHF); and chronic respiratory disease (CRD). The definition of the coexistent illnesses is detailed below in **Table III.1**.

Table III.1 – Definition of coexistent illnesses used in the present study.

Comorbidity	Definition
HIV infection	positive titer of antibodies to HIV
Immunosuppression	organ transplant and patients receiving the equivalent of ≥15 mg/day of prednisolone for ≥1 month, other immunosuppressive drugs, or TNF-α antagonists
Active cancer	any cancer except basal- or squamous cell cancer of the skin, that was active at the time of presentation
Diabetes *mellitus*	history of diabetes or fasting blood glucose concentration ≥126 mg/dL at 2 different time points
Liver failure/cirrhosis	chronic liver disease with coagulopathy and hypoalbuminaemia or a clinical or histologic diagnosis of cirrhosis
Chronic Kidney Disease	history of chronic renal disease or abnormal blood urea nitrogen and creatinine concentrations documented in the medical record
Congestive heart failure	systolic or diastolic ventricular dysfunction documented by history, physical examination, chest radiograph and/or echocardiogram
Chronic respiratory disease	COPD and structural lung disease (bronchiectasis, lung fibrosis, pneumoconiosis)

HIV – human immunodeficiency virus; TNF – tumor necrosis factor; COPD – chronic obstructive pulmonary disease

Baseline clinical features available at time of diagnosis (before patients started treatment) were collected. These included: time of symptoms onset; the presence of three respiratory (cough, hemoptysis, dyspnea) and three constitutional symptoms (fever, night sweats, weight loss); hemoglobin and C-reactive protein (CRP) values; acute hypoxemic respiratory failure (defined as

newly onset partial pressure of oxygen decrease to <60 mmHg, or arterial oxygen saturation <90%); and digital images of plain chest radiographs. Baseline chest radiographs were blindly analysed by two independent physicians according to the lesions extent, the presence of lung cavitation and pleural effusion. Disagreement between readers was resolved through a consensus read by a third physician. Most cases had microscopic examination of auramine-stained sputum slides, ranking acid-fast bacilli load as negative, 1+, 2+, or 3+ [19] and a drug susceptibility profile was also available. Deaths that occurred during the first 6 months after diagnosis were classified as TB death [16]. The survival time was calculated between dates of the first microbiological sampling (which allowed for the provisional diagnosis before culture positivity could be ascertained) and death. Patients were censored at the date of the last visit if they were lost to follow-up or at the end of TB treatment. Data were recorded as missing if information could not be ascertained by review of paper and electronic charts. Missing values within the derivation set are detailed on Table III.2. For the validation set, only cases with complete data for the predictors included in the clinical score were considered.

3. Statistical analysis

Univariate analyses were conducted for all variables comparing survivors and fatalities in the derivation cohort. Continuous variables were recategorized into binary factors. In the absence of previously described thresholds in the literature, we used the Youden index criterion to estimate the optimal cut-point when giving equal weight to sensitivity and specificity [20]. For categorical variables, comparisons were made using a Chi-square test or Fisher exact test as appropriate. For continuous variables, comparisons were made using an independent group *t*-test, or a Mann-Whitney *U*-test for non-normally distributed variables.

Models to predict death in PTB patients were derived using stepwise logistic regression with 6-month mortality as the outcome measure. Eight

clinically plausible interactions were tested (listed in Table III.3). The results of significant predictors were reported as odds ratios (ORs) and 95% confidence intervals (CI). Models were assessed for goodness-of-fit using receiving operator characteristic (ROC) curves and the Hosmer-Lemeshow test. Then, using the Heckman's selection model, values of significant variables with missing data were modeled and incorporated into the initial model to assess and correct for potential bias. This method attempts to control for the effect of nonrandom selection by incorporating both the observed and unobserved factors that affect nonresponse [21].

To derive a simple-to-compute risk score, the regression coefficients of the predictors were divided by the smallest coefficient and then rounded to the nearest integer [22]. For each patient, a total risk score was obtained by calculating the sum of individual points attributed to each of the variable.

Three methods were used to validate the CPR. Pearson's Chi-square tests for independence were performed to test the association between risk score groups and observed deaths on derivation and validation cohorts and for total sample. Association between categories was evaluated based on the adjusted residual scores, where absolute values >1.96 represent significant differences for 95% confidence level (P<0.05). Significant positive scores reveal a tendency to observe death in the considered group. To describe the accuracy of the model for predicting mortality, we reported the sensitivity, specificity and test predictive values. The area under the ROC curve (AUC) and its 95% CI were determined and compared in the derivation and validation cohorts. All the statistical analyses were performed using the SPSS software program, version 22 (IBM® SPSS®, Inc.) and STATA, version 14 (STATA Corp) for Heckman modeling.

4. Ethics

The study protocol was approved by the Health Ethics Committees of the HSJ (approval number 109-11), the North Health Region Administration (approval number 71-2014) and the Portuguese Data Protection Authority (approval number 12174-2011). The requirement to obtain informed written consent from each individual was waived, as the study was limited to the review of existing medical records. To ensure confidentiality, each case was anonymized by the assignment of a random identification number.

RESULTS

1. Study design

Between 2007 and 2013, 813 culture-confirmed new TB cases were diagnosed at the HSJ (both inpatient and outpatient), of which 142 (17.5%) were reported to have died within 6 months of diagnosis. Patients with overall lack of information (n=40), with exclusively extrathoracic TB (n=83) or <18 years (n=9) were excluded from the study (**Figure III.1, left**). A total of 681 patients were included for univariate analysis, including 121 (17.8%) fatalities within 6 months (183 days) of diagnosis, with median survival time of 33 days (range 1 to 182). Of the 560 not known to have died, 60 were lost to follow-up after a median of

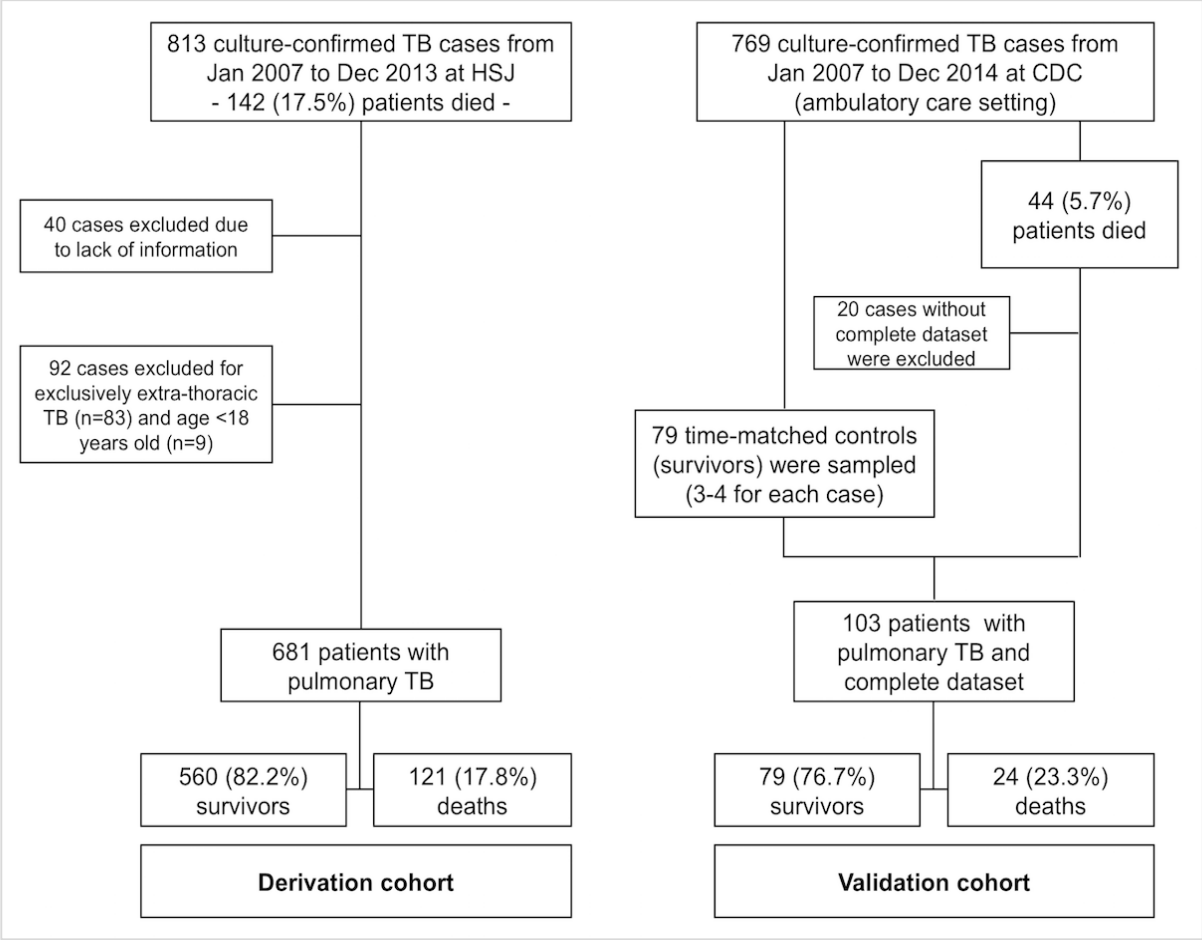

Figure III.1 – Flow chart for the selection of the participating patients, according to the STROBE guidelines. HSJ – Hospital de São João, Porto, Portugal; CDC – Chest Disease Centre (ambulatory care), Vila Nova de Gaia, Portugal.

138.5 days (range 6 to 182) and the remaining 500 were censored at the end of follow-up period. The vast majority of cases included in the study were Portuguese-born caucasians (96.5%) and only 0.4% of cases harboured multidrug-resistant strains.

The validation cohort was from an ambulatory referral centre (CDC), which followed 769 patients between 2007 and 2014 and registered a mortality rate of 5.7%. Of the 44 CDC registered deaths, only 24 patients had complete data. Seventy-nine survivors were included to match these fatalities, leading to a validation cohort of 103 patients (**Figure III.1, right**), in a 3:1 ratio (except for 7 fatal cases, which had 4 matching controls).

2. Development of a practical CPR to assess risk of death

The general characteristics of the study cohort and specific associations with death are presented in **Table III.2**.

The significant variables were then tested in multivariate logistic regression model with 6-month mortality as the dependent variable. Participants were categorized by age groups, according to a threshold based on Youden index criterion, which defined an optimal cut-point of 53.5 years (50 years, if rounded to nearest multiple of 10). Hemoglobin <12 g/dL defined anemia in both genders. Self-reporting variables with more than 20% of missing values were excluded, as the validity of these data is doubtful. This was the case for smoking, alcohol habits and time of symptoms. CRD and liver failure/cirrhosis were collinear with smoking and alcohol abuse, respectively, and worked as surrogates for those exposures. Based on clinical reasoning, malignancy was also excluded, as it related to patients with incurable active cancer and it was strongly associated with mortality itself.

Table III.2 – Study population characteristics and comparison between survivor and fatality groups. Continuous variables are presented as mean±SD or median (25th–75th percentile). The proportions in this table reflect the number of patients with each finding divided by the total number of patients for whom data were available.

Clinical feature		All (n=681)	Survivors (n=560)	Fatalities (n=121)	*P* value
Age years, median (IQR)		47 (35–64.5)	45 (33–59)	63 (46.5–76.5)	<0.001[a]
Male gender, n (%)		501/681 (73.6)	405/560 (72.3)	96/121 (79.3)	0.112
Former or current smoker, n (%)		341/555 (61.4)	274/470 (58.3)	67/85 (78.8)	<0.001[a]
Comorbidities, n (%)	Alcohol abuse	157/602 (26.1)	123/512 (24)	34/90 (37.8)	0.006[a]
	HIV positive	117/615 (19)	91/517 (17.6)	26/98 (26.5)	0.039[a]
	Immunosuppression	42/673 (6.2)	34/553 (6.1)	8/120 (6.7)	0.831
	Malignancy	43/672 (6.4)	14/553 (2.5)	29/119 (24.4)	<0.001[a]
	Diabetes *mellitus*	84/677 (12.4)	59/557 (10.6)	25/120 (20.8)	0.002[a]
	Liver failure or cirrhosis	91/669 (13.6)	67/549 (12.2)	24/120 (20)	0.024[a]
	Chronic kidney disease[b]	35/676 (5.2)	25/557 (4.5)	10/119 (8.4)	0.080
	Congestive heart failure	52/666 (7.8)	32/549 (5.8)	20/117 (17.1)	<0.001[a]
	Chronic respiratory disease	109/660 (16.5)	74/544 (13.6)	35/116 (30.2)	<0.001[a]
TB site, n (%)	Pulmonary	478/681 (70.2)	393/560 (70.2)	85/121 (70.2)	0.939
	Pleural ± pulmonary	90/681 (13.2)	75/560 (13.4)	15/121 (12.4)	
	Pulmonary + extrathoracic	113/681 (16.6)	92/560 (16.4)	21/121 (17.4)	
Time of symptoms (weeks), median (IQR)		7 (4–12)	8 (4–13)	4 (2.8–11)	0.002[a]
Main symptoms, n (%)	Cough	450/577 (78)	374/477 (78.4)	76/100 (76)	0.597
	Hemoptysis	99/571 (17.3)	90/475 (18.9)	9/96 (9.4)	0.024[a]
	Dyspnea	240/575 (41.7)	173/472 (36.7)	67/103 (65)	<0.001[a]
	Fever	345/578 (59.7)	284/479 (59.3)	61/99 (61.6)	0.668
	Night sweats	225/490 (45.9)	195/415 (47)	30/75 (40)	0.264
	Weight loss	335/539 (62.2)	271/448 (60.5)	64/91 (70.3)	0.078
Bacillary load[c], n (%)	0	138/477 (28.9)	114/384 (29.7)	24/93 (25.8)	0.823
	1+	64/477 (13.4)	52/384 (13.5)	12/93 (12.9)	
	2+	99/477 (20.8)	80/384 (20.8)	19/93 (20.4)	
	3+	176/477 (36.9)	138/384 (35.9)	38/93 (40.9)	

[a] Statistically significant results. [b] CKD stages 4 or 5. [c] Only cases of culture confirmation on sputum (the remaining subjects were diagnosed through gastric aspirate, bronchial wash, bronchoalveolar lavage, pleural fluid or biopsy cultures). IQR – interquartile range; HIV – human immunodeficiency virus; SD – standard deviation; TB – tuberculosis

Table III.2 (continuation) – Study population characteristics and comparison between survivor and fatality groups. Continuous variables are presented as mean±SD or median (25th–75th percentile). The proportions in this table reflect the number of patients with each finding divided by the total number of patients for whom data were available.

Clinical feature		All (n=681)	Survivors (n=560)	Fatalities (n=121)	*P* value
Drug resistance, n (%)	Isoniazide[d]	30/655 (4.6)	23/545 (4.2)	7/110 (6.4)	0.327
	Riphampicin	6/655 (0.9)	5/545 (0.9)	1/110 (0.9)	0.993
	Pyrazinamide[e]	6/294 (2)	5/248 (2)	1/46 (2.2)	0.945
	Ethambutol	7/655 (1.1)	5/545 (0.9)	2/110 (1.8)	0.402
Hypoxemic respiratory failure, n (%)		115/595 (19.3)	64/491 (13)	51/104 (49)	<0.001[a]
Hemoglobin g/dL, mean±SD		12.0±2.2	12.2±2.1	11.0±2.2	<0.001[a]
CRP mg/L, median (IQR)		79.4 (32.3–126.9)	74.9 (29.2–125.4)	90.5 (42.5–144)	0.011[a]
Cavitation, n (%)		265/613 (43.4)	219/494 (44.3)	47/119 (39.5)	0.339
Bilateral lung involvement, n (%)		336/598 (56.2)	248/484 (51.2)	88/114 (77.2)	<0.001[a]
Pleural effusion, n (%)		148/605 (24.5)	110/489 (22.5)	38/116 (32.8)	0.021[a]

[a] Statistically significant results. [d] Missingness of 3.8% due to contaminated culture or non-representative sampling. [e] Pyrazinamide resistance was not routinely assessed until May 2011. CRP – C-reactive protein; IQR – interquartile range; SD – standard deviation

Six interactions between variables were identified, but the resulting ORs were always similar or even lower than with significant variables alone (**Table III.3**). In addition to increased complexity, there was no benefit in terms of model performance to predict risk when these interactions were included. Hence, they were omitted from the model. Using backward selection [23], a final parsimonious model with 5 predictors (**Figure III.2**) was generated.

Table III.3 – Clinically plausible interactions tested on univariate analysis.

Variable	n	Odds ratio	95% CI	P values
Age ≥50 years	681	3.81	2.48-5.86	<0.001
Gender (male)	681	0.68	0.42-1.10	0.114
At least 1 significant comorbidity[a]	678	3.33	2.18-5.10	<0.001
HIV	615	1.69	1.02-2.79	0.014
Time of symptoms, weeks	517	0.96	0.92-0.99	0.010
Main symptoms Cough	577	0.87	0.53-1.45	0.598
Hemoptysis	571	0.44	0.22-0.91	0.027
Dyspnea	575	3.22	2.06-5.03	<0.001
Fever	578	1.10	0.71-1.72	0.668
Night sweats	490	0.75	0.46-1.24	0.265
Weight loss	539	1.55	0.95-2.52	0.079
Hemoglobin <12 g/dL	612	2.74	1.80-4.18	<0.001
Interaction terms				
Age ≥50 years * Hemoglobin <12 g/dL	612	3.82	2.50-5.85	<0.001
Age ≥50 years * ≥1 significant comorbidity	678	3.79	2.51-5.70	<0.001
Age ≥50 years * HIV	615	2.68	1.22-5.89	0.014
Gender * Hemoglobin <12 g/dL	678	3.79	2.51-5.70	<0.001
Time of symptoms * Weight loss	464	0.98	0.95-1.01	0.262
Cough * Hemoptysis	566	0.35	0.15-0.83	0.017
Cough * Dyspnea	568	2.74	1.76-4.27	<0.001
Fever * Night sweats	488	1.05	0.63-1.76	0.850

[a] At least one of these comorbidities: HIV infection, diabetes *mellitus*, liver failure or cirrhosis, congestive heart failure and chronic respiratory disease. CI - confidence interval

$$Probability\ of\ death\ = \frac{1}{(1 + e^{-Z})}$$

$$Z = -3.954 + 1.543 \times hypoxemia + 1.050 \times age + 0.899 \times bilateral + 0.813 \times comorbidity + 0.600 \times hemoglobin$$

Figure III.2 – Equations for the derived clinical prediction model that estimates TB patient-specific death probability. *"e"* is the base of the natural logarithm, each of the terms "hypoxemia", "age", "bilateral", "comorbidity" and "hemoglobin" are equal to 1 if, respectively, newly onset hypoxemic respiratory failure, age ≥50 years, bilateral lung involvement, at least 1 significant comorbidity present (HIV infection, diabetes *mellitus*, liver failure/cirrhosis, congestive heart failure, or chronic respiratory disease) and hemoglobin <12 g/dL (otherwise 0).

The equation of the prediction model and Heckman's selection equation were not independent ($\chi^2_{(1)}$= 5.12, *P*=0.023), an argument that justifies the need to apply this procedure. The marginal effects for each of the predicting variables were compared between both models (**Table III.4**).

Table III.4 – Comparison of the marginal effects between the Clinical Prediction Rule and the two-stage Heckman model.

Predictor	Marginal effects from the CPR (95% CI)	Marginal effects from the 2-stage Heckman model (95% CI)	Marginal effect absolute differences
Hypoxemic respiratory failure			
yes	0.36 (0.27-0-44)	0.40 (0.29-0.51)	0.04
no	0.13 (0.09-0.16)	0.18 (0.14-0.22)	0.05
difference	0.23	0.22	0.01
Age ≥50 years old			
yes	0.24 (0.19-0.29)	0.29 (0.23-0.35)	0.05
no	0.12 (0.08-0.15)	0.16 (0.12-0.21)	0.04
difference	0.12	0.13	0.01
Bilateral lung involvement			
yes	0.22 (0.18-0.25)	0.25 (0.20-0.29)	0.03
no	0.12 (0.07-0.16)	0.17 (0.12-0.22)	0.05
difference	0.10	0.08	0.02
At least 1 significant comorbidity[a]			
yes	0.22 (0.18-0.27)	0.26 (0.21-0.31)	0.04
no	0.13 (0.09-0.17)	0.19 (0.13-0.24)	0.06
difference	0.09	0.07	0.02
Hemoglobin <12 g/dL			
yes	0.21 (0.17-0.25)	0.26 (0.21-0.32)	0.05
no	0.14 (0.10-0.18)	0.18 (0.13-0.22)	0.04
difference	0.07	0.08	0.01

[a] At least one of these comorbidities: HIV infection, diabetes *mellitus*, liver failure or cirrhosis, congestive heart failure and chronic respiratory disease. CI - confidence interval

The maximum absolute difference found between the two models was 5% (e.g. for patients with age ≥50 years the probability of death within 6 months of diagnosis was 24% with CPR model and 29% with Heckman's model). However, the CPR model correctly identified the marginal effect associated to each predictor (e. g. when binary variable hypoxemic respiratory failure changes from 0 to 1, the death probability changes 0.23 for CPR and 0.22 with Heckman's model). Thus, the use of this model showed that missing information in the univariate significant variables had little or no effect on mortality risk assessment.

A weight for risk score was calculated for each variable, as describe above (**Table III.5**). The strongest predictors (major criteria) of mortality were hypoxemic respiratory failure, followed by age ≥50 years. Minor criteria were bilateral lung involvement, the presence of at least one of the significant comorbidities (HIV infection, diabetes, liver failure/cirrhosis, CHF, or CRD) and hemoglobin level <12 g/dL. The accuracy of the model was then tested.

Table III.5 – Multivariable logistic regression analysis for deriving tuberculosis risk score for death.

Predictor	Crude OR (95% CI)	Regression coefficient	Multivariable OR (95% CI)	Weight for risk score
Hypoxemic respiratory failure	6.7 (4.2-10.9)	1.543	4.7 (2.8-7.9)	3
Age ≥50 years old	4.2 (2.6-6.8)	1.050	2.9 (1.7-4.8)	2
Bilateral lung involvement	3.4 (2.0-5.8)	0.899	2.5 (1.4-4.4)	1
At least 1 significant comorbidity[a]	3.4 (2.1-5.4)	0.813	2.3 (1.3-3.8)	1
Hemoglobin <12 g/dL	2.5 (1.6-4.0)	0.600	1.8 (1.1-3.1)	1

[a] At least one of these comorbidities: HIV infection, diabetes *mellitus*, liver failure or cirrhosis, congestive heart failure and chronic respiratory disease. CI - confidence interval; OR - odds ratio

A ROC curve was generated (**Figure III.3**), showing that the overall sensitivity and specificity of the clinical scoring system is similar to the logistic regression model.

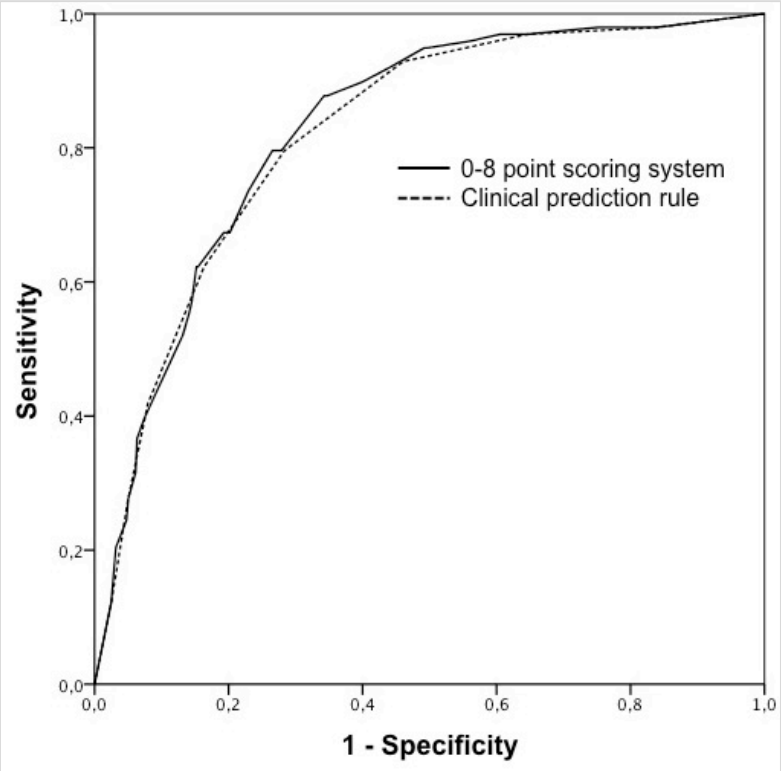

Figure III.3 – ROC curve for the logistic regression model (clinical prediction rule equation, Figure III.2) **and clinical scoring system** (0 to 8 points).

3. Development of TReAT: a scoring system to stratify the risk of death in TB patients

Based on the aforementioned clinical features and using the weights given in Table III.5, a scoring system was constructed to stratify the risk of death among PTB patients using the 2 major risk factors and the 3 minor ones, and assigning a score from 0 to +8 to each patient (**Figures III.4** and **III.5**).

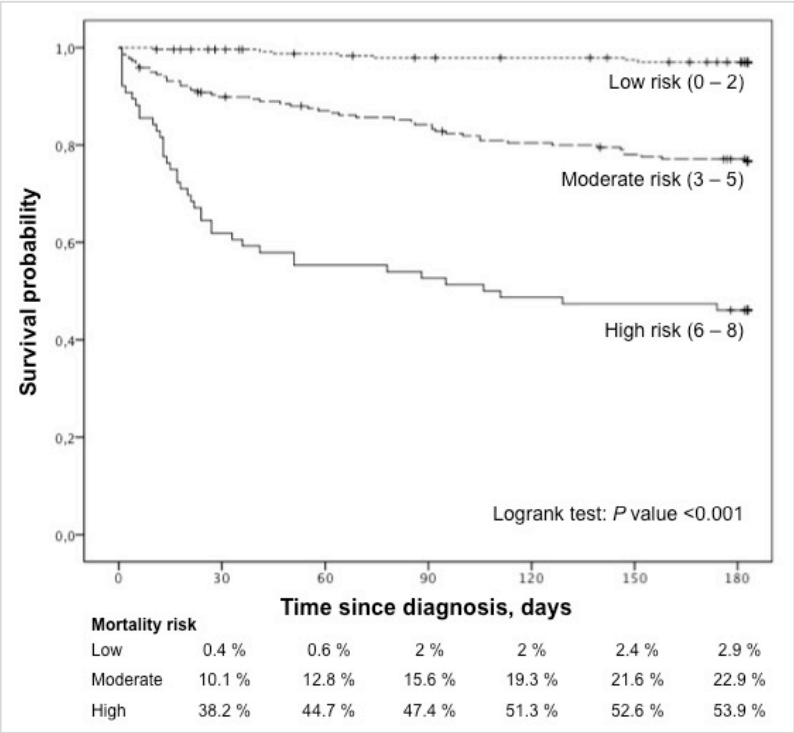

Figure III.4 – Kaplan-Meier estimates of survival in low-risk (clinical score 0-2), moderate-risk (clinical score 3-5) and high-risk (clinical score 6-8) tuberculosis patients in low-risk (clinical score 0-2), moderate-risk (clinical score 3-5) and high-risk (clinical score 6-8) tuberculosis patients. The mortality in each group at different time-points is shown below.

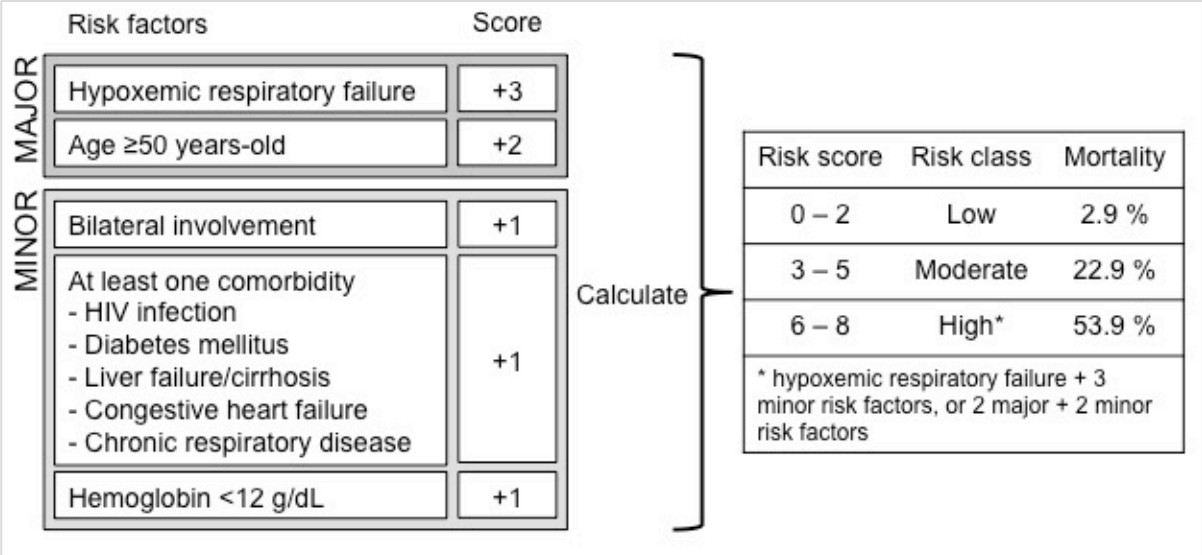

Figure III.5 – Tuberculosis risk assessment tool (TReAT), using baseline clinical features for stratifying patients with pulmonary tuberculosis into severity groups according to the probability of death at 6 months.

The sensitivities, specificities and predictive values of different score values are given in **Table III.6**. The high sensitivity and negative predictive value obtained for a score below 3 points, proved that the test performed well to identify cases of reduced death probability when scoring 0 to 2. These cases were included in the low-risk group and only a few died during follow-up (Figure III.4). The most heterogeneous group was the moderate-risk one, with a gradual decline of survival during the assessed 6 months. For a cut-off score of ≥6, the specificity and positive predictive value increased significantly. Thus, most deaths were observed in the high-risk patients scoring 6 to 8.

Table III.6 – Test characteristics with different prediction scores for mortality in the derivation cohort of patients with pulmonary tuberculosis.

Score	n (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
≥0	539 (100)	100	0	17.8	NA
≥1	466 (86.5)	98	16.1	20.6	97.3
≥2	380 (70.5)	96.9	35.4	25	98.1
≥3	294 (54.5)	92.9	54	31.0	97.1
≥4	203 (37.7)	79.6	71.7	38.4	94
≥5	133 (24.7)	62.2	83.7	45.9	90.9
≥6	76 (14.1)	41.8	92.1	53.9	87.7
≥7	49 (9.1)	27.6	95	55.1	85.5
≥8	23 (4.3)	12.2	97.5	52.2	83.3

NA – not applicable; NPV – negative predictive value; PPV – positive predictive value

4. Validation of TReAT

TReAT was validated in an independent cohort (Figure III.1, **Table III.7**). By performing the validation in a non-hospital centre, a more general applicability of the CPR was tested. Importantly, a significant association was found between group scores (low-, moderate- and high-risk) and real deaths in the validation cohort and with similar magnitude (P<0.001) to what was observed for the derivation set (**Table III.8**). Furthermore, all comparisons between sets regarding mortality within each risk group were non-significant. Association between

categories was also reinforced based on the adjusted residual scores, where absolute values >1.96 indicate that the number of cases in those cells are significantly larger in moderate and high-risk groups (positive relation), or smaller in the low-risk group (negative relation), than would be expected if the null hypothesis were true, with a significance level of 0.05. In support of the generated assessment tool (TReAT), there was no significant difference in the AUCs between the derivation (0.82, 95% CI 0.78–0.87) and the validation cohorts (0.84, 95% CI 0.76–0.93; *P*=0.72).

Table III.7 – Comparison of the distribution of predictors for derivation and validation cohorts.

Predictor	Derivation cohort (n=539)	Validation cohort (n=103)	Total sample (n=642)
Hypoxemic respiratory failure	106 (19.7%)	15 (14.6%)	121 (18.8%)
Age ≥50 years old	241 (44.7%)	43 (41.7%)	284 (44.2%)
Bilateral lung involvement	313 (58.1%)	52 (50.5%)	365 (56.9%)
At least 1 significant comorbidity[a]	252 (46.8%)	47 (45.6%)	299 (46.6%)
Hemoglobin <12 g/dL	259 (48.1%)	37 (35.9%)	296 (46.1%)

[a] At least one of these comorbidities: HIV infection, diabetes *mellitus*, liver failure or cirrhosis, congestive heart failure and chronic respiratory disease. CI - confidence interval

Table III.8 – Comparison of risk groups mortality in the derivation and validation cohorts[a].

	Derivation cohort		Validation cohort		Total sample	
	Deaths (n,%)	***ar*[b]**	**Deaths (n,%)**	***ar***	**Deaths (n,%)**	***ar***
Low risk (score 0-2)	7/245 (2.9)	-8.4	2/54 (3.7)	-4.9	9/299 (3)	-9.6
Moderate risk (score 3-5)	50/218 (22.9)	2.4	13/38 (34.2)	2.0	63/256 (24.6)	2.9
High risk (score 6-8)	41/76 (53.9)	8.7	9/11 (81.8)	4.9	50/87 (57.5)	9.8
Total	**98/539 (18.2)**		**24/103 (23.3)**		**122/642 (19)**	

[a] Association between risk groups and real deaths for derivation cohort (χ^2 = 107.3, *P*<0.001) and for validation cohort (χ^2 = 35.2, *P*<0.001) were significant. The P values for the comparisons of real mortality between sets for each risk groups are as follows: low-, *P*=0.67; moderate-, *P*=0.15; high-, *P*=0.11. [b] Absolute values >1.96 represent significant differences for 95% confidence level (*P*<0.05). *ar - Adjusted Residual* scores

DISCUSSION

This study offers, to the best of our knowledge, the first CPR for TB death prognosis in a high-income region of low to intermediate TB incidence, without expressive multi-drug resistance, comprising both hospital and ambulatory settings. The aim of this CPR is to signal confirmed cases of TB who are at higher risk of death and thus need a stricter medical supervision. Other TB scoring systems have been previously developed, but a few limitations likely hinder their use in this specific context. To the best of our knowledge, only three prognostic rules have been developed for TB. Wejse et al. proposed the first prediction rule (the Bandim TBscore) in a low-resource country (Guinea-Bissau), based on five symptoms and six clinical signs [14]. There are a few reasons why this score may not be applicable to our setting: i) it was never validated in a high-income region, which has very different epidemiological features and better healthcare access; ii) a large proportion of the patients included in the Bandim TBscore study were HIV-infected and had no antiretroviral treatment available, which could independently affect mortality; and iii) in the same study more than half of the cases were smear negative and had no culture confirmation of TB diagnosis. In our cohort, smear negative cases comprised only one third of the subjects, mostly old and HIV-infected patients, but all had culture-proven TB. Another prognostic score was developed by Horita et al. to predict in-hospital death in a context similar to ours [6]. However, the analysis was biased by excluding multidrug resistant-TB and HIV-infected subjects, which are known to increase mortality [3, 4, 24, 25]. Also, other comorbidities were not included in their score and diabetes was not significantly associated to death (possibly due to the small sample size), contrarily to the majority of other studies [26-28], including the present one. By including coexistent conditions in TReAT, the weight given to age was attenuated, as young patients with diseases, like HIV/AIDS and liver failure/cirrhosis, were also considered at risk. Moreover, all patients included in the Horita et al. study were admitted to the ward, which is hardly representative for the overall population of culture-positive TB patients, since in many European countries,

including Portugal, TB treatment is done largely outside the hospital setting. Finally, Valade et al. [15] assessed only 53 cases to propose a prognostic scoring system for TB patients admitted to the intensive care unit. It will be interesting, in future, to compare TReAT with each of these scores and analyse their relative performance and applicability to different settings.

In the present work, the fatal outcome was determined until 6 months after diagnosis, which is supported by several studies, including clinical trials [29-31], and contemplates the minimum duration of the standard multidrug treatment. Late mortality related to TB was previously described to stabilize after 6-months of treatment [30]. Nevertheless, patients died more frequently during the first 30 days after diagnosis, which did not appear to be related with diagnostic delay, since fatal cases usually had shorter time of symptoms compared to survivors (Table III.1). Our results thus suggest that high-risk subjects are usually severely ill from the beginning and should, therefore, be strictly followed and start anti-TB drugs under close monitoring. Initial care in an intensive care or high dependency unit may be appropriate for some cases, particularly because they may have a slow response to standard treatment regimens. The mortality in the derivation cohort (17.5%) was higher than the national estimate (6.4%) [1] and that of the validation cohort (5.7%). This is in line with the described worse TB outcome in hospitalized patients [32, 33]. However, in-hospital mortality due to PTB seems underestimated, when those who require advanced respiratory support face a much higher death rate (up to 70%) than subjects with respiratory failure due to other causes [34].

One of the strengths of this study was the clear definition of our predictor variables, which are not dependent on patient's recall or susceptible to clinicians' subjective judgement. The proposed prediction model is based on intrinsic patients' characteristics (age and comorbidities), disease extent (respiratory failure and one radiographic feature) and a measure of consumption and overall nutritional status (hemoglobin level). The relevance of these variables to the outcome of TB is in line with previous studies [3-7, 14, 31, 34-36].

Limitations of the current study include its retrospective nature and the fact that the validation set is relatively small. Owing to the retrospective design of

the study, a significant number of missing values were found in certain variables. The causes of non-random missing information are very heterogeneous, mainly dependent on the patient status, or on the clinicians, who were of different medical specialties, acting in different settings (emergency room, ward, ICU, outpatient clinic), and may have neglected differential data. For instance, there was a 12.6% of missingness in the "hypoxemic respiratory failure" variable. It is possible that less severe patients without any signs of respiratory distress were not assessed for hypoxemia. On the other hand, patients who were admitted at the busy emergency room and who died during the first 24-48h after admission may have had a short description of his/her clinical condition or past history. Also, 66 patients (9%) of the derivation cohort were not tested for HIV. In a recent work [37] with 7683 TB cases notified in Northern Portugal between 2006 and 2012, 879 (11%) had also unknown HIV status (usually older patients or without history of addiction). We addressed the missingness issue by applying the Heckman method and showed the little impact on the validity of the prediction model. Moreover, sample collection was based in two centres with geographical proximity. It is however important to note that these centres had very different characteristics (hospital *versus* ambulatory) and yet the accuracy of TReAT was similar. Considering the small validation set, it will undoubtedly be of interest to test this CPR prospectively to other surroundings, in whole cohorts or in multicentric studies that would allow larger sample sizes.

The WHO has recently launched the End TB Strategy, which defined the lines towards TB elimination [38]. Among the discussed requirements was the importance of monitoring treatment outcomes. As TB incidence rates decrease with a combination of near-universal access to high-quality diagnosis and treatment and general socioeconomic development, it is expected that the TB death rate will become one of the core indicators for disease control [11]. Implementation of a CPR to identify patients who are more likely to die may prompt screening initiatives in particular risk groups and point directions for further programmatic interventions. On the other hand, the great variability of reported rates of hospital admission and lengths of stay for TB [39, 40] may reflect the uncertainty among clinicians regarding the definition of severity of

illness. We expect that the proposed TReAT helps to bridge these gaps, providing more cost-effectiveness use of medical resources by selecting more appropriately patients that will need closer clinical surveillance.

REFERENCES

1. World Health Organization. Global tuberculosis report 2016. Geneva: World Health Organization; 2016.
2. Franco I, Sousa P, Gomes M, Oliveira A, Gaio AR, Duarte R. Social profile of the highest tuberculosis incidence areas in Portugal. Rev Port Pneumol. 2016;22(1):50-2.
3. Lefebvre N, Falzon D. Risk factors for death among tuberculosis cases: analysis of European surveillance data. Eur Respir J. 2008;31(6):1256-60.
4. Low S, Ang LW, Cutter J, James L, Chee CB, Wang YT, et al. Mortality among tuberculosis patients on treatment in Singapore. Int J Tuberc Lung Dis. 2009;13(3):328-34.
5. Walpola HC, Siskind V, Patel AM, Konstantinos A, Derhy P. Tuberculosis-related deaths in Queensland, Australia, 1989-1998: characteristics and risk factors. Int J Tuberc Lung Dis. 2003;7(8):742-50.
6. Horita N, Miyazawa N, Yoshiyama T, Sato T, Yamamoto M, Tomaru K, et al. Development and validation of a tuberculosis prognostic score for smear-positive in-patients in Japan. Int J Tuberc Lung Dis. 2013;17(1):54-60.
7. Lui G, Wong RY, Li F, Lee MK, Lai RW, Li TC, et al. High mortality in adults hospitalized for active tuberculosis in a low HIV prevalence setting. PLoS One. 2014;9(3):e92077.
8. Lim WS, van der Eerden MM, Laing R, Boersma WG, Karalus N, Town GI, et al. Defining community acquired pneumonia severity on presentation to hospital: an international derivation and validation study. Thorax. 2003;58(5):377-82.
9. Mello FC, Bastos LG, Soares SL, Rezende VM, Conde MB, Chaisson RE, et al. Predicting smear negative pulmonary tuberculosis with classification trees and logistic regression: a cross-sectional study. BMC Public Health. 2006;6:43.
10. Fournet N, Sanchez A, Massari V, Penna L, Natal S, Biondi E, et al. Development and evaluation of tuberculosis screening scores in Brazilian prisons. Public Health. 2006;120(10):976-83.
11. Pontali E, Centis R, D'Ambrosio L, Migliori GB. Monitoring predictors of mortality: A necessary action to reach TB elimination. Rev Port Pneumol. 2015;21(6):295-8.
12. Wisnivesky JP, Henschke C, Balentine J, Willner C, Deloire AM, McGinn TG. Prospective validation of a prediction model for isolating inpatients with suspected pulmonary tuberculosis. Arch Intern Med. 2005;165(4):453-7.
13. Solari L, Acuna-Villaorduna C, Soto A, Agapito J, Perez F, Samalvides F, et al. A clinical prediction rule for pulmonary tuberculosis in emergency departments. Int J Tuberc Lung Dis. 2008;12(6):619-24.
14. Wejse C, Gustafson P, Nielsen J, Gomes VF, Aaby P, Andersen PL, et al. TBscore: Signs and symptoms from tuberculosis patients in a low-resource setting have predictive value and may be used to assess clinical course. Scand J Infect Dis. 2008;40(2):111-20.
15. Valade S, Raskine L, Aout M, Malissin I, Brun P, Deye N, et al. Tuberculosis in the intensive care unit: A retrospective descriptive cohort study with determination of a predictive fatality score. Can J Infect Dis Med Microbiol. 2012;23(4):173-8.
16. World Health Organization. Guidelines for treatment of tuberculosis - 4rd edition Geneva: World Health Organization; 2010.
17. von Elm E, Altman DG, Egger M, Pocock SJ, Gotzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. Lancet. 2007;370:1453–7.
18. Moons KGM, de Groot JAH, Bouwmeester W, Vergouwe Y, Mallett S, Altman DG, et al. Critical Appraisal and Data Extraction for Systematic Reviews of Prediction Modelling Studies: The CHARMS Checklist. PLoS Med. 2014;11(10):e1001744.
19. Isenberg HD. Clinical Microbiology Procedures Handbook. 2nd ed. Washington D.C: ASM Press; 2004.
20. Fluss R, Faraggi D, Reiser B. Estimation of the Youden index and its associated cutoff point. Biom J. 2005;47(4):458-72.

21. Sales AE, Plomondon ME, Magid DJ, Spertus JA, Rumsfeld JS. Assessing response bias from missing quality of life data: the Heckman method. Health Qual Life Outcomes. 2004;16(2):49.
22. Toll DB, Janssen KJ, Vergouwe Y, Moons KG. Validation, updating and impact of clinical prediction rules: a review. J Clin Epidemiol. 2008;61(11):1085-94.
23. Bouwmeester W, Zuithoff NP, Mallett S, Geerlings MI, Vergouwe Y, Steyerberg EW, et al. Reporting and methods in clinical prediction research: a systematic review. PLoS Med. 2012;9(5):1-12.
24. Horne DJ, Hubbard R, Narita M, Exarchos A, Park DR, Goss CH. Factors associated with mortality in patients with tuberculosis. BMC Infect Dis. 2010;10:258. doi: 10.1186/1471-2334-10-258.
25. Millet JP, Orcau A, Rius C, Casals M, de Olalla PG, Moreno A, et al. Predictors of death among patients who completed tuberculosis treatment: a population-based cohort study. PLoS One. 2011;6(9):e25315.
26. Dooley KE, Chaisson RE. Tuberculosis and diabetes mellitus: convergence of two epidemics. Lancet Infect Dis. 2009;9(12):737-46.
27. Lee PH, Lin HC, Huang AS, Wei SH, Lai MS, Lin HH. Diabetes and risk of tuberculosis relapse: nationwide nested case-control study. PLoS One. 2014;9(3):e92623.
28. Baker MA, Lin HH, Chang HY, Murray MB. The risk of tuberculosis disease among persons with diabetes mellitus: a prospective cohort study. Clin Infect Dis. 2012;54(6):818-25.
29. Nunn P, Brindle R, Carpenter L, Odhiambo J, Wasunna K, Newnham R, et al. Cohort study of human immunodeficiency virus infection in patients with tuberculosis in Nairobi, Kenya. Analysis of early (6-month) mortality. Am Rev Respir Dis. 1992;146(4):849–54.
30. Ismail I, Bulgiba A. Predictors of death during tuberculosis treatment in TB/HIV co-infected patients in Malaysia. PLoS One. 2013;8(8):e73250.
31. Marcy O, Laureillard D, Madec Y, Chan S, Mayaud C, Borand L, et al. Causes and Determinants of Mortality in HIV-Infected Adults With Tuberculosis: An Analysis From the CAMELIA ANRS 1295-CIPRA KH001 Randomized Trial. Clin Infect Dis. 2014;59(3):435-45.
32. Perrechi MC, Ribeiro SA. Outcomes of tuberculosis treatment among inpatients and outpatients in the city of São Paulo, Brazil. J Bras Pneumol. 2011;37(6):783–90.
33. Yen YF, Yen MY, Shih HC, Deng CY. Risk factors for unfavorable outcome of pulmonary tuberculosis in adults in Taipei, Taiwan. Trans R Soc Trop Med Hyg. 2012;106(5):303–8.
34. Balkema CA, Irusen EM, Taljaard JJ, Koegelenberg CF. Tuberculosis in the intensive care unit: a prospective observational study. Int J Tuberc Lung Dis. 2014;18(7):824-30.
35. Isanaka S, Mugusi F, Urassa W, Willett WC, Bosch RJ, Villamor E, et al. Iron deficiency and anemia predict mortality in patients with tuberculosis. J Nutr. 2012;142(2):350-7.
36. Lanoix JP, Gaudry S, Flicoteaux R, Ruimy R, Wolff M. Tuberculosis in the intensive care unit: a descriptive analysis in a low-burden country. Int J Tuberc Lung Dis. 2014;18(5):581-7.
37. Reina S, Silva C, Gaio AR, Correia AM, Duarte R. HIV screening in tuberculosis patients in the northern region of Portugal. Int J Tuberc Lung Dis. 2015;19(12):1554-5.
38. Lönnroth K, Migliori GB, Abubakar I, D'Ambrosio L, de Vries G, Diel R, et al. Towards tuberculosis elimination: an action framework for low-incidence countries. Eur Respir J. 2015;45(4):928-52.
39. Taylor Z, Marks SM, Ríos Burrows NM, Weis SE, Stricof RL, Miller B. Causes and costs of hospitalization of tuberculosis patients in the United States. Int J Tuberc Lung Dis. 2000;4(10):931-9.
40. MacIntyre CR, Plant AJ, Hendrie D. Shifting the balance between in-patient and out-patient care for tuberculosis results in economic savings. Int J Tuberc Lung Dis. 2001;5(3):266-71.

CHAPTER 4 – STRATIFICATION OF TUBERCULOSIS SEVERITY AND ITS ASSOCIATION WITH PATHOGEN PHYLOGENY AND PROPERTIES

The results presented in this chapter were published:

(i) **in conferences**

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Caracterização molecular das linhagens filogenéticas de *Mycobacterium tuberculosis* num grupo de doentes de baixo risco para tuberculose do norte de Portugal (Oral communication) | Authors: Helder Novais e Bastos, Henrique Machado, Maria Isabel Veiga, Patrício Costa, Nuno S. Osório, Ivo Ferreira, Sara Magalhães, Angélica Ramos, Teresa Carvalho, Jorge Pedrosa, Fernando Rodrigues, António Gil Castro, João Tiago Guimarães, Margarida Saraiva

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ABSTRACT

A striking feature of tuberculosis (TB) is the variability of infection outcomes. The heterogeneity observed has been classically attributed to environmental and host determinants. However, studies uncovering *Mycobacterium tuberculosis* complex (MTBC) genomic diversity and its association to clinical outcomes of TB have shown the potential importance of pathogen-related factors to the disease pathogenesis. Nonetheless, the role of this variability in the clinical setting remains poorly understood. To address this question, we resorted to a cohort of 681 pulmonary TB cases and selected a group of 133 patients without known predictor or precipitator factors. After developing a clinical decision tree to stratify TB, the disease manifestation in each of these patients was classified in different severities. Corresponding MTBC strains were isolated and genotyped using single nucleotide polymorphisms (SNPs) as stable genetic markers to define the phylogeny. A highly homogeneous phylogeographical structure was observed, with the majority of isolates belonging to Lineage 4 and LAM sublineage. In parallel, we assessed the growth features of *M. tuberculosis* isolates associated with different clinical phenotypes. We found that isolates from patients with mild disease grew significantly slower than moderate or severe strains. Strikingly, isolates associated to moderate outcome had a longer lag phase and reached a highest plateau, after a steep exponential phase. To gain in-depth knowledge of the genetic basis for differential growth phenotype, we performed whole genome sequencing and genomic analysis. The heterogeneity observed was not lineage-specific, but we identified several SNPs as potentially related with the phenotype of differential growth. Functional annotation analysis of the genes harboring the identified SNPs showed a significant enrichment of the ATP binding Gene Ontology molecular function. Analysis of previous literature allowed to identify which genes were previously associated with growth suppression. Among the genes that had not been formerly implicated in phenotypes of *in vitro* growth, the membrane transport and biosynthetic pathways were highlighted. In conclusion, mutations implicated in disease severity could be related to microevolution events within the host.

Furthermore, the altered growth behaviour of moderate strains of MTBC might indicate an adaptation of the pathogen to induce a high transmissible disease without devastating effects to the host, ensuring the transmission to new hosts.

INTRODUCTION

Despite largely curable, tuberculosis (TB) continues to rank among the leading causes of death by an infectious agent, accounting for 1 death every 18 seconds, according to the latest estimates [1]. Further aggravating this scenario, one quarter of the world's population is estimated to be latently infected with the TB-causing agent *Mycobacterium tuberculosis*, thus providing a virtually impossible to eliminate reservoir [2, 3]. Recent studies start to question the concept of the existence of two opposing poles in TB outcome, active disease and latent TB infection (LTBI) [4, 5]. Instead, a continuous spectrum of TB likely exists between both extremes, with cases of subclinical disease described in patients with normal chest X-ray regardless of human immunodeficiency virus (HIV) infection status [6], and reports of human subjects with latent infection that have metabolically active lesions, as assessed by 18-fluorodeoxyglucose positron emission tomography (18-FDG PET) [7-9]. Another study that combined FDG-PET and computed tomography (CT) in asymptomatic, antiretroviral-therapy-naïve, HIV-1-infected adults with latent tuberculosis, described a subgroup of individuals presenting pulmonary abnormalities suggestive of subclinical form of TB and had an increased likelihood of progressing to active disease [10]. Finally, viable *M. tuberculosis* bacilli were recovered from incidental TB lesions discovered post-mortem [11, 12].

Following these observations, molecular and clinical biomarkers that drive the host along the range of manifestations for both latent and active forms have just started to be unveiled [8, 13-17]. The heterogeneity of TB has been classically attributed to environmental and host determinants [18]. However, studies uncovering *M. tuberculosis* complex (MTBC) genomic diversity have shown the potential importance of pathogen-related factors. Given the low mutation rate and lack of evidence for genetic recombination or horizontal gene transfer [19-21], the human-adapted members of the MTBC were long assumed to be essentially identical. Over the years, improved methods for the genotyping of clinical isolates revealed the existence of 7 different phylogenetic lineages causing human TB [22, 23], associated with different geographic regions and sympatric human

populations in cosmopolitan settings [24-27]. These facts suggest that different lineages of *M. tuberculosis* evolved in parallel and are possibly adapted to particular human populations [28]. This association may be disrupted in the case of HIV patients [29], possibly due to deficient recognition of pathogen's epitopes associated with CD4 T-cell depletion. A phylogeographical structure can also be observed at the sublineage level. Lineage 4 (L4), or *Euro-American*, is the most globally widespread, predominating throughout Europe and America, but also in some parts of Africa and the Middle East [22, 23]. This lineage comprises at least 10 sublineages, including Haarlem, Latin-American-Mediterranean (LAM), X and T families of strains [30, 31], which differ in their geographical distribution [32]. A recent work demonstrated that some L4 sublineages are geographically restricted "specialists", while others with higher epitope diversity have a global distribution, which is in agreement with the idea of being "generalists" regarding the host population range [32].

The phylogenetic classification of the MTBC provided the rational framework for a number of studies around the world linking the genotypic diversity of MTBC to diversity in clinical phenotypes. For instance, L4 causes mainly pulmonary TB [33], while Lineages 2 [33-35] and 3 [36] were reported to associate with extrapulmonary disease. In addition, more debilitating symptoms (such as weight loss) have been associated with L4 strains in Tanzania [37]. Lineage 2 (L2), or East-Asian, is another highly successful phylogenetic branch that has collected much attention in research. This lineage has been repeatedly associated with treatment failure and relapse [38-40]. Moreover, one *in vitro* study demonstrated that L2 may acquire drug resistances more rapidly than L4 [41], which was confirmed in reports from Belarus and Mongolia, where multidrug-resistant (MDR) isolates were more likely to belong to the L2 Beijing family of strains [42, 43]. Both L2 and L4 were shown to have a higher rate of progression to active TB as compared to Lineage 6 in the Gambia [44]. There are also differences in terms of efficacy of transmission. Beijing strains showed higher transmissibility in San Francisco [45], whereas Lineage 3 was reported to be less transmissible than Lineages 1, 2 and 4 in TB patients from Montreal, Canada

[46], and striking differences in transmissibility among sublineages of Lineage 5 in Benin and Nigeria were reported [47].

The purpose of the present study was to explore the phylogenetic distribution of MTBC isolates collected from a group of patients without known host-related predictor or precipitator TB factors in a main hospital in the Porto area, Portugal. Furthermore, we aimed to understand the possible links between the phylogenetic structure and bacterial growth properties of molecularly characterized strains with clinical phenotypes of severity.

MATERIALS AND METHODS

1. Study population and clinical severity stratification

A cohort of 681 culture-confirmed pulmonary TB cases diagnosed at a University-affiliated hospital (Hospital de São João - HSJ, Porto) during the period of 7 years (2007-2013) was analyzed. The overall demographic and clinical features of this population have been described in chapter 3 [48]. Patients were excluded if they had known predictor and/or precipitator TB factors (n=488). These included human immunodeficiency virus (HIV) infection, pharmacological immunosuppression, hepatitis C virus (HCV) chronic infection, alcohol abuse and non-communicable comorbid diseases (diabetes, end-stage chronic kidney failure, malignancy, autoimmune diseases, cirrhosis or chronic liver failure, heart failure, chronic obstructive pulmonary disease, and patients with structural lung disease, such as silicosis, fibrosis, or bronchiectasis). The exclusion criteria were designed to eliminate known host-related variable outcomes of infection [18, 49] (**Figure IV.1**).

The clinical records of the remaining 193 "otherwise healthy" pulmonary TB patients were retrospectively reviewed to stratify into severity. To stratify the severity of disease, a classification flowchart was developed (**Figure IV.2**) taking into consideration the site of TB involvement, baseline (before initiation of treatment) levels of hemoglobin and C-reactive protein, the severity of symptoms and the chest radiography findings for intrathoracic TB. Digital images of plain chest radiographs were blind graded by two independent clinicians (**Figure IV.3**), using a previously published decision tree [14]. The disagreements between the two clinicians were resolved through a consensus assessment by a third reader. Sixty of the 193 selected patients were unclassifiable because of lacking information regarding the described classification criteria. Thus, the final population comprised 133 patients, that were divided in 5 severity groups: mild, moderate, severe, extremely severe and disseminated disease.

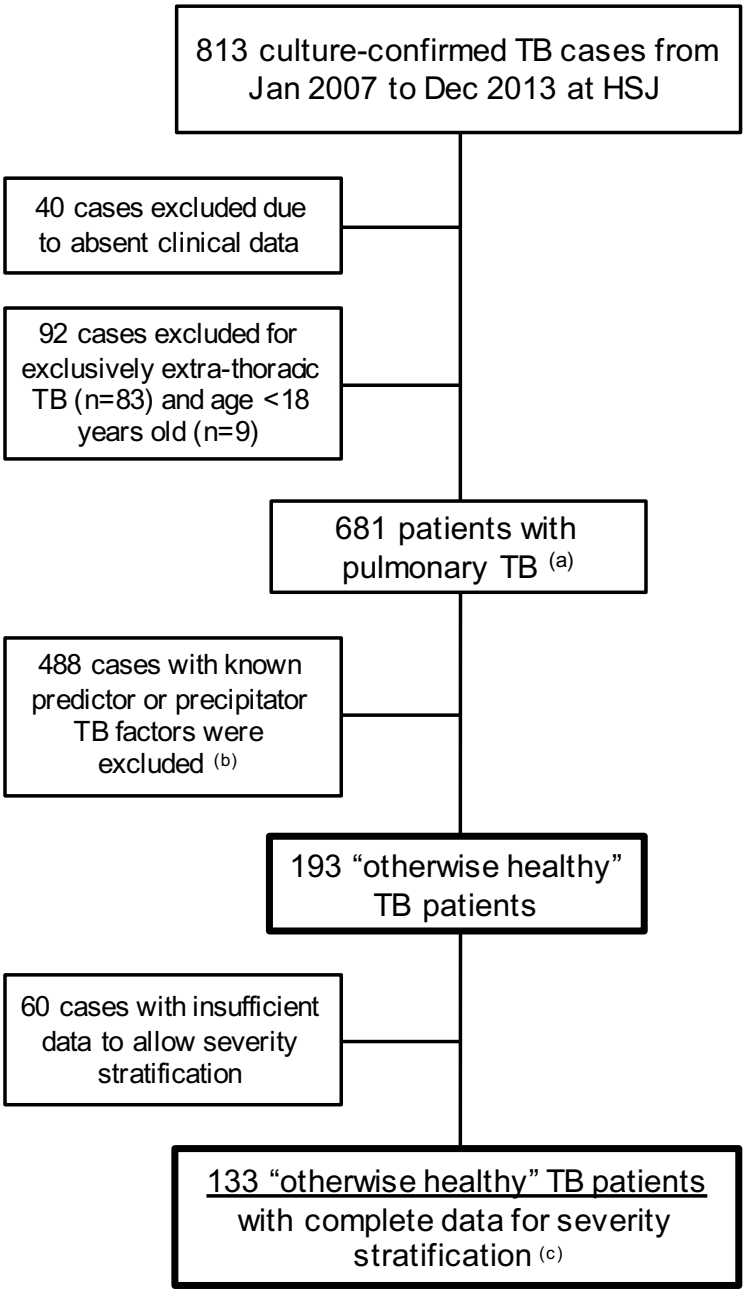

Figure IV.1 – Flow chart for the selection of the participating patients. (a) Full description of this cohort available at Bastos HN et al. [48]. (b) Predictor or precipitator factors for TB include HIV infection, pharmacological immunosuppression, HCV chronic infection, alcohol abuse, diabetes, end-stage chronic kidney failure, malignancy, autoimmune diseases, cirrhosis or chronic liver failure, heart failure, chronic obstructive pulmonary disease, patients with structural lung disease, such as silicosis, fibrosis, or bronchiectasis, and age >65 years. (c) The study was based in 133 "otherwise healthy" TB patients that had sufficient data to allow severity stratification (see Figure IV.2 for details). HSJ – Hospital de São João, Porto, Portugal; TB – tuberculosis.

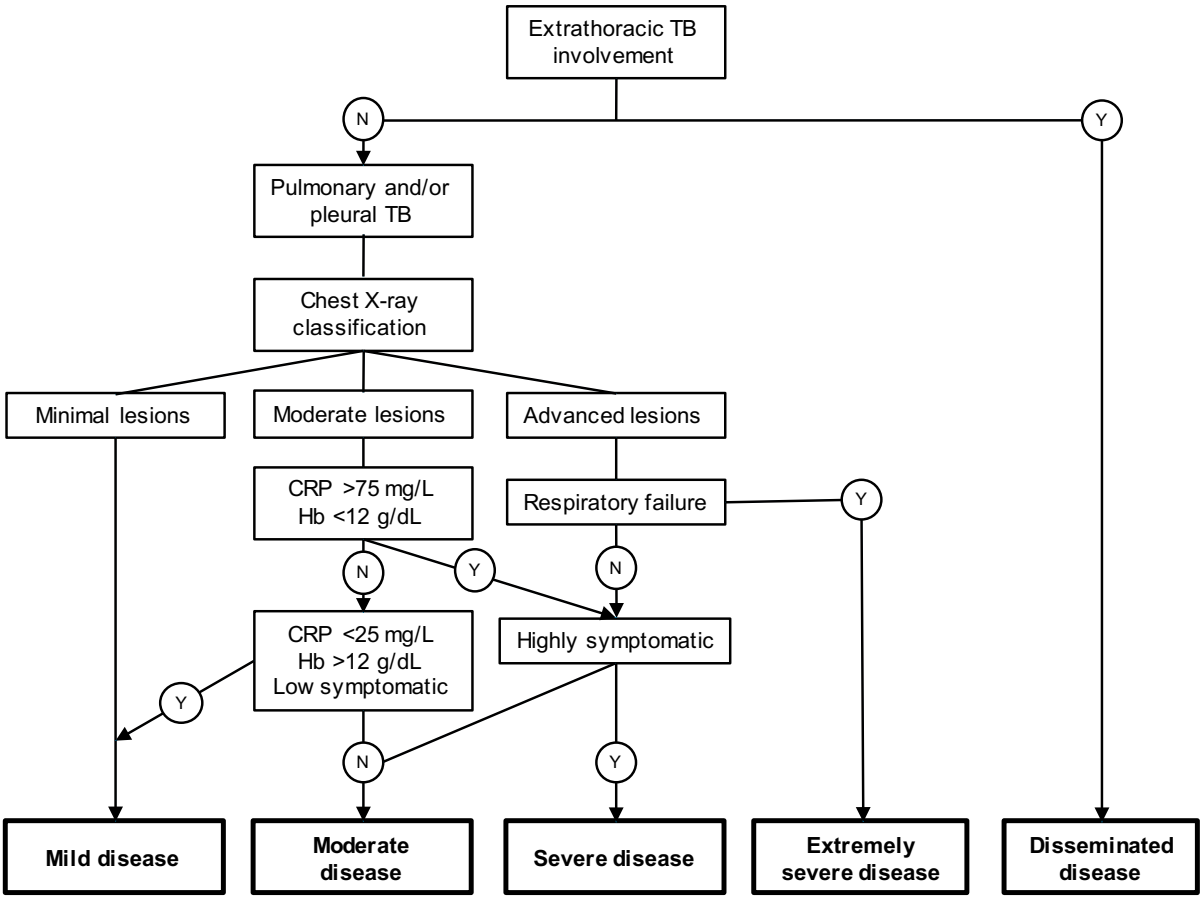

Figure IV.2 – Classification system developed to define the TB clinical phenotype. TB diagnosis was defined according to the WHO guidelines [50]. Disseminated disease was characterized by the presence of any of the following conditions: i) isolation of *M. tuberculosis* from blood, bone marrow, liver biopsy specimen, or ≥2 noncontiguous organs; ii) mycobacterial isolation from 1 organ and either histologic demonstration of caseating granulomatous inflammation from another noncontiguous organ, bone marrow and liver biopsy specimen, or radiographic finding of miliary lung lesions [51]. The dual sites involvement of cervical or axillary lymph node and lung was regarded as a locoregional disease rather than disseminated disease [52]. Grading of the radiographic extent of disease was based on the decision tree supplied in Figure IV.3. Hemoglobin <12 g/dL defined anemia in both genders. Acute hypoxemic respiratory failure was defined as newly onset partial pressure of oxygen decrease to <60 mmHg, or arterial oxygen saturation <90%. A symptom score scale (0 to 6) was calculated on the basis of the presence of 3 respiratory symptoms - cough, hemoptysis, dyspnea - and 3 constitutional symptoms - fever, night sweats, weight loss (1 point for each item) [53]. Patients presenting a symptom score >4, or a score >3 that included the presence of dyspnea, were classified as having highly symptomatic disease. CRP – C-reactive protein; Hb – Hemoglobin; N – No; TB – Tuberculosis; Y – Yes.

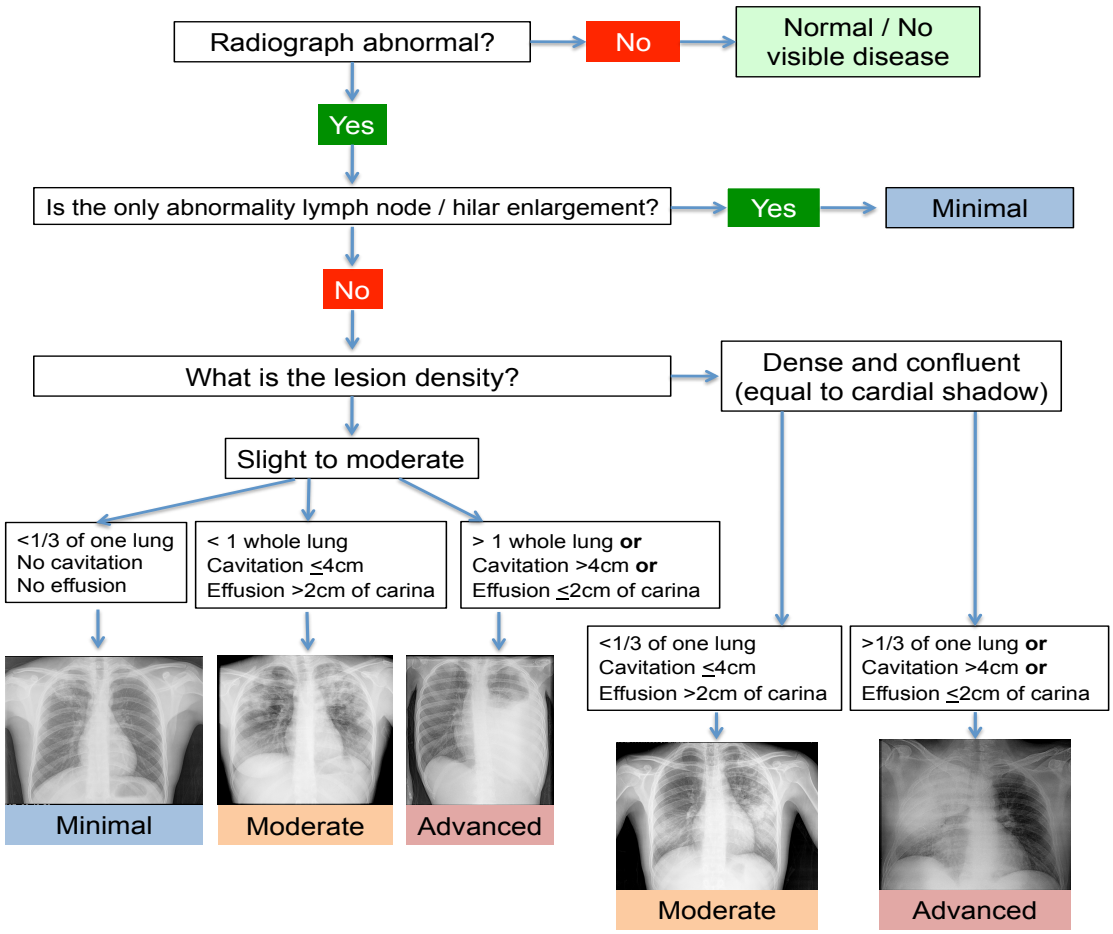

Figure IV.3 – Decision tree grading system developed to assess the radiographic extent of disease. Adaptation of the algorithm developed and expanded by Berry et al. [14] from the text description definitions given in Falk et al. [54]. Baseline chest radiographs were blindly analysed by two independent physicians according to the lesions extent, the presence of lung cavitation and pleural effusion. Disagreement between readers was resolved through a consensus read by a third physician.

2. Growth of *M. tuberculosis* clinical isolates

Bacterial samples of the selected 133 subjects were recovered from stored primary cultures of *M. tuberculosis* clinical isolates at HSJ. Two hundred µL of inoculum were plated and smeared uniformly on solid Mycobacteria 7H11 agar supplemented with Oleic Albumin Dextrose Catalase Growth Supplement (OADC) and PANTA antibiotic mixture (to prevent non-mycobacteria growth). The plates were incubated at 37 °C for 4 to 8 weeks. After this period, grown colonies were gently rubbed and transferred to 20 mL of Middlebrook 7H9 liquid medium (BD Biosciences, San Jose, USA) complemented with OADC enrichment, 2% glycerol

as a carbon source and 0.5% Tween® 80 (Sigma-Aldrich, St. Louis, USA) to prevent bacterial clumping. Cultures were incubated at 37 °C with constant 120 rpm shaking for an additional 7–10 days, to increase the bacterial yield. Upon reaching an optical density at 570 nm of at least 0.7 (mid log-phase growth stage) the bacterial suspension was aliquoted into 1 mL inoculums, which were frozen at -80 °C for further use.

3. Genomic DNA extraction from bacterial suspensions

Ten to 15 mL of the bacterial suspension was centrifuged at 3500 g for 5 minutes at 4 °C and the resulting pellet was resuspended in water to a final volume of 1000 μL and then inactivated with 500 μL a 1:2 phenol-water solution for DNA extraction. Fifty μL of 0.1 mm diameter zirconia beads and 55 μL TEN 10x buffer (1 M Tris, 500 mM EDTA, 5 M NaCl) were added to 750 μL of phenol-inactivated bacterial suspension and the mixture was subsequently homogenized in the Fast-Prep24 bead beater at 4 M/s for 30 seconds, twice. Then, 250 μL of chloroform were added to the samples, followed by mild agitation and 5-minute centrifugation at 12,000 rpm at 4 °C in order to separate the sample into an organic and an aqueous phase. The upper aqueous phases (containing mostly nucleic acids and water) were then recovered and mixed with a 1:1 volume of chloroform, followed by a repetition of the previous centrifuging step to further purify the samples. The phenol-chlorophorm extraction method was chosen since it grants a good DNA yield, a low amount of contaminants and an effective way to inactivate the bacterium [55]. The final aqueous phases were recovered and mixed with 1 mL of ice cold absolute ethanol and 40 μL of 3M sodium acetate (NaAc). The samples were then incubated at -20 °C for 30 minutes, to allow the DNA to precipitate, and centrifuged for 5 minutes at 14,000 rpm at 4 °C. The supernatant was discarded, the pellet washed in 500 μL of 70% ethanol and the samples centrifuged again for 5 minutes at 12000 rpm at 4°C. The supernatant was then removed, the DNA pellet allowed to dry and resuspended in TE buffer (1M Tris [pH 8], 500 mM EDTA). The DNA was quantified by spectrophotometry

(NanoDrop® 1000, Thermo Scientific, Wilmington, USA) and its quality assessed according to the 260/280 nm and 260/230 nm ratios. Samples with ratios close to 2 were considered pure. Finally, the samples were normalized to a standard concentration of 200 ng/μL of nucleic acid.

4. *M. tuberculosis* lineage and sublineage genotyping

M. tuberculosis lineage genotyping was performed by a custom TaqMan® real-time polymerase chain reaction (PCR) assay (Applied Biosystems, Carlsbad, USA), using single nucleotide polymorphisms (SNPs) as stable genetic markers, as previously described (**Figure IV.4**) [32, 56]. For this, 2 μL of DNA sample (10 ng/μL) were added to the real-time PCR mix containing 2.5 μL of water, 4 μL of TaqMan® Universal MasterMix II (Applied Biosystems), 1.5 μL of primer-probe sets for both ancestral and mutant genotypes (Applied Biosystems), at a concentration of 3.6 μM and 0.8 μM, respectively. Primer and probe sequences are listed in **Table IV.1**. Reactions were run in a Bio-Rad (Hercules, USA) CFX96™ thermocycler, using the following program: 95°C 10 min; 92°C 15 sec and 60°C 1 min, for 44 cycles. The fluorescence intensity of VIC and FAM channels was measured at the end of every cycle. Results were analyzed with the Bio-Rad CFX Manager™ 3.1 and genotypes were determined according to the software Allele Discrimination.

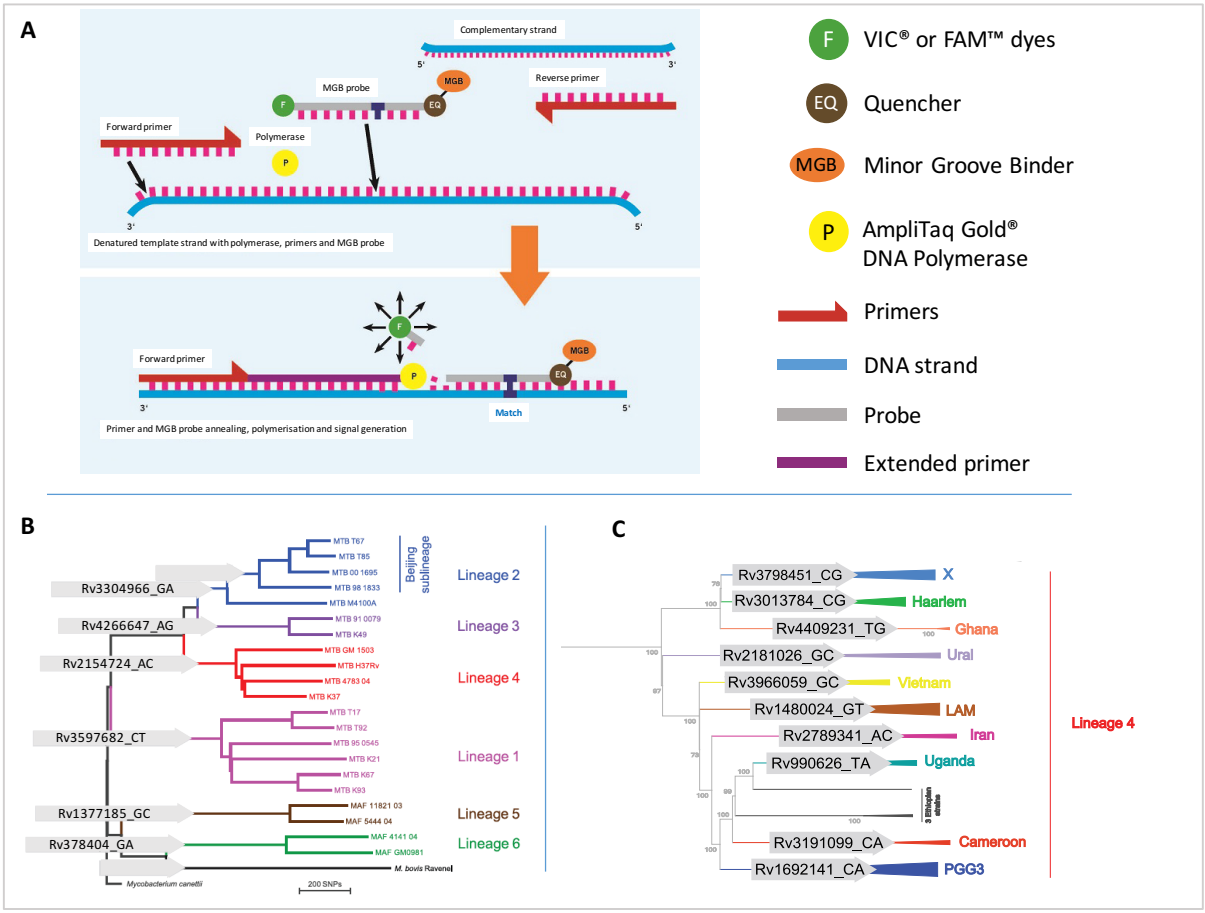

Figure IV.4 – *M. tuberculosis* lineage genotyping was performed by a custom TaqMan® real-time PCR SNP assay. (A) Allelic discrimination is achieved by the selective annealing of TaqMan® MGB probes, that contain the nucleotide complementary to the SNP in the template strand. As AmpliTaq Gold® DNA Polymerase polymerizes over the probe, its exonuclease activity will release the fluorochrome, therefore generating a signal. Adapted from TaqMan® SNP Genotyping Assays Product Bulletin [57]. **(B)** Phylogenetic tree of 22 whole genome sequences of MTBC plus *Mycobacterium canettii* as outgroup, and canonical SNPs used for TaqMan assay. Adapted from Stucki et al. [56]. **(C)** The analysis of 72 MTBC Lineage 4 genome sequences provided the basis for the definition of 10 sublineages. Sublineage names are working names (partially overlap with spoligotype families). Numbers next to lines are bootstrap values. Kindly provided by Iñaki Comas (Biomedicine Institute of Valencia) and partially published at [32]. **(B) and (C)** Rv-numbers in grey arrows represent specific SNPs for the lineages/sublineages and the nucleotide change at the position in the annotated reference sequence of H37Rv.

Table IV.1 – Primer and probes sequences for TaqMan® SNP genotyping real-time assays.

MTBC lineage/ sublineage[a]	SNP in H37Rv[b]	Forward primer	Reverse primer	Ancestral probe[c]	Mutant Probe[c]	Reference strains tested
Lineage 1	Rv3597682CT	TGTCAACGAAGGCG ATCAGA	GACCGTTCCGGCAG CTT	FAM-ACAAGGGCGACGTC-MGBNFQ	VIC-ACAAGGGCGACATC-MGBNFQ	H43; HN121; N157
Lineage 2	Rv3304966GA	CCTTCGATGTTGTGC TCAATGT	CATGCGGCGATCTCA TTGT	FAM-CCCAGGAGGGTAC-MGBNFQ	VIC-CCCAGGAAGGTACT-MGBNFQ	HN878; N52; N145
Lineage 3	Rv4266647AG	GCATGGATGCGTTG AGATGA	CGAGTCGACGCGAC ATACC	VIC-AAGAATGCAGCTTGTTGA-MGBNFQ	FAM-AAGAATGCAGCTTGTCGA-MGBNFQ	H4
Lineage 4	Rv2154724AC	CCGAGATTGCCAGC CTTAAG	GAAACTAGCTGTGAG ACAGTCAATCC	VIC- CCAGATCCTGGCATC-MGBNFQ	FAM-CAGATCCGGGCATC-MGBNFQ	H37Rv; CDC1551; H163
LAM	Rv1480024GT	CCAGGCCAGGATCC ACAT	CGTGCTGATCATCTC GATGGT	VIC-CAGGATGAACCCGAACAC-MGBNFQ	FAM-CAGGATGAACCCTAACAC-MGBNFQ	Sequenced clinical isolates
X	Rv3798451CG	GCCCGTTCTAACAAT GCTTTCC	GGAGGCGTATTGACT CTGGAT	FAM-AGGGACACTGCCCAGAA-MGBNFQ	VIC-CAGGGACACTCCCCAGAA-MGBNFQ	Sequenced clinical isolates
Haarlem	Rv3013784CG	CCCGGCCTCGTCGA A	CGAATCGCCCAGTTG CTAGT	FAM-TTGAGTTGCTCGAGCTG-MGBNFQ	VIC-TTGAGTTGGTCGAGCTG-MGBNFQ	Sequenced clinical isolates
Lineage 5	Rv1377185GC	TCCAGCAGGTGACC ATCGT	GGCCTGTGACCCGTT CAAC	VIC-CGTGGACCTCATG-MGBNFQ	FAM-CGTGGACCTGATGC-MGBNFQ	Not available
Lineage 6	Rv378404GA	CGGCCGACAGCGAG AA	CCATCACGACCGAAT GCTT	FAM-CTGCAAATCCCGCAGTA-MGBNFQ	VIC-CTGCAAATCCCACAGT-MGBNFQ	H91

[a] Nomenclature according to [19]. [b] Position of SNP in reference to the H37Rv genome (accession number: NC_000962.3). [c] In the 5' extremity either VIC or FAM fluorochromes and on the 3' extremity a minor groove binding (MGB) protein coupled with a non-fluorescent quencher (NFQ)

5. Mycobacterial growth curves from standardized inoculum

We studied and compared the growth properties of 8 isolates associated with mild, 8 with severe and 10 with moderate outcomes of TB. Matching of selected isolates was based on at least 2 out of 3 variables: age within 15 years, gender and smoking habits. For each clinical isolate, a standardized inoculum was prepared, as previously described [58]. The isolates were grown for 21 days in Middlebrook 7H9 liquid medium (BD Biosciences), as described above, and the 570 nm OD of these subcultures adjusted to 0.01–0.03. One hundred µL of this standardized inoculum were used to inoculate in duplicate BACTEC™ MGIT™ (Mycobacteria Growth Indicator Tubes) 960 tubes (BD Biosciences) supplemented with 0.8 mL of OADC. The MGIT™ 960 culture tubes contain 7 mL of modified Middlebrook 7H9 broth, a growth supplement (bovine albumin, catalase, dextrose, oleic acid and polyoxyethylene stearate) and an antibiotic mixture consisting of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (MGIT™ PANTA) [59]. After inoculation, the tubes are entered into a BACTEC™ MGIT™ 960 instrument (BD Biosciences), where cultures are incubated at 37 ºC for a maximum of 42 days.

Growth curves were generated by automatically monitoring of the MGIT cultures using the BD Epicentre software, which collected fluorescence data from each tube every 60 minutes. The amount of fluorescence is inversely proportional to the oxygen level in the culture medium, indicating the consumption of oxygen due to the growth of inoculated organisms in the culture tubes, measured as Growth Units (GU) [59]. Data were extracted and the length of the lag phase or ‘‘time to positivity’’ (the manufacturer-set threshold is 75 GU, corresponding to approximately 10^5–10^6 CFU per ml of medium) for each isolate was measured. Furthermore, the doubling time (t_d = time required for the number of cells or bacterial density to double during the exponential phase) and the peak point on the bacterial growth curve (C_{max}) were calculated. The doubling time was calculated using the following equation $t_d = \frac{ln2}{\kappa}$, where κ represents the growth rate [60]. To determine the growth rate, a graph of GU (acquired by the BACTEC™

system) as a function of time with exponential growth was plotted and the method of least squares regression using GraphPad Prism® 7 software (GraphPad Software, Inc.) was applied [61]. To validate the present methodology, the above described procedures were performed with the reference strain H37Rv.

From the same initial standardized inoculum, four 10-fold dilutions were prepared in sterile PBS 1x and 100 µL of each dilution plated on 7H11 solid medium. The plates were incubated for 3 weeks at 37 ºC to estimate the number of CFU present in the original suspension, using a binocular magnifying glass. This controlled for differences in the bacterial burden of the standardized inocula.

6. *M. tuberculosis* whole genome sequencing and analysis

Next generation sequencing (NGS) of selected strains was performed on the Illumina MiSeq or HiSeq platforms with 50-300 base pairs (bp) and 101 bp paired-end run modes respectively, using Nextera libraries. The quality of raw Illumina sequence data for each isolate was assessed using the FastQC toolkit [62]. Low quality reads were trimmed using trimmomatic software [63]. Nextera adapter sequences were removed and bases were trimmed from the ends of the reads with parameters chosen according to the information given by FastQC on “per base sequence content” and “per base sequence quality”. Reads with <20 average quality or less than 30 bases were excluded.

The reference genome sequence used for the analysis was the most recent common ancestor of MTBC [64]. Using this sequence and not the reference genome of H37Rv strain [65, 66] is advantageous since H37Rv harbors SNPs typical of Lineage 4 and from adaptation to the laboratory. At first, mapping to reference and SNP calling was done with Geneious [67]. Then, reads were again mapped using Burrows Wheeler Aligner (BWA) [68] and called using SAMtools [69] and VarScan.v2.3.7 [70, 71]. To improve accuracy, the genomic positions were filtered and classified as SNPs only when detected by both approaches, with a variant frequency ≥90% and a coverage ≥10. Genomic positions pertaining to repetitive regions, mobile elements and *pe/ppe* genes were excluded from our

analysis due to the technical difficulties in obtaining reliable nucleotide sequences in these regions using NGS methods based on short reads [72].

Calculation of a maximum likelihood phylogenetic tree was performed with RaXML [73] using 1797 concatenated genome-wide SNPs and the resulting tree was rooted to the *M. tuberculosis* ancestor. Bootstrap support was calculated based on 1000 replicates to determine the confidence of the branching. The resulting maximum likelihood tree was confirmed by a second phylogenetic tree built with a Bayesian approach. A barcode of 62 SNPs chosen to identify MTBC lineage and sublineages [74] was also used for the phylogenetic classification of the isolates.

SNPs in coding regions were categorized as non-synonymous or synonymous using Geneious [67]. Prediction of the effects of coding non-synonymous variants on protein function was performed using the Sorting Intolerant From Tolerant (SIFT) algorithm [75]. Briefly, SIFT uses sequence homology to compute the likelihood that an amino acid substitution will have an adverse effect on protein function. The underlying assumption is that evolutionarily conserved regions tend to be less tolerant to mutations, and hence amino acid substitutions or insertions/deletions in these regions are more likely to affect function [76]. A SIFT score is a normalized probability of observing the new amino acid at that position, and ranges from 0 to 1. Substituted amino acids with a SIFT score ≤0.05 was used as the cut-off value for prediction of impact on protein function.

Genes identified were compared with a set of genes previously associated with growth *in vitro* [77] and *in vivo* [78]. Functional characterization of novel gene candidates detected was based on annotations contained in the TubercuList database (http://tuberculist.epfl.ch/) and by a Gene Ontology (GO) analysis. The PANTHER (protein annotation through evolutionary relationship) enrichment tool (http://www.pantherdb.org/) was used to obtain the GO terms for molecular function [79]. A two-tailed Fisher's Exact Test was used to measure the significance of enrichment. Proteins assigned to enriched GO categories (*P value* < 0.05) were grouped according to the PANTHER classification system [80].

7. Statistical analysis

The association of SNPs and phylogenetic branches of the selected isolates with different clinical phenotype groups was assessed using a Chi-square test or Fisher exact test as appropriate. Growth curve analysis was performed with one-way ANOVA and LSD post hoc test, or Kruskal-Wallis rank test and Mann-Whitney U-test if non-normal distribution of measured variables. All the statistical analyses were performed using the GraphPad Prism® 7 software (GraphPad Software, Inc.) or SPSS software program, version 24 (IBM® SPSS®, Inc.). Values of $P<0.05$ were considered statistically significant.

8. Ethics statement

The study protocol was approved by the Health Ethics Committees of the HSJ (approval number 109-11), the North Health Region Administration (approval number 71-2014) and the Portuguese Data Protection Authority (approval number 12174-2011). To ensure confidentiality, each case was anonymized by the assignment of a random identification number. Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

RESULTS

1. Stratification of TB severity in the study group

Since the aim of this study was to relate disease outcome with bacterial properties, we reasoned that host factors known to influence the course of the infection should be kept to a minimum. Therefore, we reviewed the clinical files of a selected group of 133 "otherwise healthy" patients (Figure IV.1) and the severity of TB at presentation was assigned to each individual following the classification criteria presented in Figures IV.2 and IV.3. As shown in **Figure IV.5**, most (52.3%) TB cases within the study group were classified as moderate disease, followed by mild (23.3%) and severe (9.8%) manifestations. A smaller group of patients (5.3%) presented an extremely severe form of TB. Finally, almost 10% of the cases had evidence of disseminated disease. We then decided to focus our study in the mild, moderate and severe TB groups. This was because the extremely severe TB group represented a rare phenotype and because disseminated disease associated with significantly longer duration of disease before diagnosis (median time of symptoms was 18 weeks in this group of patients). The general characteristics of the patients within this range of severity, as well as comparisons between categories, are shown in **Table IV.2**. Of note, the vast majority of subjects studied were Portuguese-born caucasians (92.2%), which is in line with previous estimates [81] and with the full cohort [48], and only one (0.5%) case harbored multidrug-resistant strains.

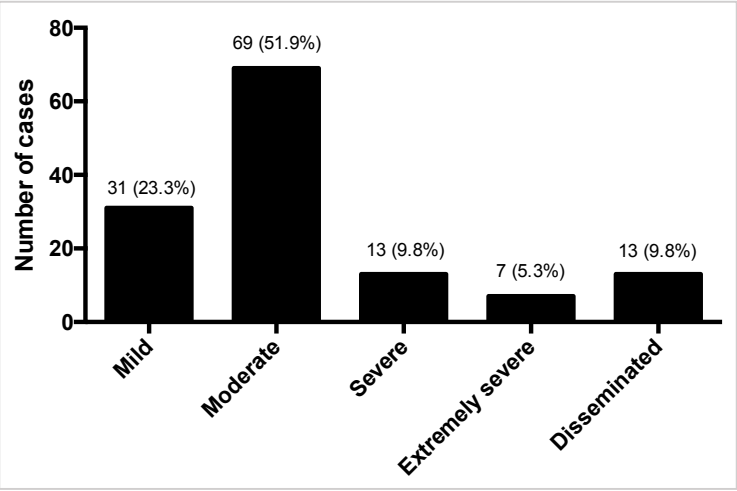

Figure IV.5 – Tuberculosis severity stratification for the study population. The clinical files for the study group of 133 were revised and the TB disease severity assigned according to the fluxogram supplied in Figure IV.2.

Table IV.2 – Study population characteristics and comparison between severity groups. Continuous variables are presented as mean±SD or median (25th–75th percentile). The proportions in this table reflect the number of patients with each finding divided by the total number of patients for whom data were available.

Clinical feature	All (n=113)	Mild (n=31)	Moderate (n=69)	Severe (n=13)	*P* value
Age years, median (IQR)	31 (25–44.5)	30 (27–39)	30 (24–45)	42 (27.5–50)	0.386
Male gender, n (%)	65/113 (57.5)	16/31 (51.6)	38/69 (55.1)	11/13 (84.6)	0.104
Smoking habits, n (%)					
Non smoker	42/98 (42.9)	17/26 (65.4)	22/61 (36.1)	3/11 (27.3)	0.64
Former smoker	5/98 (5.1)	1/26 (3.8)	4/61 (6.6)	0/11 (0)	
Active smoker	51/98 (52)	8/26 (30.8)	35/61 (57.4)	8/11 (72.7)	
Weeks of symptoms, median (IQR)	7 (3.5–12)	5 (2–10)	7 (4–12)	9 (4.5–13.5)	0.192
Main symptoms, n (%)					
Cough	97/112 (86.6)	26/31 (83.9)	58/68 (85.3)	13/13 (100)	0.399
Hemoptysis	31/112 (27.7)	13/31 (41.9)	16/68 (23.5)	2/13 (15.4)	0.119
Dyspnea	28/112 (25)	3/31 (9.7)	13/68 (19.1)	12/13 (92.3)	<0.001[a]
Fever	60/110 (54.5)	6/31 (19.4)	41/66 (62.1)	13/13 (100)	<0.001[a]
Night sweats	46/101 (45.5)	6/28 (21.4)	33/63 (52.4)	7/10 (70)	0.006[a]
Weight loss	54/106 (50.9)	8/29 (27.6)	35/65 (53.8)	11/12 (91.7)	0.001[a]
Highly symptomatic, n (%)	20/103 (19.4)	1/24 (4.2)	6/66 (9.1)	13/13 (100)	<0.001[a]
Bacillary load[b], n (%)					
0	26/83 (31.3)	12/23 (52.2)	13/52 (25)	1/8 (12.5)	0.116
1+	6/83 (7.2)	1/23 (4.3)	5/52 (9.6)	0/8 (0)	
2+	17/83 (20.5)	5/23 (21.7)	9/52 (17.3)	3/8 (37.5)	
3+	34/83 (41)	5/23 (21.7)	25/52 (48.1)	4/8 (50)	
Drug resistance[c,d], n (%)					
Isoniazide	5/111 (4.5)	2/30 (6.7)	3/69 (4.3)	0/12 (0)	0.799
Riphampicin	1/111 (0.9)	1/30 (3.3)	0/69 (0)	0/12 (0)	0.378
Pyrazinamide	1/49 (2)	0/12 (0)	1/31 (3.2)	0/6 (0)	1.000
Ethambutol	2/111 (1.8)	2/30 (6.7)	0/69 (1.8)	0/12 (0)	0.136
Hemoglobin g/dL, mean±SD	13.0±1.6	13.6±1.2	12.8±1.7	12.6±1.6	0.058
CRP mg/L, median (IQR)	66.3 (19.9–124.2)	15.8 (3.9–20.4)	89 (47.6–124.9)	160.5 (108.7–197.7)	<0.001[a]
Chest X-ray classification, n (%)					
Minimal	19/113 (16.8)	19/31 (61.3)	0/69 (0)	0/13 (0)	<0.001[a]
Moderate	52/113 (46)	12/31 (38.7)	39/69 (56.5)	1/13 (7.7)	
Advanced	42/113 (37.2)	0/31 (0)	30/69 (43.5)	12/13 (92.3)	

[a] Statistically significant results. [b] Numbers related only to cases with culture confirmation on sputum (bacillary load was not considered for the remaining subjects, who were diagnosed through gastric aspirate, bronchial wash, bronchoalveolar lavage, pleural fluid or biopsy cultures). [c] Missingness of 1.8% due to contaminated culture or non-representative sampling. [d] Pyrazinamide resistance was not routinely assessed until May 2011. CRP – C-reactive protein; IQR – interquartile range; SD – standard deviation; TB – tuberculosis

2. Lineage diversity of *M. tuberculosis* within disease severity groups

MTBC typing methods have evolved substantially over the years, from simple phenotypic approaches, like phage typing and drug susceptibility profiling, to the more recent DNA-based strain genotyping methods [82]. Despite being standard methods for transmission studies, both spacer oligonucleotide typing (spoligotyping) and variable-number tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTR) typing rely on repetitive DNA elements with high propensity for convergent evolution, making its use for the inference of phylogenies misleading [30]. Contrasting with mobile genetic elements, large sequence polymorphisms (LSP) and SNPs are considered robust phylogenetically markers of MTBC [30, 83]. In particular, SNPs are ideal markers for genotyping of MTBC, as they represent unique events and show virtually no homoplasy [30, 56].

Given these facts, we genotyped the mycobacterial isolates through a custom TaqMan® real-time PCR assay, using phylogenetically informative SNPs obtained from 22 MTBC whole genome sequences representative of lineages 1 to 6 (Table IV.1) [56]. This SNP-typing technique has an additional advantage, since it can be very accurate and fast (<2h/96 samples) [56, 84]. We first validated the method by performing a test with reference strains, which were available for all lineages, except Lineage 5. Our assays generated steady and consistent amplification curves (**Figure IV.6, A-B**). The ancestral and mutant genotypes were defined by whether or not they contained the lineage defining SNP in a specific assay. For instance, in an assay containing L4 primers and probes, the DNA of a L4 isolate (*e.g.* H37Rv) annealed with the mutant probe. On the other hand, in the same assay, the DNA of a L6 strain (*e.g.* H91) annealed with the ancestral probe.

Once the genotyping method was optimized, the bacterial lineage for the bacteria associated with each of the 113 mild, moderate and severe TB patients

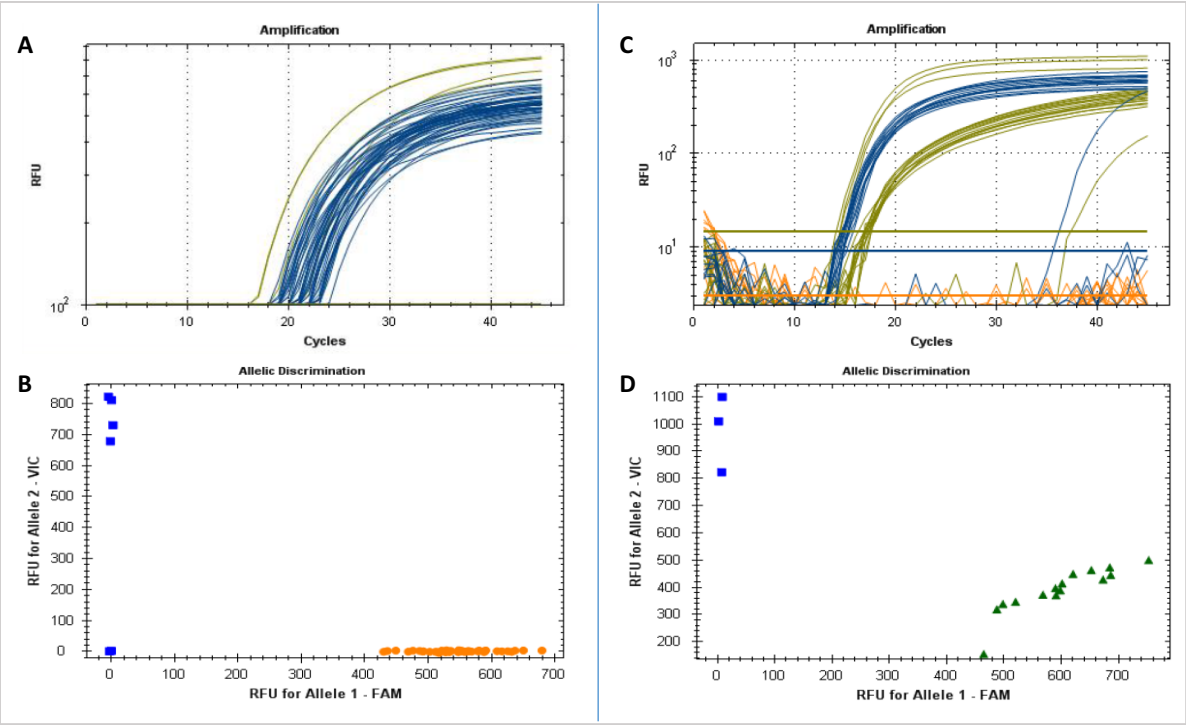

Figure IV.6 – Allele discrimination for Lineage 4 (L4, A-B) and LAM sublineage (C-D). (A) and (C) shows the amplification curves measured by relative fluorescence units (RFU). Blue curves represent FAM fluorescence (L4 positive in A or LAM positive in C) and green curves represent VIC fluorescence (L4 negative in A or LAM negative in C). **(B) and (D)** represents the genotype discrimination according to RFUs for L4 and LAM, respectively.

was determined. Sixteen clinical isolates for which the primary cultures were contaminated or not viable were not included in the study.

We found that the MTBC L4 was largely predominant (n=94, 96.9%) in our data set (**Figure IV.7, A**). The non-L4 isolates (n=3) were from lineages 1, 3 and one was of undetermined phylogeny.

We next applied the same method to define the sublineage distribution of the clinical isolates within L4, after validating the technique with our previously sequenced isolates (**Figure IV.6, C-D**). A preceding work showed that the most frequent strain groups in Portugal was the LAM sublineage, followed by Haarlem and X [31]. Thus, we screened the sublineages for all L4 isolates in the same order, in a stepwise manner. In our study population, comprising patients with no known major risk factors for TB, the overall sublineage distribution also showed LAM as the most prevalent, followed by Haarlem and X (**Figure IV.7, B**). However, when calculating the phylogenetic distribution within the different TB severity groups, we observed a decreasing frequency of LAM from mild, to

moderate and to severe direction (**Figure IV.7, C**), although without reaching statistical significance (*P*=0.796).

In all, our data shows a highly homogeneous phylogenetic structure of *M. tuberculosis* strains isolated in a major hospital of the north of Portugal, with nearly all cases belonging to L4. Moreover, lineage-defining mutations did not impact significantly the disease severity when analysing a group of patients with no known immune suppression.

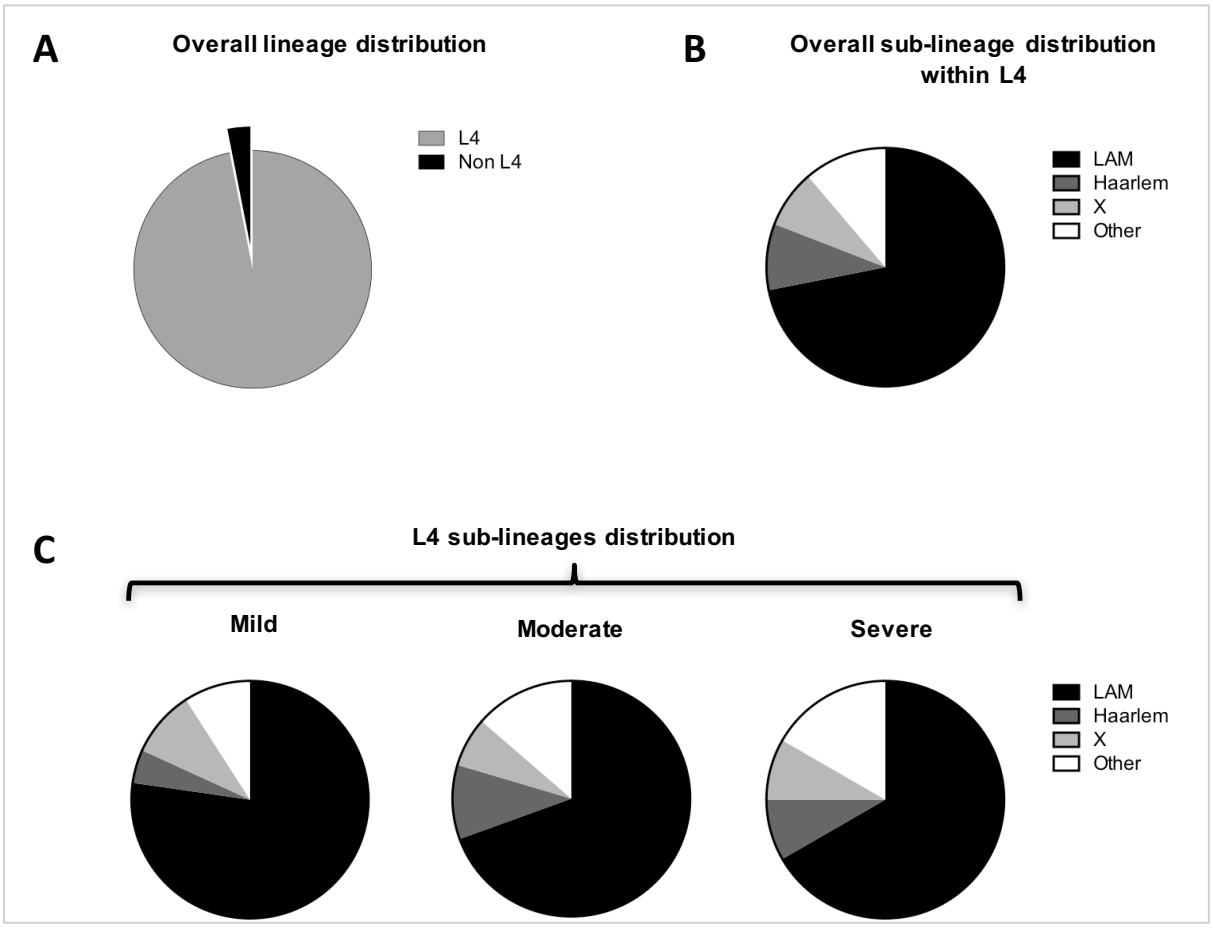

Figure IV.7 – Characterization of lineages among *M. tuberculosis* isolates and distribution of sublineage genotypes through the different clinical outcomes of TB. All (n=97) but 3 isolates belonged to the Lineage 4 and LAM sublineage was the most prevalent (71%) genotype within L4 in all severity groups, followed by Haarlem (8.6%) and X (7.5%). Clinical isolates that did not belong to these sublineages were classified as "other" (12.9%). Proportions were similar between groups (*P*=0.796).

3. Differential bacterial growth kinetics across TB severity groups

Differences in growth rates have been proposed as a possible explanation for the lower severity of *Mycobacterium africanum* as compared with *M. tuberculosis* [58]. Since we obtained different *M. tuberculosis* isolates associated with a spectrum of severity within pulmonary active TB, we reasoned that they may display differences in the growth rates. To test this hypothesis, growth curves (each performed in duplicate) for clinical isolates associated with 8 mild, 10 moderate and 8 severe TB cases were obtained using a previously published method [58, 59]. General characteristics of the selected cases are presented in **Table IV.3**.

As expected, all curves displayed a lag phase, followed by a steep exponential phase and finally reaching a high plateau (stationary phase) (**Figure IV.8**). The overall analysis of the data showed that, whereas clinical isolates associated with mild or severe TB presented some degree of dispersion (Figure IV.8, A and B), all moderate strains tested showed a very similar growth profile (Figure IV.8, C). An overlap of all growth curves obtained per isolate category further illustrates the differences between the moderate TB-associated isolates when compared to mild or severe TB-associated ones (**Figure IV.9**). The growth profile observed for the H37Rv Trudeau (Figure IV.9, blue line) provides a reference for comparison with the other groups of strains. This laboratory strain has been adapted to *in vitro* culture, with evidence of accumulating genetic diversity during serial passaging of cultures [85]. Compared to mild and severe strains, the H37Rv strain exhibits a steeper exponential growth, however without reaching the same maximum concentration level seen with the moderate strains (Figure IV.9). The length of lag phase (5.12±0.12 [mean±SD] days) and mean doubling time (21.03 hours, 95% CI 20.29—21.8 h) measured (Figure IV.8, D) were in line with previous descriptions [58, 86], which validates the current methodology.

Table IV.3 – Description of the hosts with mild, moderate or severe disease corresponding to the clinical isolates selected for growth analysis. Differences between severity groups regarding patient's gender (*P*=0.307), age *(P*=0.374) and smoking habits (*P*=0.607) were not statistically significant. The time of symptoms of the severe cases selected was significantly less than for the mild ones (6.1±3.5 *vs* 11.4±9.4 weeks, respectively, *P*=0.035) and, hence, severity of disease could not be imputable to diagnostic delay.

Severity group	Strain code	Gender	Age, years	Smoking habits	Time of symptoms, weeks	Phylogeny
Mild	2D2	Male	45	Never smoker	13	L4 (LAM)
	2I3	Female	39	Former/active smoker	12	L4 (LAM)
	3A3	Female	35	Never smoker	2	L4 (LAM)
	4I2	Female	34	Never smoker	1	L4 (LAM)
	5A9	Male	25	Former/active smoker	5	L4 (LAM)
	5D4	Male	28	Never smoker	26	L4 (LAM)
	5F8	Female	26	Never smoker	8	L4 (LAM)
	6D3	Female	30	Former/active smoker	24	L4 (LAM)
Moderate	1B2	Male	35	Former/active smoker	3	L4 (LAM)
	1C7	Female	20	Former/active smoker	17	L4 (LAM)
	1E1	Female	27	Former/active smoker	2	L4 (LAM)
	2G8	Male	25	Former/active smoker	4	L4 (LAM)
	3A7	Female	28	Never smoker	6	L4 (LAM)
	3A8	Female	31	Former/active smoker	9	L4 (LAM)
	5D3	Male	22	Never smoker	15	L4 (X)
	5D7	Male	30	Never smoker	7	L4 (LAM)
	5F9	Female	50	Never smoker	11	L4 (LAM)
	5I5	Male	22	Never smoker	13	L4 (LAM)
Severe	4D5	Male	19	Former/active smoker	9	L4 (LAM)
	5B5	Male	54	Never smoker	3	L4 (X)
	5C7	Female	49	Former/active smoker	10	L4 (LAM)
	5C8	Male	42	Never smoker	1	L4 (LAM)
	5D6	Male	43	Former/active smoker	10	L4 (other)
	5I6	Female	42	Never smoker	6	L4 (LAM)
	6C1	Male	19	Former/active smoker	7	L4 (LAM)
	6C4	Male	20	Former/active smoker	3	L4 (LAM)

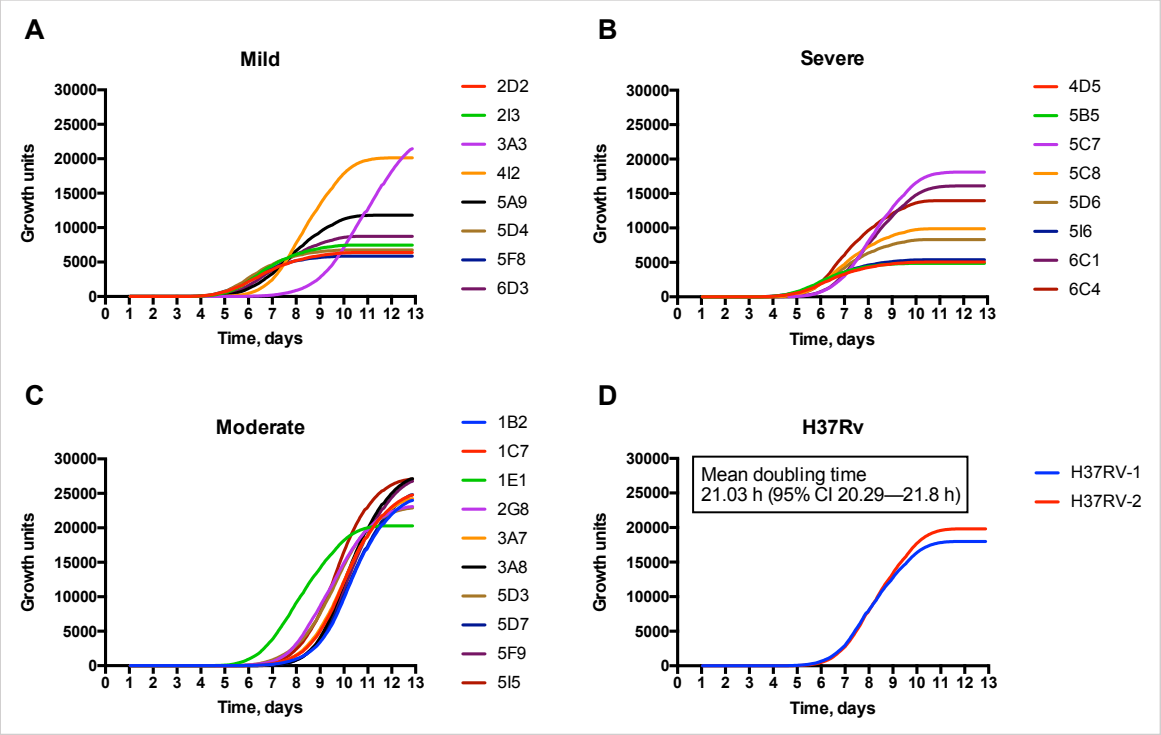

Figure IV.8 – Growth of *M. tuberculosis* strains in MGIT™ 960 tubes. Average curves for each of the duplicates inoculated in the liquid culture media are displayed to illustrate the growth characteristics of **(A)** mild, **(B)** moderate and **(C)** severe clinical isolates. Growth curves for **(D)** H37Rv inocula are shown independently.

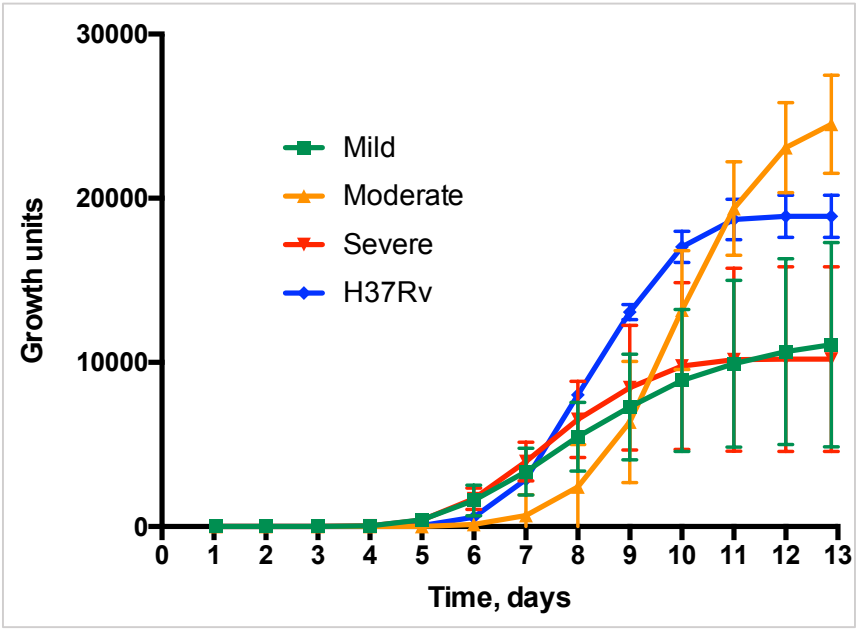

Figure IV.9 – Average growth curve in MGIT™ 960 tubes for *M. tuberculosis* strains belonging to each clinical phenotype and H37Rv. Data represent the mean and SD values (n = 16 for mild and severe isolates, n=20 for moderate isolates and n=2 for H37Rv).

To quantify the growth properties of the different isolates, we assessed the time to positivity, t_d and C_{max} for each strain and then compared the data obtained across the different severity groups. The time to positivity was 6.33±0.59 (mean±SD) days for moderate TB-associated isolates and significantly less for mild or severe TB-associated ones, 4.62±0.92 and 4.37±0.46 days, respectively (*P*<0.001) (**Figure IV.10, A**). Moderate isolates also showed higher C_{max} (24501.4±3002.2 GU, *P*<0.001) than mild (11078.1±6225.4 GU) or severe (10210.6±5624.1 GU) ones (**Figure IV.10, C**). Overall, top values of C_{max} were highly correlated with longer time to positivity (r=0.939, *P*<0.001). Mild isolates presented slower doubling time (19.98±4.03 h), when compared to moderate (15.02±2.78 h, *P*<0.001) and severe (15.94±2.50 h, *P*=0.001) ones (**Figure IV.10, B**). As these experiments were controlled by estimating the CFU present in the original suspension, the differences found in terms of time to positivity and C_{max}

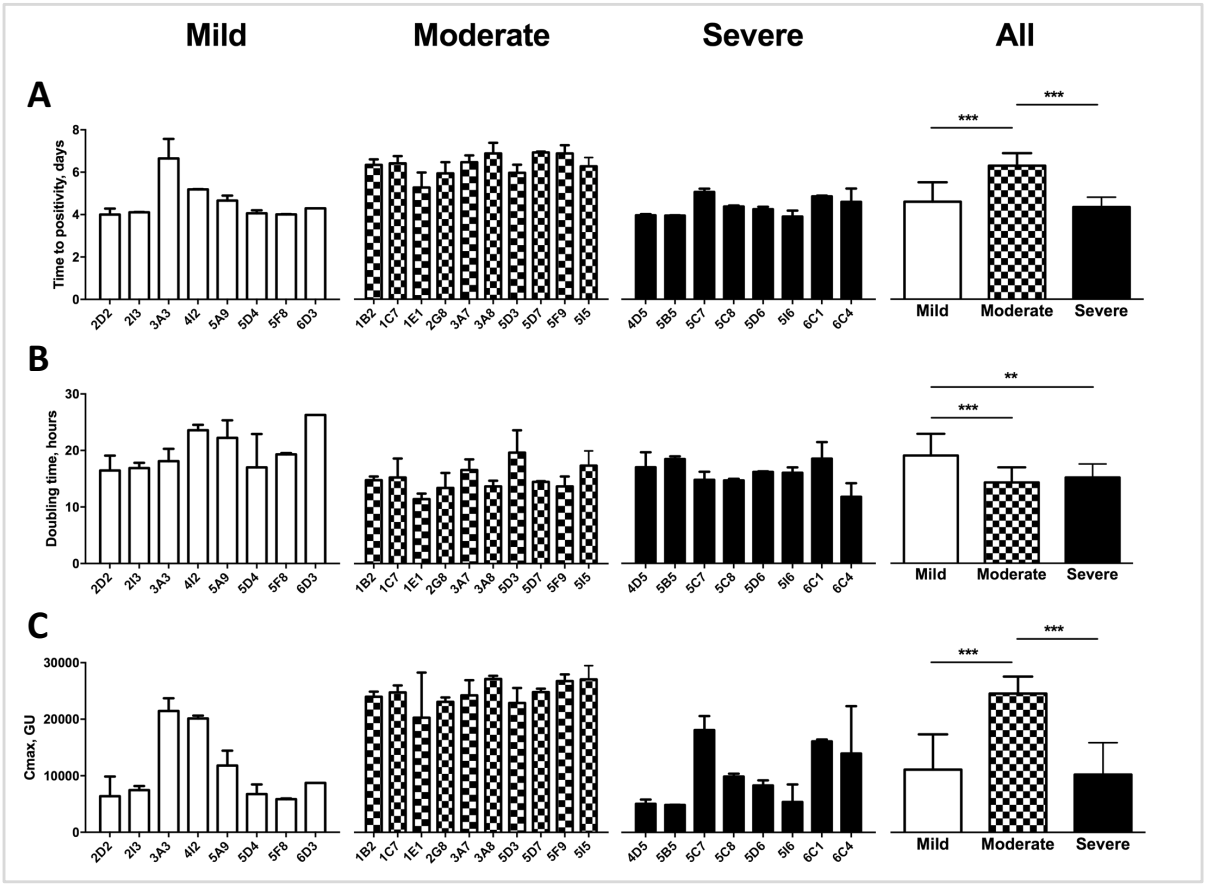

Figure IV.10 – Comparison of (A) time to positivity, (B) doubling time and (C) maximum point on the growth curve (C_{max}) for different clinical isolates. Data represent the mean and SD values (n=2 for each strain). Moderate strains showed longer time to positivity and higher C_{max} (****P*<0.001) than mild or severe strains. Mild strains presented slower doubling time than moderate (*P*<0.001) and severe (***P*=0.001) strains.

were not imputable to differential amounts of bacteria in the original *inocula* (**Figure IV.11**).

In all, our data show a striking association between differential mycobacterial growth kinetics and severity of disease. Mild phenotypes of TB correlated with slow growing *M. tuberculosis*. On the other hand, clinical isolates associated to moderate outcomes of disease presented longer lag phases, but reached a higher plateau of growth units, after a steep exponential phase.

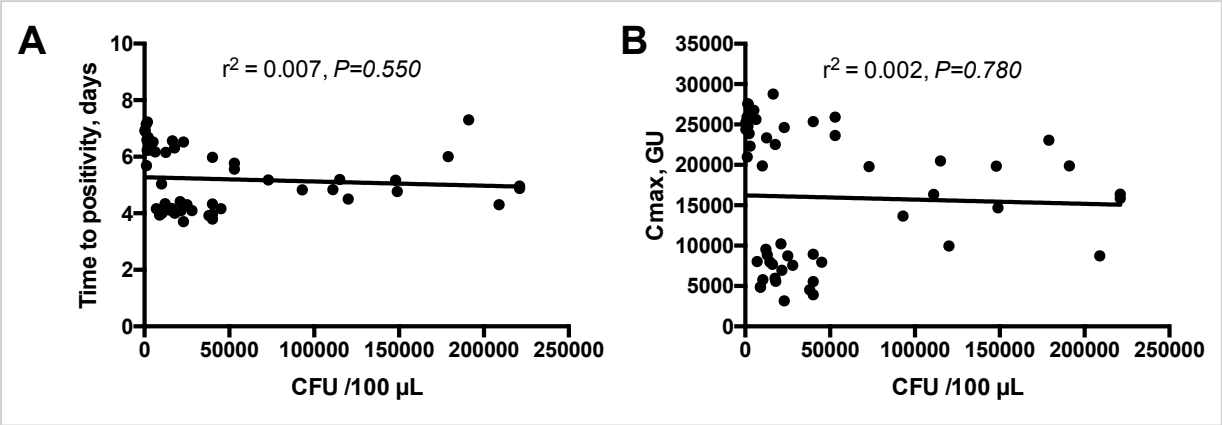

Figure IV.11 – Correlation between CFUs present in the original suspension with growth of strains inoculated into MGIT™ 960 tubes. Linear regression analysis shows a non-significant correlation between initial CFU and **(A)** days to positivity or **(B)** maximum concentration (C_{max}) on liquid media culture. Correlation coefficient and *P value* result from correlation test (Pearson) are indicated on the graph.

4. Genetic diversity underlying differential bacterial growth

To gain insight on the genetic mechanisms underlying the phenotypic differences observed we resorted to whole genome comparisons. For this, two groups of clinical isolates were defined: fast and slow growers. The fast growing group comprised all moderate strains, except one (5I5), for which the sequence was not yet available. Mild and severe strains were included in the slow growing group. Two mild isolates with discordant growth profile (3A3 and 4I2) were excluded from the analysis, and one severe strain (5I6) also lacked genome sequence for comparison.

4.1. Phylogenetic structure unrelated to growth pattern

The information for 1797 concatenated genome-wide SNPs across the 22 clinical isolates was used to construct a phylogenetic tree using maximum likelihood and Bayesian analysis (**Figure IV.12**). The phylogenetic analysis confirmed our previous SNP-typing results (Table IV.3), with the branching of the tree following the distribution of our isolates into sublineages 4.1 (X family) and 4.3 (LAM).

We then explored the distribution of fast and slow growing strains across the phylogenetic tree (Figure IV.12) and found that the majority of the represented taxa were not specifically associated with a particular growth profile. The exceptions were taxa 4.3.3 and 4.9 that are composed only by slow growers and taxa 4.3.4.2 formed by fast growers. However, the low number of isolates from these taxa (4.3.3, n=5; 4.9, n=1; 4.3.4.2, n=2) is clearly insufficient to make any generalization about the growth profile of the taxa.

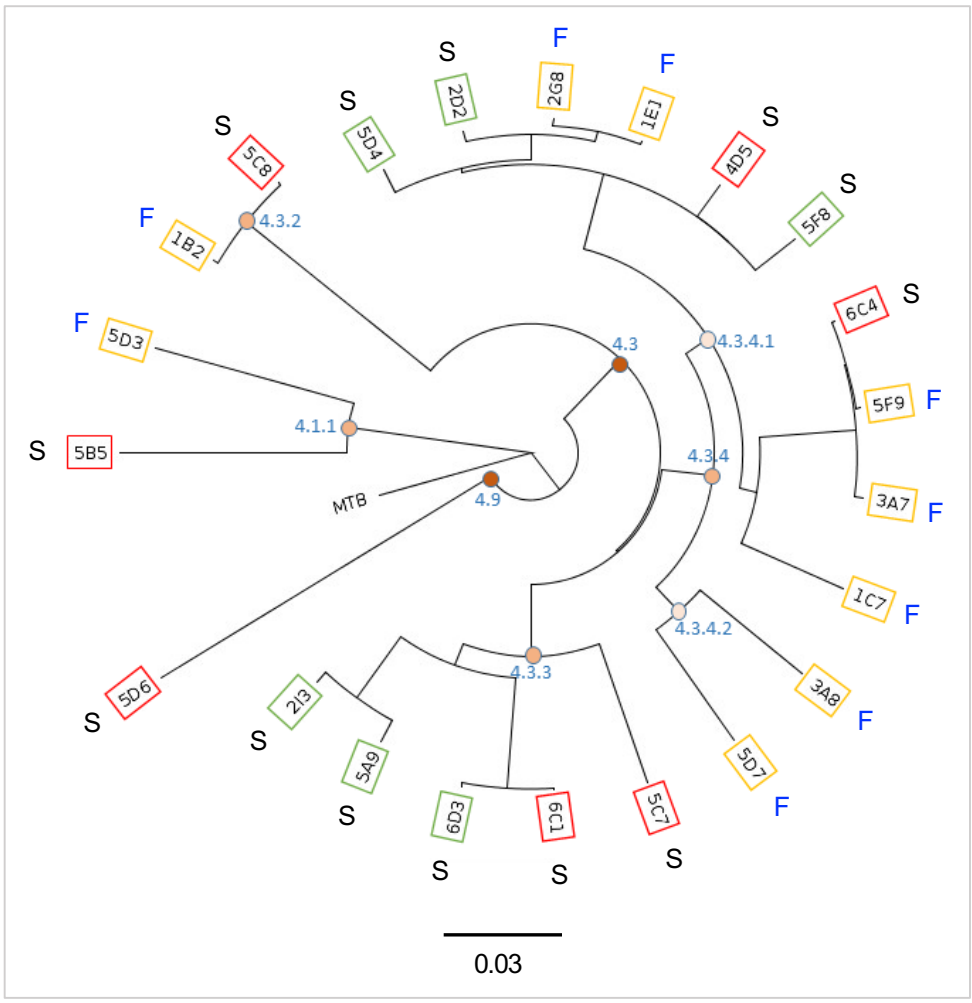

Figure IV.12 – Maximum likelihood phylogenetic tree. Constructed using the information of 1797 concatenated genome-wide SNPs across the 22 clinical isolates and rooted on the *M. tuberculosis* ancestor. Isolate identification code was colored according to severity group: green – mild, orange – moderate, red – severe. F – fast grower, S – slow grower.

4.2. Genome-wide scan of SNPs associated with growth

To map the genetic determinants with potential impact on the growth ability of *M. tuberculosis*, we performed a genome-wide search for SNPs that were differentially present when comparing different growth profiles. Allelic frequencies were derived from the relative amount of sequence reads supporting alternative alleles for any given variant position. The absolute differences between the derived allele frequencies (ΔDAF) from the two groups were calculated, ranging from 0 to 1, to find the SNPs associated to fast or slow growing group. The maximal ΔDAF of 1 would mean that a certain variant at a causative locus

was exclusive to one of the two pools. The mean ΔDAF was 0.196±0.141. **Figure IV.13-A** shows the result of plotting the absolute values of ΔDAF of 1797 genomic position where SNPs were found, comprising 1135 known genes. While we observed a significant group of SNPs with a ΔDAF <0.1 (n=458, 25.5%), suggesting little association to the growth phenotype, the larger proportion of SNPs clustered around ΔDAF 0.2, counting a total of 1252 (69.7%) SNPs within the range of ΔDAF 0.1 to 0.4. Only 87 (4.8%) SNPs had ΔDAF >0.4 and thus were more probably involved in the differential mycobacterial growth ability.

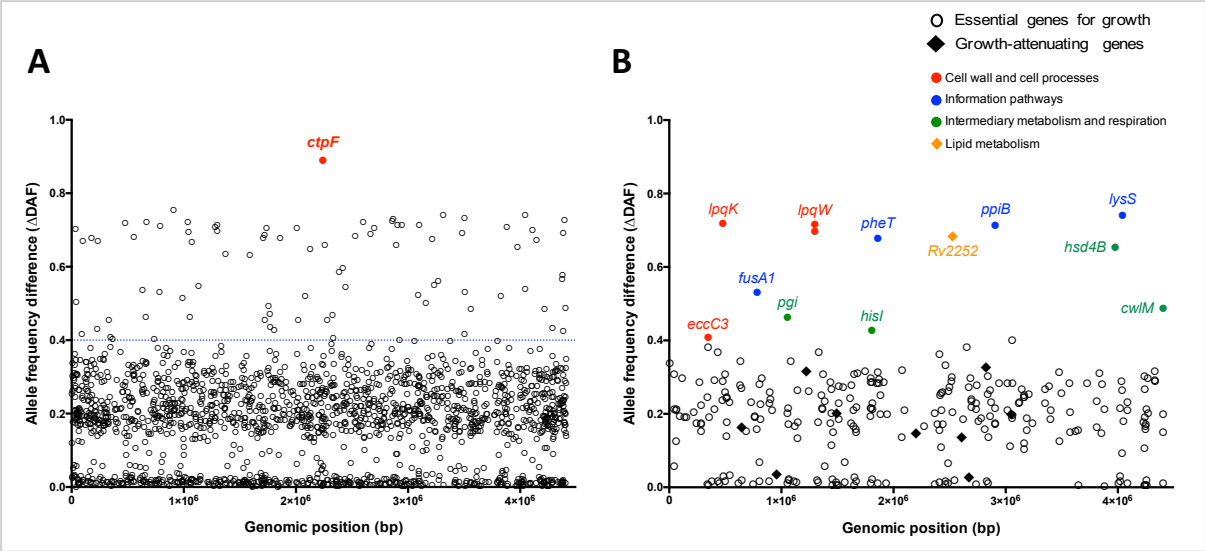

Figure IV.13 – Manhattan plot showing genome-wide association data comparing fast and slow growing strains. Each dot in the graph represents a SNP. The x axis shows the genomic position and the y axis shows the absolute allele frequency difference (ΔDAF) between fast and slow growing strains. **(A)** The clinical isolates under study presented 1797 SNPs (in 1135 known genes), taking as reference the H37Rv genome sequence. The dashed line marks the threshold of ΔDAF=0.4, that was empirically defined as the level of significant genetic differentiation between groups. Mycobacterial growth had the strongest association with the locus of the gene *ctpF*. **(B)** Focus was given for the SNPs of essential genes for optimal growth (o) and growth-attenuating genes (◊), according to previous literature [77]. Genes are highlighted in **(A)** and **(B)** with different colors that correspond to functional categories based on the classification by Tuberculist [88].

4.2.1. Unbiased analysis of the genetic basis of differential growth

We identified 87 SNPs in a total of 83 genes with large (>0.4) allelic frequency differences between fast and slow growing clinical isolates (**Supplementary information, Table IV.S1**). The highest ΔDAF found was 0.889 for the locus of gene *ctpF*, which encodes a P-type ATPase of the plasma membrane [87]. Further bioinformatic analysis using Tuberculist database [88] grouped the genes harboring SNPs with ΔDAF >0.4 within functional categories (**Figure IV.14**). Genes associated to the bacterial cell wall and cell processes, which includes the *ctpF*, were over-representated in the analysis. Members of this family are surface proteins that perform a variety of functions related to cell wall biosynthesis, active transport accross the membrane and secretion [88]. One particularly interesting set of genes, which also turned up heavily representated, are those known to have a pivotal role in *M. tuberculosis* intermediary metabolism and respiration. For example, the sulphotransferases *cysA2* and *A3* are essential for the sulphate assimilation [89], central to several cellular processes, including the biosynthesis of sulphur amino acids, as methionine, which is involved in translation initiation and methylation reactions that are abundant in mycobacteria [87]. In addition, many genes harboring SNPs identified in the present study are still of unknown function.

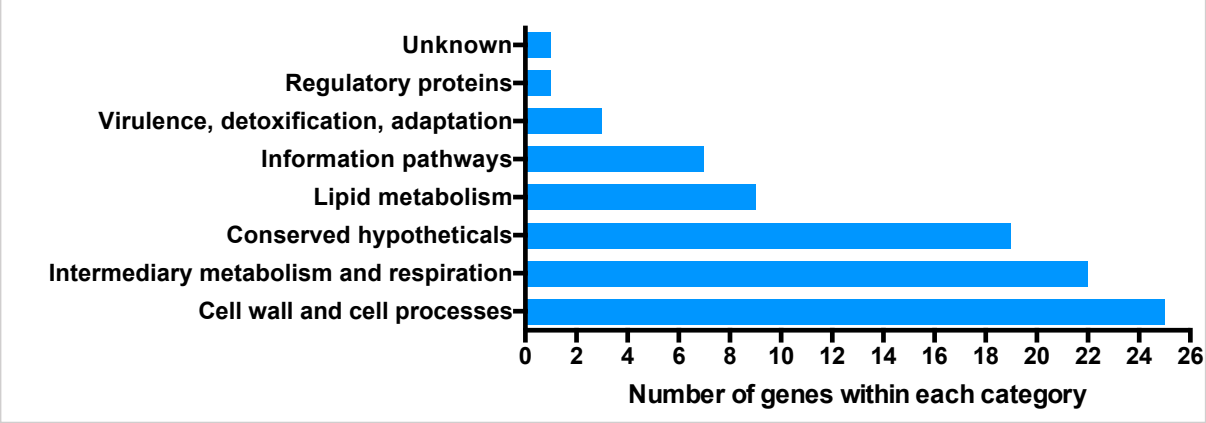

Figure IV.14 – Functional distribution of the genes identified with allelic frequencies differing more than 40% (ΔDAF >0.4) between fast and slow growing strains. Gene categories were analyzed based on the classification by Tuberculist [88].

We also performed a functional enrichment analysis on genes corresponding to the 87 SNPs with ΔDAF >0.4. GO term enrichment analysis revealed that this set of genes was significantly over-represented (*P*<0.001*)* in gene categories associated with the ATP binding (**Table IV.4**). The results suggest that the differential growth of *M. tuberculosis* is associated with the presence of SNPs in genes that couple the energy of ATP hydrolysis to a large variety of essential biological processes, comprising not only transmembrane transport, such as in cation transporter ATPase (*ctpF, ctpA*) and ATP-Binding Cassette (ABC) transporters (*pstA1*, *pstB, Rv0194, Rv2326c*), ESX/type VII secretion systems (*espG2, eccC2, eccC3, eccC5*), but also several non-transport-related processes, such as translation (*lysS, ileS, pheT*) and cell wall formation (*murE*).

Table IV.4 – Hierarchical view of GO (molecular function) for the 87 SNPs with ΔDAF >0.4. Each block of related function classes (the first block starts with ATP binding activity, the second with purine ribonucleoside binding activity) is arranged with the most specific class at the top, with less specific (general) classes indented below it. [a] Bonferroni correction for multiple testing, displaying only results with *P<0.05*.

	H37Rv (reference)	Clinical isolates				
GO molecular function complete	**Background frequency**	**Sample frequency**	**Expected**	**Fold Enrichment**	**Over/under-represented (+/-)**	***P* value[a]**
ATP binding	337	15	4.70	3.19	+	3.95E-02
• Purine ribonucleoside binding	364	16	5.07	3.15	+	2.57E-02
• • Purine nucleoside binding	364	16	5.07	3.15	+	2.57E-02
• • • Nucleoside binding	366	16	5.10	3.14	+	2.75E-02
• • • • Carbohydrate derivative binding	402	17	5.60	3.03	+	2.35E-02
• • Ribonucleoside binding	366	16	5.10	3.14	+	2.75E-02
• Adenyl ribonucleotide binding	340	15	4.74	3.17	+	4.36E-02
• • Adenyl nucleotide binding	341	15	4.75	3.16	+	4.51E-02
• • • Purine nucleotide binding	368	16	5.13	3.12	+	2.93E-02
• • Purine ribonucleotide binding	366	16	5.10	3.14	+	2.75E-02
• Purine ribonucleoside triphosphate binding	363	16	5.06	3.16	+	2.49E-02
Unclassified	5294	59	73.77	0.80	-	0.00E00

4.2.2. Essential and non-essential growth-attenuating genes

A previous work used transposon insertion and gene-disruption to identify the genes required for optimal growth of *M. tuberculosis*, and broadly classified them into two groups: essential or non-essential for *in vitro* growth [77]. In addition, the authors described a set of non-essential genes that, when inactivated through transposon insertion, resulted in mild growth attenuation, and were thus designated as growth-attenuating genes [77]. As expected most of these genes are highly conserved having a very reduced number of SNPs across the MTBC. However, in the whole genome analysis of the studied clinical isolates we found SNPs in 201 out of the 614 essential genes and in 10 of the 42 growth-attenuating genes formerly described for H37Rv (**Figure IV.13, B**). The SNPs in some of these genes had very low ΔDAF, indicating identical allele frequency in both fast and slow growing groups. This is the case of *rmlB* (ΔDAF=0.002), encoding an enzyme involved in dTDP-rhamnose synthetic pathway (fundamental to the structural integrity of mycobacterial cell wall), that has been previously demonstrated to be essential for mycobacterial growth [90]. Thirteen SNPs in 11 essential genes and in one growth-attenuating gene presented ΔDAF >0.4, of which 7 genes had non-synonymous mutations. However, only three of these genes (*Rv2252*, *pgi* and *hisI*) were affected in their protein function as predicted by SIFT analysis. A detailed description of the analysis is provided in **Table IV.5**.

4.2.3. Genes essential for intracellular survival

Another work using transposon mutants identified 126 genes required for *M. tuberculosis* to grow within primary macrophages [78]. In our study, we found at least one nucleotide change in 54 out of the 126 genes essential for intracellular survival, 6 of which were already identified for *in vitro* growth [77]. Of the remaining set of genes exclusively associated with *in vivo* growth, 4 had ΔDAF >0.4, two of which with non-synonymous mutations, though amino acid changes in the coded proteins were predicted to be tolerated by the bacteria (**Table IV.5**).

Table IV.5 – List of loci associated with *in vitro* [77] and intracellular [78] growth that have allelic frequencies differing more than 40% (ΔDAF >0.4) between fast and slow growing strains. Variant allele (mutant) was defined by comparative analysis with the reference *M. tuberculosis* genome of the laboratory strain H37Rv. Tuberculist database [88] provided the putative gene product or function. The (†) marks growth-attenuating genes and (‡) essential genes for optimal *in vitro* growth. The (*) marks essential genes for intracellular growth.

Position	Substitution, ancestral allele	SNP type	Amino acid substitution	Functional Effect, SIFT Score	ΔDAF	Gene	Putative product or function
4042761	G/A, G	Syn	D60D	Not Scored	0.74	*lysS*‡ *(Rv3598c)*	Lysine-tRNA ligase 1, involved in translation
1037355	C/T, T	Syn	T119T	Not Scored	0.722	*pstA1** *(Rv0930)*	Phosphate ABC transporter permease, involved in active transport of inorganic phosphate across the membrane
478358	T/C/G, C	Non-syn	E67K	Tolerated, 0.31	0.717	*lpqK*‡ *(Rv0399c)*	Possible conserved lipoprotein, showing some similarity to penicillin binding proteins and various peptidases e.g. D-alanyl-D-alanine carboxypeptidase protein
1297327	A/G, G	Syn	V392V	Not Scored	0.714	*lpqW*‡ *(Rv1166)*	Probable conserved lipoprotein, involved in phosphatidylinositol and phospholipid metabolism
1297999	G/T, T	Syn	S616S	Not Scored	0.695		
2906918	T/A, A	Syn	L35L	Not Scored	0.712	*ppiB*‡ *(Rv2582)*	Peptidyl-prolyl cis-trans isomerase B, accelerate the folding of proteins
2527676	A/G, G	Non-syn	G230S	Deleterious, 0.02	0.683	*Rv2252*†	Diacylglycerol kinase, involved in synthesis of phosphatidylinositol mannosides (PIMS)
1861274	A/G, A	Non-syn	R506H	Tolerated, 0.23	0.678	*pheT*‡ *(Rv1650)*	Phenylalanine-tRNA ligase subunit beta, involved in translation
101727	A/G, G	Non-syn	G382E	Tolerated, 0.07	0.668	*ctpA** *(Rv0092)*	Cation-transporting ATPase A, possibly catalyzes the transport of copper with the hydrolysis of ATP
3977226	A/G/T, G	Syn	L55L	Not Scored	0.653	*hsd4B*‡ *(Rv3538)*	Probable dehydrogenase, involved in cellular metabolism/ lipid catabolism
1588456	G/A, A	Syn	R9R	Not Scored	0.632	*lprG** *(Rv1411c)*	Lipoarabinomannan carrier protein, involved in transporting triacylglycerides across the inner cell membrane into the periplasm
784440	G/T, G	Syn	A652A	Not Scored	0.53	*fusA1*‡ *(Rv0684)*	Elongation factor G, involved in protein synthesis
40162	C/T, C	Non-syn	M347I	Tolerated, 0.43	0.503	*Rv0037c**	MFS-type transporter, possibly involved in transport of drug (macrolide?) across the membrane
4403900	A/G, A	Non-syn	M237V	Tolerated, 0.34	0.486	*cwlM*‡ *(Rv3915)*	Thought to work as peptidoglycan hydrolase, recent data suggest a role as regulator of the enzyme MurA and stimulates it to start producing peptidoglycans
1055049	C/T, C	Non-syn	R546H	Deleterious, 0.01	0.461	*pgi*‡ *(Rv0946c)*	Glucose-6-phosphate isomerase, involved in glycolysis and gluconeogenesis
1805948	T/C, C	Non-syn	T99I	Deleterious, 0	0.426	*hisI*‡ *(Rv1606)*	Phosphoribosyl-AMP cyclohydrolase, involved in histidine biosynthesis
347766	C/T, T	Non-syn	A711V	Tolerated, 0.27	0.407	*eccC3*‡ *(Rv0284)*	ESX-3 secretion system protein, involved in iron/zinc homeostasis

DISCUSSION

We studied a population of patients between 18 and 65 years-old, without known predictor or precipitator TB factors, diagnosed at a major hospital center in the north of Portugal, and proposed a classification system for disease severity taking into consideration the extension of lung involvement and baseline clinical features. Even after excluding comorbid conditions, we still determined a normal distribution of disease manifestations, with extreme cases of mild and severe TB and, with the most prevalent group presenting moderate TB. This distribution was independent of age, gender, smoking habits, duration of symptoms and drug resistance profile of the infecting bacteria. Given the common host characteristics between groups of severity, we investigated if the MTBC diversity was related to the clinical heterogeneity observed.

The publication of the complete genome sequence of *M. tuberculosis* H37Rv in 1998 [87] had an enormous impact in the biological studies for TB, supporting numerous works addressing the clinical relevance of MTBC diversity at the lineage and sublineage level [24]. Moreover, exploring the pathogen diversity has proven important for epidemiological purposes, to monitor predominant or emerging MTBC genotypes and their associations with TB outbreaks, transmission clusters and drug resistance mutations acquisition. The determination of lineages and sublineages based on SNPs provided a phylogenetically robust classification system [30]. We used a panel of SNPs that are mutually exclusive between different clades, allowing for a fast and cost-effective approach. Epidemiologic data show that most of the new TB cases in the country are Portuguese native and that immigrants comprise only 1.6 to 1.8% in the region of Porto, where our study was based [81]. This fact accounts for the highly homogeneous phylogeographical structure observed, with almost all isolates belonging to Lineage 4. Though we confirmed that Lineage 4 is also genetically diverse, the large majority of the cases were infected with strains of sublineage LAM, which is in line with previous descriptions [31, 32]. Disease severity was not exclusively related to any specific phylogenetic taxa within

Lineage 4 in the present study. However, this conclusion should be further supported by the analysis of more clinical isolates, in particular of sublineages of Lineage 4 that are less represented in the geographic area where this study was conducted.

Our findings strongly suggest that the growth patterns of MTBC strains are associated with differences in TB clinical severity. This heterogeneity in growth does not seem to be lineage-specific, but we identify in these strains a number of SNPs that might underlie the phenotypes of fast and slow growth. Whereas many genes harbouring the SNPs were already described to be essential for optimal growth [77, 78], we also provide new links between *M. tuberculosis* genotypes and varying growth phenotypes. The majority of these genes are predicted to encode products vital to cellular metabolism, but some still have no clear function assigned.

Previous experimental work exploring the molecular mechanisms underlying differential virulence of MTBC strains found differences in growth rates [58, 91-93], that possibly modulate the level of the inflammatory response generated by the host. In the present study, isolates from patients with mild disease grow significantly slower in liquid media. Conversely, moderate- and severe-associated isolates present higher growth rates, with doubling times shorter by 4-5 h, when compared to mild-associated ones. Although growth in liquid broth hardly reflects the much more complex infection pathogenesis in humans, this method has already proved to be well suited to compare the growth behaviour of different bacterial isolates [58]. The lower growth rate may be related with the decreased virulence of these isolates, generally causing non-cavitary lung disease and with minimal symptoms. Similar associations have been described previously with Lineage 3, that was reported to be less transmissible compared to other modern lineages [46] and also grows slowly in liquid broth [91, 94, 95]. Likewise, Lineages 6 (*M. africanum*) exhibits slower progression to active disease [44] and slower growth rate [58].

Strains associated to moderate TB outcomes showed a remarkably distinct growth pattern, characterized by a longer lag phase, followed by a steep exponential phase, that is related to a very short doubling time (around 15 h),

and finally reaching the highest plateau. The clinical relevance of this finding is still unclear. However, several previous studies have assessed intracellular growth of *M. tuberculosis* in human macrophages as a marker of virulence [96-99]. Furthermore, one study demonstrated that the Beijing-family strain 210 (Lineage 2), responsible for an outbreak in Los Angeles, grows more rapidly than small cluster or unique cluster strains in human macrophages [100]. In fact, the conception of "virulence" is tightly connected to the transmissibility of the infecting strain [101, 102]. *M. tuberculosis* is a human-adapted obligate pathogen, without animal or environmental reservoir, whose success lays in its ability to transmit successfully to a new human host. In order to ensure that goal, the bacteria have to be recognized and simultaneously survive to the host immune microenvironment, be able to grow intracellularly, cause lung damage and to survive the aerosolization process outside of the host [24]. So, it is possible that the moderate-associated isolates represent the most successful group of MTBC strains, that has evolved to interact with the immune cells and cause destructive cavitary lung lesions, a marker of high transmissible disease [101], but only mildly inflicting systemic deleterious effects, so that the host is able to continue at ambulatory for enough time to keep spreading the disease.

To unveil the underlying mechanisms for the observed differences in the growth profiles, we compared the genome sequences of fast growing (moderate) strains with the slow growing isolates (associated to mild and severe clinical outcome). We found variants in 1797 genomic positions. The large majority of detected SNPs formed a cluster below ΔDAF 0.4, and only 87 SNPs in 83 genes had allelic differences greater than 40% (ΔDAF >0.4).

The top ΔDAF was found for the gene *ctpF*, encoding a P-type ATPase of the plasma membrane [87], which is responsible for generating an electrochemical gradient that is necessary for transmembrane ion transport, possibly Mg^{2+} or Ca^{2+} [103]. Evidence suggests that this gene is upregulated under hypoxic environment [104] and helps to protect mycobacteria against toxic heavy-metal cation levels, as well as maintaining cell volume in hostile conditions and nutrient transport across the cell membrane [105]. The transporter function was overrepresented in the unbiased analysis (Figure IV.14). Future studies

regarding the functional impact of this gene to the observed phenotype will be required. It is interesting to note that *ctpF* was not identified as an essential gene for growth in previous studies [77]. This may be due to the fact that our approach is unveiling possible gain- or loss-of-function mutations associated with growth patterns, whereas the cited approach was based in a complete deletion mutant.

We next investigated the genes that within our 87 hits had already been determined to affect *in* vitro [77] and *in vivo* [78] growth. Of the 16 genes presenting ΔDAF >0.4 (Table IV.5), nine presented non-synonymous SNPs. In particular, three gene products (*Rv2252*, *pgi*, *hisI*) were predicted by SIFT analysis to be affected in their protein function. These three proteins are likely candidates to be responsible for the observed profile of slow growing isolates. The candidates are involved in essential biological pathways, such as protein synthesis (*hisI*), cellular metabolism (*pgi*) and biosynthesis of cell envelope components (*Rv2252*).

In addition to previously characterized pathways, many genes identified in the unbiased genome-wide analysis are conserved hypotheticals with unknown function. Further studies now require the inclusion of more isolates and validation of candidate hits, the study of the growth properties under other conditions, for example, other growth media, and the growth pattern of representative isolates in infected cells or animals. In future experiments we will also relate the growth curves obtained in the terms of growth units to effective increase in bacterial CFUs.

In summary, these findings suggest that genotypic diversity of MTBC must be, at least, partially responsible for the phenotypic heterogeneity seen in clinical presentation of TB. However, the infecting bacteria will face distinct immune status and micro-environments in different hosts, resulting in different interactions, which in turn contribute for different final outcomes of disease. The identification of the pathways that are required for mycobacterial growth provide insight into the structural and metabolic requirements of the pathogen and may direct the rational design of effective antimicrobial agents towards new molecular targets. This is of particular importance in the case of moderate-to-severe and drug-resistant strains.

REFERENCES

1. World Health Organization. Global tuberculosis report 2016. Geneva: World Health Organization; 2016.
2. Houben RM, Dodd PJ. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. PLoS Med. 2016;13(10):e1002152.
3. Dye C, Glaziou P, Floyd K, Raviglione M. Prospects for tuberculosis elimination. Annu Rev Public Health. 2013;34:271-86.
4. Barry CE 3rd, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Microbiol. 2009;7(12):845-55.
5. Lin PL, Flynn JL. Understanding latent tuberculosis: a moving target. J Immunol. 2010;185:15-22.
6. Pepper T, Joseph P, Mwenya C, McKee GS, Haushalter A, Carter A, et al. Normal chest radiography in pulmonary tuberculosis: implications for obtaining respiratory specimen cultures. Int J Tuberc Lung Dis. 2008;12(4):397-403.
7. Hofmeyr A, Lau WF, Slavin MA. Mycobacterium tuberculosis infection in patients with cancer, the role of 18-fluorodeoxyglucose positron emission tomography for diagnosis and monitoring treatment response. Tuberculosis. 2007;87(5):459-63.
8. Kim IJ, Lee JS, Kim SJ, Kim YK, Jeong YJ, Jun S, et al. Double-phase 18F-FDG PET-CT for determination of pulmonary tuberculoma activity. Eur J Nucl Med Mol Imaging. 2008;35(4):808-14.
9. Park IN, Ryu JS, Shim TS. Evaluation of therapeutic response of tuberculoma using F-18 FDG positron emission tomography. Clin Nucl Med. 2008;33(1):1-3.
10. Esmail H, Lai RP, Lesosky M, Wilkinson KA, Graham CM, Coussens AK, et al. Characterization of progressive HIV-associated tuberculosis using 2-deoxy-2-[18F]fluoro-D-glucose positron emission and computed tomography. Nat Med. 2016;22(10):1090-3.
11. Chan J, Flynn J. The immunological aspects of latency in tuberculosis. Clin Immunol. 2004;110(1):2-12.
12. Vandiviere HM, Loring WE, Melvin I, Willis S. The treated pulmonary lesion and its tubercle bacillus. II. The death and resurrection. Am J Med Sci. 1956;232(1):30-7.
13. Lin PL, Rodgers M, Smith L, Bigbee M, Myers A, Bigbee C, et al. Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. Infect Immun. 2009;77(10):4631-42.
14. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature. 2010;466(7309):973-7.
15. Weiner J 3rd, Parida SK, Maertzdorf J, Black GF, Repsilber D, Telaar A, et al. Biomarkers of inflammation, immunosuppression and stress with active disease are revealed by metabolomic profiling of tuberculosis patients. PLoS One. 2012;7(7):e40221.
16. Kaforou M, Wright VJ, Oni T, French N, Anderson ST, Bangani N, et al. Detection of tuberculosis in HIV-infected and -uninfected African adults using whole blood RNA expression signatures: a case-control study. PLoS Med. 2013;10(10):e1001538.
17. Lin PL, Ford CB, Coleman MT, Myers AJ, Gawande R, Ioerger T, et al. Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. Nat Med. 2014;20(1):75-9.
18. Esmail H, Barry CE 3rd, Young DB, Wilkinson RJ. The ongoing challenge of latent tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2014;369(1645):20130437.
19. Eldholm V, Norheim G, von der Lippe B, Kinander W, Dahle UR, Caugant DA, et al. Evolution of extensively drug-resistant Mycobacterium tuberculosis from a susceptible ancestor in a single patient. Genome Biol. 2014;15(11):490.
20. David HL. Probability distribution of drug-resistant mutants in unselected populations of Mycobacterium tuberculosis. Appl Microbiol. 1970;20(5):810-4.

21. Ford CB, Lin PL, Chase MR, Shah RR, Iartchouk O, Galagan J, et al. Use of whole genome sequencing to estimate the mutation rate of Mycobacterium tuberculosis during latent infection. Nat Genet. 2011;43(5):482-6.
22. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, Narayanan S, et al. Variable host-pathogen compatibility in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A. 2006;103(8):2869-73.
23. Firdessa R, Berg S, Hailu E, Schelling E, Gumi B, Erenso G, et al. Mycobacterial lineages causing pulmonary and extrapulmonary tuberculosis, Ethiopia. Emerg Infect Dis. 2013;19(3):460-3.
24. Coscolla M, Gagneux S. Consequences of genomic diversity in Mycobacterium tuberculosis. Semin Immunol. 2014;26(6):431-44.
25. Hirsh AE, Tsolaki AG, DeRiemer K, Feldman MW, Small PM. Stable association between strains of Mycobacterium tuberculosis and their human host populations. Proc Natl Acad Sci USA. 2004;101(14):4871–6.
26. Baker L, Brown T, Maiden MC, Drobniewski F. Silent nucleotide polymorphisms and a phylogeny for Mycobacterium tuberculosis. Emerg Infect Dis. 2004;10:1568–77.
27. Reed MB, Pichler VK, McIntosh F, Mattia A, Fallow A, Masala S, et al. Major Mycobacterium tuberculosis Lineages Associate with Patient Country of Origin. J Clin Microbiol. 2009;47(4):1119–28.
28. Gagneux S. Host–pathogen coevolution in human tuberculosis. Phil Trans R Soc B. 2012;367(1590):850–9.
29. Fenner L, Egger M, Bodmer T, Furrer H, Ballif M, Battegay M, et al. HIV Infection Disrupts the Sympatric Host–Pathogen Relationship in Human Tuberculosis. PLoS Genet. 2013;9(3):e1003318.
30. Comas I, Homolka S, Niemann S, Gagneux S. Genotyping of genetically monomorphic bacteria: DNA sequencing in Mycobacterium tuberculosis highlights the limitations of current methodologies. Plos One. 2009;4:e7815.
31. Lopes JS, Marques I, Soares P, Nebenzahl-Guimaraes H, Costa J, Miranda A, et al. SNP typing reveals similarity in Mycobacterium tuberculosis genetic diversity between Portugal and Northeast Brazil. Infect Genet Evol. 2013;18:238-46.
32. Stucki D, Brites D, Jeljeli L, Coscolla M, Liu Q, Trauner A, et al. Mycobacterium tuberculosis lineage 4 comprises globally distributed and geographically restricted sublineages. Nat Genet. 2016;48(12):1535-43.
33. Caws M, Thwaites G, Dunstan S, Hawn TR, Lan NT, Thuong NT, et al. The influence of host and bacterial genotype on the development of disseminated disease with Mycobacterium tuberculosis. PLoS Pathog. 2008;4(3):e1000034.
34. Kong Y, Cave MD, Zhang L, Foxman B, Marrs CF, Bates JH, et al. Association between Mycobacterium tuberculosis Beijing/W lineage strain infection and extrathoracic tuberculosis: Insights from epidemiologic and clinical characterization of the three principal genetic groups of M. tuberculosis clinical isolates. J Clin Microbiol. 2007;45(2):409-14.
35. Kong Y, Cave MD, Yang D, Zhang L, Marrs CF, Foxman B, et al. Distribution of insertion- and deletion-associated genetic polymorphisms among four Mycobacterium tuberculosis phospholipase C genes and associations with extrathoracic tuberculosis: a population-based study. J Clin Microbiol. 2005;43(12):6048-53.
36. Lari N, Rindi L, Cristofani R, Rastogi N, Tortoli E, Garzelli C. Association of Mycobacterium tuberculosis complex isolates of M. bovis and Central Asian (CAS) genotypic lineages with extrapulmonary disease. Clin Microbiol Infect. 2009;15(6):538-43.
37. Stavrum R, PrayGod G, Range N, Faurholt-Jepsen D, Jeremiah K, Faurholt-Jepsen M, et al. Increased level of acute phase reactants in patients infected with modern Mycobacterium tuberculosis genotypes in Mwanza, Tanzania. BMC Infect Dis. 2014;14:309.
38. Parwati I, Alisjahbana B, Apriani L, Soetikno RD, Ottenhoff TH, van der Zanden AG, et al. Mycobacterium tuberculosis Beijing genotype is an independent risk factor for tuberculosis treatment failure in Indonesia. J Infect Dis. 2010;201(4):553-7.
39. Lan NT, Lien HT, Tung le B, Borgdorff MW, Kremer K, van Soolingen D. Mycobacterium tuberculosis Beijing genotype and risk for treatment failure and relapse, Vietnam. Emerg Infect Dis. 2003;9(12):1633-5.

40. Sun YJ, Lee AS, Wong SY, Paton NI. Association of Mycobacterium tuberculosis Beijing genotype with tuberculosis relapse in Singapore. Epidemiol Infect. 2006;134(2):329-32.
41. Ford CB, Shah RR, Maeda MK, Gagneux S, Murray MB, Cohen T, et al. Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. Nat Genet. 2013;45(7):784-90.
42. Wollenberg KR, Desjardins CA, Zalutskaya A, Slodovnikova V, Oler AJ, Quiñones M, et al. Whole genome sequencing of Mycobacterium tuberculosis provides insight into the evolution and genetic composition of drug-resistant tuberculosis in Belarus. J Clin Microbiol. 2016. Epub Nov 30. doi: 10.1128/JCM.02116-16.
43. Gurjav U, Erkhembayar B, Burneebaatar B, Narmandakh E, Tumenbayar O, Hill-Cawthorne GA, et al. Transmission of multi-drug resistant tuberculosis in Mongolia is driven by Beijing strains of Mycobacterium tuberculosis resistant to all first-line drugs. Tuberculosis. 2016;101:49-53.
44. de Jong BC, Hill PC, Aiken A, Awine T, Antonio M, Adetifa IM, et al. Progression to active tuberculosis, but not transmission, varies by Mycobacterium tuberculosis lineage in The Gambia. J Infect Dis. 2008;198(7):1037–43.
45. Kato-Maeda M, Kim EY, Flores L, Jarlsberg LG, Osmond D, Hopewell PC. Differences among sublineages of the East-Asian lineage of Mycobacterium tuberculosis in genotypic clustering. Int J Tuberc Lung Dis. 2010;14(5):538-44.
46. Albanna AS, Reed MB, Kotar KV, Fallow A, McIntosh FA, Behr MA, et al. Reduced transmissibility of East African Indian strains of Mycobacterium tuberculosis. PLoS One. 2011;6(9):e25075.
47. Gehre F, Antonio M, Faïhun F, Odoun M, Uwizeye C, de Rijk P, et al. The first phylogeographic population structure and analysis of transmission dynamics of M. africanum West African 1--combining molecular data from Benin, Nigeria and Sierra Leone. PLoS One. 2013;8(10):e77000.
48. Bastos HN, Osório NS, Castro AG, Ramos A, Carvalho T, Meira L, et al. A prediction rule to stratify mortality risk of patients with pulmonary tuberculosis. PLoS One. 2016;11(9):e0162797.
49. Dheda K, Barry CE 3rd, Maartens G. Tuberculosis. Lancet. 2016;387(10024):1211-26.
50. World Health Organization. Guidelines for treatment of tuberculosis - 4rd edition Geneva: World Health Organization; 2010.
51. Wang JY, Hsueh PR, Wang SK, Jan IS, Lee LN, Liaw YS, et al. Disseminated tuberculosis: a 10-year experience in a medical center. Medicine. 2007;86(1):39-46.
52. Yew WW, Lee J. Pathogenesis of cervical tuberculous lymphadenitis: pathways to anatomic localization. Tuber Lung Dis. 1995;76:275-6.
53. Alisjahbana B, Sahiratmadja E, Nelwan EJ, Purwa AM, Ahmad Y, Ottenhoff TH, et al. The effect of type 2 diabetes mellitus on the presentation and treatment response of pulmonary tuberculosis. Clin Infect Dis. 2007;45(4):428-35.
54. Falk A, O'Connor JB. Diagnosis Standards and Classification of Tuberculosis. Association NTaRD, editor: eds Falk, A. et al; 1969.
55. Käser M, Ruf MT, Hauser J, Marsollier L, Pluschke G. Optimized method for preparation of DNA from pathogenic and environmental mycobacteria. Appl Environ Microbiol. 2009;75(2):414-8.
56. Stucki D, Malla B, Hostettler S, Huna T, Feldmann J, Yeboah-Manu D, et al. Two new rapid SNP-typing methods for classifying Mycobacterium tuberculosis complex into the main phylogenetic lineages. PLoS One. 2012;7:e41253.
57. Life Technologies. TaqMan® SNP Genotyping Assays. 2011 [Jan 30th 2017]. Available from: http://tools.lifetechnologies.com/content/sfs/brochures/cms_040597.pdf.
58. Gehre F, Otu J, DeRiemer K, de Sessions PF, Hibberd ML, Mulders W, et al. Deciphering the growth behaviour of Mycobacterium africanum. PLoS Negl Trop Dis. 2013;7(5):e2220.
59. Hillemann D, Richter E, Rüsch-Gerdes S. Use of the BACTEC Mycobacteria Growth Indicator Tube 960 automated system for recovery of Mycobacteria from 9,558 extrapulmonary specimens, including urine samples. J Clin Microbiol. 2006;44(11):4014-7.
60. Coultas MK, Hutchison DJ. Metabolism of resistant mutants of Streptococcus faecalis. IV. Use of a biophotometer in growth-curve studies. J Bacteriol. 1962;84:393-401.
61. GraphPad Software Inc. Curve fitting with Prism 6. Equation: Exponential growth 2015. Available from: http://www.graphpad.com/guides/prism/6/curve-fitting/index.htm?reg_exponential_growth.htm.

62. Andrews S. FastQC: A Quality Control tool for High Throughput Sequence Data 2010. Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc.
63. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114–20.
64. Comas I, Chakravartti J, Small PM, Galagan J, Niemann S, Kremer K, et al. Human T cell epitopes of Mycobacterium tuberculosis are evolutionarily hyperconserved. Nat Genet. 2010;42:498-503.
65. Cole ST, Barrell BG. Analysis of the genome of Mycobacterium tuberculosis H37Rv. Novartis Found Symp. 1998;217:160-77.
66. Bifani P, Moghazeh S, Shopsin B, Driscoll J, Ravikovitch A, Kreiswirth BN. Molecular Characterization of Mycobacterium tuberculosis H37Rv/Ra Variants: Distinguishing the Mycobacterial Laboratory Strain. J Clin Microbiol. 2000;38(9):3200–4.
67. Kearse M, Moir R, Wilson A, Stones-Havas S, Sturrock S, Buxton S, et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28(12):1647–9.
68. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754–60.
69. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078-9.
70. Koboldt DC, Chen K, Wylie T, Larson DE, McLellan MD, Mardis ER, et al. VarScan: variant detection in massively parallel sequencing of individual and pooled samples. Bioinformatics. 2009;25(17):2283-5.
71. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res. 2012;22(3):568-76.
72. McEvoy CR, Cloete R, Müller B, Schürch AC, van Helden PD, Gagneux S, et al. Comparative analysis of Mycobacterium tuberculosis pe and ppe genes reveals high sequence variation and an apparent absence of selective constraints. PLoS One. 2012;7(4):e30593.
73. Stamatakis A. RAxML Version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30(9):1312-3.
74. Coll F, McNerney R, Guerra-Assunção JA, Glynn JR, Perdigão J, Viveiros M, et al. A robust SNP barcode for typing Mycobacterium tuberculosis complex strains. Nat Commun. 2014;5:4812.
75. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc. 2009;4(7):1073-81.
76. Sim NL, Kumar P, Hu J, Henikoff S, Schneider G, Ng PC. SIFT web server: predicting effects of amino acid substitutions on proteins. Nucleic Acids Res. 2012;40(Web Server issue):W452-7.
77. Sassetti CM, Boyd DH, Rubin EJ. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol. 2003;48(1):77-84.
78. Rengarajan J, Bloom BR, Rubin EJ. Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. Proc Natl Acad Sci U S A. 2005;102(23):8327-32.
79. Mi H, Huang X, Muruganujan A, Tang H, Mills C, Kang D, et al. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucleic Acids Res. 2017;45(D1):D183-D9.
80. Mi H, Muruganujan A, Casagrande JT, Thomas PD. Large-scale gene function analysis with the PANTHER classification system. Nat Protoc. 2013;8(8):1551-66.
81. Franco I, Sousa P, Gomes M, Oliveira A, Gaio AR, Duarte R. Social profile of the highest tuberculosis incidence areas in Portugal. Rev Port Pneumol. 2016;22(1):50-2.
82. Schürch AC, van Soolingen. DNA fingerprinting of Mycobacterium tuberculosis: From phage typing to whole-genome sequencing. D Infect Genet Evol. 2012;12:602–9.
83. Flores L, Van T, Narayanan S, DeRiemer K, Kato-Maeda M, Gagneux S. Large sequence polymorphisms classify Mycobacterium tuberculosis strains with ancestral spoligotyping patterns. J Clin Microbiol. 2007;45(10):3393-5.
84. Ben Shabat M, Mikula I, Gerchman I, Lysnyansky I. Development and evaluation of a novel single-nucleotide-polymorphism real-time PCR assay for rapid detection of fluoroquinolone-resistant Mycoplasma bovis. J Clin Microbiol. 2010;48(8):2909-15.

85. Domenech P, Reed MB. Rapid and spontaneous loss of phthiocerol dimycocerosate (PDIM) from Mycobacterium tuberculosis grown in vitro: implications for virulence studies. Microbiology. 2009;155(Pt 11):3532-43.
86. von Groll A, Martin A, Portaels F, da Silva PE, Palomino JC. Growth kinetics of Mycobacterium tuberculosis measured by quantitative resazurin reduction assay: a tool for fitness studies. Braz J Microbiol. 2010;41(2):300-3.
87. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 1998;393(6685):537-44.
88. Lew JM, Kapopoulou A, Jones LM, Cole ST. TubercuList – 10 years after. Tuberculosis. 2011;91(1):1–7.
89. Wooff E, Michell SL, Gordon SV, Chambers MA, Bardarov S, Jacobs WR Jr, et al. Functional genomics reveals the sole sulphate transporter of the Mycobacterium tuberculosis complex and its relevance to the acquisition of sulphur in vivo. Mol Microbiol. 2002;43(3):653-63.
90. Li W, Xin Y, McNeil MR, Ma Y. rmlB and rmlC genes are essential for growth of mycobacteria. Biochem Biophys Res Commun. 2006;342(1):170-8.
91. Sarkar R, Lenders L, Wilkinson KA, Wilkinson RJ, Nicol MP. Modern lineages of Mycobacterium tuberculosis exhibit lineage-specific patterns of growth and cytokine induction in human monocyte-derived macrophages. PLoS One. 2012;7(8):e43170.
92. Homolka S, Niemann S, Russell DG, Rohde KH. Functional genetic diversity among Mycobacterium tuberculosis complex clinical isolates: delineation of conserved core and lineage-specific transcriptomes during intracellular survival. PLoS Pathog. 2010;6(7):e1000988.
93. Krishnan N, Malaga W, Constant P, Caws M, Tran TH, Salmons J, et al. Mycobacterium tuberculosis lineage influences innate immune response and virulence and is associated with distinct cell envelope lipid profiles. PLoS One. 2011;6(9):e23870.
94. Tanveer M, Hasan Z, Kanji A, Hussain R, Hasan R. Reduced TNF-alpha and IFN-gamma responses to Central Asian strain 1 and Beijing isolates of Mycobacterium tuberculosis in comparison with H37Rv strain. Trans R Soc Trop Med Hyg. 2009;103(6):581-7.
95. Newton SM, Smith RJ, Wilkinson KA, Nicol MP, Garton NJ, Staples KJ, et al. A deletion defining a common Asian lineage of Mycobacterium tuberculosis associates with immune subversion. Proc Natl Acad Sci U S A. 2006;103(42):15594-8.
96. Li Q, Whalen CC, Albert JM, Larkin R, Zukowski L, Cave MD, et al. Differences in rate and variability of intracellular growth of a panel of Mycobacterium tuberculosis clinical isolates within a human monocyte model. Infect Immun. 2002;70(11):6489-93.
97. Zhang M, Gong J, Yang Z, Samten B, Cave MD, Barnes PF. Enhanced capacity of a widespread strain of Mycobacterium tuberculosis to grow in human macrophages. J Infect Dis. 1999;179(5):1213–7.
98. Hoal-van Helden EG, Hon D, Lewis LA, Beyers N, van Helden PD. Mycobacterial growth in human macrophages: variation according to donor, inoculum and bacterial strain. Cell Biol Int. 2001;25(1):71-81.
99. Wong KC, Leong WM, Law HK, Ip KF, Lam JT, Yuen KY, et al. Molecular characterization of clinical isolates of Mycobacterium tuberculosis and their association with phenotypic virulence in human macrophages. Clin Vaccine Immunol. 2007;14(10):1279-84.
100. Theus SA, Cave MD, Eisenach KD. Intracellular macrophage growth rates and cytokine profiles of Mycobacterium tuberculosis strains with different transmission dynamics. J Infect Dis. 2005;191(3):453-60.
101. Rodrigo T, Caylà JA, García de Olalla P, Galdós-Tangüis H, Jansà JM, Miranda P, et al. Characteristics of tuberculosis patients who generate secondary cases. Int J Tuberc Lung Dis. 1997;1(4):352-7.
102. Brites D, Gagneux S. Old and new selective pressures on Mycobacterium tuberculosis. Infect Genet Evol. 2012;12(4):678-85.
103. Agranoff D, Krishna S. Metal ion transport and regulation in Mycobacterium tuberculosis. Front Biosci. 2004;9:2996-3006.
104. Pulido PA, Novoa-Aponte L, Villamil N, Soto CY. The DosR dormancy regulator of Mycobacterium tuberculosis stimulates the Na(+)/K (+) and Ca (2+) ATPase activities in plasma membrane vesicles of mycobacteria. Curr Microbiol. 2014;69(5):604-10.

105. Ayala-Torres C, Novoa-Aponte L, Soto CY. Pma1 is an alkali/alkaline earth metal cation ATPase that preferentially transports Na(+) and K(+) across the Mycobacterium smegmatis plasma membrane. Microbiol Res. 2015;176:1-6.

SUPPLEMENTARY INFORMATION

Table IV.S1 – List of loci with allelic frequencies differing more than 40% (∆DAF >0.4) between fast and slow growing strains. Variant allele (mutant) was defined by comparative analysis with the reference *M. tuberculosis* genome of the laboratory strain H37Rv. Tuberculist database provided the putative gene product or function. ABC – ATP-binding cassette; ECF – extracytoplasmic function; ESX – early secreted antigenic target (ESAT-6) secretion system; HTH – helix-turn-helix; MFS – major facilitator superfamily

Position	SNP	Mutant	∆DAF	Gene	Putative product or function
2240700	A/G	A	0.889	*ctpF*	Metal cation transporter ATPase F
909280	C/A	A	0.753	*cysA2*	Sulfotransferase involved in the formation of thiosulfate
3312632	C/T	C	0.740	*Rv2959c*	Rhamnosyl O-methyltransferase
4042761	G/A	G	0.740	*lysS*	Lysine-tRNA ligase (translation)
3503231	C/G/T/A	G	0.733	*Rv3136A*	Conserved protein (function unknown)
2868659	C/G	C	0.728	*vapB19*	Antitoxin
4395964	C/A	C	0.725	*mutT4*	Possible mutator protein (function unknown)
2857014	A/G	G	0.724	*adi*	Ornithine/arginine/lysine decarboxylase involved in the biosynthesis of spermidine from arginine
1037355	C/T	T	0.722	*pstA1*	Phosphate ABC transporter permease
667659	C/T	C	0.720	*Rv0574c*	Conserved protein (function unknown)
2599821	A/C	A	0.720	*Rv2326c*	ABC transporter, possibly involved in energy coupling to the transport system and the translocation of the substrate across the membrane
478358	T/C/G	C	0.717	*lpqK*	Lipoprotein
1297327	A/G	G	0.714	*lpqW*	Monoacyl phosphatidylinositol tetramannoside-binding protein
2946157	C/T	T	0.713	*Rv2617c*	Transmembrane protein
2906918	T/A	A	0.712	*ppiB*	Peptidyl-prolyl cis-trans isomerase B
3099269	C/A	A	0.712	*ltp1*	Lipid-transfer protein, possibly catalyzes the transfer of a great variety of lipids between membranes
790180	G/A	A	0.711	*Rv0690c*	Conserved protein (function unknown)
1281443	G/C	C	0.708	*omt*	O-methyltransferase
3881187	A/G	G	0.708	*Rv3463*	Conserved protein (function unknown)
1736638	T/C	C	0.705	*ileS*	Isoleucine-tRNA ligase (translation)
2007785	T/C	C	0.705	Unknown	Unknown
2698585	T/C	C	0.703	*Rv2402*	Trehalase, converts trehalase (storage carbohydrate) into glucose
35097	C/T	T	0.701	*bioF2*	8-amino-7-oxononanoate synthase
4109354	C/A	A	0.698	*acs*	Acetyl-coA synthetase
1297999	G/T	T	0.695	*lpqW*	Monoacyl phosphatidylinositol tetramannoside-binding protein
1041445	T/C/G	C	0.693	*pstB*	Phosphate ABC transporter (ATP-binding ability and ATPase activity)
4383094	G/A	A	0.691	*Rv3897c*	Conserved protein (function unknown)

Table IV.S1 (continuation)

3484012	G/T	T	0.690	*cysA3*	Sulfotransferase involved in the formation of thiosulfate
2664299	C/G/A	G	0.688	*mbtE*	Peptide synthetase, involved in the biogenesis of the hydroxyphenyloxazoline-containing siderophore mycobactins (related with iron shuttle)
1719322	A/G/C	G	0.686	*Rv1524*	Glycosyltransferase
2527676	A/G	G	0.683	*Rv2252*	Diacylglycerol kinase, involved in synthesis of phosphatidylinositol mannosides
557133	A/G	A	0.679	*Rv0466*	Conserved protein (function unknown)
176303	T/C	C	0.678	*Rv0149*	NADPH:quinone oxidoreductase, may act in the detoxification of xenobiotics
1861274	A/G	A	0.678	*pheT*	Phenylalanine-tRNA ligase subunit beta (translation)
1709899	C/A	A	0.676	*Rv1518*	Glycosyltransferase
3214790	T/C	C	0.675	*lppW*	Alanine-rich lipoprotein
4146330	G/A	G	0.672	*egtB*	Iron-dependent oxidoreductase
234051	A/G	G	0.669	*Rv0197*	Oxidoreductase
101727	A/G	G	0.668	*ctpA*	Cation (possibly copper) transporter ATPase A
1071797	G/C	G	0.666	*Rv0959*	Conserved protein (function unknown)
2257780	G/T	T	0.659	*Rv2008c*	Conserved protein (function unknown)
2894854	T/C	T	0.658	*Rv2570*	Conserved protein (function unknown)
3977226	A/G/T	G	0.653	*hsd4B*	Dehydrogenase, possibly involved in lipid catabolism
2126366	C/G	G	0.647	*Rv1877*	MFS-type transporter, possibly involved in transport of drug across the membrane
1373170	G/C	G	0.634	*Rv1230c*	Membrane protein
1588456	G/A	A	0.632	*lprG*	Lipoprotein
2420535	C/A	C	0.595	*murE*	UDP-N-acetylmuramoylalanyl-D-glutamate-diaminopimelate ligase (peptidoglycan biosynthesis), involved in cell wall formation
2385695	C/T	C	0.584	*metH*	Methionine synthase
4377447	G/A	G	0.577	*eccC2*	ESX-2 type VII secretion system protein
4373475	C/G	C	0.566	*espG2*	ESX-2 secretion-associated protein
3267743	A/G	A	0.559	*ppsE*	Phthiocerol synthesis polyketide synthase type I, involved in phenolpthiocerol and phthiocerol dimycocerosate biosynthesis (components of cell envelope)
3232815	G/A/C	A	0.549	Unknown	Conserved protein (function unknown)
1132368	C/T	C	0.548	*pks16*	Polyketide synthase
2443188	G/A/T	G	0.544	*Rv2180c*	Integral membrane protein
4043365	G/T	G	0.537	*Rv3600c*	Type III pantothenate kinase
632330	G/T	G	0.536	*Rv0539*	Dolichyl-phosphate sugar synthase
784440	G/T	G	0.530	*fusA1*	Elongation factor G (protein synthesis)
3504930	C/T	T	0.530	*pflA*	Pyruvate formate lyase activating protein
3089679	G/A	G	0.522	*pepR*	Zinc protease
2084526	G/A	G	0.520	*Rv1836c*	Conserved protein (function unknown)
3829152	G/A	A	0.518	*guaB3*	Inosine-5'-monophosphate oxidoreductase (GMP biosynthesis)
990533	T/C	T	0.515	*Rv0890c*	HTH-type transcriptional regulator

Table IV.S1 (continuation)

40162	C/T	C	0.503	*Rv0037c*	MFS-type transporter, possibly involved in transport of drug (macrolide?) across the membrane
3158935	G/C	G	0.500	*Rv2850c*	Magnesium chelatase, possibly introduces a magnesium ion into specific substrate
1755599	C/T	T	0.491	*plsB1*	Acyltransferase
4403900	A/G	A	0.486	*cwlM*	Peptidoglycan hydrolase
3561155	G/A	G	0.485	*Rv3193c*	Transmembrane protein
4218350	T/C	T	0.483	*Rv3773c*	Conserved protein (function unknown)
1769099	C/A	C	0.469	*treY*	Maltooligosyl trehalose synthase
2327492	C/T	T	0.467	*sigC*	ECF RNA polymerase sigma factor (promoter recognition, transcription initiation)
660859	C/T	C	0.462	*cyp135B1*	Cytochrome P450
1055049	C/T	C	0.461	*pgi*	Glucose-6-phosphate isomerase (glycolysis and gluconeogenesis)
332131	C/A	C	0.455	*Rv0276*	Conserved protein (function unknown)
2019942	A/G	A	0.454	*eccC5*	ESX-5 type VII secretion system protein, involved in secretion of the mycobacteria specific PE/PPE proteins and cell wall stability
1724120	G/A	G	0.453	*pks5*	Polyketide synthase
2278442	C/G	G	0.453	*Rv2030c*	Conserved protein (function unknown)
1771320	G/A/C	G	0.434	*treX*	Maltooligosyl trehalose synthase
4288405	G/T	G	0.432	*mmpL8*	Integral membrane transport protein, involved in the transport of lipids and required in the production of sulfolipid-1
228847	G/A	A	0.429	*Rv0194*	Multidrug ABC transporter ATPase permease, involved in multidrug resistance by efflux pump mechanism
1805948	T/C	C	0.426	*hisI*	Phosphoribosyl-AMP cyclohydrolase (histidine biosynthesis)
4385177	G/C	G	0.423	*Rv3899c*	Conserved protein (function unknown)
90502	C/T	T	0.417	*Rv0083*	Oxidoreductase
3503246	A/G	A	0.416	*Rv3136A*	Conserved protein (function unknown)
347766	C/T	T	0.407	*eccC3*	ESX-3 secretion system protein, involved in iron/zinc homeostasis
2332143	T/C	C	0.405	*Rv2075c*	Hypothetical exported or envelope protein
365358	G/A	G	0.403	*Rv0303*	Dehydrogenase/reductase
736710	C/T	T	0.402	*mmaA4*	Hydroxymycolate synthase, involved in mycolic acids modification

CHAPTER 5 – HOST AND BACTERIAL MODULATION OF THE PROTECTIVE IL-1 RECEPTOR SIGNALING IN TB

The results presented in this chapter were published:

(i) **in conferences**

2016 Annual Meeting of the SPI (Sociedade Portugusa de Imunologia), "Immunity, from cells to organisms" | Instituto Gulbenkian de Ciências (Oeiras), 1-3 June 2016
and
Tuberculosis 2016 EMBO Conference, "Interdisciplinary research on tuberculosis and pathogenic mycobacteria" | Institut Pasteur (Paris), 19-23 September 2016
Host and *Mycobacterium tuberculosis* determinants of immune cells activation (Poster presentation) | Authors: Jeremy Sousa*, Helder Novais e Bastos*, Nuno S. Osório, Maria Isabel Veiga, Henrique Machado, Filipa Cardoso, Joana Gaifem, Angélica Ramos, Teresa Carvalho, Jorge Vieira, Cristina Vieira, Fernando Rodrigues, João Tiago Guimarães, António Gil Castro, Margarida Saraiva

Keystone Symposia 2017, "New Developments in Our Basic Understanding of Tuberculosis"| Fairmont Hotel, Vancouver, 14-18 January 2017
Impact of host and *Mycobacterium tuberculosis* molecular determinants of immune cells activation to the outcome of disease| Authors: Jeremy Sousa*, Helder Novais e Bastos*, Nuno S. Osório, Maria Isabel Veiga, Henrique Machado, Filipa Cardoso, Joana Gaifem, Angélica Ramos, Teresa Carvalho, Jorge Vieira, Cristina Vieira, Fernando Rodrigues, João Tiago Guimarães, António Gil Castro, Margarida Saraiva

(*equal contributions)

ABSTRACT

A striking feature of tuberculosis (TB) is the variability of disease outcomes, including pathogen clearance, latency establishment and active disease. Immune imbalances clearly contribute to this heterogeneity, assuming a rising importance as therapeutic targets. Several studies show a central role of the cross-talk between host and bacteria in defining the immune response and the outcome of disease. To underpin the molecular mechanisms regulating this cross-talk and its impact in immunity, we are studying a cohort of TB patients, showing different severity of disease at presentation. Sixteen *Mycobacterium tuberculosis* clinical isolates associated with different clinical severity of TB were selected and PBMCs from non-treated/non-recent latent TB infected (LTBI) donors or past/cured TB patients infected. Cytokine production by the infected cells was then measured. Independently of the host genetics, we identified two distinct groups of *M. tuberculosis* isolates: high *versus* low inflammatory triggers. Furthermore, we report that PBMCs from past TB patients produced less IL-1β than those from LTBI participants in response to a variety of isolates, whereas the opposite was observed for IL-1RA. LTBI subjects elicited responses with significantly higher IL-1β/IL-1RA ratios than those from TB patients. Our findings suggest that infected hosts producing elevated IL-1β/IL-1RA ratio may be protected from active disease, offering a platform to predict those individuals in higher risk of latent to active TB transition.

INTRODUCTION

Tuberculosis (TB) still imposes high morbidity and mortality burdens in the developing world, with nearly 10.4 million new cases occurring worldwide and approximately 1.8 million deaths every year, making *Mycobacterium tuberculosis* one of the most lethal infectious agents of the history of mankind [1]. Further aggravating this scenario, one quarter of the world's population is estimated to be latently infected with *M. tuberculosis*, providing a virtually impossible to eliminate reservoir [2, 3]. Tackling TB requires fresh ways of thinking and paradigm breaking research. Recent studies have placed host-directed therapies (HDT) based in immune modulatory approaches among the most promising strategies to treat TB [4]. However, the rational development of HDTs requires a deep knowledge of the factors that modulate host-pathogen interactions and protective immunity in TB. For this, both host and pathogen determinants need to be considered. A growing body of evidence suggest that *M. tuberculosis* has learned to modulate the host immune response to its own advantage, along the millennia of coevolution with Man. For instance, the pathogen has evolved to use the macrophage intracellular compartment to replicate and to persist within lung granulomas [5]. Moreover, genome sequencing of *M. tuberculosis* showed that the known human T-cell epitopes are evolutionarily hyperconserved [6], suggesting an exploitation of host T cell responses by the bacteria. Indeed, *M. tuberculosis* appears to have evolved to elicit a "permissive" inflammatory state where cavitary pulmonary disease develops, ensuring its successful transmission [7], while the patient remains ambulatory enough to spread TB upon contact with many other individuals. Work from our group provided evidence of diversifying selection in the genome of *M. tuberculosis* in response to non-antibiotic pressure, strongly suggesting the immune system as a driver of bacterial adaptation [8]. Therefore, a certain degree of adaptation to the host immune response within a population of *M. tuberculosis* is expected. In line with this hypothesis, cellular and experimental infections with different strains belonging to the *M. tuberculosis* complex (MTBC) show the production of distinct cytokine secretion profiles and

different outcomes of infection [9-13]. However, studies linking bacterial genotypes with distinct phenotypes of immune responses and with specific clinical outcomes, in different hosts, are still scarce.

The initiation of the immune response during *M. tuberculosis* infection relies on the activation of pattern recognition receptors (PRRs) by pathogen-associated molecular patterns (PAMPs) [14]. PRRs include Toll-like receptors (TLRs), C-type lectin receptors, and the cytosolic NOD-like receptors (NLRs) recognizing microbial molecules in the intracellular compartment. Among the NLR family of proteins, NLR pyrin domain containing 3 (NLRP3) is known to induce the assembly of the inflammasome in response to mycobacterial infection [15]. Another inflammasome complex is initiated by the absent in melanoma 2 (AIM2) in response to bacterial dsDNA delivered in the cytosol of infected macrophages [16]. The activation of the inflammasome has a pivotal role in the maturation and secretion of interleukin 1-beta (IL-1β), which is a critical cytokine in host immune defence against *M. tuberculosis* [14]. In the animal model, IL-1β and IL-1-receptor deficient mice presented acute mortality and increased pulmonary bacterial burden after infection [17, 18]. On the other hand, human data showed that IL-1β and IL-1 receptor antagonist (IL-1RA), a natural competitive antagonist of IL-1 signaling, are highly expressed in the bronchoalveolar lavage fluid from pulmonary TB patients [19, 20]. However, the precise impact of IL-1RA on TB pathogenesis remains elusive. Anakinra, a recombinant human IL-1RA, has been used on a large number of patients with different inflammatory diseases [21], and still, records show little or absent risk of *M. tuberculosis* reactivation in latently infected individuals [21, 22]. Conversely, Type I IFNs that counter-regulate the IL-1 function [23], have been linked to pathologic exacerbation in TB [24].

In this study, we hypothesized that the initial events of the immune response to *M. tuberculosis* infection may be important determinants of the infection outcome and that both pathogen and host molecular elements are in place to modulate these events. In order to address this point, we analysed the cytokine expression profile induced in human peripheral blood mononuclear cells (PBMCs) by different *M. tuberculosis* isolates associated with specific clinical outcomes. As host donors, we analysed individuals who were latently infected for

at least 2 years and individuals who had pulmonary TB in the past. Independently of the host genetics, we identified two distinct groups of *M. tuberculosis* isolates: high *versus* low inflammatory triggers. Furthermore, our findings suggest that individuals producing elevated IL-1β/IL-1RA ratio may be protected from active disease. These results highlight the importance of considering both host- and bacteria-associated characteristics when designing immunomodulatory interventions for TB.

MATERIALS AND METHODS

1. *M. tuberculosis* clinical isolates selection and growth

We recovered the bacterial samples from a cohort of 133 TB cases with at least pulmonary or pleural involvement [25], diagnosed at a University-affiliated hospital (Hospital de São João - HSJ, Porto) during the period of 7 years (2007-2013). All these cases were individuals who did not present known TB precipitating or predictor factors. Details on inclusion criteria, overall demographic and clinical features of this population have been described in the former chapters. The study population was stratified in 5 severity groups, according to the classification algorithm provided in Chapter 4 (Figure IV.2). Eight *M. tuberculosis* isolates were selected from patients with a mild manifestation of TB. Eight matched strains from patients with severe TB were then selected. Matching was based on at least 2 out of 3 host variables: age within 15 years, gender and smoking habits.

Recovery of the selected isolates was performed as described in Chapter 4. The stocks were amplified in 7H9 medium for 7–10 days of growth, till reaching the mid log-phase growth stage. To determine the concentration of the *M. tuberculosis* stocks, 6 frozen aliquots were serial diluted and plated in Middlebrook 7H11 (BD Biosciences) agar plates supplemented with 10% Oleic Albumin Dextrose Catalase Growth Supplement (OADC) and 0.5% glycerol. Viable bacteria were determined by counting colony-forming units (CFUs) after 19-21 days of incubation at 37°C. Prior to infections, the frozen *M. tuberculosis* stocks were thawed, syringed and directly diluted in complete Roswell Park Memorial Institute (RPMI) medium, considering the obtained values for CFU.

2. PBMCs isolation

Venous blood was drawn into 9 mL EDTA tubes (Vacutest Kima, Italy) from the cubital vein of 19 healthy adult volunteer donors with non-treated latent TB infection (LTBI), to whom a positive interferon-gamma release assay (IGRA) was known for more than 2 years. Eight past TB patients were also enrolled in the study, corresponding to specific host-pathogen pairs associated with either mild or severe outcomes. PBMCs were isolated from whole blood on a Ficoll-Paque (Sigma-Aldrich, Missouri, USA) density gradient, according standard protocols [13]. The PBMCs interface was carefully removed into fresh RPMI-1640 medium, centrifuged and the resulting cellular pellet resuspended in 5 mL of RPMI supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate and 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Cell suspensions were then counted in trypan blue. For the phenotypic analysis of the PBMCs obtained for each donor, $1x10^6$ PBMCs were stained with specific antibodies against human CD3 (clone UCHT1, labelled with PE, BD Pharmingen), CD4 (clone RPAT4, labelled with APC, BD Pharmingen), CD19 (clone HIB19, labelled with FITC, Biolegend), CD14 (clone M5E2, labelled with FITC, BD Pharmingen) and CD16 (clone 3G8, labelled with APC Cy7, BD Pharmingen) and analysed by fluorescence-activated cell sorting (FACS). All antibodies were titrated prior to be used. A viability dye (7AAD, Biolegend) was also included in the analysis. Data was acquired in a LSRII flow cytometer and analysed using the FlowJo software (Ashland, USA).

3. PBMCs infection

Freshly isolated PBMCs were plated in 24 well-plates at a density of $0.5x10^6$ cells/well and infected with each of the selected *M. tuberculosis* isolates or with the laboratory strain H37Rv Trudeau at a multiplicity of infection (moi) of 1:1. Non-infected cells were incubated with culture medium alone, to work as

negative controls. Cell culture supernatants were collected 24 hours post-infection, filter-sterilized and frozen in 50 μL aliquots at -80 ºC for future analysis.

4. Cytokine detection by Enzyme-Linked Immunosorbent Assay (ELISA) and Multiplex analysis

Cell supernatants were screened for IL-6 and interferon (IFN)-γ by sandwich ELISA kits (eBioscience, San Diego, USA), following the manufacturer's instructions. Production of IFN-β, IL-1β, IL-1RA, IL-10, IL-12p40 and TNF-α was measured using a Multiplex kit according to the manufacturer's recommendations (ProcartaPlex®, eBioscience).

5. Statistical analysis

Cytokines were analysed using the non-parametric Mann Whitney U-test. Correlations were assessed using Spearman's rank correlation. Receiver operating characteristic (ROC) curve was used to determine the cutoff point yielding the highest combined specificity and sensitivity (Youden index criterion) [26], and discriminative ability was evaluated by the area under the ROC curve (AUC) and its 95% confidence interval (CI). All the statistical analyses were performed using the GraphPad Prism® 7 software (GraphPad Software, Inc.) and SPSS software program, version 24 (IBM® SPSS®, Inc.). Values of $P<0.05$ were considered statistically significant.

6. Ethics statement

The study protocol was approved by the Health Ethics Committees of the HSJ (approval number 109-11), the North Health Region Administration (approval number 71-2014) and the Portuguese Data Protection Authority (approval number 12174-2011). Written informed consent was obtained from all patients and LTBI donors prior to draw samples of venous blood. To ensure confidentiality, each case was anonymized by the assignment of a random identification number. Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

RESULTS

1. Group definition and experimental design

To investigate a possible relation between the clinical outcome of TB and the inflammatory phenotype induced by different *M. tuberculosis* isolates in immune cells, we selected 8 clinical isolates associated with mild TB outcomes and another 8 associated with severe TB, according to matching criteria described before (**Table V.1**). The isolates are geographically matched with the hosts, to ensure that mutual adaptations may be revealed. To avoid deviations in the clinical outcome due to imbalanced host immunity, the selected *M. tuberculosis* isolates were obtained from TB patients who did not present known TB precipitating or predicting factors, such as HIV, metabolic disease, or general immune suppression.

These isolates were then used to infect PBMCs obtained from individuals who were latently infected for at least 2 years (as measured by IGRA+ data), who did not undergo preventive antibiotherapy and who did not develop TB. PBMCs from individuals who had pulmonary TB in the past and have been successfully cured upon antibiotherapy were also included. These two groups are respectively representative of individuals who are resistant or susceptible to active TB progression upon infection. By choosing IGRA+ participants we also minimized innate resistance to TB, likely existing in some IGRA- individuals. As controls, non-stimulated cells and cells infected with the laboratory reference strain H37Rv Trudeau were included.

Table V.1 – Description of the hosts with mild or severe disease corresponding to the clinical isolates selected for functional analysis. Differences between selected mild and severe cases regarding gender (*P*=0.315), age *(P*=0.577) and smoking habits (*P*=0.619) distribution were not statistically significant. The time of symptoms of the severe cases selected was significantly less than for the mild ones (6.1±3.5 *vs* 11.4±9.4 weeks, respectively, *P*=0.035) and, hence, severity of disease could not be imputable to diagnostic delay. The "*" marks the selected host-pathogen pairs assessed in the functional analysis for cytokines induction (see Figure V.7).

Severity group	Strain code	Gender	Age, years	Smoking habits	Time of symptoms, weeks	Phylogeny
Mild	2D2*	Male	45	Never smoker	13	L4 (LAM)
	2I3	Female	39	Former/active smoker	12	L4 (LAM)
	3A3*	Female	35	Never smoker	2	L4 (LAM)
	4I2*	Female	34	Never smoker	1	L4 (LAM)
	5A9	Male	25	Former/active smoker	5	L4 (LAM)
	5D4	Male	28	Never smoker	26	L4 (LAM)
	5F8*	Female	26	Never smoker	8	L4 (LAM)
	6D3	Female	30	Former/active smoker	24	L4 (LAM)
Severe	4D5*	Male	19	Former/active smoker	9	L4 (LAM)
	5B5*	Male	54	Never smoker	3	L4 (X)
	5C7*	Female	49	Former/active smoker	10	L4 (LAM)
	5C8	Male	42	Never smoker	1	L4 (LAM)
	5D6*	Male	43	Former/active smoker	10	L4 (other)
	5I6	Female	42	Never smoker	6	L4 (LAM)
	6C1	Male	19	Former/active smoker	7	L4 (LAM)
	6C4	Male	20	Former/active smoker	3	L4 (LAM)

The production of several cytokines was measured at 24h post-infection. The stimulation time of 24 hours was chosen based on previous studies that defined this period as the most appropriate to assess monocyte-derived cytokines [27, 28]. By deciding to stimulate PBMCs, rather than isolated immune cell populations, we were able to capture the interactions between different immune cell types (for instance, between monocytes and T cells), making it closer to the natural immune response. However, to account for possible differences in the PBMC compartment of each participant, the frequency of live cells, of lymphocytes and of myeloid cells was monitored by flow cytometry (**Figure V.1**). Although different donors showed different PBMCs composition, the observed variations were within the expected range and no skew was observed between LTBI and past TB participants.

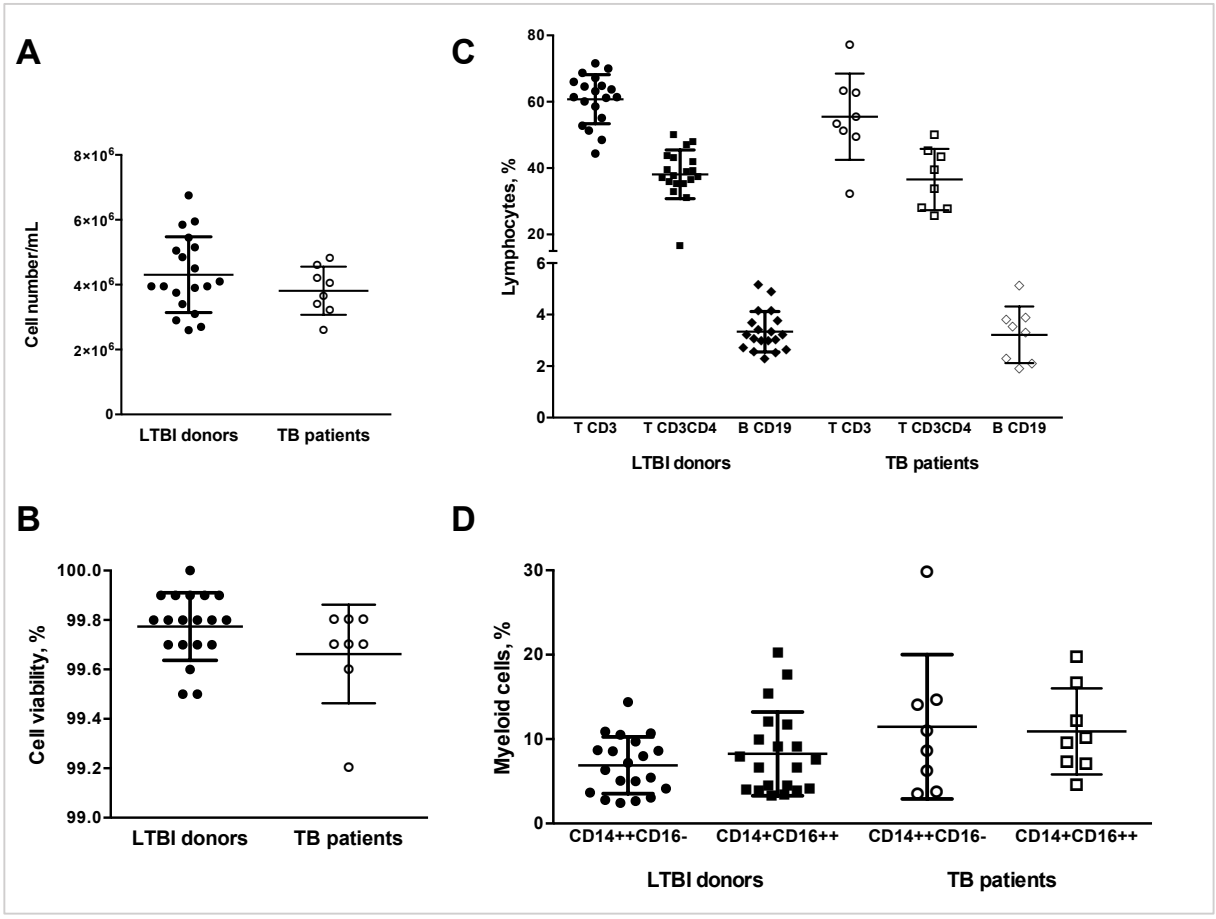

Figure V.1 – General characteristics of the PBMCs isolated from 19 non-treated LTBI donors and 8 TB patients. Each dot in the graphs corresponds to one participant. The cellular concentration in the isolated PBMCs obtained per donor was determined by trypan blue cell counting and the viability and cellular composition determined by FACS as indicated in the Material and Methods section. **(A)** Cellular concentration per mL obtained for each donor. **(B)** Viability of PBMCs isolated for each donor. **(C)** Frequency of T cells (CD3+ and CD3+CD4+), B cells (CD19+) and **(D)** monocytes (classical CD14++CD16- and non-classical CD14+CD16++).

2. Heterogeneity of host responses to clinical isolates associated with different TB outcomes

We started by investigating a possible correlation between the outcome of TB and the capacity of the infecting *M. tuberculosis* isolate to induce a particular type of cytokine production in PBMCs. For that we focused on the response of LTBI donors. A heatmap was calculated, considering the response of 10 LTBI participants to each of the isolates tested (**Figure V.2**). For all tested cytokines (IL-1β, IL-6, IL-12p40, IFN-β, IL-1RA, IL-10), some isolates clearly induced higher production of cytokines than others, suggesting the presence of bacterial determinants of the immune response, independently of the host background. Interestingly, among the top three inducers of cytokine production were isolates that associate with mild TB, whereas among the lowest three inducers were isolates associated with a severe manifestation of TB (Figure V.2). This led us to compare the overall PBMC response to the group of isolates related to mild *versus* those related to severe TB presentation (**Table V.2**). It was interesting to observe that *M. tuberculosis* isolates associated with severe disease consistently induced lower levels of pro-inflammatory (IL-1β, IL-6, IL-12p40) and anti-inflammatory (IL-1RA, IL-10) cytokines than did mild isolates ($P<0.001$). Of note, H37Rv infections of PBMCs led to significantly higher production of cytokines by every donor as compared to those detected for the clinical isolates (**Figure V.3**). The same result was observed in previous studies and is likely to reflect a laboratorial adaptation of this strain towards the induction of high inflammatory responses [29]. An exception to this finding was the production of IL-1RA, which did not vary significantly for the H37Rv infection.

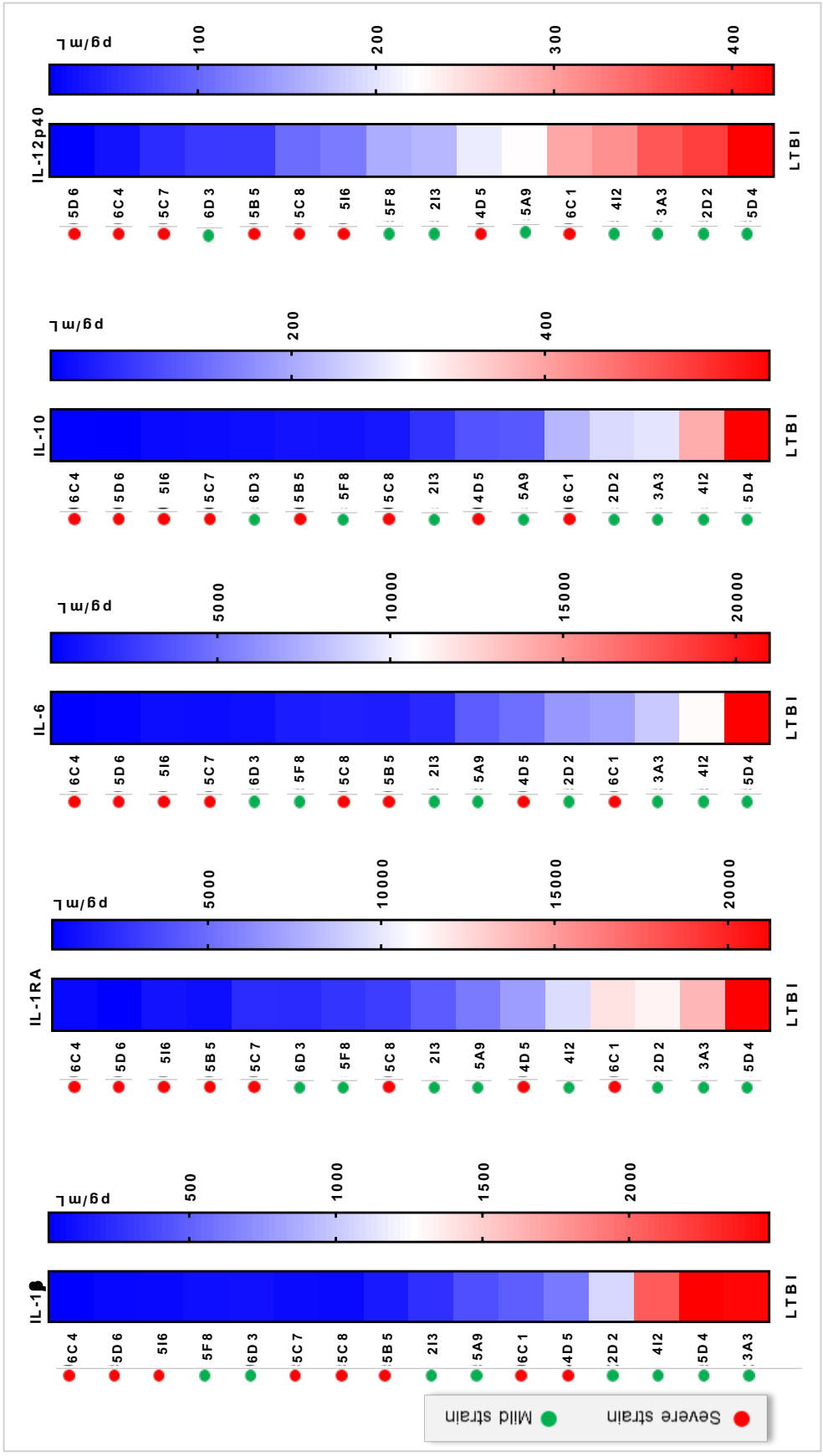

Figure V.2 – *M. tuberculosis* isolates can be divided in high and low inducers of responses in a host-independent way. PBMCs of 10 LTBI subjects were infected with each of the 16 clinical isolates associated with either mild or severe outcome (moi 1:1). 24 hours post-infection supernatants were harvested and filtered and the production of the indicated cytokines assessed by immunoassay. The heatmap shows the strain-related hierarchy in the inflammatory response. The clinical isolates are ranked according to the median concentration of each cytokine. Colored dots at left indicate the mild and severe strains.

Table V.2 – *M. tuberculosis* isolates associated with severe disease induced significantly less pro-inflammatory (IL-1β, IL-6, IL-12p40) and anti-inflammatory (IL-1RA, IL-10) cytokine production by infected PBMCs than isolates associated with mild disease. IFN-β production was below detection level. Non-stimulated cell also had cytokines below detection level. The cytokines concentration values are presented as median (25th–75th percentile). IQR – interquartile range.

Cytokines	Mild strains	Severe strains	*P* value
IL-1β, median (IQR) pg/mL	661.7 (147.7–2137.6)	116.0 (37.0–386.6)	<0.001
IL-1RA	7021.3 (3535.3–15746.9)	2126.0 (879.2–5207.4)	<0.001
IL-6	4257.4 (1331.3–9816.7)	1052.3 (275.6–3978.3)	<0.001
IL-10	138.6 (39.7–384.3)	31.5 (10.9–85.1)	<0.001
IL-12p40	228.7 (105.4–427.9)	101.8 (42.2–213.5)	<0.001

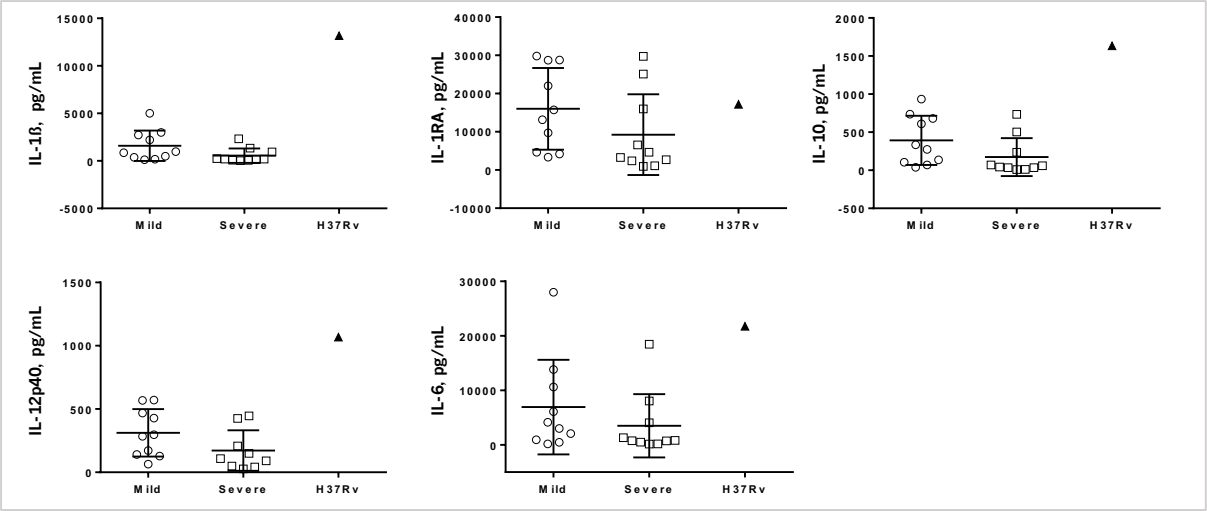

Figure V.3 – Cytokine production induced by mild, severe or H37Rv Trudeau infection of PBMCs. The graph presents the data obtained from infection of PBMCs from one donor with each of the *M. tuberculosis* isolates or H37Rv Trudeau (moi 1:1). The 24 hours post-infection supernatants were harvested and filtered and the production of cytokines measured by Multiplex or ELISA (IL-6) assay. Data is a representative example for all donors.

3. The strain-related hierarchy in inflammatory response is maintained across multiple human donors

The data presented in the previous section strongly support the presence of bacterial factors determining heterogeneous host immune responses. Remarkably, some dispersion was found between the cellular responses of the different donors to each strain, as shown for IL-1β in **Figure V.4**, suggesting an interplay between bacteria and host factors.

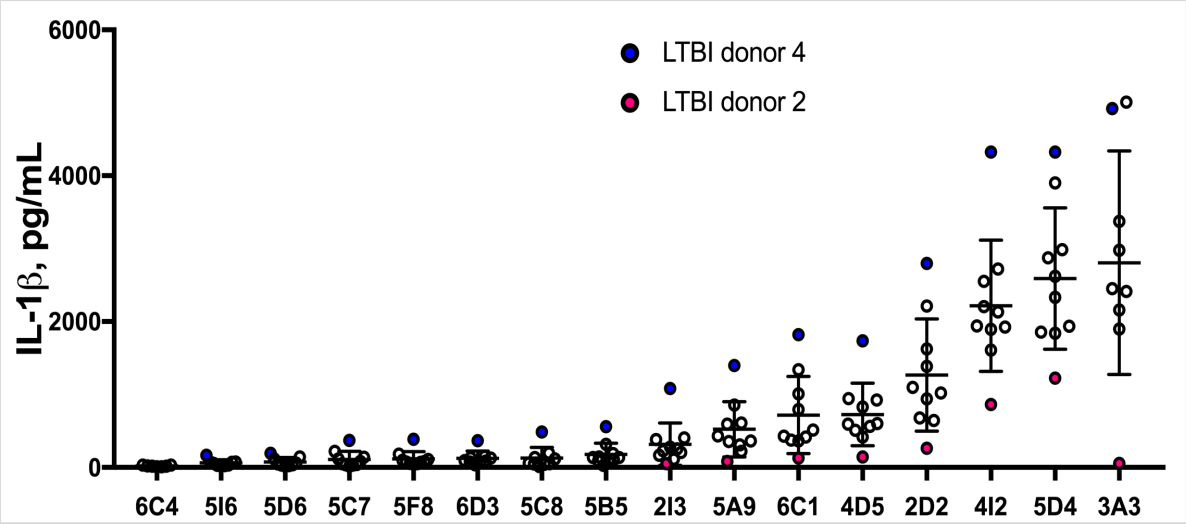

Figure V.4 – Strain-related hierarchy in inflammatory response is maintained across multiple human donors. Scatter plot representation of IL-1β PBMCs production from 10 different LTBI subjects. The response to each strain was ranked according to the average response across the 10 donors. The results show that some strains are low cytokine inducers (6C4 to 5B5), while others are high inducers (2I3 to 3A3). The hierarchy of IL-1β response is maintained across independent donors. It is also evident that some subjects are consistently high responders, such as the LTBI donor 4, and others are exceptionally low responders, even for high-inducing strains, as is the LTBI donor 2. Similar findings were observed for all the other cytokines tested.

When heatmaps based on the host immune response were generated, an overall hierarchy of cytokine production was also found, where low and high responders could be identified among the LTBI participants, independently of the infecting bacteria (**Figure V.5**).

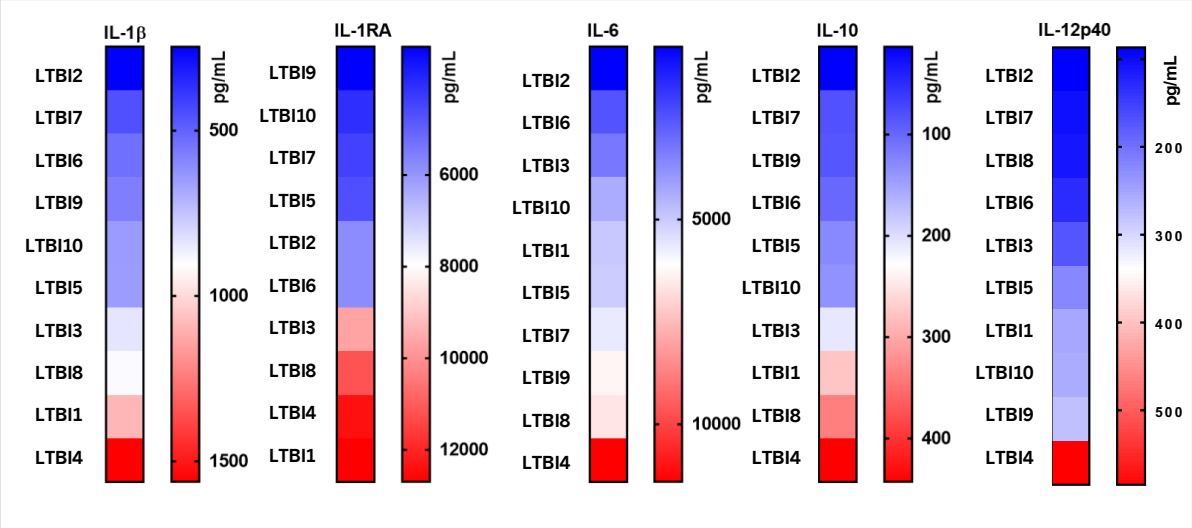

Figure V.5 – Host immune response to all isolates also shows some hierarchy. Heatmap representation of average cytokines production from 10 different LTBI subjects. LTBI donor 2 was a low responder regardless of the strain and cytokine measured, while LTBI donor 4 was a top responder.

It is interesting to note that the pattern of response of the host PBMCs was similar in what respected pro- and anti-inflammatory cytokine production. As an example, a highly significant correlation between production of IL-12p40 and IL-10 (Spearman rank correlation coefficient = 0.788, $P<0.001$) and between IL-1β and IL-1RA ($r_s = 0.882$, $P<0.001$) was observed (**Figure V.6**). This suggested that a common pathway for cytokine production is in place in the different individuals, rather than a specific pro- or anti-inflammatory signalling.

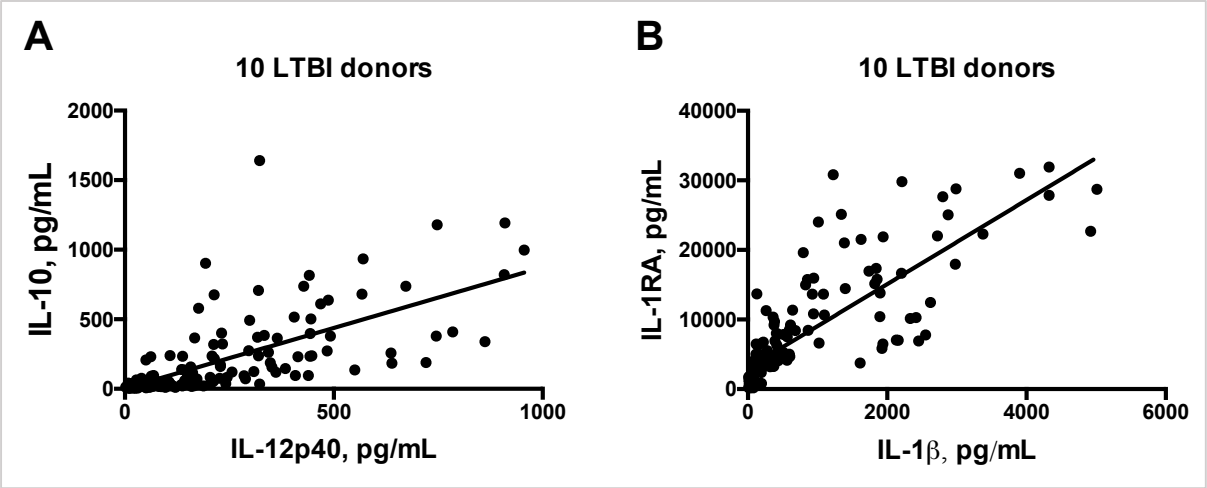

Figure V.6 – Linear regression analysis shows significant correlations between pro- and anti-inflammatory cytokine responses to the different strains used in the study. (A) There was a positive correlation between the levels of IL-12p40 and IL-10 (r_s = 0.788, *P*<0.001), and **(B)** the same was observed between IL-1β and its natural antagonist IL-1RA (r_s = 0.882, *P*<0.001).

4. PBMCs from past active TB patients respond to *M. tuberculosis* infection differently than those from LTBI donors

Next, we investigated whether the hierarchy of responses observed upon infection of PBMCs isolated from non-treated LTBI volunteers with *M. tuberculosis* clinical isolates was maintained after infection of PBMCs from past TB patients. For this, we selected 4 mild TB-associated isolates and 4 severe TB-associated ones and infected PBMCs isolated from another 9 LTBI donors or from 8 past TB patients. As shown before (Figure V.1), the PBMC compartment of each participant was monitored and no differences found in terms of total cell number, cell viability, frequency of lymphocytes or frequency of myeloid cells. We found that the high-inducing (2D2, 3A3, 4I2) and low-inducing (5B5, 5C7, 5D6) isolates retained this same feature in the past TB patients group, with the remaining isolates (5F8 and 4D5) expressing a more heterogeneous profile within the average (**Figure V.7**).

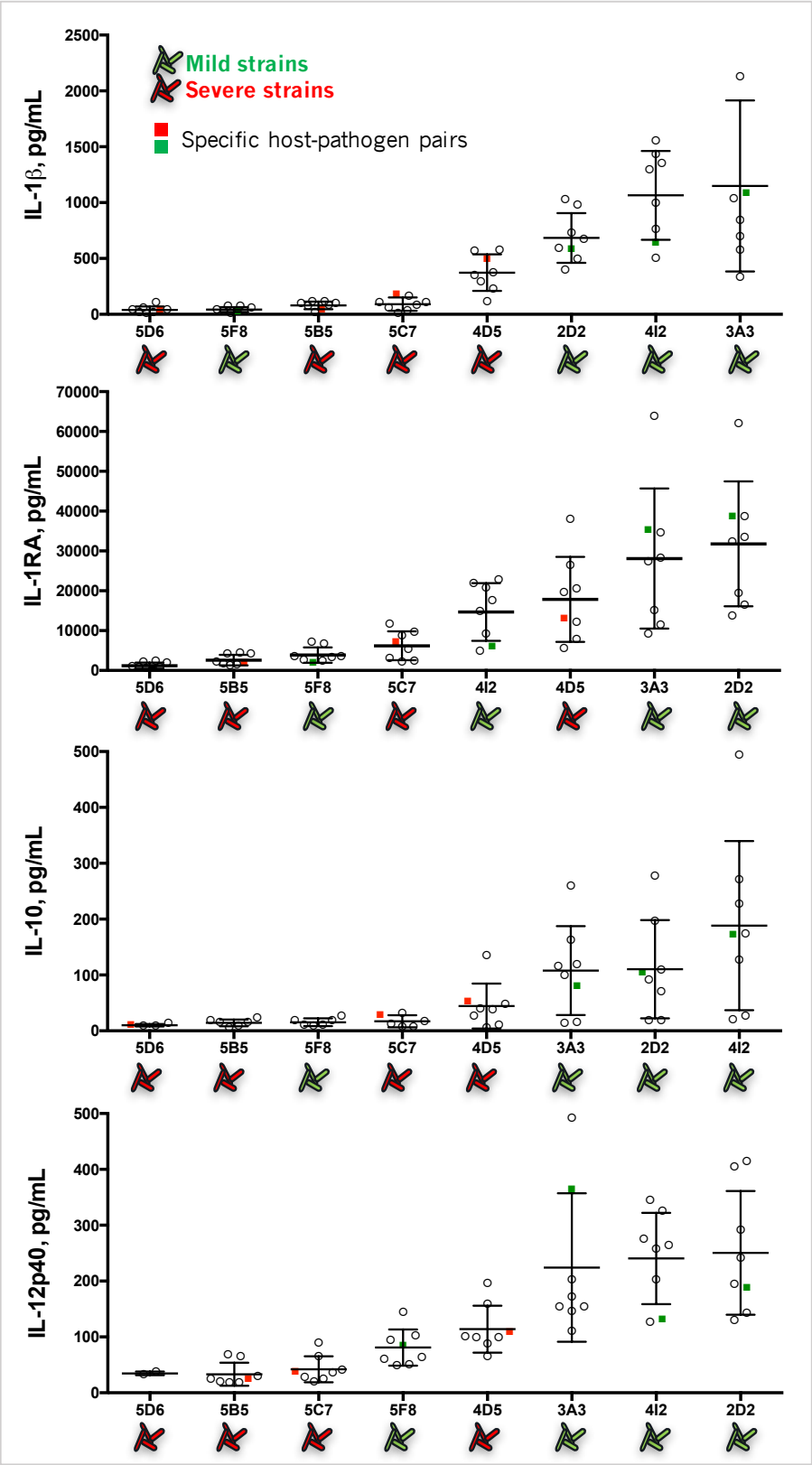

Figure V.7 – The intensity of the response is not related to specific host-pathogen pairs. PBMCs of 8 past TB patients were infected with each of the corresponding strains, associated with either mild (n=4) or severe (n=4) outcome. The response of specific host-pathogen pairs (colored square ■) was concordant with the overall profile for each strain. Missing dots correspond to cases where cytokine production was below detection level.

Therefore, the isolates cytokine-inducing ranking (highest to lowest) was almost overlapping in both past TB patient and LTBI groups. This further argues on the existence of bacterial factors determining the intensity of the immune response. Since the recruited participants had been previously infected with one of the selected isolates, we questioned if a pattern of response was in place for specific host-pathogen pairs. As shown in Figure V.7, this was not the case, with the response of specific host-pathogen pairs being repetitively within the overall range provided by the other hosts.

We then compared the cytokine production after infection of PBMCs from LTBI or past active TB participants with isolates associated with either mild (2D2, 3A3, 4I2, 5F8) or severe (4D5, 5B5, 5C7, 5D6) TB outcomes. This comparison was performed considering data from 19 non-recent/non-treated LTBI subjects and in 8 previous active TB patients. PBMCs from subjects with LTBI secreted significantly more IL-1β, IL-12p40 and IL-10 than those from past TB patients (**Table V.3**). In depth analysis confirmed the same overall trend across all mild (**Figure V.8, A**) and severe (**Figure V.8, B**) strains, though statistical significance was not always achieved for each isolate. It is important to mention that these differences were not observed in the case of IL-1RA and TNF, where similar levels were produced by PBMCs from LTBI or past TB donors.

Table V.3 – *M. tuberculosis* isolates induced significantly less production of IL-1β, IL-12p40 and IL-10 cytokines by infected PBMCs belonging to TB patients, when compared with LTBI donors. PBMCs of 19 LTBI subjects and 8 TB patients were infected with each of the 8 selected strains associated with either mild or severe outcome. Cytokines' concentration values are presented as median (25th–75th percentile). IQR – interquartile range.

Cytokines	LTBI donors	TB patients	*P* value
IL-1β, median (IQR) pg/mL	407.1 (85.3–1361.3)	171.0 (55.3–662.8)	0.007
IL-1RA	6612.6 (2937.5–13626.5)	7386.3 (2362.5–20223.8)	0.218
IL-10	63.2 (20.8–235.9)	26.2 (12.6–110.7)	0.004
IL-12p40	157.4 (63.6–319.7)	101.3 (46.7–194.3)	0.008
TNF-α	154.6 (56.0–434.6)	171.9 (70.6–351.2)	0.976

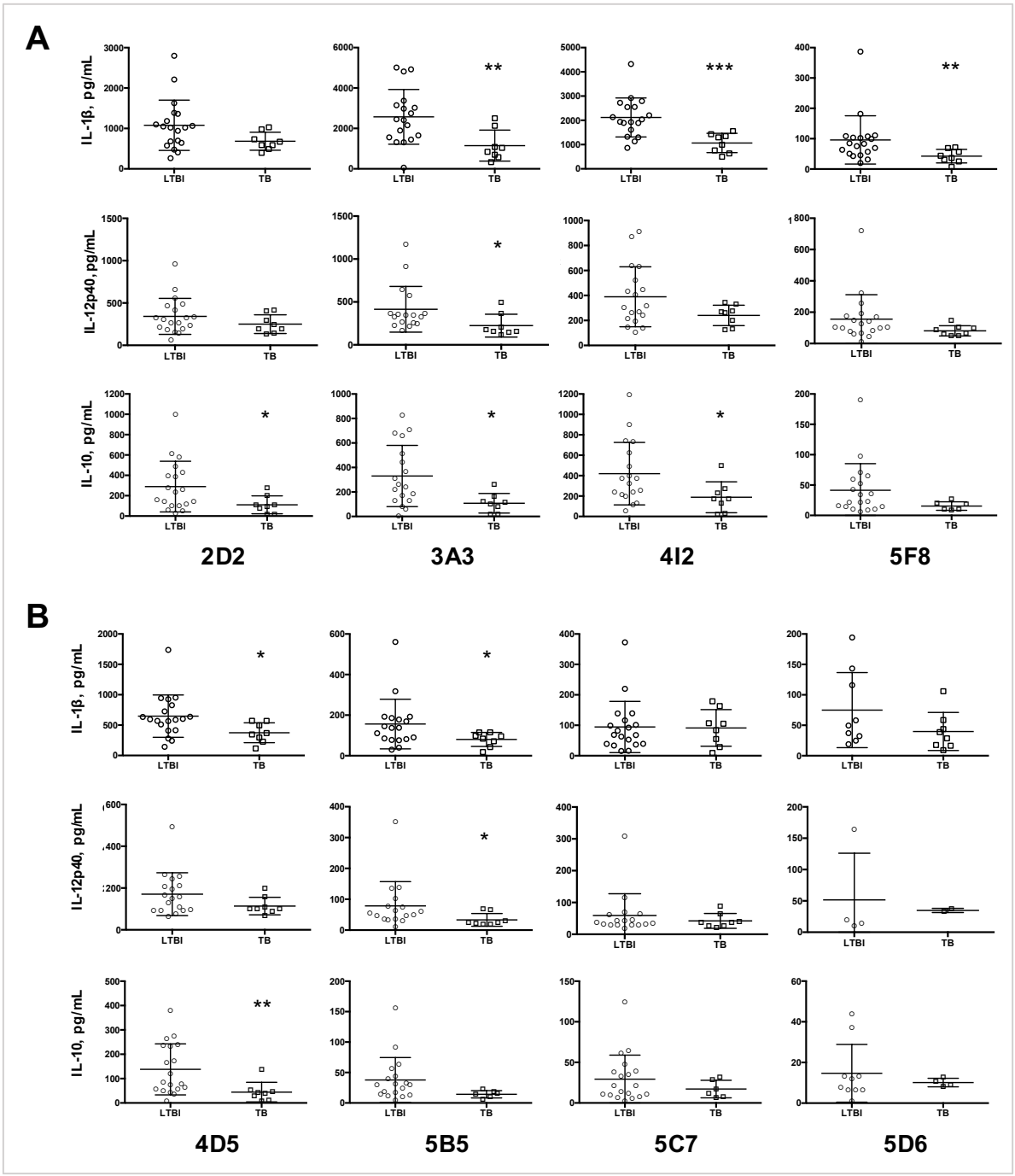

Figure V.8 – Scatter plot representation of the production of IL-1β, IL-12p40 and IL-10 by PBMCs from LTBI donors or past TB patients. (A) Host responses to isolates associated with mild outcome and **(B)** for isolates associated with severe outcome. Each dot represents one infection (missing dots = below detection level). **P*<0.05, ***P*<0.01, ****P*<0.001.

5. TB patients have imbalanced IL-1 signaling

As shown before, PBMCs from past TB patients produced less IL-1β than those from LTBI participants in response to a variety of isolates, whereas no differences were observed for IL-1RA (Table V.3 and Figure V.8). Considering that a competent IL-1 receptor signalling depends on the balance IL-1β/IL-1RA, we next compared the ratio of these two cytokines across the different donors and for different *M. tuberculosis* isolates. Strikingly, we found that with LTBI participants consistently displayed high IL-1β/IL-1RA ratios when compared to past active TB ones (**Figure V.9**). In particular, within the LTBI participants, two subgroups could be distinguished: one associated with a high IL-1β/IL-1RA ratio across all the isolates and another with a ratio comparable to that observed in past active TB participants. This finding led to the hypothesis that an elevated IL-1β/IL-1RA ratio in *M. tuberculosis*-infected PBMCs, may in fact be a biomarker of protection from latent to active TB transition.

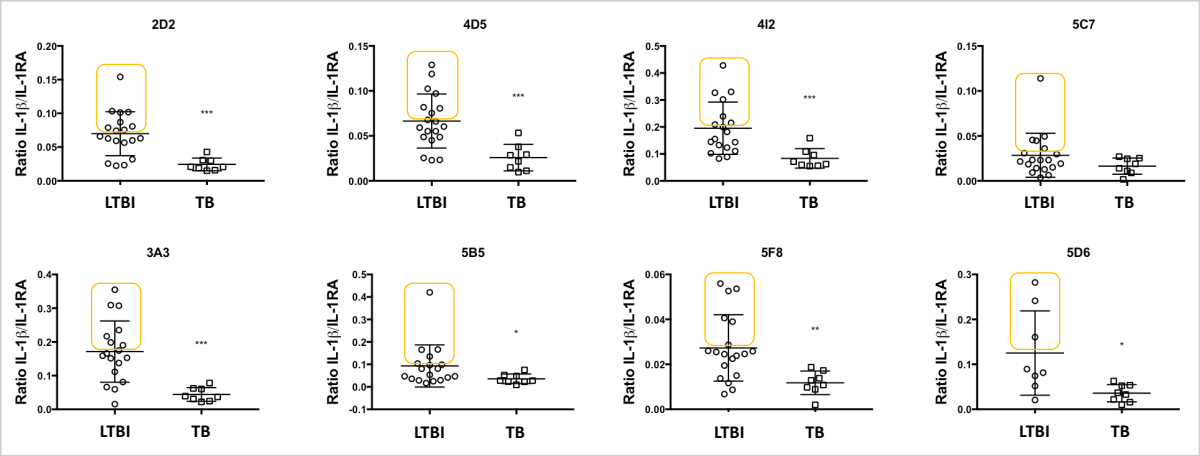

Figure V.9 – Comparison of IL-1β/IL-1RA ratio responses between LTBI and TB groups. All tested strains, except 5C7, elicited an immune response in LTBI subjects characterized by a significantly high IL-1β/IL-1RA ratio, which seems to be a hallmark of protection against active disease.

6. Diagnostic performance of IL-1β/IL-1RA ratio to predict protection

To evaluate the diagnostic performance of IL-1β/IL-1RA ratio in discriminating the PBMC response from LTBI *versus* past active TB, a ROC curve was generated (**Figure V.10, A**). The AUC was 0.752 (95% CI 0.685–0.819) and 0.063 was chosen as the optimum cutoff point, based on Youden's index method [26]. Individuals whose PBMCs displayed a IL-1β/IL-1RA ratio below this threshold are more similar to past active TB ones and may therefore be at a higher risk of developing active disease, with a sensitivity of 90.6%, a specificity of 50.7%, a positive predictive value of 46% and a negative predictive value of 92.1%. These numbers were derived from the values presented in **Table V.4**. It is interesting to note that either cytokine alone did not discriminate the host groups upon ROC curve analysis (**Figure V.10, B**), showing that correlated processes are likely better determinants of infection outcomes than individual molecules.

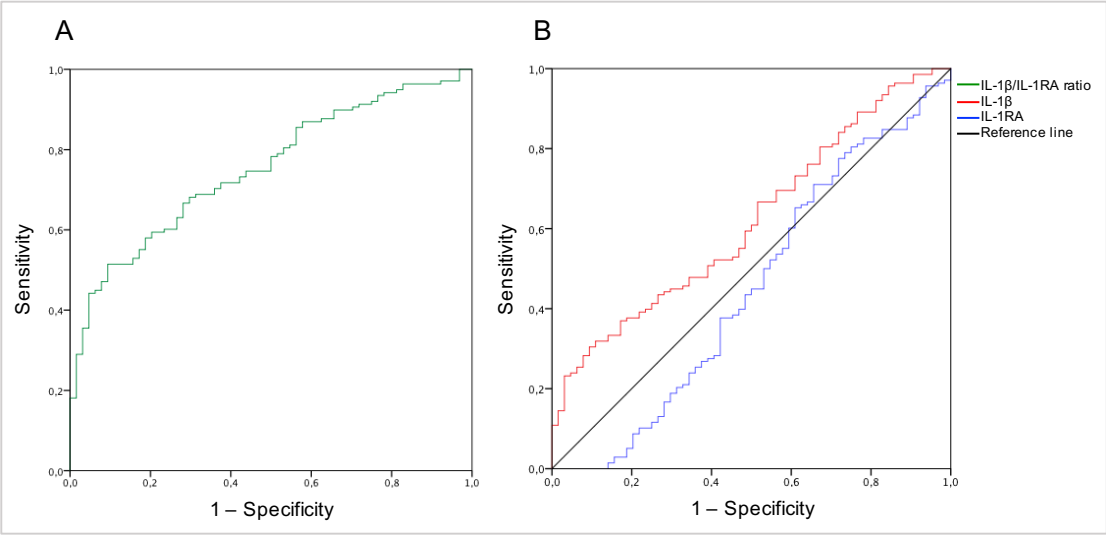

Figure V.10 – ROC curve for the use of IL-1β/IL-1RA ratio as a classifier to distinguish LTBI individuals at risk to active TB transition. The AUC analysis for the IL-1β/IL-1RA ratio **(A)** revealed an accuracy of 0.752 (95% CI 0.685–0.819; *P*<0.001), which was significantly improved when compared to the analysis for either cytokine alone **(B)**: IL-1β AUC 0.619 (95% CI 0.539–0.699; *P*=0.007) and IL-1RA AUC 0.449 (95% CI 0.358–0.541; *P*=0.246).

Table V.4 – Diagnostic performance of IL-1β/IL-1RA ratio to distinguish LTBI individuals at risk to active TB transition. A ratio below 0.063 was considered as "positive test", or risk value. Given this cutoff point, subjects at risk of having active disease (TB) may be identified with a sensitivity of 90.6%[a] and a specificity of 50.7%[b].

IL-1β/IL-1RA ratio	LTBI, n (%)	TB, n (%)	Total
≥0.063 (–)	70 (50.7[b])	6 (9.4)	76
<0.063 (+)	68 (49.3)	58 (90.6[a])	126
Total	138	64	202

DISCUSSION

Previous studies provided the evidence that *M. tuberculosis* strains differ in their ability to stimulate host immune cells [9-13, 29-34]. However, the classical setup for these studies involves the use of *M. tuberculosis* of different lineages to infect immune cells of unrelated hosts. In our study, we resorted to a collection of *M. tuberculosis* clinical isolates for which the outcome of TB is fully characterized and used them to infect PBMCs of geographically related PBMCs. Moreover, the PBMCs obtained belonged to two different groups of participants. On one hand, we recruited individuals who were IGRA+ for longer than 2 years, had not taken therapy for latent TB and had not developed an active TB episode. We consider this group to encompass individuals who are naturally resistant to active TB infection. On the other hand, we also recruited individuals who, despite being immunocompetent, had active TB, but were subsequently cured through successful antibiotherapy. This second group, of past TB patients, encompasses susceptible individuals, but because they are now cured, allows for the elimination of confounding effects caused by acute inflammatory responses related with increased bacterial burdens during active TB. Overall, our experimental setup allows for the investigation of different long-lasting questions in TB:

i) can we draw links between the inflammatory phenotype generated by a specific *M. tuberculosis* isolate and the outcome of TB?

ii) is there a specific response observed for matched pairs of host-*M. tuberculosis* infecting isolate?

iii) can we find differences between the responses of PBMCs from each group to *M. tuberculosis* infection and correlate them with resistance *versus* susceptibility to disease?

To answer these questions, we started by comparing the inflammatory response triggered in PBMCs from LTBI donors by isolates associated with mild or severe outcomes of TB. The classification basis for TB outcomes has been described in Chapter 4. Our data clearly defined a *M. tuberculosis* hierarchy with some isolates inducing low cytokine production for all cytokines tested, in a host-

independent fashion. Our results were consistent with previous reports associating high virulent strains with low inflammatory responses [11, 31, 35-42], highlighting the notion that certain isolates of *M. tuberculosis* appear to have acquired immune evasion strategies to imbalance the immune system in their benefit [43]. Since a certain degree of association was observed between the intensity of the inflammatory response triggered and the outcome of TB, it is tempting to speculate that the initial threshold of the immune response may define the course of infection. If the immune response does not reach a certain threshold of activation, the development of the adaptive response may be delayed [44], the growth of *M. tuberculosis* will not be concealed and the bacterial burden will raise rapidly to a level that would associate with a fast-evolving severe TB. From the evolutionary point of view, that strategy may be advantageous in the context of high human population densities of the modern world, since even when causing potentially fatal disease, the isolates have already provoked lung pathology that ensued its propagation to new hosts. Conversely, a vigorous host immune response after infection may be protective. As a practical example, CDC1551, which is a representative of Lineage 4, was shown to induce high levels of pro-inflammatory cytokines in monocyte cultures [9, 36]. During an outbreak with this strain, despite an unusually high frequency of seroconversion in exposed persons, the rate of new TB cases was not correspondingly high [45]. Thus, despite initial strong immune response, these isolates should be able to induce a complex network of counter-regulating pathways that limit the pathological damage at a later stage. Corroborating this hypothesis, our results showed that high-inflammatory isolates induce both pro- and anti-inflammatory cytokines in similar proportions, as shown with the significant correlations between IL-1β and IL-1RA levels, or between IL-12p40 and IL-10. This observation is in line with previous reports [12, 29] and suggest common induction signals. It is important to highlight that the immune responses were not uniform within each group of severity. While top inducers were always isolates associated with mild outcome, and the least inducers were recovered from severe cases, within the average we observed low-inflammatory mild isolates (such as 6D3) and high-inflammatory severe isolates (such as 6C1). This is not surprising considering that the

complexity of TB is regulated by multiple and largely unknown factors. It will now be important to search for the bacterial determinants associated with high *versus* low cytokine triggering. This can be done through whole genome analysis, as performed in Chapter 4 when investigating genetic players of differential growth.

In what respects the response triggered by specific host-bacteria pairs, our findings demonstrate that the response of PBMCs to the bacteria that previously infected that host is no different to that obtained for other bacteria. This can be justified by the close proximity of the isolates used, that namely belong to the same lineage and, most of them, same sublineage too.

Finally, when comparing the response of PBMCs from LTBI *versus* past active TB donors across different isolates, several conclusions could be drawn. Firstly, we clearly found that certain subjects were high responders whereas others were exceptionally low responders. One possible explanation for this fact may be linked to a differential innate recognition of *M. tuberculosis* strains related with molecular diversity in human TLR genes [46] or a variety of other genetic polymorphisms in the innate immune system that modulate the host response [47]. Resorting to whole genome and functional analysis, we are now investigating the host determinants underlying the observed differences. Another important conclusion came from the comparison of the PBMC responses of LTBI donors with those of past TB patients. We observed that PBMCs from past TB patients secreted significantly less IL-1β, IL-12p40 and IL-10 when infected with a series of isolates. However, their production of IL-1RA was not significatively different. Considering that several studies show that higher IL-1β [48] and IL-10 levels [49, 50] were associated to active disease, our data suggest that bacteria clearance with antibiotherapy brings the host immune response back to a close to basal level. Thus, differences found between LTBI and cured TB donors are more likely reflecting their inherent characteristics, rather than alterations due to infection. In this context, the finding that the IL-1β/IL-1RA is differently modulated in LTBI and past active TB participants could be explored from a biomarker point of view. This would make sense in light of the protective role for IL-1β in TB. Particularly, it is possible that a high IL-1β/IL-1RA ratio associates with protection from progression to active TB, as individuals who had past TB consistently present low

ratios. Long term follow-up of the LTBI participants presenting ratios below 0.063 would be useful to verify if those individuals will develop active disease. A larger prospective longitudinal study will be needed to validate our findings. Similar experiments in other geographical settings would also be important. Our findings also call for deeper studies on the mechanisms modulating the production of IL-1β and IL-1RA upon infection in cells from these different backgrounds. We are now investigating the bases for a higher IL-1β production in some individuals, namely a differential activation of the inflammasome, which is a critical pathway regulating IL-1β production.

In summary, we show that the initial events of the immune response to *M. tuberculosis* infection may be important determinants of the infection outcome and that both pathogen and host molecular elements are in place to modulate these events. Understanding these determinants will help in a better definition of the immune response triggered to different bacteria and in defining possible immunomodulatory strategies in the context of host-directed therapies.

REFERENCES

1. World Health Organization. Global tuberculosis report 2016. Geneva: World Health Organization; 2016.
2. Houben RM, Dodd PJ. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. PLoS Med. 2016;13(10):e1002152.
3. Dye C, Glaziou P, Floyd K, Raviglione M. Prospects for tuberculosis elimination. Annu Rev Public Health. 2013;34:271-86.
4. Zumla A, Maeurer M; Host-Directed Therapies Network, Chakaya J, Hoelscher M, Ntoumi F, Rustomjee R, et al. Towards host-directed therapies for tuberculosis. Nat Rev Drug Discov. 2015;14(8):511-12.
5. Hossain MM, Norazmi MN. Pattern recognition receptors and cytokines in Mycobacterium tuberculosis infection--the double-edged sword? Biomed Res Int. 2013;2013:179174.
6. Comas I, Chakravartti J, Small PM, Galagan J, Niemann S, Kremer K, et al. Human T cell epitopes of Mycobacterium tuberculosis are evolutionarily hyperconserved. Nat Genet. 2010;42:498-503.
7. Rodrigo T, Caylà JA, García de Olalla P, Galdós-Tangüis H, Jansà JM, Miranda P, et al. Characteristics of tuberculosis patients who generate secondary cases. Int J Tuberc Lung Dis. 1997;1(4):352-7.
8. Osório NS, Rodrigues F, Gagneux S, Pedrosa J, Pinto-Carbó M, Castro AG, et al. Evidence for Diversifying Selection in a Set of Mycobacterium tuberculosis Genes in Response to Antibiotic- and Nonantibiotic-Related Pressure. Mol Biol Evol. 2013;30(6):1326-36.
9. Manca C, Tsenova L, Barry CE 3rd, Bergtold A, Freeman S, Haslett PA, et al. Mycobacterium tuberculosis CDC1551 induces a more vigorous host response in vivo and in vitro, but is not more virulent than other clinical isolates. J Immunol. 1999;162(11):6740-6.
10. Dormans J, Burger M, Aguilar D, Hernandez-Pando R, Kremer K, Roholl P, et al. Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different Mycobacterium tuberculosis genotypes in a BALB/c mouse model. Clin Exp Immunol. 2004;137(3):460–8.
11. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, et al. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha/beta. Proc Natl Acad Sci U S A. 2001;98(10):5752-7.
12. Carmona J, Cruz A, Moreira-Teixeira L, Sousa C, Sousa J, Osorio NS, et al. Mycobacterium tuberculosis strains are differentially recognized by TLRs with an impact on the immune response. PLoS One. 2013;8(6):e67277.
13. Portevin D, Gagneux S, Comas I, Young D. Human macrophage responses to clinical isolates from the Mycobacterium tuberculosis complex discriminate between ancient and modern lineages. PLoS Pathog. 2011;7(3):e1001307.
14. Dubois H, Wullaert A, Lamkanfi M. General strategies in inflammasome biology. Curr Top Microbiol Immunol. 2016;397:1-22.
15. Mishra BB, Moura-Alves P, Sonawane A, Hacohen N, Griffiths G, Moita LF, et al. Mycobacterium tuberculosis protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. Cell Microbiol. 2010;12(8):1046-63.
16. Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, et al. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat Immunol. 2010;11(5):395–402.
17. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, Cheever A, et al. Caspase-1 independent IL-1beta production is critical for host resistance to mycobacterium tuberculosis and does not require TLR signaling in vivo. J Immunol. 2010;184(7):3326-30.
18. McElvania Tekippe E, Allen IC, Hulseberg PD, Sullivan JT, McCann JR, Sandor M, et al. Granuloma formation and host defense in chronic Mycobacterium tuberculosis infection requires PYCARD/ASC but not NLRP3 or caspase-1. PLoS One. 2010;5(8):e12320.
19. Condos R, Rom WN, Liu YM, Schluger NW. Local immune responses correlate with presentation and outcome in tuberculosis. Am J Respir Crit Care Med. 1998;157(3 Pt 1):729-35.

20. Tsao TC, Li L, Hsieh M, Liao S, Chang KS. Soluble TNF-alpha receptor and IL-1 receptor antagonist elevation in BAL in active pulmonary TB. Eur Respir J. 1999;14(3):490-5.
21. Lopalco G, Rigante D, Giannini M, Galeazzi M, Lapadula G, Iannone F, et al. Safety profile of anakinra in the management of rheumatologic, metabolic and autoinflammatory disorders. Clin Exp Rheumatol. 2016;34(3):531-8.
22. Cantarini L, Lopalco G, Caso F, Costa L, Iannone F, Lapadula G, et al. Effectiveness and tuberculosis-related safety profile of interleukin-1 blocking agents in the management of Behçet's disease. Autoimmun Rev. 2015;14(1):1-9.
23. Guarda G, Braun M, Staehli F, Tardivel A, Mattmann C, Förster I, et al. Type I interferon inhibits interleukin-1 production and inflammasome activation. Immunity. 2011;34(2):213-23.
24. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature. 2010;466(7309):973-7.
25. World Health Organization. Guidelines for treatment of tuberculosis - 4rd edition Geneva: World Health Organization; 2010.
26. Fluss R, Faraggi D, Reiser B. Estimation of the Youden index and its associated cutoff point. Biom J. 2005;47(4):458-72.
27. van Crevel R, van der Ven-Jongekrijg J, Netea MG, de Lange W, Kullberg BJ, van der Meer JW. Disease-specific ex vivo stimulation of whole blood for cytokine production: applications in the study of tuberculosis. J Immunol Methods. 1999;222(1-2):145-53.
28. Li Y, Oosting M, Deelen P, Ricaño-Ponce I, Smeekens S, Jaeger M, et al. Inter-individual variability and genetic influences on cytokine responses to bacteria and fungi. Nat Med. 2016;22(8):952-60.
29. Wang C, Peyron P, Mestre O, Kaplan G, van Soolingen D, Gao Q, et al. Innate immune response to Mycobacterium tuberculosis Beijing and other genotypes. PLoS One. 2010;5(10):e13594.
30. Krishnan N, Malaga W, Constant P, Caws M, Tran TH, Salmons J, et al. Mycobacterium tuberculosis lineage influences innate immune response and virulence and is associated with distinct cell envelope lipid profiles. PLoS One. 2011;6(9):e23870.
31. Sarkar R, Lenders L, Wilkinson KA, Wilkinson RJ, Nicol MP. Modern lineages of Mycobacterium tuberculosis exhibit lineage-specific patterns of growth and cytokine induction in human monocyte-derived macrophages. PLoS One. 2012;7(8):e43170.
32. López B, Aguilar D, Orozco H, Burger M, Espitia C, Ritacco V, et al. A marked difference in pathogenesis and immune response induced by different Mycobacterium tuberculosis genotypes. Clin Exp Immunol. 2003;133(1):30-7.
33. Dunn PL, North RJ. Virulence ranking of some Mycobacterium tuberculosis and Mycobacterium bovis strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice. Infect Immun. 1995;63(9):3428-37.
34. Aguilar D, Hanekom M, Mata D, Gey van Pittius NC, van Helden PD, Warren RM, et al. Mycobacterium tuberculosis strains with the Beijing genotype demonstrate variability in virulence associated with transmission. Tuberculosis. 2010;90(5):319-25.
35. Li Q, Whalen CC, Albert JM, Larkin R, Zukowski L, Cave MD, et al. Differences in rate and variability of intracellular growth of a panel of Mycobacterium tuberculosis clinical isolates within a human monocyte model. Infect Immun. 2002;70(11):6489-93.
36. Manca C, Reed MB, Freeman S, Mathema B, Kreiswirth B, Barry CE 3rd, et al. Differential monocyte activation underlies strain-specific Mycobacterium tuberculosis pathogenesis. Infect Immun. 2004;72(9):5511-4.
37. Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, Murray PJ, et al. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res. 2005;25(11):694-701.
38. Tsenova L, Ellison E, Harbacheuski R, Moreira AL, Kurepina N, Reed MB, et al. Virulence of selected Mycobacterium tuberculosis clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli. J Infect Dis. 2005;192(1):98-106.
39. Chacón-Salinas R, Serafín-López J, Ramos-Payán R, Méndez-Aragón P, Hernández-Pando R, Van Soolingen D, et al. Differential pattern of cytokine expression by macrophages infected in vitro with different Mycobacterium tuberculosis genotypes. Clin Exp Immunol. 2005;140(3):443-9.

40. Rocha-Ramírez LM, Estrada-García I, López-Marín LM, Segura-Salinas E, Méndez-Aragón P, Van Soolingen D, et al. Mycobacterium tuberculosis lipids regulate cytokines, TLR-2/4 and MHC class II expression in human macrophages. Tuberculosis. 2008;88(3):212-20.
41. Subbian S, Bandyopadhyay N, Tsenova L, O'Brien P, Khetani V, Kushner NL, et al. Early innate immunity determines outcome of Mycobacterium tuberculosis pulmonary infection in rabbits. Cell Commun Signal. 2013;11:60.
42. Reiling N, Homolka S, Walter K, Brandenburg J, Niwinski L, Ernst M, et al. Clade-specific virulence patterns of Mycobacterium tuberculosis complex strains in human primary macrophages and aerogenically infected mice. MBio. 2013;4(4):e00250-13.
43. Newton SM, Smith RJ, Wilkinson KA, Nicol MP, Garton NJ, Staples KJ, et al. A deletion defining a common Asian lineage of Mycobacterium tuberculosis associates with immune subversion. Proc Natl Acad Sci U S A. 2006;103(42):15594-8.
44. Rakotosamimanana N, Raharimanga V, Andriamandimby SF, Soares JL, Doherty TM, Ramarokoto H, et al. Variation in IFN-{gamma} responses to different infecting strains of Mycobacterium tuberculosis in AFB smear positive patients and household contacts in Antananarivo, Madagascar. Clin Vaccine Immunol. 2010;17(7):1094–103.
45. Valway SE, Sanchez MP, Shinnick TF, Orme I, Agerton T, Hoy D, et al. An outbreak involving extensive transmission of a virulent strain of Mycobacterium tuberculosis. N Engl J Med. 1998;338(10):633-9.
46. Velez DR, Wejse C, Stryjewski ME, Abbate E, Hulme WF, Myers JL, et al. Variants in toll-like receptors 2 and 9 influence susceptibility to pulmonary tuberculosis in Caucasians, African-Americans, and West Africans. Hum Genet. 2010;127:65-73.
47. Azad AK, Sadee W, Schlesinger LS. Innate immune gene polymorphisms in tuberculosis. Infect Immun. 2012;80:3343–59.
48. Prabhavathi M, Kabeer BS, Deenadayalan A, Raja A. Role of QuantiFERON-TB Gold antigen-specific IL-1β in diagnosis of active tuberculosis. Med Microbiol Immunol. 2015;204(5):567-74.
49. Tebruegge M, Dutta B, Donath S, Ritz N, Forbes B, Camacho-Badilla K, et al. Mycobacteria-Specific Cytokine Responses Detect Tuberculosis Infection and Distinguish Latent from Active Tuberculosis. Am J Respir Crit Care Med. 2015;192(4):485-99.
50. Jeong YH, Hur YG, Lee H, Kim S, Cho JE, Chang J, et al. Discrimination between active and latent tuberculosis based on ratio of antigen-specific to mitogen-induced IP-10 production. J Clin Microbiol. 2015;53(2):504-10.

CHAPTER 6 – GENERAL DISCUSSION

In 2015 tuberculosis (TB) remained the main cause of death by a single infectious agent and one of the top 10 causes of death overall [1]. Not only the high number of active TB patients, but also the massive reservoir of latently infected individuals hamper TB control, putting at risk the elimination target set to 2050 [2, 3]. To effectively address these two groups, an understanding of the course of infection in different individuals is needed, so that treatment measures could be applied in a rational way to those at higher risk of disease. Indeed, clinical biomarkers of progression along the continuous spectrum of TB, from latent to active forms, are just starting to be unveiled [4-9] and many observations indicate a role of the host immune response in this process [10-17]. Overall, available evidence shows that the immune condition of an infected host is going to be shaped by genetics and by extrinsic factors that alter the local micro-environment [18], but also by the heterogeneity of the infecting bacteria [19]. Altogether, this *troika* determines the threshold of the immune response generated during infection and consequently the disease outcome. Modulating these thresholds and uncovering the links between host, pathogen and micro-environments should allow for the discovery of solid correlates of protection, molecular markers for disease prognosis, and the development of safe and effective host-directed therapies (HDT) to TB [20-22].

The starting point of the current project was to understand which mechanisms underlie TB clinical heterogeneity. We approached this question from different angles, by combining the study of the pathogen properties, the host immune response and the clinical features of TB, within a cohort of 681 culture-confirmed pulmonary TB cases diagnosed at the Hospital de São João, a major healthcare center in Porto, Portugal, between 2007 and 2013. Such a cohort, where for each patient the clinical files and the infecting *Mycobacterium tuberculosis* isolate were available, provided the foundations for this work.

We started by examining the clinical files for all patients with culture-confirmed TB, which led to the establishment of a clinical prediction rule to stratify TB patients according to their risk of death [23]. The proposed prediction model was named TReAT (Tuberculosis Risk Assessment Tool) and presented as

major strengths (1) the clear definition of the predictor variables (age; presence of comorbidities; disease extent determined by respiratory failure and one radiographic feature; and a measure of consumption or overall nutritional status, the hemoglobin level), and (2) an external validation in an independent cohort, attesting the general applicability to both hospital and ambulatory settings. In addition to understand the main severity factors in TB, which generally are confirmed with previous studies [24-33], we provided a prognosis tool to identify high-risk cases, that often have rapidly progressive disease and are at major risk of dying within the first 30 days after diagnosis. We discuss that this particular group need a stricter medical supervision and may benefit on the admission in high-dependency units. As TB incidence rates decrease with a combination of near-universal access to high-quality diagnosis and treatment and general socioeconomic development, it is expected that the TB death rate will become one of the core indicators for disease control [34]. In this context, the implementation of TReAT may improve treatment outcomes. Current efforts are being made to plan a prospective validation study in a different location.

After focusing on the host clinical indicators associated with the prognosis of death in TB patients, in the second part of the project we wanted to assess the impact of *M. tuberculosis* diversity on the disease clinical severity. We started by developing a clinical decision tree to classify the severity of the disease and by applying it to the group of individuals that in our cohort did not present known predictor or precipitator TB factors [35]. The disease presentation in these "otherwise healthy" TB patients was classified into 5 possible outcomes: mild, moderate, severe, extremely severe and disseminated. Whereas disseminated TB in immunocompetent hosts appeared to correlate with the natural course of disease, individuals within the mild, moderate or severe presented similar time of symptoms. Thus, these 3 categories most likely reflect an outcome of disease resulting from different factors or events, and not simply reflecting the course of infection. The most common outcome for the "otherwise healthy" group was the moderate TB. It is possible that the moderate isolates represent the most successful group of *M. tuberculosis* complex (MTBC) strains, that has evolved to interact with the immune cells and cause destructive cavitary lung lesions, a

marker of high transmissible disease [36], but only mildly inflicting systemic deleterious effects, so that the host is able to continue at ambulatory for enough time to keep spreading the disease. Further studies are now being pursued to decipher how the presence of major TB comorbidities, as HIV and diabetes, alter this disease presentation.

Next we investigated the *M. tuberculosis* phylogeny within the "otherwise healthy" individuals and its phylogenetic distribution along the disease severity. We found a highly homogeneous phylogenetic composition, with almost all isolates belonging to Lineage 4 (Euro-American) and within this family, the majority of them to sublineage Latin-American-Mediterranean (LAM). This is in line with previous descriptions for Portugal [37, 38]. These results are also consistent with the fact that the vast majority of subjects included in the study were Portuguese-born Caucasians, thus further supporting the described phylogeographical structure of MTBC population [39]. The phylogenetic classification of the MTBC provided the rational framework for a number of studies around the world linking the genotypic diversity of MTBC to heterogeneity in clinical phenotypes [40-53]. In the present study, disease severity was not related to any specific phylogenetic taxa, although a decrease on the prevalence of the LAM sublineage was observed from mild to moderate to severe forms of disease. This is indicative that any bacterial contribution to the outcome of disease in "otherwise healthy" TB patients is not due to ancestral events, but instead to the occurrence of microevolution. We are currently addressing the distribution of the different phylogenies in patients with a disrupted immune system (such as HIV) to investigate for the loss of any sympatric interactions between *M. tuberculosis* and its host in our specific cohort. We are also performing whole genome analysis to compare isolates associated with mild or severe disease, with the goal of finding traceable variations that could be used as prognosis of disease outcomes.

Although no association was found between the ancestry of the infecting isolate and the clinical outcome of TB, our results showed a correlation between the growth patterns of MTBC strains and the TB clinical severity. Isolates obtained from patients with mild disease grew significantly slower in liquid media. These

isolates generally caused non-cavitary lung disease and minimal symptoms. We propose that limited growth may be one of the features required for decreased virulence. Similar associations have been described previously with Lineage 3, that was reported to be less transmissible compared to other modern lineages [54], and also grows slowly in liquid broth [55-57]. Likewise, Lineage 6 (*Mycobacterium africanum* West-Africa 2) exhibits slower progression to active disease [51] and slower growth rate [58]. Although growth in liquid broth only partially reflects the much more complex infection pathogenesis in humans, this method has already proved to be well suited to compare the growth behaviour of different bacterial isolates [58]. Conversely, moderate and severe strains present higher growth rates, with doubling times shorter by 4-5 h, when compared to mild isolates. In particular, isolates associated to moderate outcomes showed a remarkably distinct growth pattern, characterized by a longer lag phase, followed by a steep exponential phase, that is related to a very short doubling time (around 15 h), and finally reaching the highest plateau. The clinical relevance of this finding is still unclear, but may contribute for the success of this group of strains, as described above in the context of its prevalence in the present cohort. Previous studies have assessed intracellular growth of *M. tuberculosis* in human macrophages as a marker of virulence [59-62]. Furthermore, one study demonstrated that the Beijing-family strain 210 (Lineage 2), responsible for an outbreak in Los Angeles, grows more rapidly than small cluster or unique cluster strains in human macrophages [63]. It will now be important to understand if the growth differences reported hold in infected cells and organisms. For this, we will perform experiments using *in vitro* macrophage culture systems and the mouse model of infection.

To unveil the underlying mechanisms for the observed differences in the growth profiles, we compared the genome sequences of fast growing (moderate) strains with the slow growing isolates (associated to mild and severe clinical outcome). We found single nucleotide polymorphisms (SNPs) in 1135 genes, with varying levels of allelic differences between groups. Only 83 (7.3%) genes had allelic differences greater than 40% (ΔDAF >0.4), while the large majority of SNPs formed a cluster below ΔDAF 0.4. The top ΔDAF was found for the gene *ctpF*,

encoding a P-type ATPase of the plasma membrane [64], which is responsible for generating the electrochemical gradient necessary for transmembrane ion transport, possibly Mg^{2+} or Ca^{2+} [65]. Previous evidence suggest that this gene is upregulated under hypoxic environment [66] and helps to protect mycobacteria against toxic heavy-metal cation levels, as well as maintaining cell volume in hostile conditions and nutrient transport across the cell membrane [67]. The transporter function was overrepresented in the unbiased analysis. Future studies will be dedicated to the functional implications of our bioinformatics predictions.

When we investigated the group of genes already determined to affect *in* vitro [68] and *in vivo* [69] growth, of the 16 genes presenting ΔDAF >0.4, nine presented non-synonymous SNPs. In particular, three gene products (*Rv2252*, *pgi*, *hisI*) were predicted by SIFT analysis to be affected in their protein function. These three proteins are the most likely candidates responsible for the observed profile of fast growing isolates. The candidates are involved in essential biological pathways, such as protein synthesis (*hisI*), cellular metabolism (*pgi*) and biosynthesis of cell envelope components (*Rv2252*).

In addition to previously characterized pathways, many genes identified in the unbiased genome-wide analysis are conserved hypotheticals with unknown function. Further biological validation on these loci is warranted. This is important, because the identification of the pathways that are required for mycobacterial growth provide insight into the structural and metabolic requirements of the pathogen and may direct the rational design of effective antimicrobial agents towards new molecular targets in the moderate-to-severe and drug-resistant strains.

Finally, in the third part of this work, we studied the architecture of the immune response triggered by the different isolates of *M. tuberculosis* associated with mild and severe phenotypes in peripheral blood mononuclear cells (PBMCs) from latent TB infected (LTBI) donors or past TB patients. Although many studies provide evidence that *M. tuberculosis* strains differ in their ability to stimulate the host immune cells [57, 70-79], the links of inflammatory phenotypes with clinical outcomes remain elusive. Our findings clearly show that even within a relatively homogeneous group of *M. tuberculosis* isolates, diversity in the cytokine

production triggered in PBMCs existed. Certain isolates of *M. tuberculosis* appear to have acquired immune evasion strategies to reduce the cellular response at early stages of infection. As these low inflammatory isolates tended to be associated with severe outcomes of TB, it is possible that this adaptation occurs in the pathogen benefit, with a permissive immune response allowing the rapid mycobacterial growth that induces exacerbated lung pathology [56, 57, 59, 76, 80-86]. From the evolutionary point of view, that strategy may be advantageous in the context of high human population densities of the modern world, since even when severe strains cause potentially fatal disease, the isolates have already provoked large pulmonary cavitations that ensued its propagation to new hosts. To gain more insight in this association, a higher number of isolates needs to be tested.

Interestingly, this strain-related hierarchy in the response induced in PBMCs upon infection was maintained across multiple human donors, independently of being LTBI or past TB participants. This strongly suggests the existence of bacterial molecular determinants associated with high *versus* low triggers. As before, whole genome comparisons might prove useful to identify and study these molecular signatures. Also, comparing the progression of infection with two extreme bacterial examples in mice might provide important information about the consequences of the differential initial interaction to the course of the immune response, bacterial burdens and tissue pathology.

Despite the importance of the bacteria in determining the intensity of the immune response, we also observed that some donors were consistently high responders and others were exceptionally low responders, even for high-inducing strains. Therefore, host factors also play a role in determining the final cytokine response against each clinical isolate. It is plausible that a diverse range of mutations might influence the innate immune recognition of the pathogen, something that is now under study in the lab.

One of the most interesting findings in this work relates to the identification of the IL-1β/IL-1RA ratio as a possible discriminator of latently infected individuals. We found that independently of the *M. tuberculosis* isolate used to infect PBMCs, two possible responses were observed: a high IL-1β/IL-1RA ratio,

which was only observed in a subgroup of LTBI participants; and a low IL-1β/IL-1RA ratio, which was observed in some LTBI participants and in all past TB ones. These two groups could be discriminated with a ROC curve analysis, showing that despite the still low numbers, a robust result is already obtained. Assuming a genetic basis for this observation, we are impelled to think that individuals with a high ratio may be at a less risk of progressing to active TB disease. However, because in active TB a high production of IL-1β has been reported [87], it is also possible that latent participants with a high ratio may actually represent a subgroup of individuals still undergoing a dynamic fight with the bacteria, providing evidence for trained immunity [88]. To clarify these various possibilities similar experiments performed with more participants in these two groups and also with IGRA negative and active TB participants will be required. In any case, it is tempting to speculate that an imbalance of the IL-1 receptor signalling might be a biomarker of susceptibility to active disease after infection. This in turn awaits validation in prospective studies, being now planned in the lab. Clarifying this issue is of utmost importance as it would provide a platform for rational management of LTBI, namely by highlighting those individuals who would benefit more from preventive antibiotherapy.

In summary, this work combined the study of clinical data, infecting bacteria and human PBMC responses to *M. tuberculosis*. A new clinical prediction rule for the risk of death in TB patients was developed and validated, and a new classification tree for TB severity was proposed. On the pathogen side, we unveiled the differential growth of clinical isolates associated with moderate outcomes of TB as a distinctive feature. On the host side, we suggest the ratio IL-1β/IL-1RA as a possible biomarker of disease resistance *versus* susceptibility to TB. This way, we believe that our findings present new platforms for active and latent TB management and open new avenues for basic research, to unveil host and pathogen determinants of TB outcomes.

REFERENCES

1. World Health Organization. Global tuberculosis report 2016. Geneva: World Health Organization; 2016.
2. Houben RM, Dodd PJ. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. PLoS Med. 2016;13(10):e1002152.
3. Dye C, Glaziou P, Floyd K, Raviglione M. Prospects for tuberculosis elimination. Annu Rev Public Health. 2013;34:271-86.
4. Kim IJ, Lee JS, Kim SJ, Kim YK, Jeong YJ, Jun S, et al. Double-phase 18F-FDG PET-CT for determination of pulmonary tuberculoma activity. Eur J Nucl Med Mol Imaging. 2008;35(4):808-14.
5. Lin PL, Rodgers M, Smith L, Bigbee M, Myers A, Bigbee C, et al. Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. Infect Immun. 2009;77(10):4631-42.
6. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature. 2010;466(7309):973-7.
7. Weiner J 3rd, Parida SK, Maertzdorf J, Black GF, Repsilber D, Telaar A, et al. Biomarkers of inflammation, immunosuppression and stress with active disease are revealed by metabolomic profiling of tuberculosis patients. PLoS One. 2012;7(7):e40221.
8. Kaforou M, Wright VJ, Oni T, French N, Anderson ST, Bangani N, et al. Detection of tuberculosis in HIV-infected and -uninfected African adults using whole blood RNA expression signatures: a case-control study. PLoS Med. 2013;10(10):e1001538.
9. Lin PL, Ford CB, Coleman MT, Myers AJ, Gawande R, Ioerger T, et al. Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. Nat Med. 2014;20(1):75-9.
10. Cooper AM, Mayer-Barber KD, Sher A. Role of innate cytokines in mycobacterial infection. Mucosal Immunol. 2011;4(3):252-60.
11. Cooper AM. Cell-mediated immune response in TB. Annu Rev Immunol. 2009;27:393-422.
12. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. Annu Rev Immunol. 2013;31:475–527.
13. Dorhoi A, Reece ST, Kaufmann SH. For better or for worse: the immune response against Mycobacterium tuberculosis balances pathology and protection. Immunol Rev. 2011;240(1):235-51.
14. Sia JK, Georgieva M, Rengarajan J. Innate Immune Defenses in Human Tuberculosis: An Overview of the Interactions between Mycobacterium tuberculosis and Innate Immune Cells. J Immunol Res. 2015;2015:747543.
15. Nunes-Alves C, Booty MG, Carpenter SM, Jayaraman P, Rothchild AC, Behar SM. In search of a new paradigm for protective immunity to TB. Nat Rev Microbiol. 2014;12(4):289-99.
16. Jasenosky LD, Scriba TJ, Hanekom WA, Goldfeld AE. T cells and adaptive immunity to Mycobacterium tuberculosis in humans. Immunol Rev. 2015;264(1):74-87.
17. Cliff JM, Kaufmann SH, McShane H, van Helden P, O'Garra A. The human immune response to tuberculosis and its treatment: a view from the blood. Immunol Rev. 2015;264(1):88-102.
18. Esmail H, Barry CE 3rd, Young DB, Wilkinson RJ. The ongoing challenge of latent tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2014;369(1645):20130437.
19. Coscolla M, Gagneux S. Does M. tuberculosis genomic diversity explain disease diversity? Drug Discov Today Dis Mech. 2010;7(1):e43.
20. Zumla A, Maeurer M; Host-Directed Therapies Network, Chakaya J, Hoelscher M, Ntoumi F, Rustomjee R, et al. Towards host-directed therapies for tuberculosis. Nat Rev Drug Discov. 2015;14(8):511-12.
21. Zumla A, Maeurer M, Host-Directed Therapies Network (HDT-NET) Consortium. Host-directed therapies for tackling multi-drug resistant tuberculosis: learning from the Pasteur-Bechamp debates. Clin Infect Dis. 2015;61(9):1432–8.

22. Wallis RS, Hafner R. Advancing host-directed therapy for tuberculosis. Nat Rev Immunol. 2015;15(4):255–63.
23. Bastos HN, Osório NS, Castro AG, Ramos A, Carvalho T, Meira L, et al. A prediction rule to stratify mortality risk of patients with pulmonary tuberculosis. PLoS One. 2016;11(9):e0162797.
24. Lefebvre N, Falzon D. Risk factors for death among tuberculosis cases: analysis of European surveillance data. Eur Respir J. 2008;31(6):1256-60.
25. Low S, Ang LW, Cutter J, James L, Chee CB, Wang YT, et al. Mortality among tuberculosis patients on treatment in Singapore. Int J Tuberc Lung Dis. 2009;13(3):328-34.
26. Walpola HC, Siskind V, Patel AM, Konstantinos A, Derhy P. Tuberculosis-related deaths in Queensland, Australia, 1989-1998: characteristics and risk factors. Int J Tuberc Lung Dis. 2003;7(8):742-50.
27. Horita N, Miyazawa N, Yoshiyama T, Sato T, Yamamoto M, Tomaru K, et al. Development and validation of a tuberculosis prognostic score for smear-positive in-patients in Japan. Int J Tuberc Lung Dis. 2013;17(1):54-60.
28. Lui G, Wong RY, Li F, Lee MK, Lai RW, Li TC, et al. High mortality in adults hospitalized for active tuberculosis in a low HIV prevalence setting. PLoS One. 2014;9(3):e92077.
29. Wejse C, Gustafson P, Nielsen J, Gomes VF, Aaby P, Andersen PL, et al. TBscore: Signs and symptoms from tuberculosis patients in a low-resource setting have predictive value and may be used to assess clinical course. Scand J Infect Dis. 2008;40(2):111-20.
30. Isanaka S, Mugusi F, Urassa W, Willett WC, Bosch RJ, Villamor E, et al. Iron deficiency and anemia predict mortality in patients with tuberculosis. J Nutr. 2012;142(2):350-7.
31. Marcy O, Laureillard D, Madec Y, Chan S, Mayaud C, Borand L, et al. Causes and Determinants of Mortality in HIV-Infected Adults With Tuberculosis: An Analysis From the CAMELIA ANRS 1295-CIPRA KH001 Randomized Trial. Clin Infect Dis. 2014;59(3):435-45.
32. Lanoix JP, Gaudry S, Flicoteaux R, Ruimy R, Wolff M. Tuberculosis in the intensive care unit: a descriptive analysis in a low-burden country. Int J Tuberc Lung Dis. 2014;18(5):581-7.
33. Balkema CA, Irusen EM, Taljaard JJ, Koegelenberg CF. Tuberculosis in the intensive care unit: a prospective observational study. Int J Tuberc Lung Dis. 2014;18(7):824-30.
34. Pontali E, Centis R, D'Ambrosio L, Migliori GB. Monitoring predictors of mortality: A necessary action to reach TB elimination. Rev Port Pneumol. 2015;21(6):295-8.
35. H. Esmail, C. E. Barry, 3rd, D. B. Young, R. J. Wilkinson. The ongoing challenge of latent tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2014;369(1645):20130437. doi: 10.1098/rstb.2013.0437. PubMed PMID: 24821923; PubMed Central PMCID: PMC4024230.
36. Rodrigo T, Caylà JA, García de Olalla P, Galdós-Tangüis H, Jansà JM, Miranda P, et al. Characteristics of tuberculosis patients who generate secondary cases. Int J Tuberc Lung Dis. 1997;1(4):352-7.
37. Lopes JS, Marques I, Soares P, Nebenzahl-Guimaraes H, Costa J, Miranda A, et al. SNP typing reveals similarity in Mycobacterium tuberculosis genetic diversity between Portugal and Northeast Brazil. Infect Genet Evol. 2013;18:238-46.
38. Stucki D, Brites D, Jeljeli L, Coscolla M, Liu Q, Trauner A, et al. Mycobacterium tuberculosis lineage 4 comprises globally distributed and geographically restricted sublineages. Nat Genet. 2016;48(12):1535-43.
39. Gagneux S. Host–pathogen coevolution in human tuberculosis. Phil Trans R Soc B. 2012;367(1590):850–9.
40. Caws M, Thwaites G, Dunstan S, Hawn TR, Lan NT, Thuong NT, et al. The influence of host and bacterial genotype on the development of disseminated disease with Mycobacterium tuberculosis. PLoS Pathog. 2008;4(3):e1000034.
41. Kong Y, Cave MD, Zhang L, Foxman B, Marrs CF, Bates JH, et al. Association between Mycobacterium tuberculosis Beijing/W lineage strain infection and extrathoracic tuberculosis: Insights from epidemiologic and clinical characterization of the three principal genetic groups of M. tuberculosis clinical isolates. J Clin Microbiol. 2007;45(2):409-14.
42. Kong Y, Cave MD, Yang D, Zhang L, Marrs CF, Foxman B, et al. Distribution of insertion- and deletion-associated genetic polymorphisms among four Mycobacterium tuberculosis phospholipase C genes and associations with extrathoracic tuberculosis: a population-based study. J Clin Microbiol. 2005;43(12):6048-53.

43. Lari N, Rindi L, Cristofani R, Rastogi N, Tortoli E, Garzelli C. Association of Mycobacterium tuberculosis complex isolates of M. bovis and Central Asian (CAS) genotypic lineages with extrapulmonary disease. Clin Microbiol Infect. 2009;15(6):538-43.
44. Stavrum R, PrayGod G, Range N, Faurholt-Jepsen D, Jeremiah K, Faurholt-Jepsen M, et al. Increased level of acute phase reactants in patients infected with modern Mycobacterium tuberculosis genotypes in Mwanza, Tanzania. BMC Infect Dis. 2014;14:309.
45. Parwati I, Alisjahbana B, Apriani L, Soetikno RD, Ottenhoff TH, van der Zanden AG, et al. Mycobacterium tuberculosis Beijing genotype is an independent risk factor for tuberculosis treatment failure in Indonesia. J Infect Dis. 2010;201(4):553-7.
46. Lan NT, Lien HT, Tung le B, Borgdorff MW, Kremer K, van Soolingen D. Mycobacterium tuberculosis Beijing genotype and risk for treatment failure and relapse, Vietnam. Emerg Infect Dis. 2003;9(12):1633-5.
47. Sun YJ, Lee AS, Wong SY, Paton NI. Association of Mycobacterium tuberculosis Beijing genotype with tuberculosis relapse in Singapore. Epidemiol Infect. 2006;134(2):329-32.
48. Ford CB, Shah RR, Maeda MK, Gagneux S, Murray MB, Cohen T, et al. Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. Nat Genet. 2013;45(7):784-90.
49. Wollenberg KR, Desjardins CA, Zalutskaya A, Slodovnikova V, Oler AJ, Quiñones M, et al. Whole genome sequencing of Mycobacterium tuberculosis provides insight into the evolution and genetic composition of drug-resistant tuberculosis in Belarus. J Clin Microbiol. 2016. Epub Nov 30. doi: 10.1128/JCM.02116-16.
50. Gurjav U, Erkhembayar B, Burneebaatar B, Narmandakh E, Tumenbayar O, Hill-Cawthorne GA, et al. Transmission of multi-drug resistant tuberculosis in Mongolia is driven by Beijing strains of Mycobacterium tuberculosis resistant to all first-line drugs. Tuberculosis. 2016;101:49-53.
51. de Jong BC, Hill PC, Aiken A, Awine T, Antonio M, Adetifa IM, et al. Progression to active tuberculosis, but not transmission, varies by Mycobacterium tuberculosis lineage in The Gambia. J Infect Dis. 2008;198(7):1037–43.
52. Kato-Maeda M, Kim EY, Flores L, Jarlsberg LG, Osmond D, Hopewell PC. Differences among sublineages of the East-Asian lineage of Mycobacterium tuberculosis in genotypic clustering. Int J Tuberc Lung Dis. 2010;14(5):538-44.
53. Gehre F, Antonio M, Faïhun F, Odoun M, Uwizeye C, de Rijk P, et al. The first phylogeographic population structure and analysis of transmission dynamics of M. africanum West African 1--combining molecular data from Benin, Nigeria and Sierra Leone. PLoS One. 2013;8(10):e77000.
54. Albanna AS, Reed MB, Kotar KV, Fallow A, McIntosh FA, Behr MA, et al. Reduced transmissibility of East African Indian strains of Mycobacterium tuberculosis. PLoS One. 2011;6(9):e25075.
55. Tanveer M, Hasan Z, Kanji A, Hussain R, Hasan R. Reduced TNF-alpha and IFN-gamma responses to Central Asian strain 1 and Beijing isolates of Mycobacterium tuberculosis in comparison with H37Rv strain. Trans R Soc Trop Med Hyg. 2009;103(6):581-7.
56. Newton SM, Smith RJ, Wilkinson KA, Nicol MP, Garton NJ, Staples KJ, et al. A deletion defining a common Asian lineage of Mycobacterium tuberculosis associates with immune subversion. Proc Natl Acad Sci U S A. 2006;103(42):15594-8.
57. Sarkar R, Lenders L, Wilkinson KA, Wilkinson RJ, Nicol MP. Modern lineages of Mycobacterium tuberculosis exhibit lineage-specific patterns of growth and cytokine induction in human monocyte-derived macrophages. PLoS One. 2012;7(8):e43170.
58. Gehre F, Otu J, DeRiemer K, de Sessions PF, Hibberd ML, Mulders W, et al. Deciphering the growth behaviour of Mycobacterium africanum. PLoS Negl Trop Dis. 2013;7(5):e2220.
59. Li Q, Whalen CC, Albert JM, Larkin R, Zukowski L, Cave MD, et al. Differences in rate and variability of intracellular growth of a panel of Mycobacterium tuberculosis clinical isolates within a human monocyte model. Infect Immun. 2002;70(11):6489-93.
60. Zhang M, Gong J, Yang Z, Samten B, Cave MD, Barnes PF. Enhanced capacity of a widespread strain of Mycobacterium tuberculosis to grow in human macrophages. J Infect Dis. 1999;179(5):1213–7.
61. Hoal-van Helden EG, Hon D, Lewis LA, Beyers N, van Helden PD. Mycobacterial growth in human macrophages: variation according to donor, inoculum and bacterial strain. Cell Biol Int. 2001;25(1):71-81.

62. Wong KC, Leong WM, Law HK, Ip KF, Lam JT, Yuen KY, et al. Molecular characterization of clinical isolates of Mycobacterium tuberculosis and their association with phenotypic virulence in human macrophages. Clin Vaccine Immunol. 2007;14(10):1279-84.
63. Theus SA, Cave MD, Eisenach KD. Intracellular macrophage growth rates and cytokine profiles of Mycobacterium tuberculosis strains with different transmission dynamics. J Infect Dis. 2005;191(3):453-60.
64. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 1998;393(6685):537-44.
65. Agranoff D, Krishna S. Metal ion transport and regulation in Mycobacterium tuberculosis. Front Biosci. 2004;9:2996-3006.
66. Pulido PA, Novoa-Aponte L, Villamil N, Soto CY. The DosR dormancy regulator of Mycobacterium tuberculosis stimulates the Na(+)/K (+) and Ca (2+) ATPase activities in plasma membrane vesicles of mycobacteria. Curr Microbiol. 2014;69(5):604-10.
67. Ayala-Torres C, Novoa-Aponte L, Soto CY. Pma1 is an alkali/alkaline earth metal cation ATPase that preferentially transports Na(+) and K(+) across the Mycobacterium smegmatis plasma membrane. Microbiol Res. 2015;176:1-6.
68. Sassetti CM, Boyd DH, Rubin EJ. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol. 2003;48(1):77-84.
69. Rengarajan J, Bloom BR, Rubin EJ. Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. Proc Natl Acad Sci U S A. 2005;102(23):8327-32.
70. Manca C, Tsenova L, Barry CE 3rd, Bergtold A, Freeman S, Haslett PA, et al. Mycobacterium tuberculosis CDC1551 induces a more vigorous host response in vivo and in vitro, but is not more virulent than other clinical isolates. J Immunol. 1999;162(11):6740-6.
71. Carmona J, Cruz A, Moreira-Teixeira L, Sousa C, Sousa J, Osorio NS, et al. Mycobacterium tuberculosis strains are differentially recognized by TLRs with an impact on the immune response. PLoS One. 2013;8(6):e67277.
72. Portevin D, Gagneux S, Comas I, Young D. Human macrophage responses to clinical isolates from the Mycobacterium tuberculosis complex discriminate between ancient and modern lineages. PLoS Pathog. 2011;7(3):e1001307.
73. Krishnan N, Malaga W, Constant P, Caws M, Tran TH, Salmons J, et al. Mycobacterium tuberculosis lineage influences innate immune response and virulence and is associated with distinct cell envelope lipid profiles. PLoS One. 2011;6(9):e23870.
74. Wang C, Peyron P, Mestre O, Kaplan G, van Soolingen D, Gao Q, et al. Innate immune response to Mycobacterium tuberculosis Beijing and other genotypes. PLoS One. 2010;5(10):e13594.
75. Dormans J, Burger M, Aguilar D, Hernandez-Pando R, Kremer K, Roholl P, et al. Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different Mycobacterium tuberculosis genotypes in a BALB/c mouse model. Clin Exp Immunol. 2004;137(3):460–8.
76. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, et al. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha/beta. Proc Natl Acad Sci U S A. 2001;98(10):5752-7.
77. López B, Aguilar D, Orozco H, Burger M, Espitia C, Ritacco V, et al. A marked difference in pathogenesis and immune response induced by different Mycobacterium tuberculosis genotypes. Clin Exp Immunol. 2003;133(1):30-7.
78. Dunn PL, North RJ. Virulence ranking of some Mycobacterium tuberculosis and Mycobacterium bovis strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice. Infect Immun. 1995;63(9):3428-37.
79. Aguilar D, Hanekom M, Mata D, Gey van Pittius NC, van Helden PD, Warren RM, et al. Mycobacterium tuberculosis strains with the Beijing genotype demonstrate variability in virulence associated with transmission. Tuberculosis. 2010;90(5):319-25.
80. Manca C, Reed MB, Freeman S, Mathema B, Kreiswirth B, Barry CE 3rd, et al. Differential monocyte activation underlies strain-specific Mycobacterium tuberculosis pathogenesis. Infect Immun. 2004;72(9):5511-4.

81. Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, Murray PJ, et al. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res. 2005;25(11):694-701.
82. Tsenova L, Ellison E, Harbacheuski R, Moreira AL, Kurepina N, Reed MB, et al. Virulence of selected Mycobacterium tuberculosis clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli. J Infect Dis. 2005;192(1):98-106.
83. Chacón-Salinas R, Serafín-López J, Ramos-Payán R, Méndez-Aragón P, Hernández-Pando R, Van Soolingen D, et al. Differential pattern of cytokine expression by macrophages infected in vitro with different Mycobacterium tuberculosis genotypes. Clin Exp Immunol. 2005;140(3):443-9.
84. Rocha-Ramírez LM, Estrada-García I, López-Marín LM, Segura-Salinas E, Méndez-Aragón P, Van Soolingen D, et al. Mycobacterium tuberculosis lipids regulate cytokines, TLR-2/4 and MHC class II expression in human macrophages. Tuberculosis. 2008;88(3):212-20.
85. Subbian S, Bandyopadhyay N, Tsenova L, O'Brien P, Khetani V, Kushner NL, et al. Early innate immunity determines outcome of Mycobacterium tuberculosis pulmonary infection in rabbits. Cell Commun Signal. 2013;11:60.
86. Reiling N, Homolka S, Walter K, Brandenburg J, Niwinski L, Ernst M, et al. Clade-specific virulence patterns of Mycobacterium tuberculosis complex strains in human primary macrophages and aerogenically infected mice. MBio. 2013;4(4):e00250-13.
87. Condos R, Rom WN, Liu YM, Schluger NW. Local immune responses correlate with presentation and outcome in tuberculosis. Am J Respir Crit Care Med. 1998;157(3 Pt 1):729-35.
88. Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, et al. Trained immunity: A program of innate immune memory in health and disease. Science. 2016;352(6284):aaf1098.