Universitat Autònoma de Barcelona

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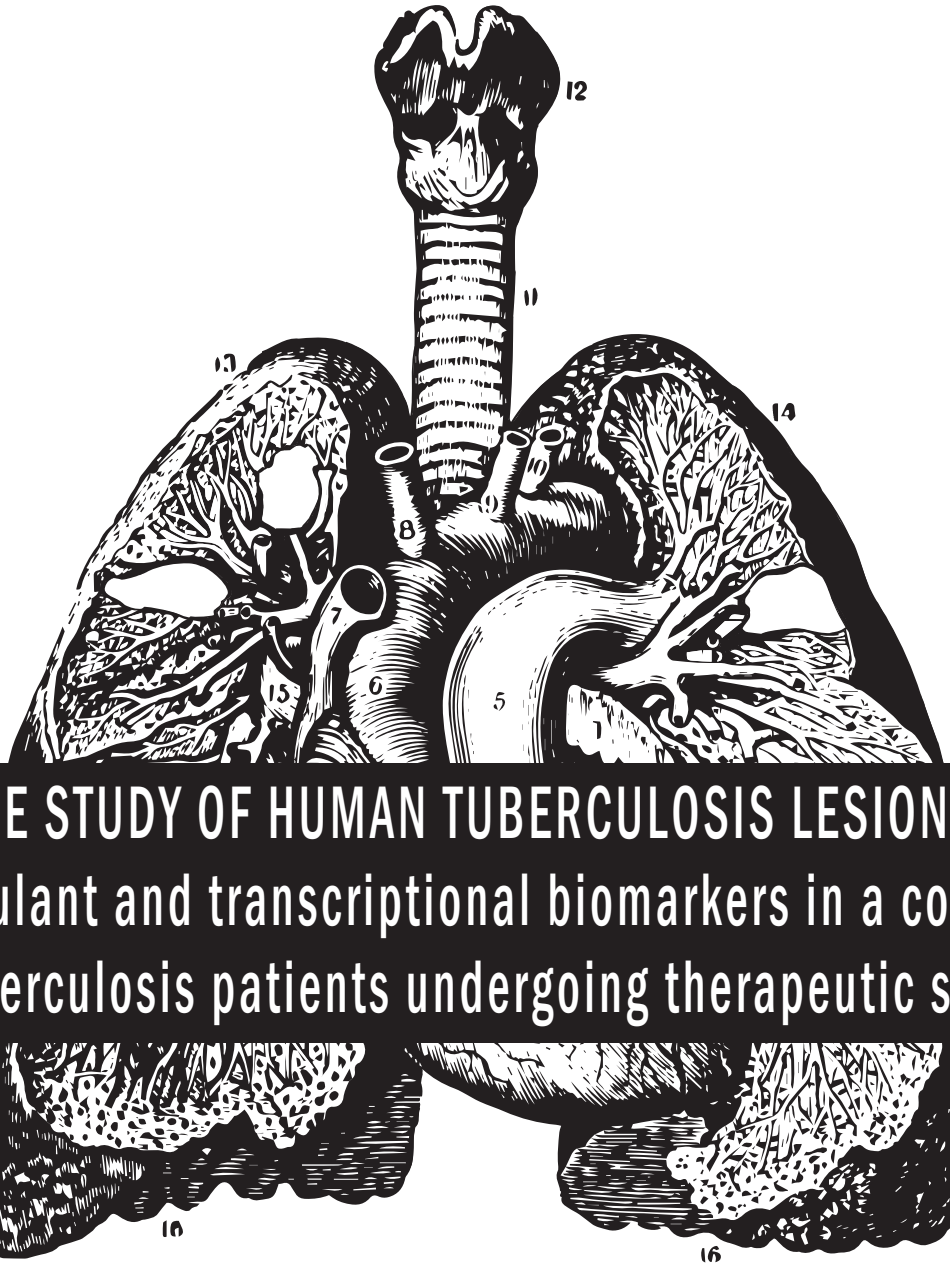

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PhD THESIS - 2020

PROGRAMA DE DOCTORAT EN MICROBIOLOGIA
MENCIÓ INTERNACIONAL
UNIVERSITAT AUTÒNOMA DE BARCELONA

DOCTORAL THESIS

Programa de Doctorat en Microbiologia
Menció Doctor Internacional – International Doctoral Research Component
Departament de Genètica i Microbiologia
Universitat Autònoma de Barcelona
2020

The Study of Human Tuberculosis Lesions: circulant and transcriptional biomarkers in a cohort of tuberculosis patients undergoing therapeutic surgery

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Fundació Institut d'investigació en Ciències de la Salut Germans Trias i Pujol (IGTP)

Unitat de Tuberculosi Experimental (UTE)

Doctoral thesis to obtain the Ph.D. in Microbiology by the Department of Genetics and Microbiology from Universitat Autònoma de Barcelona

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"Crying won't help you,
praying won't do you no good"

Robert Plant
When the levee breaks - Led Zeppelin

ACKNOWLEDGEMENTS | AGRAÏMENTS

This project has received funding from the Spanish Government-European Regional Development Fund (FEDER) [CP13/00174, CPII18/00031] and [PI16/01511], from the "CIBER Enfermedades Respiratorias" (CIBERES) Network, the "Spanish Society of Pneumology and Thoracic Surgery" (SEPAR) [16/023], and the "Agència de Gestió d'Ajuts Universitaris i de Recerca" (AGAUR) [2017 FI_B_00797].

I would like to start by saying thanks to doctor Sergo Vashakidze. Without him, and his team, this thesis would not exist. I feel deeply grateful to him for being very kind during all these years and all the help he has given us tirelessly. It has been an honor to have collaborated with such an expert on the TB field and hopefully, we will do more science together in the future. Also, I would like to thank all the commitment of doctor Myrsini Kaforou with this project. She hosted me in London for three months and she showed me a whole new world behind a screen. I would like to thank her invaluable help in a key part of this study but especially for helping me with the tons of work I had to do. I'm super proud of what I learned on bioinformatics and the feel that this is something really interesting and I love, which is thanks to you. I feel very lucky to have learned from an expert like you and all your lessons from this part of the path. Thank you so much Sergo and Myrsini!

No podria començar d'altra manera que poder donar les gràcies per haver arribat fins aquí als meus pares. Mama, papa, els anys han volat però des que vaig posar el primer peu a la universitat fins ara només he rebut suport i comprensió per part vostre, i us voldria agrair tot l'esforç, la molta paciència i dedicació que m'heu brindat per a que pugui arribar aquest moment. L'educació, la cultura de l'esforç, la tenacitat, la persistència i la curiositat per voler comprendre aquest món boig en el que vivim són els millors regals que m'heu fet des que vaig néixer i encara no sé com us ho podré agrair mai. No hi ha prou pàgines en aquesta tesi que pugui omplir amb paraules boniques cap a vosaltres. Simplement dir-vos que us estimo molt i que aquesta tesi us la dedico a vosaltres dos.

No puc deixar d'agrair a les meves iaies, la iaia Quimeta i la iaia Joana de totes les coses bones que m'han passat a la vida. Si algú pot presumir que té dues mares jo puc dir que en tinc tres. Des de ben petit m'heu tractat com si fos el vostre fill i us

heu esmerçat amb tot el que teníeu perquè jo sigui una persona feliç. No us ho podré agrair prou mai. També vull recordar als meus avis, el Pere i en Paquito, que de ben segur que estarien molt orgullosos de mi i traurien pit davant els seus amics dient que "el meu net és doctor i els vostres no".

A la meva germana Anna, que sempre, sempre hi ets quan et necessito. No puc tenir una millor germana gran, que es preocupi tant per mi i que sempre vulgui el millor per mi. Sempre seràs la millor companyia per riure'ns de les bogeries del papa i la mama. A les preciositats de les meves nebodes, la Laia i l'Arlet, m'agradaria també dedicar-vos part d'aquesta tesi a vosaltres dues. Espero que tot aquest llibre que ha escrit el vostre tiu us faci despertar algun dia la curiositat per la ciència i la vida, i la recerca de la veritat. Aquí em tindreu sempre, sempre, sempre pel que necessiteu. No em puc oblidar a la resta de la família, tiet Jordi, tia Esther, als meus cosins Jordi, Sergi i Jordina, així com la Yana, el l'Anton, al Félix i a l'Enric. Gràcies, gràcies, i més gràcies per tot a tots. No em podeu fer més feliç!

Gran part del mèrit de tot això que hi ha escrit darrere aquestes pàgines és també gràcies a l'Anna, el meu amor. Sé que han estat uns mesos molt durs pels dos, confinats a casa, separats, escrivint aquesta interminable tesi, però no obstant sempre has estat al meu costat donant-me forces quan més ho necessitava. Sé que he posat en risc la teva inesgotable paciència per culpa dels meus deliris derivats d'aquest tros de paper, de les meves pors, de les meves inseguretats, però de nou sempre et trobava. No hi ha hagut dia que no t'hagi tingut ben a la vora per ajudar-me en el que fos, per escoltar-me, per donar-me consell. És per això que una part d'aquesta tesi també és teva. No ho podria haver aconseguit sense tu. T'estimo molt Anna. I et prometo que mai, mai més tornaré a escriure una tesi doctoral.

Un pilar bàsic de la meva vida són els meus amics. Els meus amics de tota la vida. Els meus germans i germanes. La meva estranya família. Amb molta probabilitat, no seria qui sóc sense vosaltres. Potser seria millor persona, qui sap. No obstant, vull donar-vos les gràcies per haver-me acompanyat en tot aquest viatge. Heu estat una peça clau i bàsica per no perdre el control. Ha estat divertit, però no repetiré. Gràcies Alex, Ernest, Eric (Posho), Ibon, Esteve, Isaac, Erik, Ana, Alfredo, Ivan, Jou, Roger, David, Maria, Sara, Ester i Alba. Sou increïbles, companys! Sóc molt afortunat de tenir-vos com a amics. Us estimo moltíssim!

A les feres, les meves feres. La meva segona família. Tot va començar amb vosaltres seient al final de les classes de Bellvitge, i mireu fins a on hem arribat. Qui ens ho havia de dir! Això se'ns n'ha anat de les mans. Estic molt feliç d'haver arribat fins aquí però molt més haver-ho fet amb vosaltres ben a prop. No podria haver tingut més sort d'haver-me topat amb una colla farcida de bones persones, normalment, com vosaltres, amics. Gràcies Nacho, Inma, Edu, Lidia, Helena, Lili, Laura, Mireia i Txell. Gràcies família. Us estaré sempre molt agraït, per tot. Us estimo infinit!

Si soc on soc i he arribat fins a on he arribat és gràcies a la Cris Vilaplana. Moltes gràcies, per no dir que infinites, per la grandiosa oportunitat que em vas donar. No podria haver anat a parar a unes millors mans que les teves. Gràcies per tot el que m'has donat, gràcies per totes les lliçons que m'has ensenyat, gràcies per escoltar-me i entendre'm tantes i tantes vegades, i sobretot gràcies per confiar en mi. Em sento molt afortunat que t'hagi tingut com a directora de tesi però encara més de poder tenir-te com una persona de referència tant per a la meva carrera professional com per a la vida en general. I per últim, gràcies per la paciència que tens i que sovint et faig perdre, però això ja va inclòs en aquest ofici, imagino.

Vull donar les gràcies també a en Pere-Joan, que des del primer dia em va obrir les portes de la UTE i m'ha tractat com un més de la família. Em sento francament molt afortunat d'haver pogut aprendre de tu i sobretot que m'ho hagi passat tan i tan bé tots aquests anys. Tampoc podria haver arribat fins aquí sense la incansable ajuda de la Lili. Et dec tantíssim que no sabria per on començar. Només puc, hores d'ara, donar-te les gràcies per ser així (excepte quan no m'estimes) i ajudar-me tantíssim sempre. Ha estat una sort immensa poder haver treballat tant de temps amb una amiga com tu. Gràcies Lili, ets lo millor de lo millor. Si algú es mereix una especial dedicatòria és la Laura. Sense tu Laura la rutina diària hauria estat més monòtona i avorrida. Sense tu molts dels problemes que he tingut probablement no s'haurien solucionat tan fàcilment. Sense tu no hauria estat el mateix haver passat per la UTE. Laura t'haig d'agrair moltíssim tota la teva ajuda, paciència i comprensió. Em sento molt afortunat d'haver pogut treballar amb tu i molt orgullós de tenir-te com a amiga. Part d'aquesta tesi doctoral també vull que te la sentis com a teva. I no em puc oblidar de la resta de la UTE, Paula, Marta, Ester i Kaori gràcies

per estimar-me sempre que heu pogut i gràcies per no liquidar-me sempre que heu volgut. Ha estat molt divertit i em sap greu que arribi a la fi.

I a la resta de gent de l'IGTP, gràcies Erika, Meri, Gemma, Yaiza, Jorge, Maria Rosa, Eli, Vio, Maribel, Eugeni, Marina, Alfonsina, Marta Montguió, Laura, Emi, Marta Carrió, Lucía, Quim, Marco, Gerard, i tanta més gent que tan m'heu donat tant durant tots aquests anys. No hauria estat igual sense tots vosaltres.

GRÀCIES A TOTS! THANK YOU ALL!

INDEX

ABSTRACT

Tuberculosis (TB) is the worldwide leading cause of death among infectious diseases. Therapeutic surgery is still an invaluable tool to resolve the most complicated TB cases, for instance, if the patient is experiencing persistent lung cavities despite good adherence to chemotherapy. Scientific efforts are focused on identifying which clinical-epidemiological factors lead a patient to evolve poorly during treatment and to identify biomarkers that can predict the treatment response, health status, and fatal outcomes. Nonetheless, these strategies are not reflecting the in situ lung pathology, and therefore, the local host-to-pathogen response is misrepresented. We hypothesized that the study of the local and systemic immune responses from patients undergoing therapeutic surgery for their pulmonary TB could help us to identify potential TB biomarkers and essential information regarding the role of the host in the mechanisms associated to the generation of the TB lesions. Clinical-epidemiological data of a cohort of 137 patients undergoing therapeutic surgery for pulmonary TB were retrospectively analyzed according to the macroscopic features of the removed TB lesions. Next, in a new cohort of 40 patients also receiving therapeutic surgery, we assessed the levels of circulating immune markers and we performed RNA-seq upon the fresh human TB granuloma biopsies, to be correlated with the pathophysiological phenotype of the participants together with the macroscopic lesions' characteristics. We found the persistence of *Mycobacterium tuberculosis* in surgical TB cavitary biopsies despite microbiological clearance in culture. Sex and toxic habits are important factors that may determine the evolution of the disease. Circulant biomarkers correlated with the size of the lesion, with multi-drug resistant forms, and factors considered to indicate the worst disease outcomes. We noted an immunosuppressive effect exerted by the presence of the TB lesions, suggested by the immune-markers and the human TB granuloma transcriptome. Finally, we generated a 6056-gene signature of the human TB granuloma and a list of genes of interest gathering the total-RNA expression of the main pathophysiological traits of the cohort. The proposed platform of circulant biomarkers and its genes should be further validated and assessed in other active TB patients to confirm the potential use as a prognostic tool.

RESUM

La tuberculosi (TB), és la major causant de morts associades a malalties infeccioses al món. La cirurgia terapèutica segueix sent una eina essencial en els casos més complicats de TB, per exemple, si el pacient mostra persistència de lesions cavitàries malgrat bona adherència als antibiòtics. Avui dia, la recerca en TB està centrada en identificar factors clínics-epidemiològics que condueixen al pacient a evolucionar negativament durant el tractament i identificar biomarcadors que puguin predir l'estat de salut i una mala prognosi. Aquestes estratègies no reflecteixen la malaltia pulmonar in situ, i per tant, la resposta local de l'hostatger al patogen no està representada. En aquesta tesi doctoral vàrem hipotetizar que l'estudi de la resposta immunològica local i sistèmica de pacients que rebran cirurgia terapèutica per la seva TB pulmonar podria ajudar a determinar nous biomarcadors així com informació essencial respecte els mecanismes de resposta de l'hostatger en la generació de les lesions tuberculoses. Es van analitzar retrospectivament dades clínic-epidemiològiques considerant les característiques macroscòpiques de les lesions obtingudes en una cohort de 137 pacients tuberculosos sotmesos a cirurgia. En una nova cohort de 40 pacients sotmesos a cirurgia terapèutica, vam avaluar els nivells de marcadors immunològics circulants i vam realitzar RNA-seq en biòpsies fresques de granuloma tuberculós humà, per ser correlacionades amb el perfil fisiopatològic dels participants juntament amb les característiques macroscòpiques de les lesions extirpades. Vam detectar persistència del *Mycobacterium tuberculosis* en biòpsies de lesions malgrat negativitat microbiològica en cultiu. El sexe i hàbits tòxics son factors importants que podrien determinar l'evolució de la TB. Biomarcadors circulants correlacionen amb la mida de la lesió, formes multi-resistents i factors considerats de mal pronòstic. Es va detectar un efecte immunosupressor induït per la presència de les lesions, suggerit per marcadors immunològics i el transcriptoma de la lesió tuberculosa. Vam generar una signatura de 6056 gens del granuloma tuberculós humà i una llista de gens d'interès que aglomeren l'expressió gènica de les característiques fisiopatològiques de la cohort. La plataforma de biomarcadors circulants i els gens haurien de ser validats i avaluats en altres pacients amb TB i confirmar la seva potencial utilitat com a eina de prognosi.

ABBREVIATIONS

AEC	Airway Epithelial Cells
AFB	Acid-Fast Bacilli
ALB	Serum Albumin
AM	Alveolar Macrophages
ATT	Anti-tuberculosis Treatment
AUC	Area Under the Curve
BCG	Bacille Calmette-Guérin
BMI	Body Mass Index
BSL-3	BioSafety Level 3
C	Center of the Lesion
CRP	C-Reactive Protein
CT Scan	Computed Tomography Scan
CXCL	C-X-X motif chemokine
CXR	Chest X-Ray
DC	Dendritic Cells
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DOT	Directly Observed Therapy
DST	Drug-Susceptibility Testing
DS-TB	Drug-Sensitive Tuberculosis
E	External Wall of the Lesion
eCRF	electronic Case Report Form
EPTB	Extrapulmonary Tuberculosis
ES	Enrichment Score
FDR	False Discovery Rate
FFPE	formalin-fixed paraffin-embedded
GDA	General Discriminant Analysis
GSEA	Gene Set Enrichment Analysis
H	Healthy lung parenchyma
HBV	Hepatitis B Virus

HCV	Hepatitis C Virus
HDT	Host-Directed Therapies
HIV	Human Immunodeficiency Virus
HK	Housekeeping
HRZE	Isoniazid, Rifampicin, Pyrazinamide and Ethambutol
I	Internal Wall of the Lesion
IFN-γ	Interferon-Gamma
IGRA	Interferon-Gamma Release Assay
IGTP	Institute for Health Science Research Germans Trias i Pujol
IL	Interleukin
IP-	Interferon Gamma-Induced Protein-
IPA	Ingenuity Pathways Analysis
LAM	Alere Determine TB lipoarabinomannan strip test
LCM	Laser Capture Microdissection
lfcSE	Log Fold Change Standard Error
LLL	Left Lower Lobe
Log2FC	Log 2-Fold Change
LPAs	Line-Probe Assays
LTBI	Latent Tuberculosis Infection
LUL	Left Upper Lobe
MDR-TB	Multi-Drug Resistance Tuberculosis
MDSC	Myeloid-Derived Suppressor Cells
MMP-	Matrix Metalloproteinase
Mtb	*Mycobacterium tuberculosis*
N	Nodulus
NCTLD	National Center for Tuberculosis and Lung Diseases
NES	Normalized Enrichment Score
NET	Neutrophil Extracellular Trap
NGS	Next Generation Sequencing
NK	Natural Killer
NTM	Non-Tuberculous Mycobacteria
OPN	Osteopontin
Padj	Adjusted p-value

PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PMN	Polymorphonuclear Cells
PPD	Purified Protein Derivative
PRR	Pattern Recognition Receptors
PTB	Pulmonary Tuberculosis
RIF	Rifampicin
RINe	RNA Integrity Number equivalent
RLL	Right Lower Lobe
RNA	Ribonucleic Acid
RNA-seq	Ribonucleic Acid Sequencing
ROC	Receiver Operator Characteristics
ROS	Reactive Oxygen Species
RR-TB	Rifampicin Resistance Tuberculosis
RUL	Right Upper Lobe
SCC	Sputum Culture Conversion
sCD14	Soluble CD14
SDE	Significantly Differentially Expressed
SH-TBL	Study of the Human Tuberculosis Lesions
SNP	Single Nucleotide Polymorphisms
STAR	Spliced Transcripts Alignment to a Reference
TB	Tuberculosis
Th	T helper
TLR	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor-alpha
TST	Tuberculin Skin Test
UTE	Experimental Tuberculosis Unit
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR-TB	Extensively Drug-Resistance Tuberculosis

1 | INTRODUCTION

Description: "Tuberculosis: the head of the Medusa representing the disease as an exhibition advert against tuberculosis in Basel." Year 1883-1938.

Author: Robert Strüdel, Gewerbmuseum Basel, Switzerland (Wellcome Library no. 5134i; Photo number: L0034019)

1.1 Tuberculosis: the disease and epidemiology

Tuberculosis (TB) is a communicable infectious disease, caused by *Mycobacterium tuberculosis* (*Mtb*) bacteria. Is an acid-fast, intracellular bacillus that was discovered by the German physician and microbiologist doctor Robert Koch, in 1882, which led him to win the Nobel prize in Medicine or Physiology in 1905 (1).

According to the annual World Health Organization (WHO) report, last year 10 million people developed TB and 1.5 million people died of it (2). It is placed among the top 10 deathliest diseases worldwide, but in the context of the infectious disease, TB is the major cause of death from a single infection, even surpassing the deceases caused by the Human Immunodeficiency Virus (HIV) per year. Beyond these facts, it must be highlighted that approximately a quarter of the worldwide population is considered infected with the *Mtb* bacilli according to estimations, and between the 3-10% of cases will end developing the disease during their life (3).

TB incidence is not homogeneous. Countries with a better social-economic structure are facing on average 10 new cases per 100.000 inhabitants. On the other hand, high-incidence areas are mainly found in low-income countries, such as South Africa or the Philippines, where TB is considered an epidemic, registering 500 cases per 100.000 people annually (3).

To face these numbers, the WHO designed the End TB Strategy to end the TB epidemic by the end of 2035, reducing the number of TB deaths and the incidence rate by 95% and 90%, respectively. This strategy involves three defined pillars: efficient prevention; bold policies and support from the society; and an intensified research and innovation (4).

1.2 *Mtb* pathogenesis

Mycobacterium tuberculosis is an airborne pathogen so it must travel through the air as an aerosol droplet or droplet nuclei. The aerosol is generated through exhaled breath, especially when talking, sneezing, coughing and signing from an active TB individual, hence from someone showing symptomatology of a Pulmonary TB (PTB).

This is the main condition that is required to start an infective cycle, although many other factors are influencing the success of the *Mtb* path (5):

1. Space-time factors: a sufficient close contact in a crowded and not enough ventilated space during a prolonged time with an active TB individual is a potential situation of infection (e.g. household contacts).
2. The aerosol quality: the active TB subject must be capable to produce enough quantity of particles containing enough quantity of the bacilli to internalize in the host's respiratory tract.
3. Host's protective factors: the bacilli must settle in the pulmonary alveolus and infect the alveolar macrophages, but the quality of the alveolar surfactant together with an efficient immune response may become a strong response to avoid the infection.
4. Host genetics, nutrition, comorbidities, sex, and the age: characteristics beyond the immune response can define an individual as susceptible or protective relative to the *Mtb* infective cycle (6,7).

1.2.1 The spectrum of the disease

Lungs are the major gate to host the most of TB infections. The first infection with no prior exposition, or primary TB, occurs when the *Mtb* deposition takes place in the lung's parenchyma after an inhalation, to be hosted in middle or lower pulmonary lobes. Alveolar macrophages, which role is to keep clean the alveolar environment to allow efficient gas exchange, will phagocyte the mycobacteria (5,8).

After the primary infection, the fate of the mycobacteria is bound to the host innate immunity, which in some cases can either prevent the TB progression to finally allow the *Mtb* elimination or to progress to active TB. However, a single episode of *Mtb* infection may not be enough to develop the active disease. The individuals to have greater chances to develop an active disease are those who are continuously exposed to a pulmonary TB case, as well as to consider the age and/or those who are immunocompromised, suffering from other infections such as HIV, or important comorbidities, like Diabetes Mellitus (DM). Thus, it is required a chronic reinfection process, independently or in combination with other critical coinfections and/or comorbidities (9,10).

If the innate and adaptive immune systems fail and an individual is likely to be reinfected, the picture change to render two intricate scenarios. The first, an efficient pro- and anti-inflammatory stimulus can block the growth of the bacilli at the milieu of infection. Consequently, the immune system will be able to keep the bacilli "dormant" and the individual may remain asymptomatic and presumably noncontagious, a situation defined as a Latent TB Infection (LTBI). The second scenario takes place when the innate and adaptive immune systems are impaired, with or without the dormancy period, and the bacilli may be reactivated to start growing with no opposition, causing a chronic granulomatous infection. In consequence, a TB lesion is forming, and therefore the subject progressing into active TB (11,12).

It is estimated that only a few percentages (3-10%) of LTBI subjects will progress to active TB within two years after infection, presenting symptoms of the disease and therefore transmission capacity (13). Furthermore, the systemic dissemination of the bacilli can sometimes occur to invade different organs than lungs, mainly given among children, immunocompromised subjects and/or bound to a genetic component (14). We refer to this category of active TB as Extrapulmonary TB (EPTB).

A consensus was achieved regarding the binary paradigm of the *Mtb* infection, active TB against LTBI, which is no longer valid. Tuberculosis is a complex disease, where the pathogen dynamics together with the host's immune response creates a broad spectrum of clinical manifestations, composed in general by subjects resistant to the bacilli even under a chronic exposure; those who are infected initially but with the innate capacity to sterilize the bacilli; LTBI individuals infected but asymptomatic and at risk to become a progressor; the group having TB with the subtle and/or occasional presence of symptoms although with transmission capacity; individuals with an active disease showing typical TB symptoms; and those with a fulminant TB, thus with a severe clinical presentation (8,15–17) (**Figure 1**).

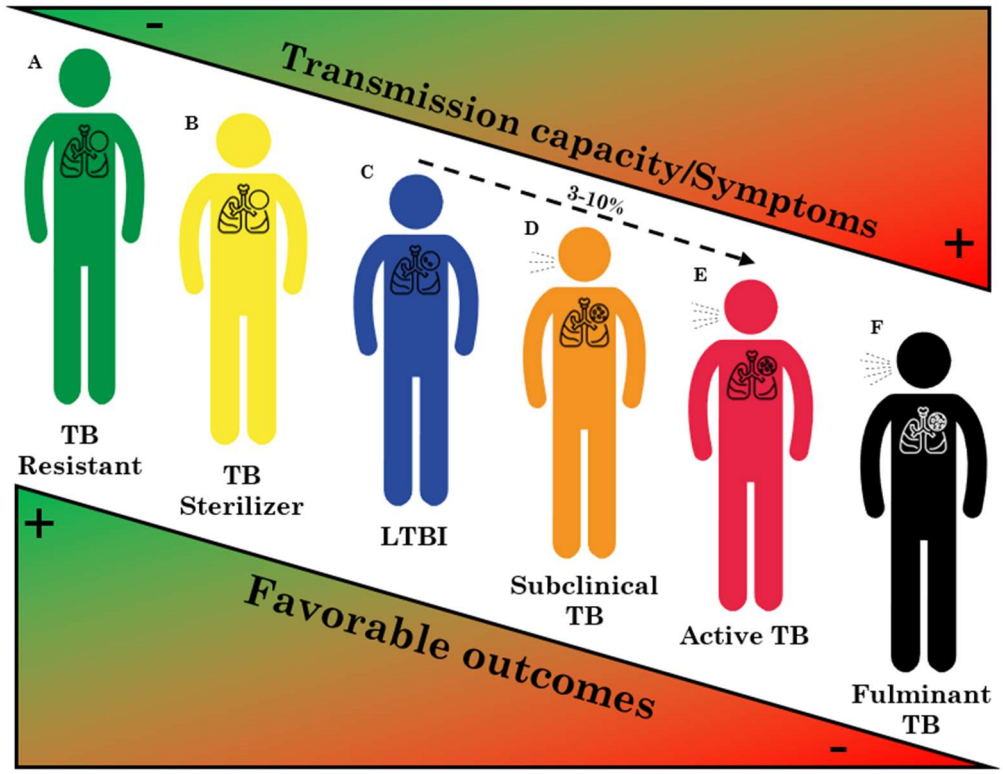


Figure 1. **The spectrum of the TB disease.** (**A**) TB resistant, patients with the capacity to avoid the infection. (**B**) TB sterilizer, initially infected but with the innate capacity to sterilize the bacilli. (**C**) LTBI, a latent infection at risk to progress to active forms of the disease. (**D**) Subclinical TB, patients that may not show symptoms, but are culture-positive although having low bacillary loads and therefore with low chances to disseminate TB. (**E**) Active TB, patients experiencing typical symptoms of TB and with transmission capacity. (**F**) Fulminant TB, including patients with a severe clinical picture and a very poor prognosis.

1.2.2 The clinical picture

Signs and symptoms and its severity found in PTB can be variable according to what stage of the disease the individual is found, the extension of the TB, and genetic factors. The *Mtb* bacilli have a very slow division rate, one every 24 hours. That explains why the disease is developed very slowly and the clinical picture is exhibited once TB is partially or quite advanced. Despite everything, the frequency of symptoms may be variable when primary TB and post-primary are compared (**Table 1**).

Table 1. Percentage of clinical symptoms by patients suffering from active TB. Adapted from Lyon SM *et al.,* 2017 (18).

	Estimate percentage of affected patients	
Symptoms	**Primary**	**Reactivation or Post-primary TB**
Cough	25-37	42
Fever	18-42	37-79
Weight loss	No data	7-24
Hemoptysis	8	9

Among adolescents and adults, individuals having a primary TB do not tend to show symptoms or are minimally symptomatic, whereas reactivation or post-primary TB presents fever as the most frequent symptom, subtle at the onset but prominent once the TB lesion/s progress in the lungs. When the subject restores temperature, occasionally during sleep, sweating appears, a pathophysiological process known as night sweats. The patient may also suffer from weakness, asthenia, and anorexia, with a consequent weight loss. Mild to severe cough appear when the TB lesion grows and reaches the tracheobronchial tree wall to be broken and potentially disseminate throughout the rest of the bronchial tree. Hence the TB lesion, namely a TB cavity, is consolidated which is usually accompanied by hemoptysis and sputum production (18).

1.3 Tools for management of TB patients and treatment

1.3.1 LTBI and active TB diagnosis

Since LTBI is generally characterized for being an asymptomatic stage of the disease, to clinicians it is difficult to determine if an individual is infected or not given the dormant state of the mycobacteria. There are only a few tools to help determine the presence of the *Mtb*, which are mainly tested evaluating the host's immune response.

The first approach is an *in vivo* assay, the largely used Tuberculin Skin Test (TST) which measures the cell-mediated immunity. The TST uses the *Mtb* Purified Protein Derivative (PPD), in which 0.1 mL of this substance containing the PPD is intradermally injected. Within 48 to 72 hours after administration, it is expected a

skin induration if positive, where the diameter of the reaction is measured in millimeters. A second approach is the Interferon-Gamma Release Assay (IGRA) that works under the principle that T-cells from the host are sensitive to the *Mtb* antigens presence by inducing circulating Interferon-Gamma (IFN-γ). Hence, this test works as an *in vitro* approach, measuring IFN-γ production by harvested T-cells from peripheral blood, which are under stimulation to several *Mtb* specific antigens for 16 to 20 hours. The sensitivity of both tests is quite similar although TST has a serious gap when immunocompromised subjects and children or even the elderly are inspected. Furthermore, specificity falls when patients are Bacille Calmette-Guérin (BCG) vaccinated or had a history of a previous TB, so cross-reactivity may happen, especially when TST is performed. Therefore, IGRAs have a better specificity so both tests are preferred to be used in combination. Even though, the experience for years from using these two approaches points toward the need to find a golden standard to overcome these and many other weaknesses that can be found in the LTBI screening (19–21).

On the other hand, the diagnosis of active TB is mainly guided by the clinical examination, plus a complementary radiological exploration to provide more evidence to the manifestations of the disease. Posterior microbiological tests will aid in providing proof of the presence of the bacteria and confirming the active TB diagnosis. In this context, the TST and the IGRA tests are useless for the TB diagnosis, as they are intended to detect LTBI individuals, as well as none of them are useful enough for the LTBI and the active TB discrimination, so other techniques are needed. Namely, these microbiological techniques are involved in the molecular and non-molecular detection of the bacilli, those based on the *Mtb* culture, and the microscopy.

1.3.1.1 TB diagnosis through the Chest X-Ray

In general, TB lesions cannot be observed until they reach a discernible 10 mm diameter mass on a Chest X-Ray (CXR) (22). Typical active TB lesions are mainly localized in the apical upper lobes or the superior segment of the lower lobes. Classically, upper sites of lesion involvement have been attributed to a greater

oxygen concentration because of a gravity phenomenon and worse lymphatic drainage than other lung areas, which favors the TB lesion progression (23).

Cavities are the most radiographically evident feature found upon PTB patients and they can be seen as a single or multiple lesion/s (24). They are characterized by being patchy, thick-walled with an irregular consolidated perimeter. The surrounding lung parenchyma can present other signs, such as bronchiectasis, infiltrates, and other nodular opacities (**Figure 2**). During treatment monitoring through periodic CXR, walls tend to become thinner, and the cavity heals as a linear, fibrotic scar or with calcification, especially evident in cases of extended disease (25,26). Miliary TB is a TB form product of the lymphohematogenous dissemination of *Mtb*, which evolves creating new disseminated lesions in the lungs. Radiologically, multiple pulmonary lesions as diffuse nodules from 1 to 3 mm in size will be spotted (27,28).

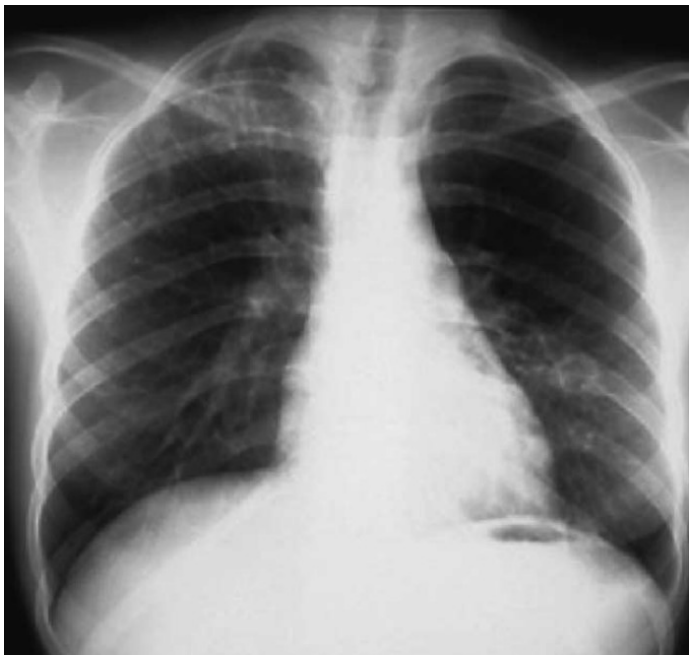


Figure 2. **Post-primary TB chest radiography.** Multiple involvements, infiltrates in the right upper lobe, cavity present in left lung lingula. Adapted from Andreu *et al.,* 2004 (25).

One of the greatest problems using the CXR is that it is impossible to establish if a radiographically healed lesion is still containing bacilli, which its persistence is a

risk of *Mtb* transmission and reactivation. Consequently, in radiological terms, the CXR is not a good correlator of a hypothetical sterilized cavity (29). In fact, the WHO recommends that the CXR should be used as a supplementary aid for the TB screening, prevalence surveys, and as a complementary tool for microbiological tests (30).

1.3.1.2 *Mtb* microscopy and culture

For decades, the golden standard tool for TB diagnosis is the sputum culture. This technique is the definitive test to provide a diagnosis of TB by the *Mtb* culture given its high sensitivity. Cultures can be performed either in liquid media or the widely used Löwenstein-Jensen solid media. The mycobacteria growth generally depends on the bacilli burden that carries the patient's sputum, but at least requires three weeks of the plates under incubation at 37ºC. The liquid media helps to speed the *Mtb* growth and identifying other Non-Tuberculous Mycobacteria (NTM). The NTM is a source of false-positives cases when a TB case is suspected and this kind of tool requires additional equipment and trained personnel. Poor countries therefore usually have not access to this technology (31,32).

Sputum microscopy smear examinations for the Acid-Fast Bacilli (AFB) detection, thanks to the Ziehl-Neelsen dye, is probably the most common tool used worldwide because of its cost-effective nature, rapid and can be performed in any health-care basic laboratory, requiring only a conventional light microscope. Although it is a technique with low sensitivity, especially among children and immunocompromised patients known for showing a low bacillary load, AFB smear is essential in any microbiology laboratory as it is in the front-line approach for TB diagnosis, and more importantly, given the fact that not all countries, especially those with a high TB burden, cannot afford any of the mentioned molecular diagnosis tools or supporting facilities. Therefore, this a strongly recommended diagnosis tool by the WHO in low and middle-income settings (33–35).

1.3.1.3 Molecular and non-molecular TB diagnosis techniques

The spearhead of the molecular-based tests to diagnose TB are those that work under the principle of the Polymerase Chain Reaction (PCR). Among the PCR based technology instruments, the Xpert® *MTB*/RIF test and its cartridges are the main

reference diagnosis tool. It is an automated real-time PCR that identifies specific regions from the *Mtb* genes, which enables to detect the presence of the bacilli in sputa samples and very importantly, to assess a potential drug-resistance to a first-line TB antibiotic as Rifampicin (RIF) is (36).

Recently, a new version of the Xpert, the Xpert® *MTB*/RIF Ultra, has appeared improving from its predecessor the sensitivity among paubacillary TB cases, like childhood TB, EPTB, and especially among immunocompromised patients. One of the greatest advantages is that results can be obtained in less than 80 minutes. Likewise, this upgrade includes new genetic targets that increase the chances for detecting RIF resistance as well as *Mtb*, yet a certain grade of specificity is sacrificed (37). In combination with a properly designed algorithm according to the setting's characteristics, the WHO strongly endorses the universal use of the Xpert® Ultra arguing a huge step forward into diagnosing a broader spectrum of active TB adults and children cases (38). The downside part of such technology is that requires sufficient trained personnel, facilities under constant maintenance, and a not minor detail, electricity. Consequently, not all hospitals nor health-care centers can afford an Xpert core, although pricing efforts have been made especially for endemic TB countries.

A remarkable example of a non-molecular based test is the Alere Determine TB lipoarabinomannan strip test (hereinafter LAM), commercially available for any health-care center since its competitive price is accompanied by moderate results within 25 minutes (Alere, Waltham, MA, USA). The LAM assay principle lies in the detection, from only 60 μL of the patient's urine, the lipoarabinomannan *Mtb* cell wall antigen. The best pool of patients that are getting a greater diagnosis performance was given among severe HIV-positive hospital inpatients, with an advanced immune depression, reaching a 94% specificity (39).

1.3.2 TB drugs and testing tools

Although massive national BCG programs were priorly implemented as a prophylactic anti-TB tool, the first gamechanger measure was the apparition of antibiotics during the early 1950s. Nowadays, with the current anti-TB Treatment (ATT), almost 90% of cases receiving antibiotics will have a favorable outcome.

Nevertheless, active TB patients are submitted to the intake of multiple drugs for really long periods, which usually entails the ATT withdrawal in many patient's cases. Abandoning treatments before a resolution is one of the major causes of reactivation/relapse TB and in the worst scenario possible, the creation of *Mtb* strains resistant to drugs (40).

The resistance to antibiotics is mainly a man-made phenomenon. Bad antibiotic prescriptions, including a wrong active substance or an unfitted drug regimen, or as previously said, a bad adherence to ATT are the main reasons for this phenomenon, named as secondary resistance or acquired. In the TB scenario, there is a pool of mycobacteria that by natural selection and/or antibiotic pressure can resist the effect of the ATT by inducing Single Nucleotide Polymorphisms (SNPs), insertions, and/or deletions on its chromosomal genes (41). A very likely scenario, which is enhanced when a correct TB patient management is not fulfilled. The secondary resistance is a frightening community health problem, not only for the patient management and at the public health expenses level, but these mycobacteria can disseminate and initiate a new infective cycle of the resistant strains. When this occurs is known as primary resistance (42).

Drug susceptibility testing (DST) is one of the cornerstones in the context of the management of TB patients. The WHO encourages DST to all patients to efficiently guide treatment and very importantly, to improve treatment outcomes. The traditional way into DST testing is by sputum culture in different media from clinical isolates, but as known this technique is slow providing results as it takes at least 12 weeks having the *Mtb* grown (43). Hence, DST delays are directly impacting the correct treatment initiation, may affect the prognosis of the patient and this situation consequently promotes further *Mtb* transmissions to patient's contacts (44).

As an alternative to culture-based methods, the WHO recommends the universal use of the Xpert® *MTB*/RIF Ultra, as previously explained to detect both the *Mtb* and rifampicin resistance on sputum samples (37). This tool can provide a rifampicin resistance diagnostic in two hours but does not cover the whole spectrum of the patient's drug susceptibility profile. Another molecular test based on PCR

technology is the line-probe assays (LPAs). The LPAs can test for resistance to isoniazid and rifampicin and second-line drugs as fluoroquinolones or injectables (45,46). Although LPAs are rapid tests, this technique as well as the Xpert are involving specialized technicians and supporting facilities, which undermines drug resistance surveillance in low-resource settings. Besides, both tests are targeting a limited number of resistance genetic variants, and importantly both lack of comparable sensitivity to culture assays (43).

Finally, the only diagnostic test that can provide a full profile of the patient's drug-resistance in *Mtb* isolates is the Whole Genome Sequencing (WGS). By computational methods, WGS individually checks known *Mtb* Deoxyribonucleic Acid (DNA) mutations in clinical isolates and therefore offers a complete genotypic report of those affected genes that are potentially encoding for a first-line drug-resistance phenotype. Current results are performing in a quite similar correlation of sensitivity and specificity to culture media tests when certain antibiotics are tested (47). Nonetheless, WGS requires further research and validation, automatization, and good prizing in terms of equipment are still lagging, reasons that are undermining this technique to be included as a routine tool for TB patient management.

1.3.3 Active TB treatments

Those individuals that have been infected with *Mtb* strains that are sensitive to short term antibiotics of the available spectrum against TB are classified as Drug Sensitive TB (DS-TB). As recommended by the WHO, the current DS-TB drug regimen for new PTB cases involves several oral antibiotics: isoniazid, rifampicin, pyrazinamide, and ethambutol (HRZE) during two months (intensive phase), followed by isoniazid and rifampicin (HR) for four months (continuation phase), whereas previously treated patients receiving this time eight months-long retreatment, including first-line drugs (2 HRZE + Streptomycin/1 HRZE/5 HRE) in settings where drug-resistant TB is not prevalent (33,48). On the other hand, the group of patients belonging to the drug-resistant *Mtb* strains comprises a subclassification in which TB patients are categorized according to its drug-

resistance (**Table 2**) and therefore a properly fitted drug-regimen must be followed, according to the WHO recommendations (33,49).

Table 2. Drug-resistance tuberculosis definitions. Adapted from the WHO's "Treatment of tuberculosis guidelines" (33).

Drug-Resistance type	Definition
Mono-Resistance	Resistant to a single first-line ATT drug only
Poly-Resistance	Resistant to more than one first-line ATT drug, different than both isoniazid and rifampicin
Multidrug Resistance (MDR-TB)	Resistant to at least both isoniazid and rifampicin
Extensively Drug-Resistance (XDR-TB)	Resistant to any fluoroquinolone and to at least one of three second-line injectable ATT drug (capreomycin, kanamycin and amikacin), in addition to MDR-TB
Rifampicin Resistance (RR-TB)	Resistant to rifampicin detected using phenotypic or genotypic methods, with or without resistance to other ATT drugs. Including any resistance to rifampicin, in the form of mono-resistance, poly-resistance, MDR/XDR-TB

1.3.3.1 Surgery as an adjuvant treatment of TB

Surgical interventions as a treatment for TB are dated even before the discovery of the *Mtb* complex. For two centuries, the surgical approach was one of the few available tools to fight TB. Nonetheless, the success of the pulmonary resection was questionable, experiencing frightening operative mortality rates between 20% to 40% (50). The apparition of ATT chemotherapy radically changed the outlook. Surgery was mostly abandoned in most industrialized countries, namely Europe, North America, Australia, and Japan, simply because the incidence and prevalence of TB decreased thanks to new drug-based strategies. There was a consensus that curing TB was almost possible in every individual without the need of indicating therapeutic surgery (51).

However, the scenario changed with the onset of the MDR/XDR-TB strains and its worldwide spread, as this form gathers the most numbers of incurable cases and higher fulminant TB rates in PTB (52,53). Under this emergency, the WHO elaborated a deep review over the pulmonary TB surgery bibliography, as nowadays is the main reference guideline for settings in which surgery is presumably needed to cope with the high burden of MDR/XDR-TB infections (54). This is especially

highlighted in the former Soviet Union countries, such as Russia and Georgia[1], were resistant forms are a serious health public problem, and therefore therapeutic surgery is a common therapeutic tool.

The therapeutic pulmonary TB surgery is an invasive procedure in which the TB lesion/s is removed. In general, the most recurrent type of operation performed is lung resections of different sizes (from lobectomy to pneumonectomy). Among the many reasons that indicate therapeutic surgery, one of the most recurrent aspects is those cases of extended TB disease, with the presence of big cavities and with important radiological infiltration. Such recommendation is highly estimated, in general, because cavitary TB may complicate the antibiotic penetration through the lesion and therefore host a high number of bacilli within the cavity (50). This fact is underlined with the discovery of living *Mtb* bacilli that were cultured from cavitary surgical specimens, taken from patients that were preoperatively negative in sputum culture during and after treatment completion. In addition, cavities may be directly linked to the development of drug resistance given its intricate architecture for the drug perfusion into the center of the lesion, where the TB patient presumably hosts an important number of living bacilli. (55,56).

The main indications for the surgical approach for PTB are herein summarized and can be found at the WHO's "The role of surgery in the treatment of pulmonary TB and multidrug- and extensively drug-resistant TB" guideline (54):

1. Emergency indications: the death of the patient is irreversible unless surgery is performed (i.e. extensive lung hemorrhage).
2. Urgent indications: irreversible TB progression and/or recurrent hemoptysis, although adequate ATT regimen and symptomatologic treatment.
3. Elective indications: persistence of sputum positivity after four to six months of ATT; localized TB cavitary lesions; ATT failure in MDR/XDR-TB forms; complications and sequelae of the PTB.

[1] Official Georgian guidelines are written in Georgian and Russian, although they are entirely based on the WHO surgery recommendations found in "The Role of surgery in the treatment of pulmonary TB and multidrug- and extensively drug-resistant TB" (WHO, 2014).

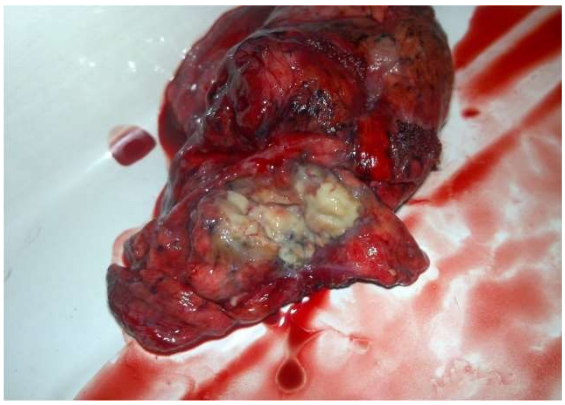


Figure 3. Resected TB lung cavitary lesion with caseus necrosis.

Several publications have reported the benefits of therapeutic surgery to cope with severe complications like hemoptysis or important residual sequelae after ATT (56,57). There is a systematic review that highlights the benefits of a combined ATT chemotherapy plus PTB surgery as an adjuvant in MDR-TB subjects, showing good performance on the therapy success rate (88%-92% of the cases) (58) and some other reporting positive outcomes for the patients that underwent therapeutic surgery (59–61). Even though, current PTB surgical experience is based on observational retrospective studies; there is not any recent randomized clinical trial in which PTB surgery efficacy is assessed (both in short and long-term evaluating surgery success); consensus is not always found between official national guidelines in terms of main indications for PTB surgery; and finally, there is also another study in which no meaningful results are found in favor of surgery in comparison of those who did not receive it when MDR/XDR-TB patients were treated (62).

In the WHO's guideline is stated that is not possible to make a strong evidence-based endorsement upon the role of the pulmonary TB surgery given that the overall role of the therapeutic surgery is not fully defined, sometimes controversial and importantly the overall insights are based on observational studies, as any recent randomized controlled trial is included. Even though, the three previous summarized surgery indications points are based on the recommendations of the Task Force designated by the WHO that participated with the guideline writing. These recommendations are specially orientated towards to MDR/XDR-TB severe

cases while warning that further research is needed in the PTB therapeutic surgery context.

1.3.4 Treatment monitoring and outcomes

Treatment response monitoring requires periodic clinical examination, serial CXR, laboratory techniques, and if possible, the patient interrogation to find out if the TB chemotherapy intake has been strictly followed since the last visit. Bad adherence to treatment is related to the induction of MDR/XDR-TB forms and poor TB treatment outcomes (e.g. treatment failure and relapse), that's why strategies such as the Directly Observed Therapy (DOT) have a direct impact over the TB resolution (63). The DOT strategy simply consists of the observation of the patient taking the TB treatment, by a health-care worker, family, or community member. This strategy has proved better disease outcomes (64).

A proper drug regimen prescription should make improve the symptoms of TB within the first weeks of ATT. The most worrying factors are those cases in which the patient presents persistent fever, weight loss, or the recurrence of any other TB classical symptoms. These clues may point towards a potential treatment failure or masked comorbidities, so further explorations are required under these circumstances (65). Moreover, associated ATT side effects may take part in the clinical picture during treatment and lead to failing therapy, especially during the intensive phase. In most cases, toxicity events among drug-resistant subjects are related to liver malfunctions, that's why all MDR/XDR-TB patients should be checked from hepatic circulant markers, especially patients declaring alcohol use (49). In general, children, adult females, and HIV co-infection are a pool of TB patients closely related to side effects. For these reasons, it is highly recommended tailored monitoring in those patients experiencing any adverse effect during the ATT course (66).

The CXR is only a good tool for tracking the patient's evolution in the worst TB cases. During treatment monitoring, is recommended that CXR should be taken every three months, given the fact that radiological changes are not so evident in short periods, yet they are useful to be compared with previous CXR if there is an

undesirable clinical shift. Also, they do not gather enough features to ensure a total bacilli clearance at the end of treatment (67).

The main source for the TB treatment monitoring and endorsed by the WHO is the sputum sample. Serial sputum culture is required for treatment monitoring and as a good evolution surrogate marker (65). In DS-TB, the sputum conversion status at month two is considered to be a correlator of disease improvement (68), even if among MDR-TB patients the conversion at month six has been proposed as a better timepoint (69,70). In consequence, the sputum culture conversion is the current best reference as an indicator of disease improvement, and the only available tool to assess if lung sterilization is happening by the bactericide effect of the anti-TB drugs.

The WHO guidelines provide definitions to classify the end of treatment outcomes or those events that may affect the course of the disease during the ATT. These definitions are divided into two types of patients: patients having a DS-TB and by patients being treated as drug-resistant TB, hence requiring second-line drug treatment (71) (**Table 3**).

1.4 The immune response against the *Mtb* aggression

The *Mtb* infective cycle that triggers the TB development is outlined at the end of this section in **Figure 4**. The infection by *Mtb* starts when the pathogen can penetrate to the host's lower respiratory tract airways by inhalation and settle in the pulmonary alveolus. There, after reaching contact with alveolar surfactant, the first set of cells to identify the bacilli are the Airway Epithelial Cells (AECs). Although the AECs role is basically to act as physical barriers and they do not belong to the immune system cells team, they display several countermeasures against the *Mtb* after recognizing the pathogen through Pattern Recognition Receptors (PRRs), by inducing changes in the airways surface liquids thus improving the antimicrobial characteristics (72).

The first defense line cells belonging to the early response or the innate immune system is headed by the resident alveolar macrophages (AMs) along with neutrophils and dendritic cells (DCs). The AMs will phagocyte the *Mtb*, recognized

by several types of PRRs (the Toll-Like Receptors (TLR), lectin receptors, mannose receptors, and the nod-like receptors) and constitute the phagosome within the macrophage (73).

Table 3. Treatment outcomes for drug-sensitive and drug-resistant patients. Adapted from the 2020 update WHO "Definitions and reporting framework for TB" (71).

Outcome	Definitions	
	Drug-Sensitive TB	**Drug-Resistant TB**
Cured	Patient bacteriologically TB confirmed at the beginning of ATT with smear or culture negative last month of ATT and on at least one previous occasion	Treatment completion with no evidence of failure and three or more consecutive culture taken at least 30 days apart are negative after the intensive phase
Treatment completed	The ATT is completed without evidence of failure but with no proof of sputum smear or culture conversion to negative	The ATT is completed without evidence of failure but no record that three or more consecutive cultures taken at least 30 days apart are negative after the intensive phase
Treatment failed	Sputum smear or culture is positive at month 5 or later during the ATT	Treatment terminated or need for permanent regimen change of at least two anti-TB drugs
Died	Passing for any reason during the course of the ATT	Passing for any reason during the course of the ATT
Lost to follow-up	The ATT was not started or the ATT was interrupted for two consecutive months or more	The ATT was interrupted for two consecutive months or more
Not evaluated	Treatment outcome is not assigned	Treatment outcome is not assigned
Treatment success	The sum of cured and treatment completed	The sum of cured and treatment completed

Following the *Mtb* internalization, the AMs become either the replication niche for the *Mtb* as they can skip the phagosome-lysosome union (and therefore ensuring its survival) as well as apoptosis; or the place homing the bacilli within the phagosome and inducing a sufficient immune response by accepting its persistence and therefore a latent infection phase (74,75). The capacity to evade the innate immune response of apoptosis is related to virulent strains of *Mtb* (76).

The *Mtb* replication rhythm in the AM although being slow will manage to create a sufficient number of descendants that will trigger the AMs necrosis. The interstitial space can also be affected by infecting epithelial cells or using the dying AMs as a carrier (77). More AMs and other phagocytes will gather to the milieu of infection (mainly in the alveolar walls) substituting the pool of necrosed and *Mtb* will be phagocyted again. This process will be repeated chronically until the infected AMs can induce a sufficient potent inflammatory response (5,74,78). Infected AMs can release several pro-inflammatory cytokines, such as the Tumor Necrosis Factor (TNF-)α, Interleukin (IL-)6, and IL-1ß (79).

The AMs inflammatory cascade can act as chemoattractant signals firstly to other AMs, and secondly inducing the polymorphonuclear cells (PMN) recruitment, mainly composed by neutrophils and monocytes, from the pulmonary vasculature to the interstitial space were more bacilli freely escaped (5). Neutrophils are also capable to phagocyte the *Mtb* by PRRs, whereas monocytes will differentiate to macrophages. In addition, neutrophils use several mechanisms to fight the *Mtb* aggression, including phagocytosis, degranulation, induction of Reactive Oxygen Species (ROS) as well as the Neutrophil Extracellular Traps (NETs) release (80,81). Consequently, AMs and neutrophils are the very first actors and key partners in protecting/fighting against the *Mtb* aggression by inducing antimicrobial agents and stimulating the attraction and activation of the DCs response.

The DCs have a pivoting role in facilitating the activation of the T-cells adaptive immune response via the antigen-presentation system after phagocyting the bacilli. The DCs have also co-stimulating activity and the capacity to produce cytokines and chemokines (82). The force combination of DCs, AMs, and neutrophils will be determinant to hold the disease progression until the adaptive immune response is activated to deploy all the resources to control the infection, but there is a delay in its activation.

First, $CD4^+$ T lymphocytes will be activated in the lymphatic nodes by the stimulation of cytokines and chemokines released by the AMs. $CD4^+$ T cells are key as they are an important pool of cells capable of releasing IFN-γ. The pro-inflammatory IFN-γ cytokine plays a central role in controlling the *Mtb* infection as

initiates the metabolic cascade responsible to fight the bacilli. Importantly, induces the production of TNF-α by the AMs and catalyzes a positive feed-back inducing the attraction and activation of more AMs, thus increasing the chances of bacterial clearance (83,84). Secondly, DCs carrying *Mtb* will migrate to peripheral pulmonary lymph nodes for the T-cell priming, but in the same time, the bacteria have had enough time to continuously replicate gathering more and more innate system cells to the milieu of infection, generating a powerful host response called TB lesion or granuloma. It is estimated that the efficient activation of the adaptive system takes between eight to 12 days after the prime infection (81,85,86).

Once in the draining lymph node, DCs will activate an antigen-specific response to drive the naïve T helper (Th) cells differentiation. Several subpopulations of naïve Th cells will be activated, namely the Th1, Th2, Th17 response as well as T regulator cells and in a certain grade the $CD8^+$ T lymphocytes (84,87,88). The effector Th1 cells will essentially fight the infection releasing IFN-γ. Hence, IFN-γ will support protecting mechanisms such as the phagocytosis stimulation, the phagosome maturation, the production of reactive nitrogen intermediates (iNOS), and promoting apoptosis and autophagy and therefore priming the antigen presentation, which approximately occurs after 14 to 17 days post-infection, and last but not least, allow the bacilli destruction (77,89). Chemokines such as the C-X-X motif chemokine 10 (CXCL10), also known as the Interferon Gamma-Induced Protein 10 (IP-10), will mediate the activated T-cells back to the infection site in the lungs (90).

1.4.1 The cytokines production is driven by the *Mtb* infection

Cytokines, as well as chemokines, play an essential role in the disease as they are mediators of the most of the early and late events in the infection control, displaying a complex network of dis- and regulations during the course of TB.

IFN-γ is the main pro-inflammatory cytokine giving the importance of its protective role against the infection. As previously exposed, the T-cells are the main IFN-γ producers but only when they are displayed. During the early stage of the infection, Natural Killer (NK) cells are mostly producing these cytokines, followed by AMs (91).

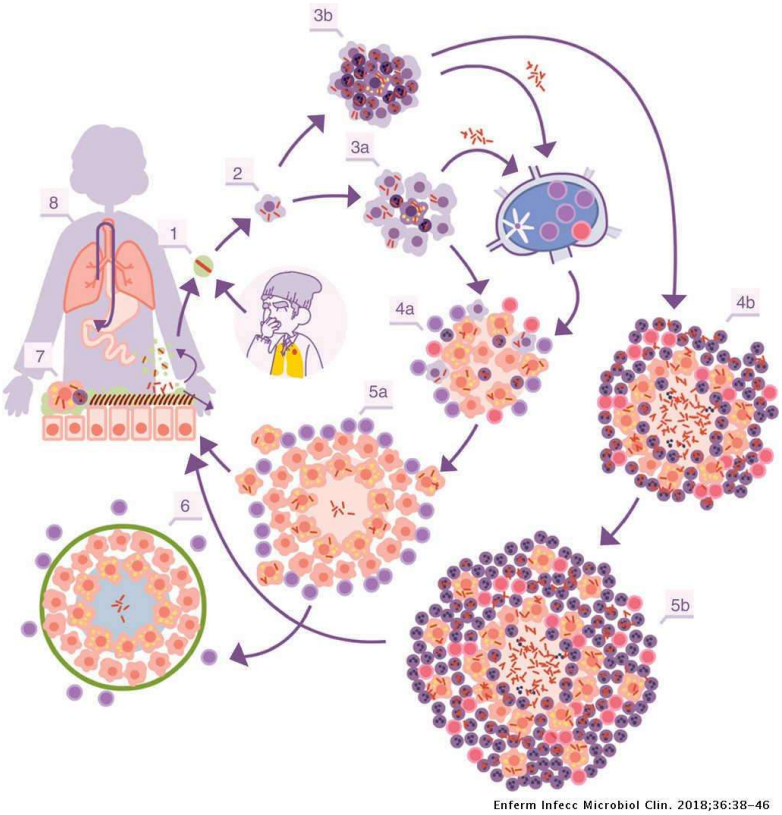


Figure 4. **The infective cycle of *Mycobacterium tuberculosis*.** (1) The bacilli penetrate the superior airways until reaches the pulmonary alveolus. (2) *Mtb* phagocytosis by the alveolar macrophages (AM) and internal replication. (3) AM destruction, local *Mtb* dissemination, and immune response dominated by monocytes (3a) or polymorphonuclear cells (PMN) (3b), to be later drained to the pulmonary lymph nodes were T-cell priming happens. The lesion can act as a pole of attraction, gathering more PMN and lymphocytes depending on either a stronger Th1 response (4a) or an impaired immune response (4b). A Th1 dominated immune response will be able to control the bacillary load as well as a proper lymphocytaire drainage (5a) until the lesion is effectively controlled by its encapsulation (6). If the lesion growth can not be controlled, the bacillary burden found is heavier, requiring more effective drainage (5b). Free bacilli can escape through the alveolar fluid (7) and be drained towards the gastrointestinal tract (8), or become part of new aerosols and restart a new infective cycle (1). Adapted from Cardona, 2018 (5).

TNF-α is another inflammatory cytokine that can be found at the site of infection, induced by the monocytes, AMs, and DCs as the result of the presence of *Mtb*. TNF-α has an important paper in the granuloma formation, as well as inducing the macrophage activation and immunoregulatory properties (92). IL-1ß inflammatory cytokine alike TNF-α is also released by the monocytes, AMs, and DCs and is

localized in the infection foci (73). IL-6 is released mainly by AMs, and has been found to have pro- and anti-inflammatory functions, exhibiting the release inhibition of TNF-α and IL-1ß and therefore exacerbating the *Mtb* infectiveness (93). On the other hand, IL-6 may be enhancing the early IFN-γ production proving its protective role (94). IL-12 is a key participant as it is a mediator between the innate and adaptive immune systems. IL-12 production is mainly given among the phagocytic cells group and executes its protective functions through the induction of the IFN-γ Th1 response to maintain control of the infection (95).

Contrarily, the anti-inflammatory group of cytokines either block or inhibit the action of the inflammatory cytokines array. IL-10 is produced in the early infection stages by macrophages following *Mtb* phagocytosis, as well as regulatory T-cells. IL-10 depresses the production of IFN-γ, TNF-α, and IL-12, thus compromising the bacterial clearance capacity and the production of other cytokines. In addition, IL-10 can block the DCs migration towards the lymph nodes and the T-cells way backward to the lungs (90,96). Similarly, IL-4 has suppressing effects over the IFN-γ production and macrophages fraction activation (97).

1.4.2 The human tuberculosis granuloma constitution

The hallmark of the human PTB is the constitution of the granuloma. This structure is the outcome of the host-pathogen interactions, and exhibits a potent duality, as the bacilli are enclosed within the granuloma avoiding its systemic dissemination, but such protection requires slow and chronic tissue damage and destruction, favoring mid and long-term poor aftermaths. The main condition for the granuloma formation is the settlement of the bacilli in the pulmonary alveoli, or in the lung's interstitial space. There, local inflammation signals will gather the first-line defense cells, AMs, neutrophils, and DCs, which will amplify the stimuli by releasing cytokines and chemokines attracting more AMs and T-cells (98).

A sustained, chronic release of TNF-α in the early granuloma foci is key as it is required to increase and retain the pool of AMs and T-cells and other cytokines and chemokines to keep this area as a constant chemoattractant core (87). At this stage, vascularized structures appear driven by the induction of the Vascular Endothelial Growth Factor (VEGF) response, stimulated by a local and continuous recruitment

of macrophages, DCs, and lymphocytes, which are needed to aggregate and control the infection milieu (99). As the disease evolves, local cells differentiate to specialized entities as a strong anti-*Mtb* response: granuloma macrophages can fuse to constitute multinucleated giant cells; to epithelioid macrophages; and to foamy macrophages (100,101). Cell differentiation goes hand in hand with the stratification of the granuloma. A macrophage-rich layer is assembled surrounding the central area where the infected macrophages and uncontained bacilli are meet. The center of the granuloma is then enclosed by a secondary fibrous layer, to finally meet how the lymphocyte fraction is pushed and aggregated outside the fibrous layer, so no physical contact is given with the infected macrophages (**Figure 5**) (87,98,101,102).

It has been suggested that this spatial-compartmentalization is the best strategy that the host can conduct; peripheral lymphoid follicle-like structures orchestrate the local host defense from the outside because the cell fraction from the inner granuloma may not provide a sufficient potent immune-response to fight the bacilli (103). The most likely scenario is that this granuloma architecture may represent the sufficient stabilizing structure to control the infection and even allowing its resolution. Nonetheless, the ability to achieve such quality of the granuloma is tied to the host's characteristics, but very importantly, this property is linked to the lung's infection site and the bacillary load. Therefore, two types of granulomas can be distinguished which its fate may be set at the onset of the immune-response post-infection: the proliferative granuloma and the exudative lesions (22).

The proliferative lesion is characterized by gathering a low bacillary load and is mainly constituted by epithelioid cells and fibroblasts. Primed by a Th1 response, this lesion is soon controlled as it favors its fibrosis and calcification mediated by the interlobular lung septum (104). On the other hand, the exudative lesion, or local neutrophilic condensations, is driven by a high bacillary load and is mainly localized in the lungs' upper lobes (22,105). Anatomically, lesions that progress in this area have less lymphatic drainage thus less immune surveillance; and higher alveolar pressure which reduces the capillary density favoring the *Mtb* interstitial infiltration (23,106,107). This situation displays a good environment for the progression of the exudative lesion, enhancing the chances of developing neighbor

lesions as a result of bronchogenic dissemination. Very importantly, high bacillary loads generate higher probabilities of necrosis, which leads to favor "soft" caseum or caseous liquefaction and finally cavitation, which is the main cause of the *Mtb* airborne transmission (22,101).

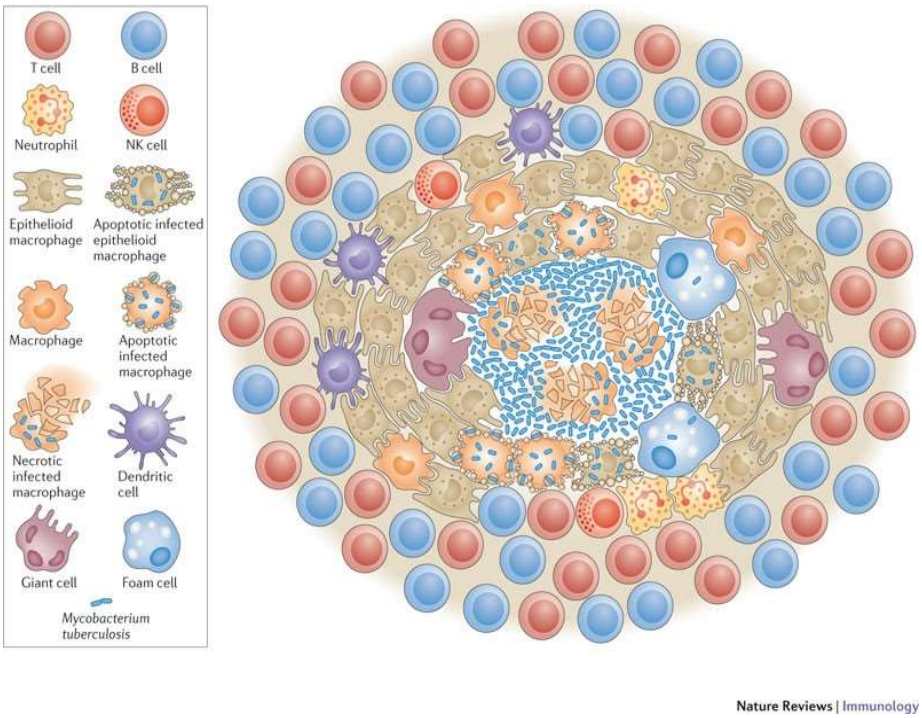


Figure 5. The cellular components and architecture of the human tuberculosis granuloma. The center of the lesion or necrotic foci is harboring the most number of bacilli, free or sequestrated by the foamy macrophages, which are the most frequent cellular fraction placed at the limit of this area, followed by other macrophages such as the AMs. Several sub-class of differentiated macrophages are contributing to building a second layer or wall surrounding the center of the lesion trying to avoid extended local dissemination of the bacilli, as well as a strong presence of fibroblasts inducing tissue changes. Finally, the rim of the lesion is led by the peripheral lymphocyte fraction, which tends to aggregate and infiltrate around the secondary layer, composing a third differentiated layer. Adapted from Ramakrishnan, 2012 (101).

The chronic aggression that occurs during the host-pathogen interaction within the granuloma sometimes leads to the necrosis of the surrounding tissue and therefore its destruction as a final outcome, especially in the center of the exudative lesion. Foamy macrophages, significantly present in this compartment at the onset of the lesion generation, can act as a pole of attraction for neutrophils, and later are an

important reservoir of *Mtb* as they help their drainage out of the lesion, thus having a role in the bronchogenic dissemination (108,109). Eventually, this cell class may die given the harmful responses induced by the high bacillary load. The accumulation of their high-lipidic cargo will start accumulating in the extracellular milieu, thus the aggregation of such amount of lipid cellular-debris will eventually constitute the caseum found in exudative-class TB granuloma lesions (87). Macroscopically, caseum have been classified as "hard" or "dry", which is linked with lower bacillary loads, and on the other hand, "soft" or "fresh" or classically known as liquefaction, harboring high bacillary loads, especially given in exudative lesions (110).

1.5 Biomarkers in TB

As part of the End-TB Strategy targets, the WHO is encouraging the scientific community to intensify the research upon the discovery, development, and fast implementation of new tools capable to overcome current limitations in the LTBI and TB patient management (4). In this context, biomarkers have emerged as an invaluable tool, as objectively can be measured and correlated as indicators of biological or pathological processes and as responses to therapeutic interventions (111,112). Hence, biomarkers may reveal the actual disease state of the patient and make accurate risk predictions such as the LTBI individual progressing to TB, to assess the prognosis of the TB patient under ATT or as surrogate endpoints in clinical trials. Furthermore, biomarkers can be critical as they may reveal protection upon vaccination, or they could be used as targets for host-directed therapies (HDT) (113). The first attempts into characterizing biomarkers at the different stages of the disease were reduced into the classical dogma: classify patients having LTBI, active TB, and healthy individuals. Nonetheless, current biomarker research has highlighted the spectrum of the TB disease and how they may be altered by other factors that modify the host-pathogen interactions (114).

The WHO elaborated a list rating the potential main nine current needed tests, the so-called target product profiles (TPPs), according to ten criteria to identify the priorities on investment, research, and development considering the stakeholders, the impact on the TB transmission, the reduction on morbi-mortality, the market

potential and its scalability. Biomarker-based tests for non-sputum samples to the TB diagnosis ranked among the most important TPPs. Other potential applications for biomarkers are including tests for the treatment monitoring and assessing the disease resolution, which was also listed as a priority. These tests must be highly sensitive, rapid, at low cost, and available for any health-care center (115,116).

Although sputum-based tests are the cornerstone for most of the tools related to the TB diagnosis and patient prognosis evaluation, this source gathers several limitations. Sputum is a relatively easy sample to access, but not all individuals have the capacity to produce sputa, such as children, subjects with HIV/TB coinfection, or patients suffering from comorbidities, such as DM. Furthermore, sputum does not always carry the sufficient *Mtb* burden to be detected, especially when the patient is under treatment, and it is barely useful in the EPTB diagnosis. In addition, sputum decontamination is an essential part of the *Mtb* culture protocol as it is required to avoid cross-contaminations, so this may induce the reduction of the number of viable bacilli (114,117,118). Such situations often induce patients to be empirically treated. To overcome these obstacles, other biological samples such as blood, urine, or even saliva are emerging as a good replacement for sputum-based tests as accessing them is barely invasive and may provide meaningful information facilitating the patient characterization and guiding treatment decisions.

Biomarkers can be subclassified into de detection of *Mtb* cellular components; the antibody response upon the presence of *Mtb* antigens; the cellular immune response to *Mtb* antigens; and those based on the "omics" technologies (i.e. transcriptomics, proteomics, and metabolomics).

A good example of the detection of *Mtb* cellular components is the urinary LAM. LAM testing has been proposed also as a good biomarker to monitor response to ATT, especially indicated in HIV-endemic areas. A study exhibited a potential capacity to identify pulmonary TB/HIV coinfected individuals at high risk of mortality and those with an especial monitoring need at the end of the intensive phase. Furthermore, urinary LAM levels decreased after 6 months of ATT (119).

In this thesis, we are focused on the host's systemic immune response against the *Mtb* by assessing circulant biomarkers that could be potentially associated with worsening factors and poor treatment outcomes. In the same way, we are evaluating the host's ribonucleic acid (RNA) expression by transcriptomics, characterizing the host's gene expression at the tissue and whole blood level.

1.5.1 Biomarkers for prognosis: State-of-the-art

The only currently established and accepted marker of sterilizing activity by the scientific community in clinical trials is the sputum culture conversion (120). Currently, the sputum culture is essential as it is widely used as a surrogate endpoint, becoming the only available tool to assess the efficacy on new drugs, clinical trials on shortening drug regimen especially in the context of drug-resistant TB, and finally as the only way to predict long-term outcomes (121). During ATT monitoring, the predictive capacity of treatment failure and relapse of the *Mtb* culture performs at low sensitivity and poor specificity (122,123). This technique is also incapable to successfully predict or to stratify those patients that may exhibit a symptomatologic worsening. Also, both researchers and clinicians complain that this technique is extremely time-consuming as the sample grows within weeks, especially during ATT, which can be detrimental for the patient as may lead to failing treatment. Besides, the two months for the sputum culture conversion may indicate simply that the bacillary burden that carries the sample is reduced rather than lung sterilization happened (121).

Thus, and according to experts, non-sputum-based host's biomarkers appear as a potential alternative, or when in combination with the sputum culture or other approaches, a supplementary aid to cope with such limitations and with enough potential to become reliable surrogate endpoints.

1.5.1.1 Circulant host's biomarkers

In this thesis, we based our research on many characteristics considered as being related to poor prognosis and its potential association with circulant biomarkers according to recent literature:

1. General aspects linked to poor prognosis: these aspects, being considered in some cases together, included epidemiological traits (sex, age, weight, toxic habits…), clinical features, radiological findings, and microbiological status.
2. Sputum smear conversion: considering the AFB smear grading and sample negativity.
3. Radiographic severity: the presence/persistence of lung cavities, the number of lesions, and other radiographical abnormalities in the CXR.
4. Evaluation of treatment response: although our biomarkers study is following a cross-sectional design, the timepoint at our analysis was made could add more insights to future treatment response studies.
5. Culture conversion after two months of treatment: considering the patient's sputum culture negativity after two months of ATT.

Current literature regarding circulant proteins and peptides that can constitute a biosignature as treatment monitoring biomarkers and/or determine disease severity is wide. To better focus into this topic and to better fit to this thesis thread, the literature research was centered in recent publications studying adult PTB patients; HIV free or excluded from the analysis; unstimulated plasma/serum or urine as the biological sources of biomarkers; and used multiplex platforms and/or other sandwich immunoassays to quantify circulant cytokines, chemokines and/or other proteins that we specifically studied in this project.

1.5.1.1.1 Biomarkers correlators of poor prognosis

Among the review articles, many researchers have attempted classifying PTB patients according to their chances of suffering from bad outcomes during the ATT course or predicting symptoms. Poor prognosis has been associated with several circulant biomarkers. In a study, the C Reactive Protein (CRP) was found higher in patients that had severe TB (considering poor clinical and radiological findings) but survived during the ATT. These findings were correlated with mortality at the termination of standard treatment (124).

IP-10 (CXCL10) is a biomarker extensively used in this context. Patients suffering from worse symptomatology were found to have significantly higher IP-10 levels at the diagnosis moment in comparison with those with mild symptoms at treatment

initiation (125). Patients having a good response treatment, this is improving symptoms and with proved sputum and smear conversion at month-2, had a significant reduction of IP-10 (126). Similarly, patients experiencing from the persistence of TB symptomatology during treatment was found a discrete IP-10 reduction at week-12 of treatment completion, in comparison with those that did not present symptoms. The author attributed this to vitamin D status (127). IP-10 was also used to predict the risk of relapse but failed into this attempt (128).

When sex differences are evaluated considering worsening factors such as weight loss, duration of cough, and AFB positivity, the Matrix Metalloproteinase (MMP)-8 protein appears as being significantly elevated among the male participants (129). In another study, the pro-inflammatory IL-12p40, IFN-γ, TNF-α, IL-1ß, and IL-6, and the anti-inflammatory IL-10 were not found to correlate with the participants' age, although a negative association was found with the Body Mass Index (BMI) repeatedly after two and four months of therapy (130). Other studies evaluating BMI did not find correlations of CRP at baseline (131). Similarly, MMP-8 was not also found linked to weight, as well as to the cough duration (129).

1.5.1.1.2 Biomarkers correlators of sputum smear

IP-10 has also been proposed as a monitor of AFB smear grades. Significant higher IP-10 concentrations in positive sputum smear subjects than negative individuals have been reported in several publications (132,133). IP-10 have also significant correlations with the AFB grading (this is to +1, +2 +3) (128,134,135) when sputum is collected at baseline for diagnosis. A comparison between individuals converting smear in less than two weeks and others within the range of two to eight weeks revealed that IP-10 levels were statistically different at the moment of conversion. Fast smear converters, therefore, presented a faster IP-10 fall (136).

At the diagnosis moment, plasma levels of the pro-inflammatory IL-17, IL-6, and IFN-γ cytokines were statistically increased in patients having a positive AFB in comparison with negative cases (137). Some circulant cytokines have also correlated with the bacterial load in the smear. IFN-γ, TNF-α, and IL-6 and the anti-inflammatory cytokines IL-10 and IL-4 serum levels correlated with the AFB smear grading, being high (+3) bacterial loads for IFN-γ, IL-6, and IL-10 and moderate

(+2) with TNF-α levels (130). Other articles performed a similar approach founding statistical tight correlations with the AFB grading on IL-1ß and IL-6 (118). On the other hand, others did not found associations with AFB smear grades in IL-2 (138), and IL-1ß, IL-6, and TNF-α (139). No statistical differences between positive and negative smears at diagnosis when MMP-8 was studied (140) and CRP (131), although contradictory results were found regarding the CRP role (132,141), and even finding CRP correlated with the sputum smear load (142).

1.5.1.1.3 Biomarkers correlators of radiographic severity

In the context of severity traits that can be evaluated through the CXR, many publications involving cytokines and other proteins were found. The IP-10 chemokine as well as TNF-α in some studies were significantly increased as the radiographic severity was grading to poor features from those with minimal to moderate levels, (135,143–145), although IFN-γ has not found related with poor radiological signs (135). On the other hand, IL-10 levels were found significantly increased in patients having abnormal CXR explorations (146). Other studies did not find an association with TNF-α and the extent of the disease (147). MMP-8 plasma levels were found to increase in PTB patients having a higher radiographical affectation score in comparison to those with a low score (140).

The presence of TB lung cavitation in the CXR has been significantly linked with several cytokines, namely TNF-α, IL-1ß, and IL-6, and a negative correlation is found in TNF-α and IL-6 (148). Also CRP and IP-10, in many publications, were related to the presence of cavities in CXR (136,142,149). In a clinical trial evaluating the rifampin effect, at baseline moment measures revealed that CRP, IL-1ß, MMP-8, and MMP-9 were tightly associated with the presence of cavities, as well as to cavities that in size were bigger than 4 cm. The authors also divided the patients according to the extent of the disease in CXR and found out that those with greater lung involvement had strong correlations with IL-1ß, IL-6, and IP-10 (118). IL-2 levels were found unchanged regarding the lung involvement evaluation (138). CRP appears as being associated with bilateral involvement (141), as well as its levels are significantly decreased among PTB patients exhibiting a radiographical

improvement during ATT at month two (142), although others did not find any relationship regarding CRP levels (131).

In a study uniquely composed by MDR/XDR-TB participants, also some clinical traits correlated with radiological findings. Patients having a poor radiological aspect at baseline were correlated with higher smear grades as well as the time to sputum conversion, yet considering that in this study was set at 71 days. Similarly, only taking into account having cavities, this trait was strongly correlated with patients having a positive culture at baseline. Authors point out that overall severity traits spotted in CXR were associated in fact with the bacillary load (150).

1.5.1.1.4 Biomarkers correlators of treatment response

IP-10 has demonstrated being involved in multiple factors related to prognosis. It is also linked to treatment response, as significant changes between before treatment initiation and completion were found (128), as well as a progressive statistically significant decrease can be seen throughout several treatment timepoints (week-8, week-12, month-6) (125,127,151). In a study where researchers combinate the evaluation of IP-10 in plasma and urine, was found that IP-10 levels in serum were not statistically different between timepoints, although in urine IP-10 levels after 2 months of ATT were higher than at diagnosis moment, but these levels dropped to similar diagnosis levels at the end of ATT month-6 (152). Some cytokines also significantly increase during ATT, especially at month-6 such as IL-2 (138).

Serum pro-inflammatory IL-12p40, IFN-γ, TNF-α, IL-1ß, and IL-6 were evaluated during the ATT course at baseline, two, four, and six-months post-ATT. All five cytokines significantly decreased during ATT achieving a similar level from healthy individuals included in the study. The most promising cytokine was IL-6, showing the most evident and rapid change, especially highlighted at the 4-month time point (130) and also found at week-8 (118). Anti-inflammatory IL-10 remained elevated at month-2 followed by a decline after four- and six-months post-treatment. On the other hand, IL-4 had no alteration over time (130). Also, IL-10 was found significantly decreasing during treatment, at month-3, and month-6 of ATT (146).

Other publications found this reduction in IFN-γ at month-2 (139) and at month-6 (144,153); and TNF-α at month-2 (139) and month-6 of ATT (144,153).

CRP levels were significantly reduced during treatment, being this circulant protein the most notable change after two months of ATT (131,142), and even at month-8 in a rifampin clinical trial (118). CRP behaved similarly in other studies at the same time points, even under DOTs (154). At the beginning of treatment, CRP levels correlated with IL-6, whereas after two months, IFN-γ correlated to CRP, authors arguing that this may be due to chronic systemic inflammation (142). CRP levels were found positively correlated to plasma IL-17 (155). Regarding tissue remodeling proteins, MMP-1 and MMP-8 had a significant decrease after eight weeks of treatment (118).

1.5.1.1.5 Biomarkers correlators of culture conversion at month-2

The combination of IP-10 and other plasmatic biomarkers was studied to find out if some of these biomarkers or in combination can predict at the very beginning if the sputum culture conversion will occur. None of the proteins were found significantly associated with culture conversion at week 8 of ATT (151). On the other hand, to predict an 8-week sputum culture positivity, a predictive model found that the combination of age, a TNF family protein, and adding the week-8 CRP levels successfully achieved this discrimination (151).

In another study, MMP-8 and other chemokines levels measured at month-2 of treatment were found significantly different between patients with persistent negative culture than fast responders. On the other hand, an association was found with those patients being persistently culture negative at the end of the intensive phase considering an AFB smear at diagnosis and high MMP-8 levels at month-2 (140). Pro-inflammatory IL-6 plasmatic levels were significantly higher among slow converters at pre-treatment and treatment completion in comparison with rapid converters (139).

In a study only including drug-resistant PTB patients, was found a significant positive correlation between subjects with delayed sputum culture conversion (71 days) and baseline levels of CRP, and IP-10, among others. To predict the delay

after 71 days, a combination of IP-10 and CRP were involved with the baseline sputum smear status biosignature. After two months under treatment, both fast and slow responders experienced a significant reduction in IP-10 and CRP levels (132). The combination of CRP, as well as IL-1ß, includes important statistical links with the treatment response and sputum conversion at month-2. Next, these researchers attempted to predict the early conversion using the combination of IL-6, MMP-8, and IFN-γ, although the AUC was low (118).

1.5.1.2 High-throughput approaches: transcriptomic biomarkers in whole blood

Literature regarding the treatment response and prediction of short and long-term outcomes by using transcriptomics is scarce. To date, the bulk of publications using a whole-genome expression profiling approach are focused on the study of the LTBI progression to TB and the TB diagnosis (156). In general, all studies found below were conducted either on a microarray or ribonucleic acid-sequencing (RNA-seq) platform, and upon drug-susceptible adult TB patients receiving standard treatment criteria, HIV free and sampling was done at different time points during the ATT course. Reviewed articles are summarized at the end of this section in **Table 4.**

The first attempt into characterizing by transcriptomics changes throughout ATT was conducted by Berry *et al.* using a microarray platform. A TB 393-transcripts signature was built after collecting whole blood from sputum-culture confirmed TB patients. The cohort was a composite of UK and South African patients. By calculating a "molecular distance to health" (MDTH) (157) and assessing the radiographic extent of the disease, were able to correlate the 393-blood signature in TB patients with different degrees of disease severity, being greater the difference among those with advanced disease. The specific gene expression was found decreasing during treatment, especially after two months and disappearing at 12 months after ATT, showing the "molecular distance to health" being lower than at recruitment and proving improved radiographic signs. The Ingenuity Pathways Analysis (IPA) and modular analysis revealed that the 393-transcripts signature most recurrent pathways were linked to neutrophil-driven interferon-inducible gene sets (mainly both IFN-γ and type I IFN-αß signaling), accompanied with a

decreased presence of B-cells and T-cells, whereas myeloid transcripts were abundant. The authors highlight an important role of neutrophils during the onset of the disease. In addition, an 86-transcript signature was found to distinguish TB from other infections (158).

Following a similar strategy, Bloom *et al.* observed that meaningful changes in the host blood transcriptome were detectable after two weeks after ATT initiation. Using a cohort of DS-TB South African adults with confirmed sputum-culture for TB, whole blood was collected before ATT initiation, at several time points during the six-month treatment, when all patients were discharged as being microbiologically cured, and after 12 months. A 320-transcripts treatment signature was generated comparing pre-treatment RNA expression with treatment termination at six months. This signature showed to significantly and rapidly change at two weeks measurement and onwards. Next, this signature was validated in a UK cohort with similar behavior. Also, by IPA, the 320-transcripts signature exhibited how the host's innate immunity genes were significantly overrepresented together with genes related to the complement system and Toll-like receptors (159).

Ottenhoff and colleagues extracted the total-RNA from unharvested PBMCs in an Indonesian cohort during several stages, specifically before ATT initiation and during it, and until reaching week 28, when the disease had a favorable resolution in all the TB participants. Posterior to the microarray, an 875-transcripts signature of significantly expressed genes was obtained when time zero were compared to their matched healthy controls group. Further analysis revealed the differences among each timepoint but the greater similarity was found between the end of treatment moment with the healthy individuals. Clustering analysis in up and down-regulated gene sets exhibited 56 transcripts with enhanced activity related to type 1 interferon and were found decreasing during the treatment course, whereas 88 involved in immunity and defense and chemokine-mediated signaling had an increment the more closer to the TB resolution and healthy volunteers (160).

Another example of research upon early changes during ATT by transcriptomics is the one performed by Cliff *et al.* Up to 27 South African adults were recruited at the TB diagnosis moment with positive smear and moderate to severe TB according to

the CXR signs. Peripheral blood was prospectively collected after one, two, four, and 26 weeks of standard DS-TB treatment when were discharged with positive outcomes. Meaningful changes in gene expression were detected early, right before the first week under ATT. 1261 genes were found to be statistically differently regulated, which the majority were infra expressed. At the end of ATT, 549 genes were at least 2-fold significantly differentially expressed when compared with diagnosis moment. The authors declared that these substantial changes during treatment reflect the lung clearance from the bacilli presence. Genes belonging to the interferon module reflected a progressive expression decrease during the treatment course, especially palpable after the first week on treatment. Down-regulated modules from month six compared to recruitment moment were B-cells, cytotoxic cells, T-cells, and ribosomal proteins (161).

Thompson and colleagues were the first using whole blood to RNA-sequencing technology to find out resultant meaningful transcripts for calculating unfavorable TB outcomes during treatment. In total, 131 positive sputum-culture newly-diagnosed adults were recruited in South Africa, divided into "cured" as individuals achieving sputum conversion by month 6 after DS-TB standard treatment and "failed" if at month 6 culture was still positive. Whole blood was collected at different time points until reaching 24 weeks after the treatment was initiated. Results indicated that genes belonging to the named DISEASE signature change significantly different during the treatment course among treatment failures, individuals that convert sputum culture in less than two months, and cured subjects. The second built signature, the FAILURE was able to predict those cases of treatment failure evaluating the gene expression before treatment initiation. This signature was especially driven by the presence of down-regulated mitochondrial genes in the failure group respective to cures at recruitment. Finally, a five-genes signature, the RESPONSE5, allowed both the treatment response monitoring and the prediction of ATT failure. These findings correlated with pulmonary affectation assessed through PET/CT scanning. The authors also declared that the overall of their findings suggests a certain link between biological processes underlying progression to TB and disease resolution (162). A later study from this consortium based on the same settings and additional Brazilian and

Peruvian cohorts found a real-time PCR derived signature, the RISK6 signature, which was able to correlate with the extent of the disease in the lung as well as exhibited a good performance as a treatment response biomarker panel. It predicted before ATT initiation a potential treatment failure as well as discriminated with good efficiency failures and cured cases at the end of the treatment course (163).

Table 4. Summary of reviewed articles providing gene-derived signatures evaluating the treatment response during treatment and/or the extent of de disease according to radiological findings. Only microarray and RNA-seq platforms were considered.

Author	Year	Cohort Country	Source	Subjects	Genome-wide platform	Generated signature	Description
Berry	2010	United Kingdom (London)	Whole blood	Active TB	Microarray	393-transcript	MDTH diminishes during ATT. Correlation with the extent of the disease
		South Africa		LTBI Healthy controls		86-gene	Signature distinguish TB from other diseases
Bloom	2012	South Africa	Whole blood	Active TB	Microarray	664-transcript	MDTH diminishes at 2 weeks of ATT and onwards
		United Kingdom (London)		LTBI		320-transcript	Changes in expression at 2 weeks onwards after ATT initiation
Ottenhoff	2012	Indonesia (Jakarta)	Unharvested PBMCs	Active TB Healthy controls	Microarray	875-transcript	Dissimilarities in the gene expression pattern between baseline and week-8 of ATT from week-28 and healthy controls
Cliff	2013	South Africa	Whole blood	Active TB	Microarray	1261-gene	Rapid expression changes after the first week of ATT
						780-gene	Sustained changes at two and four weeks of ATT from diagnosis moment
Thompson	2017	South Africa	Whole blood	Active TB	RNA-seq	DISEASE	Progression to active TB and resolution of the disease during ATT
						FAILURE	Indicates the potentiality to fail ATT
						RESPONSE5	ATT response monitoring and prediction of ATT failure
Warsinske	2018	South Africa	Whole blood	Active TB	RNA-seq	3-gene TB score	Correlation with lung pathology at diagnosis

Another project using RNA-seq created a 3-gene TB score, product of a multicohort analysis intended for the active TB diagnosis (164). This score later proved that can indicate potential individuals with lung pathology as well as the capacity to work as a treatment response monitor, especially during advanced stages of ATT in which

the bacillary burden in sputum is significantly reduced. According to results, the 3-gene TB score, measured at baseline, correlated with the lung inflammation in PTB patients, exhibiting a higher 3-gene TB score at the time of diagnosis (165).

1.5.1.3 High-throughput approaches: transcriptomics biomarkers from the human tuberculosis lesions

In the context of the genome-wide expression studies in human tuberculosis lesions, only a few researchers have had the chance to conduct this kind of approach. The first study of such characteristics was conducted by Kim and colleagues. The framework was to characterize how the TB lesions induce necrotic caseation and lung cavitation and which are the main genes responsible for this process. In this study, TB caseous lesions were surgically removed from South African TB patients with extensive lung cavitation and tissue degeneration. The surgery indication was due to poor response to ATT. TB lesions were formalin-fixed paraffin-embedded (FFPE) and total-RNA was later obtained from Laser Capture Microdissection (LCM)-derived materials. Uninvolved lung parenchyma tissue from the same donors was used as a control. Microarray analysis revealed overlapped patterns of the human TB lesions transcriptome with other diseases with a similar destructive pattern, such as cancer and metabolic diseases. An up-regulated lipid metabolism was found in the TB lesion transcriptome together with the association of TNF-α, suggesting a local potent pro-inflammatory aggression. Some lipid metabolism-related genes with a high RNA expression were found encoding important proteins related to fatty acids regulation, triacylglycerides, and the inhibition of lipid catabolism. A further exploration upon TB lesions underwent to confocal microscopy, indicated how these proteins may be related to the host's lipid sequestration by foamy macrophages during the cavitary lesion progression and how are gradually accumulated in the center of the granuloma, constituting the caseous necrosis which becomes an ideal nest for the *Mtb* reproduction (166).

Another study lead by some of the previous authors recruited six South African patients that underwent therapeutic surgery. Indications were mainly given because three of them had a bad TB prognosis because of persistent culture positivity despite good ATT adherence, while the three others were a potential

relapse case, given that a considerable worsening in their symptomatology was happening despite having sterilized culture. Two of them were MDR/XDR-TB. Resected TB lung specimens, together with healthy uninvolved lung parenchyma, were FFPE and total-RNA isolated after sectioning the samples through LCM. Microarray expression analysis exhibited 11651 significantly differentially expressed (SDE) genes from the healthy controls. The Gene Ontology (GO) posterior exploration revealed a general up-regulation of inflammation, cellular immunity, and tissue damage and remodeling in TB lesions. Differences in gene expression between cavitary and fibrotic lesions were also assessed. Fibrotic nodules harvested a higher number of meaningfully expressed genes than cavitary lesions, although when common transcripts were analyzed together, up to 1.185 of them were five-fold over-expressed in cavitary granulomas. GO indicated that chemokine and NOD-like signaling and cytokines receptor signaling were the main affected networks in TB cavitary lesions, whereas MHC-class I-mediated antigen processing in fibrotic nodules. As reported in the same way earlier, lipid metabolism and lipoproteins were found up-regulated in both lesions types (166,167).

1.6 Current challenges and potential approaches

In view of all the exposed in previous sections, some of the main underlined challenges in the TB research are herein summarized:

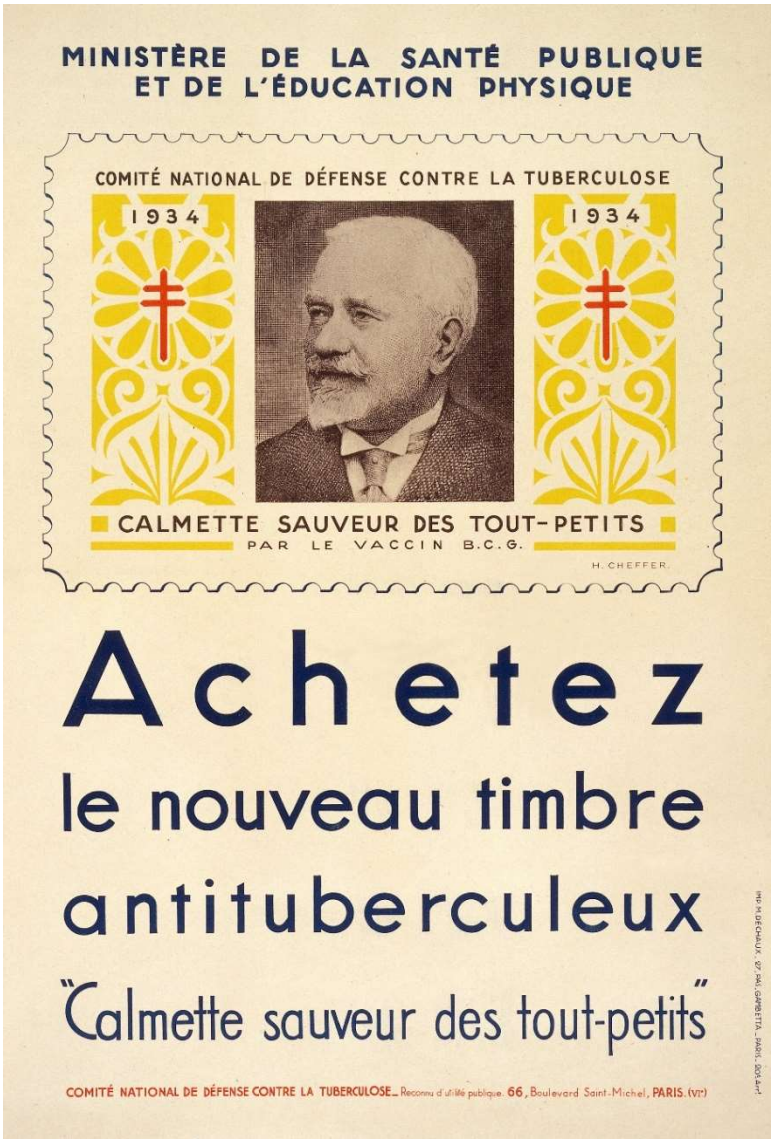
1. Surgery is a useful approach to repress the spreading of the disease, although there are not enough objective indicators to endorse its use.
2. The sputum culture is the main validated biomarker to assess treatment efficacy and microbiological status. Considering the limitations of the sputum and the evidence supporting that the culture does not show the real microbiological status, alternative biomarkers from different sources are urgently needed.
3. Mostly, current biomarkers studies include only patients' samples from the onset of the disease until the treatment completion exhibiting a cured status. In consequence, some stages of the TB spectrum are sometimes misrepresented.

4. Research upon circulant and transcriptional biomarkers is limited to the systemic behavior of the disease. None of them are including the in situ molecular profile and phenotypic characteristics of the TB lesions.

Considering the above points, potential approaches are below exposed:

1. Clinical signals and alternative biomarkers that point to the need for PTB surgery could benefit the prognosis of all TB patients.
2. Heterogenous TB patients' cohorts in terms of disease status, clinical-epidemiological, and microbiological features are needed to be more inclusive with the broad TB spectrum and refine the abilities of the non-sputum based biosignatures.
3. The study of the human TB lesions could provide evidence on the microbiological status of the TB patient and help to build and shaping new biosignatures. The generated insights about the local host(human)-pathogen interactions may become a key factor to guide the fate of the PTB patients, especially among prognosis-based studies.

2 | HYPOTHESIS AND OBJECTIVES



Description: "In the battle against tuberculosis, a portrait of Calmette, the inventor of the BCG, as the savior of children through his novel vaccine promotes the purchase of fund-raising stamps for the tuberculosis research." Year 1934.

Author: Color lithograph after H. Cheffe (Wellcome images: Library reference: ICV No 51428 and BSIP 1275105; Photo number: L0032190).

This thesis hypothesizes that the study of local and systemic immune responses from patients undergoing therapeutic surgery from their PTB could help us to identify potential TB prognostic biomarkers and to understand the role of the host in the mechanisms associated with the generation and evolution of the TB cavitary lesions. To achieve the general aim of this thesis, four specific objectives were established:

1. To describe and characterize the general and clinical forms and characteristics of a cohort of TB patients undergoing therapeutic surgery as well as the macroscopic and microbiological features of the human TB lesions to determine potential severity factors.
2. To correlate a set of circulant biomarkers with demographic and clinical forms of a cohort of TB patients undergoing therapeutic surgery.
3. To obtain the human TB granuloma transcriptome and a list of genes derived from the differential expression of the human TB lesions and whole blood according to the pathophysiological phenotype of the TB patients.
4. To characterize the systemic and in situ host immune response in patients undergoing therapeutic surgery.

To accomplish the proposed set of objectives, two specific approaches were designed:

1. Study I: a retrospective description of the clinical characteristics of a cohort of TB patients that underwent therapeutic surgery and their relationship with the features of the removed TB lesions. The main aim of this study was to examine the demographical, the clinical features, and the macroscopic and microbiological status in surgical specimens of the TB patients that required surgery and to evaluate its impact on patients' prognosis.
2. Study II: a cross-sectional study recruiting patients requiring PTB therapeutic surgery to study the differential gene expression in fresh TB lesions specimens and whole blood as well as the circulant markers levels. The main aim of this study was to determine potential diagnostic and/or prognostic TB biomarkers through the correlation to the host's clinical and microbiological features as well as to characterize and evaluate the host's immune status at this stage of the disease.

3 | MATERIALS AND METHODS

Description: "*Fight tuberculosis - obey the rules of health* - A poster from the Works Progress Administration created between 1936–1941 warns the citizens to prevent tuberculosis by having good sleeping habits, eating properly, and getting sufficient exposure to sunlight."

Author: WPA Federal Art Project, Dis. 4

3.1 The Study of Human Tuberculosis Lesions obtained in therapeutic surgery project (SH-TBL)

This thesis presents two clinical studies, the "Retrospective study of clinical and lesions characteristics of patients of patients undergoing surgical treatment for their tuberculosis in Georgia" and "Evaluation of the immune response and the transcriptomic profile in tuberculosis patients undergoing therapeutic surgery" hereinafter named as Study I and Study II respectively, both belonging to the project "The Study of Human Tuberculosis Lesions" (SH-TBL). Both studies were conceived and conducted by the Experimental Tuberculosis Unit (UTE), based in the Institute for Health Science Research Germans Trias i Pujol (IGTP) in Badalona (Catalonia), and in collaboration with the National Center of Tuberculosis and Lung Diseases (NCTLD) in Tbilisi (Georgia).

Study II was divided into two sub-studies, called "Evaluation of the immune response in tuberculosis patients before and after receiving therapeutic surgery in Georgia" and "Transcriptional analysis of the human tuberculosis lesions obtained after therapeutic surgery", hereinafter named Sub-Study A and Sub-Study B respectively. Sub-Study A and Sub-Study B methods and results are independently showed.

The SH-TBL project was registered at the ClinicalTrials.gov database under the code NCT02715271 as a clinical study. The protocol of the whole project, the research methodology, as well as associated documents (Informed Consent Sheet, Informed Consent Form), were reviewed and approved by the ethics committee from the NCTLD (IRB00007705 NCTLD Georgia #1, IORG0006411) and the IGTP ethics committee (EC: PI-16-171) to ensure the compliance with all current national and European laws on clinical studies.

The project was funded by the Spanish Government-FEDER Funds through CP13/00174, CP18/00031, and PI16/01511 grants; the "CIBER Enfermedades Respiratorias" Network (CIBERES); and by the "Spanish Society of Pneumology and Thoracic Surgery" (SEPAR) through grant 16/023; an FI grant (2017 FI_B_00797) conceded by the "Agència de Gestió d'Ajuts Universitaris i de Recerca"

(AGAUR); and a scholarship from “Education, Audiovisual and Culture Executive Agency” (EACEA).

3.2 Study I – Retrospective study of clinical and lesions characteristics of patients undergoing surgical treatment for their Tuberculosis in Georgia

3.2.1 Study I design

Data from 137 Georgian patients that underwent therapeutic surgery from their TB were recorded retrospectively and anonymously during the years 2014 and 2015 in the NCTLD according to the Georgian national guidelines, based on WHO guidelines (54).

The surgical team noted down all the gathered patients’ information on a custom spreadsheet created ad hoc. The data were listed considering participants' general clinical and epidemiological aspects, information from the current TB episode together with surgical aspects. Furthermore, removed cavitary lesions were macroscopically characterized to be included in the analysis. WHO definitions were used whenever possible. All the recorded information, definitions, and possible answers are enclosed in **Table 5.**

3.2.2 Statistical analysis

GraphPad Prism software (GraphPad version 6, CA, USA) was used to draw the figures. Statistical analysis was done using the independent samples Student’s t-test to compare continuous variables. The associations with other categorical variables were tested with the Chi-square test or the Fisher exact test. All tests were two-tailed, and p-values less than 0.05 were considered statistically significant.

Table 5. General clinical and TB related data, surgical and macroscopic TB lesions features measures.

Group	Category	Variables
Demographic and epidemiological data	Age (years old)	years old
	Gender	male/female
	Smoking habit	on daily or almost daily basis, weekly, monthly, less than monthly, never
	Alcohol intake	on daily or almost daily basis, weekly, monthly, less than monthly, never
General clinical data	Presence of comorbidities	diabetes mellitus, HIV infection, immunosuppression other than HIV-infection, renal failure, chronic obstructive pulmonary disease (COPD) previous to TB, Hepatitis C Virus infection, hepatic cirrhosis, others
Clinical data regarding the current TB episode	History of previous treatment	new patient, relapse patient: treatment after failure, treatment after loss to follow-up patient, other previously treated patients, unknown previous TB treatment
	Anatomical site of TB	pulmonary/extrapulmonary
	Drug-sensitivity	Drug Sensitive (DS-TB), Rifampicin Resistant only, Monoresistance to any other drug other than rifampicin, polydrug resistance, Multi-Drug Resistant (MDR), extensively-Drug resistance (XDR)
	Date of diagnosis of the present TB episode	dd/mm/yy
	Date of treatment initiation	dd/mm/yy
	Number of lesions found in chest X-Ray	n
	Bacteriologically cured or not at the time of surgery according to WHO's guidelines	yes/no
	Time to negativization of sputum culture	recorded in months, and analyzed as always negative, negative at ≤2 months or negative at >2 M
	Time from TB diagnosis to surgery	days
Data regarding the surgery performed	Main indication for surgery	still lesions in X-ray assays, clinically not cured, microbiologically not cured, others (spontaneous pneumothorax, pulmonary hemorrhage, pleural empyema)
	Number of operated lesions	n
	Type of surgery performed	segmentectomy (for lesion resection or removing lung segment), lobectomy (for lung resection or removing lung lobe), pulmonectomy (for removal of the whole lung)
	Date of the surgery	dd/mm/yy
	Post-surgery complications	open answer
Data on macroscopic characteristics of the TB lesions removed (surgical specimens)	Lesion size	in cm, considering the maximum length
	Type of lesion operated	tuberculoma, cavitation
	Presence of necrosis	no necrosis, necrosis: moderate growth of connective tissue alternating with unmodified lung tissue, cirrhosis: complete substitution of lung tissue by the connective tissue with damage of vessels and bronchi
	Type of necrosis	fresh: necrosis macroscopically looking like of a liquid consistency, dry: necrosis macroscopically looking like of a dry consistency, both: coexistence of fresh and dry necrosis
	Presence of bacilli in AFB	yes/no
	Positivity of culture of samples in solid medium (Lowenstein-Jensen) for *M. tuberculosis*	yes/no
Outcome	Official final outcome according to WHO definitions	treatment completed, treatment success, cured, lost to follow-up, treatment failed, still not known, not evaluated, death (if death, date of death in dd/mm/yy recorded)

3.3 Study II – Evaluation of the immune response and the transcriptomic profile in tuberculosis patients undergoing therapeutic surgery

3.3.1 Study II design

Study II is a cross-sectional study that in total 40 Georgian adults were recruited to compose the SH-TBL Cohort. The participants consecutively joined the study in the NCTLD, from May 2016 to May 2018.

All patients recruited in this study received therapy according to national guidelines (54) and at the moment of the surgery, the whole cohort had a bacteriological conversion of sputum and was negative by smear microscopy and culture. The participant inclusion criteria were if patients required surgery because of persistent radiological signs of cavitary lesions in the CXR and computed tomography (CT) scan, according to official guidelines' surgery recommendations (54). Thoracic surgery indication was made by the NCTLD Resistant Tuberculosis Treatment Committee, composed of two surgeons and 18 pulmonary TB specialists.

At diagnosis of the current TB episode, the cohort started standard treatment regimen for their PTB according to Georgia national guidelines for DS-TB, MDR-TB, and XDR-TB strains (48,49). During the ATT course, participants exhibited consecutive negative sputum cultures, as well as negative AFB smear, demonstrating bacilli clearance. Written informed consent was provided to all patients before being enrolled.

3.3.2 Data and samples collection

Pre-surgical fresh morning urine and peripheral blood samples for the immunological study and RNA sequencing were obtained at recruitment (moment hereinafter named as pre-surgery), together with a CXR, TB related data from the current episode, socio-epidemiological information, and other clinical aspects such as comorbidities. During the surgical procedure, TB lesions were removed and kept for *Mtb* detection and host's RNA sequencing. Data from the surgery and the macroscopic description of the lesion piece was also noted down. At hospital

discharge, new peripheral blood and urine samples were collected, moment hereinafter named as post-surgery, and possible complications after surgery were recorded. All data collected during the pulmonary TB episode, at recruitment, and data related to the surgery and at discharge moment was uploaded by NCTLD physicians on OpenClinica (version 3.1), an electronic case report form (eCRF) platform (168). Granuloma lesion surgical pieces retrieved during surgery were taken to check the microbiological status of the patient on-site through biopsy culture and AFB stain.

Both sub-studies A and B will use all the collected clinical and epidemiological aspects from the SH-TBL cohort to evaluate the immune response and to elaborate a transcriptomic profile through RNA-sequencing.

3.3.3 Sub-Study A – Evaluation of the immune response in tuberculosis patients before and after receiving therapeutic surgery in Georgia

3.3.3.1 Samples collection

In total, 80 Vacutainer® with EDTA Tubes (Becton Dickinson & Company, NJ, USA) enclosing peripheral blood and 80 15mL sterile falcon tubes containing fresh morning urine were collected. Half of the samples were gathered in every study participant before receiving therapeutic surgery in the NCTLD, and the resting half at the hospital discharge moment. Vacutainer® tubes were inverted 10 times and then centrifuged at 4ºC for 10 minutes at 2000g for the plasma obtaining by the NCTLD technicians. Plasma was placed in properly labeled sterile cryotubes and then frozen at -80°C within one hour after collection, for finally being batch-shipped together with urine samples on dry ice to the IGTP, in Badalona. Serial plasma and urine aliquots were made in new sterile cryotubes and frozen at -80°C in the UTE laboratory. For the following experiments, the aliquots were unfrozen so that no more than three freeze-thaw cycles were performed.

3.3.3.2 Immunological studies

Ten cytokines, IFN-γ, IL-1ß, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17a, and TNF-α, employing urine and plasma samples, and MMP-8 and MMP-9, employing

plasma samples only, were measured through Millipore's MILLIPLEX MAP human high sensitivity T cell magnetic Bead Panel (cat#HSTMAG-28SK, EMD Millipore Corporation, Billerica, MA, USA) and R&D's Human MMP Premixed Magnetic Luminex Performance Assay (cat#FCSTM07-2, R&D Systems, MN, USA), respectively, using a sandwich immunoassay format, following manufacturer's instructions.

Detection of the whole analytes set required of urine and plasma aliquots being completely thawed; prior dilution upon pre-surgery and post-surgery neat plasma samples were not necessary since the assay has built-in two-fold dilution; urine was centrifuged 10 minutes at 600g to allow debris precipitation. MMP's detection required 10 minutes 1500g centrifugation at 4ºC and a 50-fold dilution for both plasma samples' time points. For quantification of the analyte concentrations, each plate included a seven-point calibration curve (prepared by serial dilutions of the combined calibration standard) and a blank run in duplicate. Each cytokine's plate with the standards and the wells were incubated overnight with the beads on a shaker. Detection antibodies and Streptavidin-Phycoerythrin was added with intermittent washings. Both cytokines and MMP's plates were read in a Luminex® 200 instrument calibrated with an xPOTENT® Calibration Kit (EMD Millipore Catalog #40-276) and verified with the Performance Verification Kit (EMD Millipore Catalog #40-275). The xPonent software Version 3.1 (Luminex Corporation, TX, USA) was used to calculate concentrations for the control and unknown samples based on a five-parameter logistic (5-PL) fit the calibration data.

The levels of IP-10, CRP, and the soluble CD14 (sCD14) on plasma and urine were determined by sandwich ELISA (cat#DIP100, DCRP00, and DC140 respectively, Quantikine ELISA Kits, R&D Systems, MN, USA) following manufacturer instructions. Plasma samples were diluted 1:1 for IP-10, 200-fold dilution for sCD14, and 1:400 for CRP detection upon pre-surgery samples, while a 1:800 dilution was used for the post-surgery samples. For CD5L plasma levels samples were diluted 1:100 (except for three samples that a 1:500 dilution was needed) and the human AIM/CD5L/Spa ELISA kit (Medical & Biological. Laboratories Co., Ltd, Aichi, Japan) was used. ELISA plates were read in a Varioskan Flash spectral scanning multimode reader (Thermo Scientific, MA, USA). All urine samples were

centrifuged 10 minutes at 600g to allow debris precipitation. To determine the levels of the proteins, a calibration curve was generated using the simple linear regression model relying on spreadsheets using Microsoft Excel 2019 MSO (16.0.8431.2120) for all the markers.

3.3.3.3 Statistical analysis

Differences between the concentrations of biomarkers were analyzed and plotted using Graph Pad Prism 7 (La Jolla, CA, USA) applying Student's t-test, the Mann-Whitney U test, Wilcoxon's Matched-Pairs signed rank test and Fisher's exact test. Tests were two-tailed. P-values of less than 0.05 were considered to be statistically significant. Correlations were analyzed with FactoMineR through R Studio (version 3.6.2); the strength of the relationship measured by the correlation ratio (eta) for the association of binary variables with continuous ones; and by the Chi-Square statistic for the association between binary variables. The ability of combinations of selected host biomarkers and clinical/epidemiological factors to predict the features of lesions was assessed by plotting Receiver Operator Characteristics (ROC) and, because of the modest sample size (n=40), applying General Discriminant Analysis (GDA) with leave-one-out cross-validation.

3.3.4 Sub-Study B – Transcriptional analysis of the human tuberculosis lesions obtained after therapeutic surgery

Sub-Study B was conducted in collaboration with the St. Mary's Hospital (Imperial College) Department of Medicine, Paediatrics Unit in London (UK) thanks to an agreement between Dr. Myrsini Kaforou and the UTE. A three months stay was funded at the St. Mary's thanks to the FI personal grant (2017 FI_B_00797) conceded by the AGAUR and by CIBERES' "Ayudas de Perfeccionamiento y Movilidad", to learn biocomputational techniques and to analyze Sub-Study B raw results.

3.3.4.1 Samples collection

Peripheral blood was collected into PAXgene® Blood RNA Tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland), kept for two hours at room temperature and later to be stored at -80ºC, both before volunteers underwent surgery and at hospital discharge. During therapeutic surgery, a 3.2 cm diameter (median) piece of

granuloma TB lesion sample and a pulmonary Nodulus (N) sample were removed, together with unaffected, at the naked eye, Healthy lung parenchyma (H) following the surgeon criteria. Pulmonary nodulus is defined as a primary lesion product of the dissemination of a single or multiple initial lesions. They are rounded shape and their diameter ranges from 1 to 1.5 cm. They can be found single or multiple, and the inside usually contains liquefying necrosis at the onset, to become harder through time and calcifications may be developed after a few years. Both N and H were taken from the same lung where granuloma was removed. Granuloma samples were then divided into three ~0.5 cm^3 parts: the Center of the lesion (C), the Internal Wall of the lesion (I), and the External Wall of the lesion (E), following surgeon criteria (**Figure 6**).

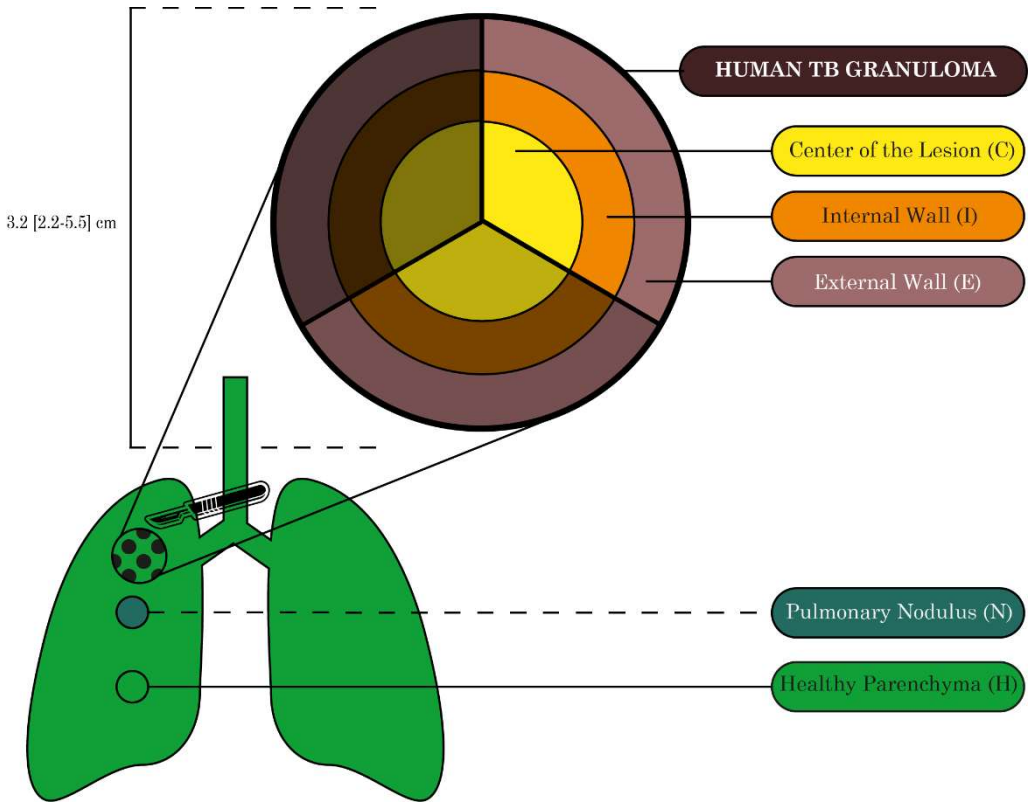


Figure 6. **The human cavitary TB lesions, also named as TB granuloma, parts scheme.** During surgery, TB lesions, ranging from 2.2 to 5.5 cm, were kept and surgeons collected samples from each of the three parts. Additionally, healthy pulmonary lung parenchyma was surgically removed to perform as injured tissue control, plus pulmonary nodulus, for further studies.

Next, fresh tissue samples were immediately placed in labeled cryotubes and kept on RNAlater solution (Qiagen, Hilden, Germany) at 4ºC overnight to be removed the following morning, for finally being stored at -80ºC until the shipment to the IGTP from the NCTLD. In total, 278 SH-TBL's biological samples were collected, intended for the total-RNA transcriptomic analysis. In terms of peripheral blood, 39 pre-surgery PAXgene® tubes were obtained, and 40 more when inpatients were discharged. Regarding tissue samples, a single piece of each lesion part was removed, so 39 samples from the C and 40 I and E, respectively, were gathered, as well as from N and H (**Table 6**).

3.3.4.2 The Total-RNA obtaining

Total-RNA whole blood from PAXgene® tubes was isolated through the PAXgene® Blood miRNA Kit (PreAnalytiX GmbH, Hombrechtikon, Switzerland) following the manufacturer's instructions. Tissue total-RNA isolates were obtained from 39 pre-surgery and 14 post-surgery samples, and 39 C, 40 I, 40 E, 40 H, and 14 N tissue samples in the Comparative Medicine and Bioimage Centre of Catalonia (CMCiB) BioSafety Level 3 (BSL-3) laboratory (**Table 6**).

Table 6. The SH-TBL Sub-Study B samples collection. The number of blood and granuloma tissue samples throughout the SH-TBL Cohort, total-RNA isolation, and constitution of the Discovery Set and Validation Set.

	SH-TBL samples Collection	Total-RNA samples obtained	Discovery Set		Validation Set	
			Rejected due to low RINe	RNA-seq Illumina	Rejected due to low RINe	NanoString nCounter®
Peripheral blood						
Pre-Surgery PAXgene®	39	39	0	13	0	26
Post-Surgery PAXgene®	40	14	0	14	0	0
Biopsy samples						
Center of the Lesion (C)	39	39	6	8	6	19
Internal Wall of the Lesion (I)	40	40	1	13	0	26
External Wall of the Lesion (E)	40	40	0	14	0	26
Healthy lung Parenchyma (H)	40	40	1	13	1	25
Pulmonary Nodulus (N)	40	14	1	13	0	0
TOTAL	278	226	9	88	7	122

For an optimal RNA recovery, tissue mother samples are required to be split to 0.16-0.21g single pieces and placed in new tubes. Single frozen tissue samples were then turned into powder by mechanical cryofracturing using a BioPulverizer device (Biospec Products, OK, USA) after being liquid nitrogen-cooled. The powdered-tissue was then transferred to 2mL Lysing Matrix D tubes (MP Biomedicals, CA, USA) together with lysis solution for a better grinding and homogenization performance over a FastPrep® instrument (MP Biomedicals, CA, USA). The mirVana miRNA Isolation Kit (Thermo Fisher Scientific, MA, USA) allowed the total-RNA isolation and recovery, followed by a genomic DNA digestion through DNA-free DNA Removal Kit (Thermo Fisher Scientific, MA, USA) considering manufacturer's instructions, respectively.

Quantitative and qualitative RNA integrity number equivalent (RINe) values were obtained mediated by an Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA). We considered as an optimal minimal value of seven RINe score for blood RNA. We could not have the same consideration regarding the ideal score with tissue samples like blood RNA. Reasonably, such integrity in affected, necrotic surgical tissue specimens was not always found. For that reason, and considering the difficult availability of this tissue collection, a minimum of a four RINe cutoff was finally given to the tissue set. Under this precept, it must be highlighted the fact that low RNA integrity is an important bias factor in RNA-Seq experiments where gene expression is intended to be tested between different groups. This may lead to misrepresent the real transcript activity and become false outcomes for the population under study.

In order to perform Next Generation Sequencing (NGS) upon the SH-TBL cohort's isolated total-RNA, patients were matched according to sex and drug susceptibility. In total, 14 patients were selected for this purpose and were established as the Discovery Set (**Table 7**). As set by the RINe cutoff, six out of 14 C samples were discarded, along with a sample from the I, N, and H. To sum up, 61 tissue samples went on to the library's construction, not forgetting 13 pre-surgery blood tubes (a patient's tube was not collected) and 14 post-surgery (**Table 6**).

The total-isolated-RNA from the Discovery Set was diluted to reach a 25 ng/µl aliquot and then was shipped to Macrogen (Seoul, South Korea) where sequencing was performed. Libraries were constructed by the TruSeq Stranded Total RNA LT Sample Prep Kit (Human Mouse Rat) (Illumina, CA, USA) following the TruSeq Stranded Total RNA Sample Prep guide (Part #15031048 Rev. E), including a prior removal of ribosomal RNA through the RNA Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat) (Illumina, CA, USA).

Table 7. The number of patients that constituted the SH-TBL Discovery Set matching for drug-sensitivity and sex.

	Drug-Sensitivity			
	DS-TB	**MDR-TB**	**XDR-TB**	**TOTAL patients**
Males	3	3	1	7
Females	3	3	1	7
TOTAL patients	6	6	2	14

3.3.4.3 RNA-Sequencing and preprocessing raw data

NGS was performed on an Illumina platform HiSeq 4000 (Illumina, CA, USA), at 50 million reads per sample, 100bp paired-end reads. Read-pairs, forward and reverse fragments, from raw-data reads were obtained in *.fastq* format. Preprocessing of raw data included quality control with MultiQC (version 1.9) (169). *Per base sequence quality* and *per sequence quality* were assessed, reaching positives values. As part of the Illumina library construction protocols, extra nucleotides are included at the end of the complementary DNA fragments to conduct the sequencing. The *per base sequence content* allowed to detect the residual presence of these Illumina library product fragments or adapters at the reads edges as low-quality regions. Finally, the *per sequence GC content* assessed the average GC bases within raw data reads to track the presence of ribosomal/globin RNA.

Before further steps in the preprocessing the reads, adapters were trimmed off from the edges of the read with Trimmomatic (version 0.39) (170). Next, the mapping required of generating a human genome index as it is needed for the genome assembly. The human genome sequences GRCh38.89 and human genes annotations were downloaded from the ENSEMBL web repository. The genome index was generated through the Spliced Transcripts Alignment to a Reference (STAR)

package (version 2.7.5b) (171). Once the human index was built, *.fastq* paired-reads files from every sample were gathered for the alignment against the human reference genome. Likewise, STAR was used again for such purpose, following the gene counts quantification mode for stranded RNA-seq data. For the following downstream analysis, using the coordinate-sorted mode, the BAM format for files was used for a more efficient size compression during the genome alignment, significantly reducing the sorting steps in time. Finally, SAMtools package allowed to calculate the percentage of successful reads alignment against the reference genome (version 1.10) (172).

3.3.4.4 Raw counts statistical analysis

The overall pipeline for the data handling, plotting and statistical computing, mainly involving the differential expression analysis, was conducted through the software R Studio "A Language and Environment for Statistical Computing" (R Foundation for Statistical Computing) (version 3.6.2) (173), integrating the desired packages for the pertinent purpose. After the mapping process through STAR, a gene count data-table was obtained including every TB cavitary lesion part and nodulus sample as well as pre/post-surgical blood samples belonging to the Discovery Set. Next, the mapped gene counts were annotated to the human reference genome according to the ENSEMBL gene ID, produced as part of the alignment output. Separately, blood and tissue gene raw counts that in mean were below or equal to five were discarded as they were considered as potential unspecific signals and would potentially interfere with the gene differential expression analysis.

The differential expression analysis from the tissue and blood counts-tables was conducted using the DESeq2 Bioconductor package (version 1.28.1) (174). As input, DESeq2 takes the raw counts data-table from STAR and runs an internal gene counts normalization before the differential expression analysis (run by the *estimateSizeFactors* function). DESeq2 normalization involves the raw counts divided by sample-specific size factors which are determined by the median ratio of gene counts relative to the geometric mean per each gene from the raw counts data-table. As the differential expression analysis output, DESeq2 provides a table

containing the significantly differentially expressed (SDE) genes and the non-SDE by their gene ID, and which can be sorted by several columns mostly reporting:

1. baseMean: the per gene average of the raw counts' internal normalization taken from the samples under the analysis.
2. log2FoldChange (log2FC): Is the logarithm, in basis 2, of the fold-change between the two factors under comparison. Output log2FC values are based on calculations of factor level 1 against factor level 2. The values refer to up or down-regulations of genes in samples belonging to factor level 1.
3. LogFC Standard Error (lfcSE): is the standard error estimate for the log2FC estimate.
4. The P-value for the statistical significance of the change between factor level 1 vs. factor level 2.
5. Adjusted p-value (padj): Is the Benjamini-Hochberg adjusted p-value. All the genes were considered as SDE when the padj value was equal or less than 0.05.

The differential expression examination for two factors levels under an experimental design was employed through the contrast argument. The contrast argument is the linear combination of the estimated log2FC, testing if differences between the groups are equal to zero. For the granuloma spatial transcriptomic expression, the contrast argument was not used as the hypothesis was that the TB lesion is compartmentalized in three parts, hence dimensions are above two. Gene intersections were explored with the online tool Venny (175). The R package pheatmap (version 1.0.12) allowed to generate the heatmaps and dendrograms for the genes and samples hierarchical clustering after the raw-counts normalization according to the above-mentioned DESeq2 function. Hierarchical clustering allowed to group the patient's samples and genes according to their molecular profiles, regardless of the pathophysiological characteristics of the cohort participants. The hierarchical clustering was executed following the Euclidean distance method, which is defined as the distance between two points with an "ordinary" space line in the Euclidean space. The DISCO package (version 0.6) was used to study the concordance and discordance between different datasets (176). The disco.score function enables to compare homologous gene pairs from different datasets,

different tissues, platforms, and even different organisms to find orthologue genes, taking as a reference both p-values and logFC or log2FC. The product of the analysis is a score reflecting if orthologous SDE gene pairs are concordantly or discordantly regulated between datasets.

During the quality control steps on the raw-counts data, a supposedly male patient according to the clinical data was found expressing significantly X chromosome typical genes instead of transcripts coding from Y chromosome. As was not possible to relate this to a sampling error, this patient's full set of counts per sequenced tissue part were excluded from the clinical variables comparisons analysis, but from the tissue genotypical characterization. Whole blood counts were found matching with the patient's clinical record, so counts were included in the analysis.

3.3.4.5 The gene set analysis and statistics

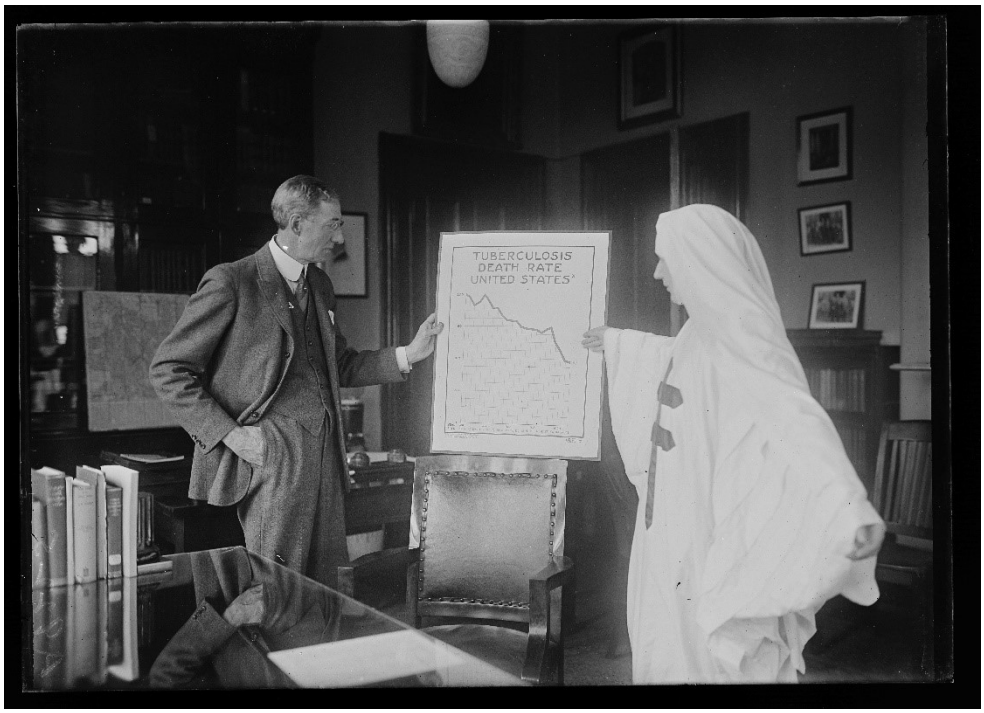
After conducting the differential expression analysis, resultant SDE genes were biologically analyzed through the Gene Set Enrichment Analysis (GSEA) software (The Broad Institute, Harvard, USA) (version 4.0.3) (177). GSEA enables into cluster large sets of genes by their interaction with common biological functions and disease phenotypes from *a priori* defined gene set collection. Therefore, GSEA provides comprehension of the regulation of the affected gene cluster that may be linked with the phenotypes that are under comparison. GSEA allowed to link the resultant SDE from the analysis to dysregulated gene sets (this is up/down-regulated) according to a database, and therefore to phenotypically characterize potential malfunctions in the SH-TBL cohort regarding the sample source.

New two-column data-tables were created from prior DESeq2 comparisons under analysis, gathering only the SDE genes by their annotation (ESNSEMBL) and log2FC. Next, were ranked from the highest positive expression to downwards according to the log2FC, whereas the spatial transcriptomics analysis log2FC was not included as involved a three-dimension expression gradient. The file format was set following GSEA instructions for RNA-seq projects. The gene sets database was established with the [Hallmarks] option and the chip platform as "Human ENSEMBL Gene ID MSigDB.v7.0.chip". In each of the gene sets details, up and down-regulated genes enriched in phenotype were checked for biological functions.

All the genes that were enriched as noted by the Core enrichment option were picked-up.

After running the gene set enrichment analysis, GSEA statistics firstly include the Enrichment Score (ES), which indicates the degree to which a gene set is found overrepresented at the beginning or end of the ranked list of the genes present in the provided gene-signatures. Higher positive ES corresponds to a stronger positive correlation of the gene set at the top of the ranked list, whereas negative involves the gene set enrichment at the end of the list. The normalized ES (NES) is calculated after normalization for gene set size, which allows to compare analysis across gene sets. Statistical significance for every enrichment score per gene set was given by the nominal p-value, considering values <0.05 as significant. False Discovery Rate (FDR) was set at 25%, reducing the probability that a gene set with a given NES becomes a false positive result. The rank metric score indicates the position of the gene within the gene set after a calculated score. The Running ES is the ES in the corresponding gene set at this point in the ranked list of genes.

4 | STUDY I RESULTS



Description: "Tuberculosis death rate in the United States by the year 1923."

Author: Harris & Ewing Collection (Library of Congress)

Results from Study I are presented below, belonging to the peer-reviewed article named "Retrospective study of clinical and lesion characteristics of patients undergoing surgical treatment for Pulmonary Tuberculosis in Georgia" and published in the International Journal of Infectious Diseases (DOI: 10.1016/j.ijid.2016.12.009) and adapted to be presented in this thesis. The full original article is included as Annex I.

The Study I patient's data was analyzed considering the following factors:

1. Demographic, epidemiological, and general clinical features
2. TB data
3. Surgery aspects
4. TB lesions macroscopic characteristics
5. TB treatment outcomes

4.1 Demographic, epidemiological, and general clinical features

All 137 patients belonging to the Study I underwent therapeutic surgery for their TB in the NCTLD during the years 2014 and 2015. The study cohort was constituted by 96 males (69.8%) and 41 females (30.8%). Men's median age was 43.5 years, which was higher than women's, the median age being 28 years old.

Regarding toxic habits, 49 patients were smokers, 48 on a daily or mostly daily basis representing 48.9% and 2.4% of total male and female populations, respectively. On alcohol intake, the majority of women (92.6%) declared never drinking alcohol. In men, alcohol use increased with age. While men declaring themselves abstemious (13.5%) had a median age of 26 years old, those declaring drinking on less than monthly (33.3%), monthly (52%) or on a weekly basis (8.3%) had a higher median age (45.6 years old).

25% of men presented comorbidities: 15 suffered from Hepatitis C Virus Infection (HCV), five presented DM, three had HCV and DM and a single patient showed HCV plus HIV infection.

4.2 TB data

Approximately half of the patients included in the study were DS-TB (56.9%), and the other half, MDR or XDR (40.1%). Mono resistance was rare. **Table 8** shows the number of cases for each category, as well as the number and percentage according to sex. The percentage of men and women was very similar regardless of the sensitivity to drugs of the involved strains.

The 92.70% of total recorded cases were pulmonary TB exclusively, while in a 7.30% pleura involvement (as empyema) was found (being more frequent in males, although not statistically significant). Pleura involvement was present in 80% of MDR/XDR-TB cases.

Table 8. Distribution of tuberculosis patients by drug-sensitivity according to the World Health Organization classification.

Drug-sensitivity (WHO)	TOTAL	Males		Females	
		N	%	N	%
DS-TB	78	54	69.23	24	30.76
Mono-Resistant	2	2	100	0	0
Rifampicin only	2	2	100	0	0
MDR/XDR-TB	55	38	69.09	17	30.09
MDR-TB	42	33	78.57	9	21.42
XDR-TB	13	5	38.46	8	61.53

Before surgery, CXR exhibited the presence of single lesions (only cavities and tuberculomas were considered) for most of the patients (76%), while a 19.20% showed two lesions, and the 4.80% up to three.

Most participants of the study undergoing surgery were considered bacteriological cured according to WHO criteria (96.30%). No statistically significant differences according to sex or drug susceptibility were found in the non-microbiological cured group of patients.

When microbiological characteristics were evaluated, differences were found between subjects having always a negative sputum culture and ≤2 months converters, being statistically significant ($p \leq 0.0001$), regardless of sex and drug-

sensibility. The culture was always negative in a non-despicable 31.30% of the cases, and up to 40.60% of male patients when stratified by sex. **Figure 7** shows proportions of time to culture conversion cases, in totals, and stratified by sex and strain's drug affinity to ATT. As women included in this cohort were younger, male participants were divided into ≤43 and >43 years old, to check if the sex itself could be an influence free from the age input.

A high percentage of patients negativized the culture in ≤2 months (51.82%), and they were younger than those who had it always negative (median age of 33 vs 43 years old, respectively; $p<0.0001$). When stratified by sex, this difference was only seen in males (37 years old as median age vs 47 years old; $p=0.022$). Statistically significant differences in terms of negativization of culture were encountered between DS-TB and MDR/XDR-TB, both in total ($p\leq0.0001$) and when stratifying the results by age and gender (males ≤43, $p=0.0009$; males >43, $p=0.0007$; and females, $p=0.0169$).

Nearly half of DS-TB cases negativized the culture at ≤2 months (47.43%), while in MDR/XDR-TB cases, the proportion of patients with negative results at >2 months (29.09%) were increased but due to a decrease of always negative results (a 44.87% in DS-TB vs a 12.73% in MDR/XDR-TB). The results showed that despite all women being ≤43 years old, females and males behaved differently, both in DS and MDR/XDR-TB cases, even if these differences were statistically significant only in DS-TB ($p\leq0.0001$).

4.3 Surgery aspects

The main indication for surgery was the presence of lesions (cavities and tuberculomas) in the CXR (73.72%), despite good treatment adherence. In the case of MDR/XDR TB, patients had sputum conversion but high risk of failure or relapse according to WHO's recommendations (54). The other indications were those cases considered as not microbiologically cured (2.9%) and a miscellanea that we called "others" group (23.3%) and which included severe complications of the TB episode (spontaneous pneumothorax, pulmonary hemorrhage, pleural empyema).

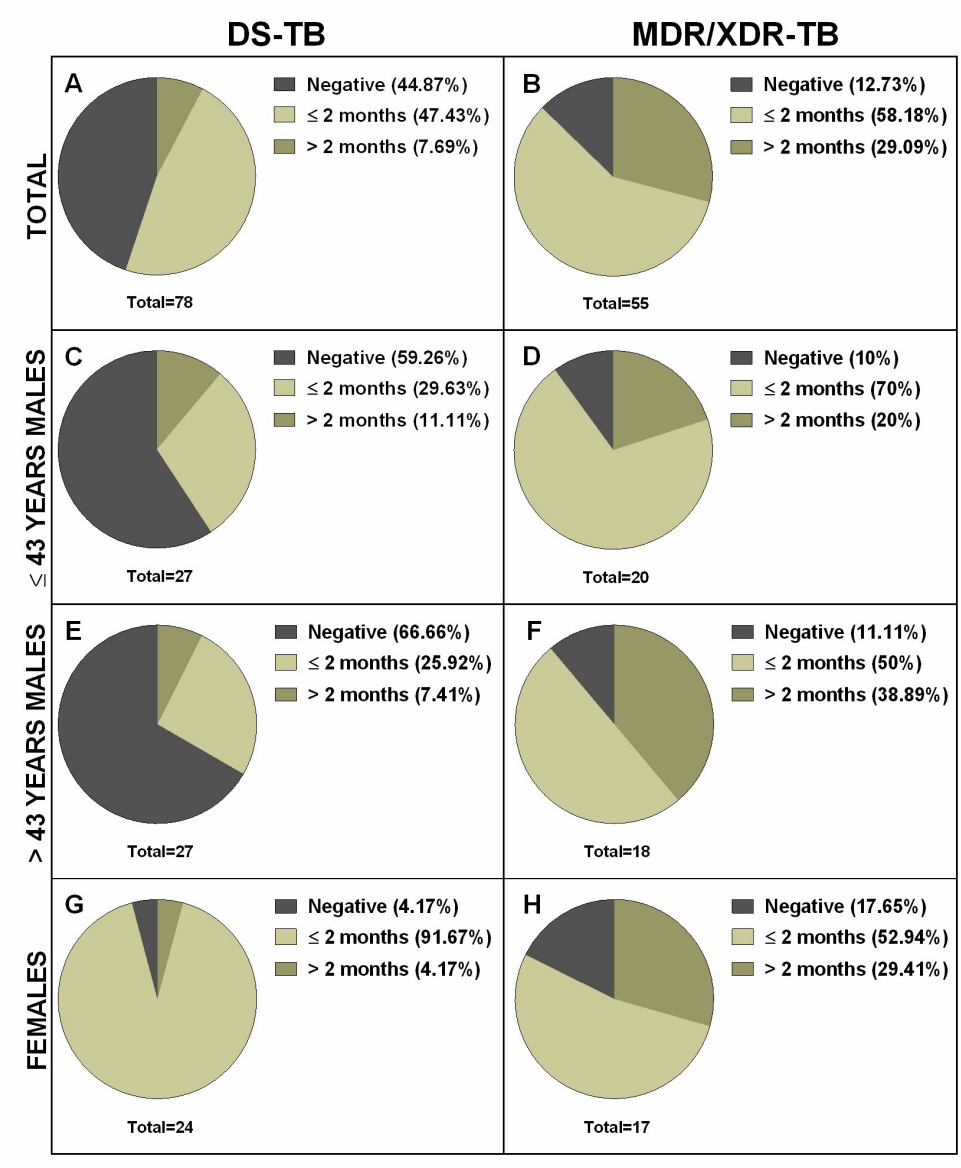


Figure 7. **Time in sputum culture conversion**. Time to SCC as: always negative, less or equal to two months (≤2 months) and more than two months (>2 months) are represented in percentages, for DS-TB and MDR/XDR-TB cases. Total values and results stratified by sex (males (≤ or > 43 years old) and females) are graphically represented.

This group was significantly bigger in males (31.2%) than for females (4.8%), something expected for men undergoing surgery being older and having comorbidities. When dividing the patients according to drug-sensitivity of their TB, the main indication of surgery was still the presence of remaining lesions in CXR,

even if the percentage was higher for MDR/XDR-TB cases (85.45% vs 65.38%). Surgery because complications of TB happened in 32.05% in DS-TB cases vs a 12.73% in MDR/XDR-TB. Differences encountered between the two groups were statistically significant (p=0.0326).

Men were operated earlier compared to women, according to days between diagnosis and surgery date, the difference being statistically significant (208.5 and 263 days, respectively; p=0.0037) as shown in **Table 9.** Sex was important especially among DS-TB cases, showing a remarkable increase in days between diagnosis and surgery date in women's cases (81.5 for men vs 225.5 for women; p=0.0002). Statistically significant differences were found between DS-TB and MDR/XDR-TB, both considering total patients as well as when stratifying by sex.

Table 9. Days between diagnosis and surgery date. Statistics: [a] All patients including: mono-resistant, rifampicin only resistant, DS-TB and MDR/XDR-TB participants. [b] Statistically significant differences between males and females, p=0.0037. [c] Statistically significant differences between DS-TB Males and DS-TB females, p= 0.0002. [d] Statistically significant differences between TOTAL DS-TB and TOTAL MDR/XDR-TB, p<0.0001. [e] Statistically significant differences between DS-TB males and MDR/XDR-TB males, p<0.0001. [f] Statistically significant differences between DS-TB females and MDR/XDR-TB females, p=0.0005.

Drug-Sensitivity (WHO)	**Median (25% - 75%)**		
	TOTAL	**Males**	**Females**
All patients[a]	233 (100 - 252.5)	208.5 (50.5 - 344)[b]	263 (210 - 373.5)[b]
DS-TB	178.5 (38.25 - 258.75)[d]	81.5 (17.5 - 225.25)[c, e]	225.5 (192.5 - 275)[c, f]
MDR/XDR-TB	351 (233 - 455)[d]	308.5 (215.75 - 405.5)[e]	373 (266.5 - 654)[f]
MDR-TB	305.5 (232 - 399.75)	307 (225 - 400.5)	304 (237 - 511)
XDR-TB	532 (276 - 666)	454 (166 - 610)	533 (372.25 - 891.5)

No statistically significant differences were encountered between DS-TB and MDR/XDR-TB according to age, neither in total or stratified by sex, even if MDR/XDR-TB operated patients tended to be younger than DS-TB: 34 (28-48) vs 43 years old (26.75-50.25).

Differences between DS-TB and MDR/XDR-TB individuals were found in terms of history of previous treatment (p=0.0053). While relapse patients were more frequent among DS-TB cases (26.47% vs 3.77% for MDR/XDR-TB), more operated

MDR/XDR-TB cases were new (64.15% vs 55.88% for DS-TB), had a history of previous ATT (26.41% vs 16.18% for DS-TB) or were under ATT after lost follow-up (5.66% vs 1.47% for DS-TB).

Regarding the surgical procedure, segmentectomy was the most frequent approach (56.93%), regardless the gender of the patients, followed by lobectomy (30.66%), pleural surgery (9.49%) and pulmonectomy (2.92%), which was performed in more MDR/XDR-patients than DS-TB (n=3 vs n=1).

In the majority of cases, only one lesion was removed (69.34%), followed by those with two lesions (17.52%), up to three (3.65%), and with pleura involvement (9.49%). In the resected lung tissue, in addition to the cavity or tuberculoma, there could be conglomerates foci, single foci, areas of fibrosis, bulla, or bronchiectasis. No statistically significant differences were found on the number of operated lesions when stratifying the results by gender, age, or drug sensitivity.

Only six patients suffered post-surgery complications, five of them being males of a median age of 41 years old. Hemorrhage was the most frequent complication (n=3). Other complications were pneumonia plus acute respiratory failure (n=1), bronchopleural fistula (n=1), and delayed unfolding of the operated lung (n=1). Half of these patients (n=3) were new patients and a half (n=3) were relapsed patients, having undergone treatment after failure. Half of the cases were DS-TB, and the other half, MDR-TB (n=2) or XDR-TB (n=1, a female case).

4.4 TB lesions macroscopic characteristics

The median size for all lesions was 2.4 cm (ranging from 2 to 2.8 cm). No statistically significant differences in terms of lesions' size were encountered, neither according to sex, age, type of necrosis (fresh, dry, both), or drug-sensitivity.

Although 132 (96.3%) out of the 137 patients were microbiologically cured according to WHO standard criteria, in 123 (93.1%) of them, the presence of necrosis in the lesion was still found. Necrosis was macroscopically considered fresh in 71.54% of cases, dry in the 6.5%, and both fresh and dry in 21.95% (**Figure 8**). **Figure 9** depicts these results according to sex and sensitivity to TB drugs. Results showed statistically significant differences due to drug-sensitivity in all cases but men of

≤43 years old (total, p=0.0136; >43 males, p=0.0418); females, p=0.0412). Sex also showed to be an important factor for the presence of a different type of necrosis, as men of >43 years old showed statistically significant different results from women, both for DS-TB (p=0.05) and MDR/XDR-TB (p=0.0288). Statistically significant differences were also encountered between men of ≤43 and >43 years old regardless of drug sensitivity (DS-TB, p=0.014; MDR/XDR-TB, p=0.0106), suggesting age as a potentially important role.

Patients being considered bacteriology non-cured according to WHO, presented a higher percentage of fresh necrosis (60%), even if the sample size of this group (n=5) was too small to extract conclusions. Fibrosis/cirrhosis was found in 89% of lesions, with a majority of cases being fibrotic (98.3%).

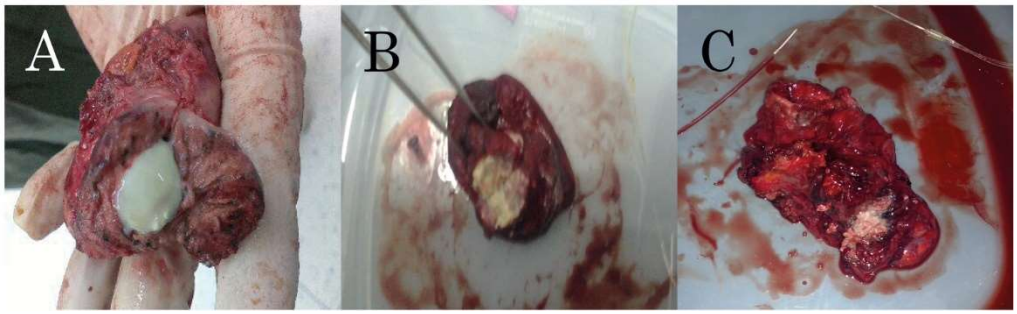


Figure 8. Types of necrosis in surgical specimens. Macroscopic photographs of removed surgical specimens showing fresh necrosis (A), both fresh and dry necrosis (B), and dry necrosis (C).

Table 10 reports the positive results obtained for AFB smear stain and culture on samples from TB surgical specimens, among those patients operated for the presence of lesions in CXR, with cavities or tuberculomas, and considered bacteriologically cured according to WHO definitions. Even if any statistical analysis is difficult to be performed and interpreted because of the small numbers obtained, a tendency on a higher percentage of positive culture in fresh necrosis when compared to both fresh and dry necrosis and in those patients always having negative sputum cultures can be seen.

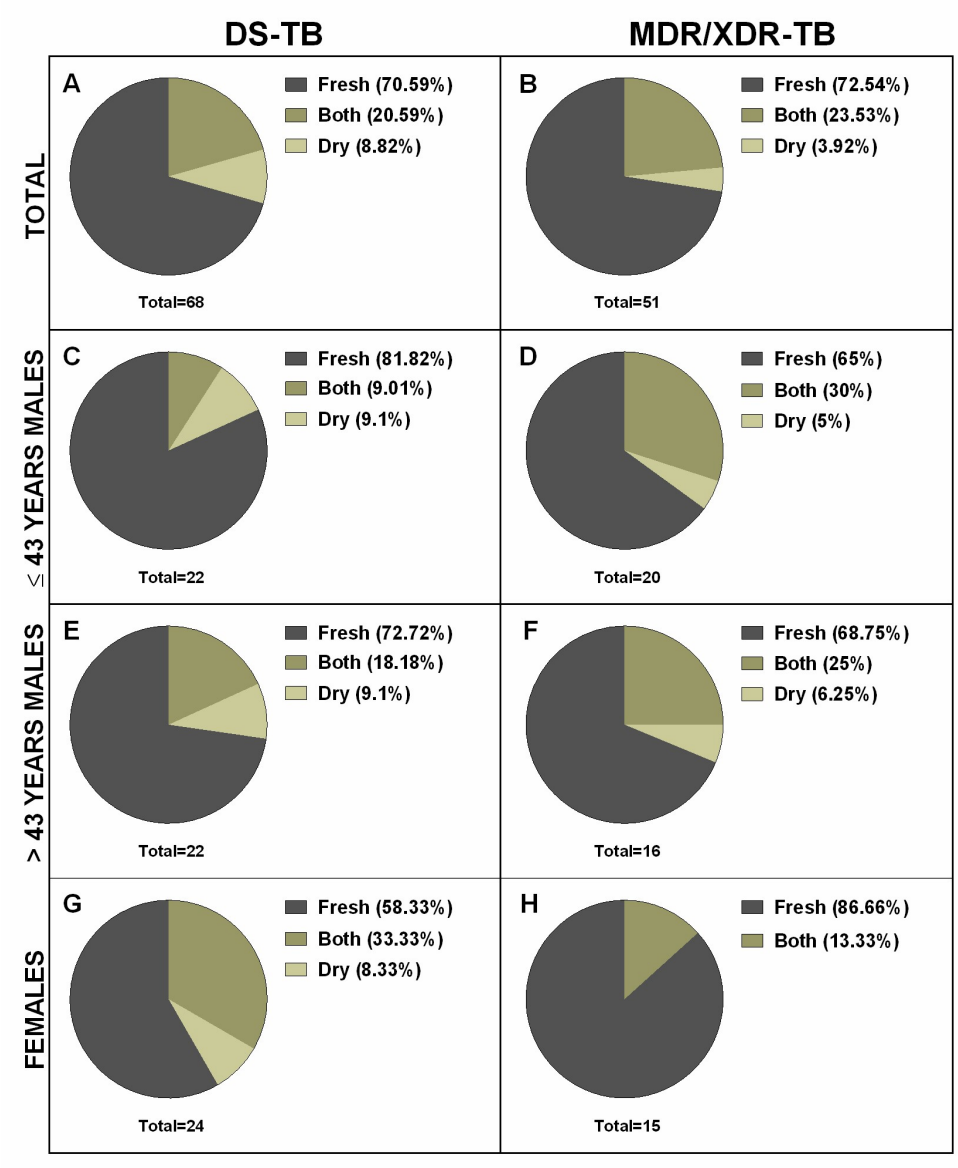


Figure 9. **Necrosis type found in TB lesions.** Percentages of presence of the different type of necrosis (fresh, dry and both) are represented for DS-TB and MDR/XDR-TB cases. Total values and results stratified by gender (males (of more or less than 43 years old) and females) are graphically represented.

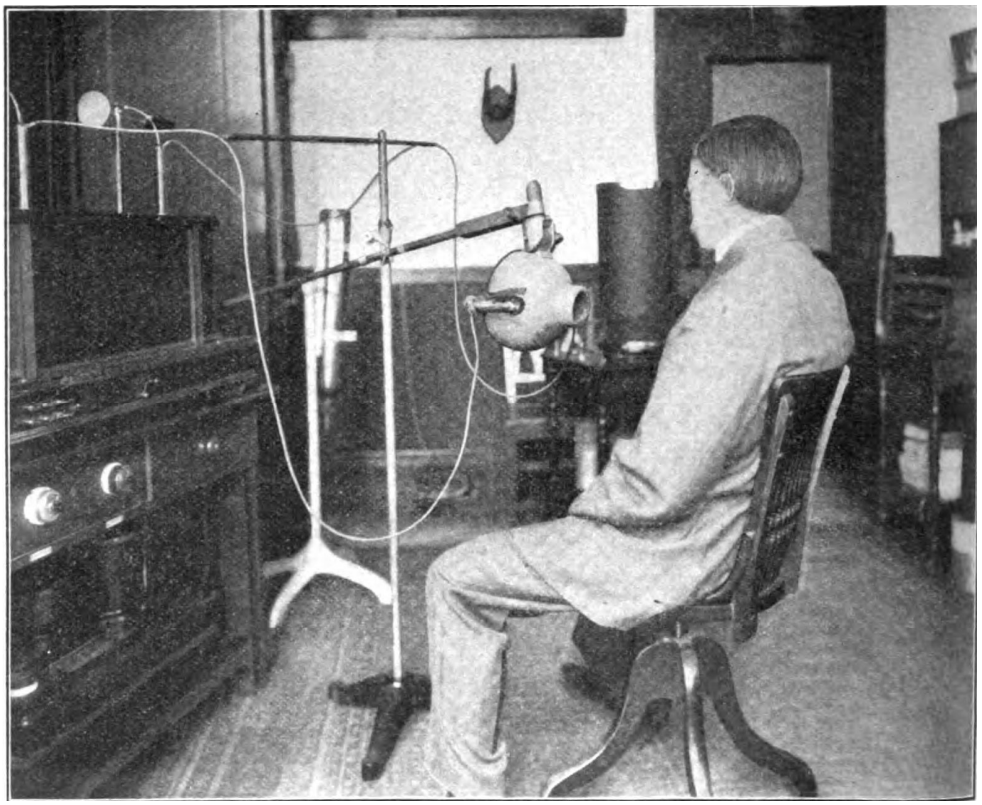
Table 10. The percentage of Acid-Fast Bacilli and culture positive in samples from surgical specimens.

	TOTAL	Lesions in the CXR	Cavities and tuberculomas	WHO Bacteriologically Cured	Samples from surgical tissue specimens	
					AFB +	Culture +
Characteristics according to Drug-Resistance						
DS-TB	78	51 (65.38%)	51 (100%)	50 (98.04%)	28 (56%)	7 (14%)
MDR/XDR-TB	55	47 (85.45%)	47 (100%)	47 (100%)	31 (65.96%)	11 (23.40%)
Characteristics according to sex						
Males >43 years old	48	29 (60.42%)	29 (100%)	28 (96.55%)	19 (67.86%)	3 (10.71%)
Males ≤43 years old	48	34 (70.83%)	34 (100%)	34 (100%)	19 (55.88%)	6 (17.65%)
Females	41	38 (92.68%)	38 (100%)	38 (100%)	23 (60.53%)	9 (23.68%)
Characteristics according to the type of necrosis						
Fresh necrosis	88	70 (79.55%)	70 (100%)	70 (100%)	41 (58.57%)	15 (21.43%)
Dry necrosis	8	6 (75%)	6 (100%)	6 (100%)	5 (83.33%)	0
Both	27	23 (85.18%)	23 (100%)	22 (95.65%)	15 (68.18%)	3 (13.64%)
Characteristics according to time to sputum conversion						
Always negative	43	15 (34.88%)	15 (100%)	14 (93.33%)	9 (64.28%)	5 (35.71%)
≤2 months	71	68 (95.77%)	68 (100%)	68 (100%)	42 (61.76%)	9 (13.23%)
>2 months	23	18 (78.26%)	18 (100%)	18 (100%)	10 (55.55%)	4 (22.22%)

4.5 TB treatment outcomes

While the percentage of cured patients was similar (30.8% for the DS-TB and 24.5% for MDR/XDR-TB) as well as those not evaluated (1.4% vs 1.8%); the categories lost to follow-up (7.3% vs 16.9%), treatment completed (47.0% vs 1.8%) and the outcome still not known (8.8 vs 35.8) showed very different values. For the patients with the outcome still not known, at the moment of the analysis were under ATT. Moreover, 1.8% and <0% of failed treatment were recorded for MDR/XDR-TB and DS-TB, respectively. No statistically significant differences were found when stratifying the results by age or sex.

5 | STUDY II RESULTS



Description: “A tuberculosis patient exposed to X-Ray as a novel treatment in 1910. The radiation exposure was for seven to ten minutes, three times a week. The X-Ray were applied in the chest and the back alternatively to reduce skin burns. The lead shield around the tube was the only precaution measure applied at that time, as radiation overexposure was not recognized as being harmful for the human body.”

Author: Noble M. Eberhardt (Noble M. Eberhardt, "Where electricity stands in the practice of medicine Ch. 2 - Electricity in Tuberculosis" in Popular Electricity, Popular Electricity Publishing Co., Vol. 2, No. 10, February 1910, p. 656, fig. 7).

Sociodemographic statistics, general clinical aspects as well as TB related features, including the microbiological findings from the SH-TBL Cohort participants are presented below. Data referred in this chapter is linked with the results presented in Sub-Study A (Chapter 6) and Sub-Study B (Chapter 7).

5.1 Characteristics of the SH-TBL Cohort and their TB lesions

Table 11 reports the characteristics of the patients included in the cohort regarding clinical and epidemiological features, characteristics of the current TB episode, and pre-surgical CXR findings according to sex-related differences. All patients had converted their sputum cultures at the time of surgery. The median range from TB diagnosis to surgery was 10 months. Surgery was performed according to indications of the official guidelines (54). Statistical differences were only found when the mean age was compared, finding males as significantly older than women (37.74-29.57 years, respectively) and regarding toxic habits. Almost half of the male participants were alcohol users (47.32%), whereas none of the female participants declared such habit. On the other hand, only two women were smokers (9.52%), which is in contrast to males (68.42%). In the context of TB comorbidities, HCV, HBV, and HIV serostatus were checked, revealing that six patients were HCV+ and two HBV+, while the entire cohort was HIV negative. On the other hand, three male patients were the only diabetics from the cohort (7.5%).

The TB symptomatology was persistent in the 30% of the cohort during the prior four weeks to the surgery date, and 13 patients had a previous TB history. Although no significant differences were encountered, the 71.80% of the SH-TBL cohort was able to convert the sputum culture in less or equal to two months during standard treatment regardless of the drug sensitivity strain, whereas the 28.20% required more than two months. Note that a patient's conversion status was not recorded at the moment of the analysis.

According to the radiological examinations (**Table 12**), most of the TB lesions were involved in the right upper lobe (52.5%). Very importantly, the whole 40 patients SH-TBL cohort was found to have TB cavitary lesions on CXR. 20% of the patients

had more than two lesions as well as radiological signs of infiltration. The presence of bronchiectasis was more evident among women than men (15.79%-42.86%, respectively).

Table 11. Demographic, clinical, and TB-related characteristics for patients at the time of surgery. Comparisons refer to males vs. females. ↓Patient's sputum culture conversion test result was not recorded at the moment of the analysis. Statistics: [a] Student's t-test, [b] Mann-Whitney U-test, [c] Fisher's exact test. *statistically significant differences.

Variable	Category	Overall	Male	Female	p-value
N		N = 40	n = 19	n = 21	
Clinical and epidemiological characteristics					
Age (mean (SD))		33.45 (11.76)	37.74 (12.53)	29.57 (9.755)	0.0364* [a]
BMI (median [range])		21.5 [16.53-32]	22.5 [16.53-31.7]	20.49 [17.3-32]	0.2163 [b]
Smoker (%)	yes no	15 (37.5) 25 (62.5)	13 (68.42) 6 (31.58)	2 (9.52) 19 (90.48)	0.002* [c]
Alcohol (%)	yes no	9 (22.5) 31 (77.5)	9 (47.37) 10 (52.63)	0 (0.0) 21 (100.0)	0.003* [c]
Diabetes (%)	yes no	3 (7.5) 37 (92.5)	3 (15.79) 16 (84.21)	0 (0.0) 21 (100.0)	0.0981 [c]
HCV (%)	yes no	6 (15) 34 (85)	4 (21.05) 15 (78.95)	2 (9.52) 19 (90.48)	0.3976 [c]
HBV (%)	yes no unrecorded	2 (5) 23 (95) 11	1 (7.69) 11 (92.31)	1 (5.88) 12 (94.12)	1 [c]
Characteristics of current TB episode					
TB symptoms (%)	yes no	12 (30) 28 (70)	5 (26.32) 14 (73.68)	7 (33.33) 14 (66.67)	0.736 [c]
Months from TB diagnosis to surgery (median [range])		10 [5-60]	11 [5-53]	10 [5-60]	0.462 [b]
Drug sensitivity (%)	DS-TB MDR-TB XDR-TB MDR/XDR-TB	15 (37.5) 18 (45) 7 (17.5) 25 (62.5)	7 (36.84) 9 (47.37) 3 (15.79) 12 (63.16)	8 (38.1) 9 (42.85) 4 (19.05) 13 (61.9)	1 [c]
Patient history (%)	New patient Relapse	27 (67.5) 13 (32.5)	14 (73.68) 5 (26.32)	13 (61.9) 8 (38.1)	0.510 [c]
Months for sputum culture conversion (median [range])	 unrecorded↓	2 [1-7] 1	2 [1-6]	2 [1-7]	0.164 [b]
Fast (≤2 months) and slow (>2 months) time to sputum culture conversion	Fast converters Slow converters unrecorded↓	28 (71.8) 11 (28.2) 1	11 (61.12) 7 (38.88)	17 (80.95) 4 (19.05)	0.2849 [c]

Table 12. **Chest X-Ray features for patients at time of surgery**. Comparisons refer to males vs. females. Statistics: [a] Fisher's exact test, [b] Mann-Whitney U-test. *statistically significant differences.

Variable	Category	Overall	Male	Female	p-value
N		N = 40	n = 19	n = 21	
CXR findings					
Localization of lesions within the lung (%)	Left Upper Lobe Right Upper Lobe Left Lower Lobe Right Lower Lobe LUL + LLL	11 (27.5) 21 (52.5) 4 (10) 3 (7.5) 1 (2.5)	5 (26.31) 10 (52.63) 3 (15.79) 1 (5.26) 0 (0.0)	6 (28.57) 11 (52.38) 1 (4.76) 2 (9.52) 1 (4.76)	0.666 [a]
Multiple lesions in the CXR (%)	yes (≥2) no (<2)	8 (20) 32 (80)	5 (26.31) 14 (73.68)	3 (14.28) 18 (85.71)	0.442 [a]
Presence of cavities (%)	yes no	40 (100) 0 (0.00)	19 (100) 0 (0.00)	21 (100) 0 (0.00)	1 [a]
Infiltrates (%)	yes no	8 (20) 32 (80)	4 (21.05) 15 (78.95)	4 (19.05) 17 (80.95)	1 [a]
Signs of bronchogenic dissemination (%)	yes no	6 (15) 34 (25)	1 (5.26) 18 (94.74)	5 (23.81) 16 (76.19)	0.185 [a]
Calcified granulomas (%)	yes no	15 (37.5) 25 (62.5)	7 (36.84) 12 (63.16)	8 (38.10) 13 (61.90)	1 [a]
Bronchiectasis (%)	yes no	12 (30) 28 (70)	3 (15.79) 16 (84.21)	9 (42.86) 12 (57.14)	0.088 [a]
Pleural involvement (%)	yes no	8 (20) 32 (80)	4 (21.05) 15 (78.95)	4 (19.05) 17 (53.13)	1 [a]
Days from surgery to discharge (median [range])		10 [7-91]	10 [9-21]	10 [7-91]	0.362 [b]

The macroscopic characterization of the lesions and the presence of bacilli are presented in **Table 13**. Patients were classified as fast (≤2M) or slow responders (>2M) based on the time to SCC of before or at 2 months (M) or later. None of the examined variables was found significant. Larger lesions were found to be located in the right upper lobe (data not shown). A large majority (84.62%) of resected lesions presented fresh necrosis (**Figure 10**), although they were more present among the fast responders' group (82.29% vs 71.73%). Surgery confirmed that 87.18% of the removed lesions were cavities, but five surgical specimens were considered as tuberculomas. The AFB bacilli staining in surgical samples were positive 28 patients (71.79%) and recovered in culture from two of them (5.12%). The center of the lesion harbored the highest number of positive *Mtb* AFB smears (18 vs 21), although when a complete surgical piece (including all the lesion parts) was stained, 16 samples were found positive. No statistical differences nor remarkable variations between fast and slow responders were found in terms of the presence of the bacilli in tissue samples.

Table 13. Attributes of resected lesions. Comparisons refer to patients presenting microbiological conversion at two months or less against those who required more than two months to respond to treatment. ⸸Patient's sputum culture conversion test result was not recorded at the moment of the analysis. Statistics: [a] Mann-Whitney U-test, [b] Fisher's exact test.

Variable	Category	Overall	Fast Responders	Slow Responders	p-value
N		N = 39⸸	n = 28	n = 11	
Size of resected lesion (mm) (median [range])		32 [22-55]	32 [22-55]	32 [22-40]	0.75 [a]
Necrosis (%)	Fresh necrosis	33 (84.62)	25 (89.29)	8 (73.73)	0.32 [b]
	Not fresh necrosis	6 (15.38)	3 (10.71)	3 (27.27)	
Cavitation (%)	Cavitation	34 (87.18)	23 (82.14)	11 (100)	0.29 [b]
	Tuberculoma	5 (12.82)	5 (17.86)	0 (0.00)	
Presence of bacilli in AFB smear					
Center of the lesion (%)	positive	18 (48.65)	14 (50)	4 (36.36)	0.49 [b]
	negative	21 (53.85)	14 (50)	7 (63.64)	
Internal wall of the lesion (%)	positive	4 (10.26)	4 (14.29)	0 (0.00)	0.30 [b]
	negative	35 (85.75)	24 (85.71)	11 (100)	
External wall of the lesion (%)	positive	5 (12.82)	2 (7.14)	3 (27.27)	0.15 [b]
	negative	34 (87.18)	26 (92.86)	8 (72.73)	
Peripheral nodulus (%)	positive	3 (7.69)	1 (3.57)	2 (18.18)	0.18 [b]
	negative	36 (92.30)	27 (96.43)	9 (81.82)	
Complete surgical piece (CIE) (%)	positive	16 (41.03)	13 (46.43)	3 (27.27)	0.47 [b]
	negative	23 (58.97)	15 (53.57)	8 (72.73)	
AFB by patient (%)	positive	28 (71.79)	21 (75)	7 (63.64)	0.69 [b]
	negative	11 (28.21)	7 (25)	4 (36.36)	
Biopsy culture positivity					
Biopsy culture conversion by patient (%)	positive	2 (5.12)	1 (3.57)	1 (9.09)	0.489 [b]
	negative	37 (94.87)	27 (96.43)	10 (90.91)	

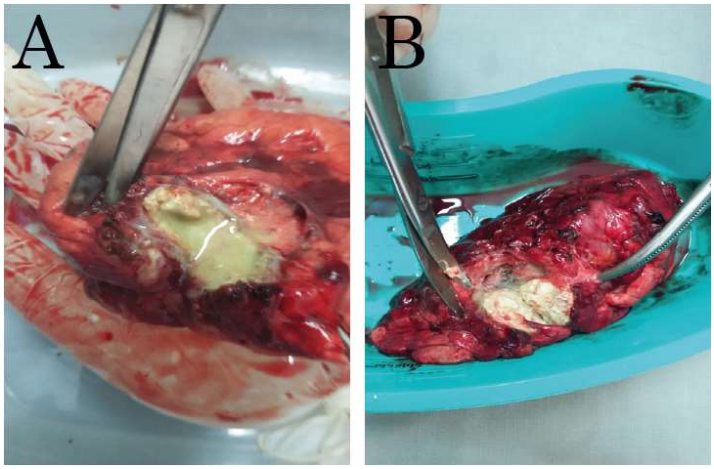


Figure 10. Cavitary TB lesions removed from two SH-TBL cohort participants. Fresh necrosis (A) and dry necrosis (B).

6 | SUB-STUDY A RESULTS

Description: "Fight Tuberculosis. Tuberculosis is a national danger, fighting it is a patriotic duty. Tuberculosis can and must be vanquished. The public authorities and private initiatives have joined battle against this evil. Support them: help them. The enemies of tuberculosis: the doctor, sun and fresh air, rest, healthy food. Precautions a tuberculosis sufferer must take: collecting and destroying his spittle; covering his mouth when coughing or sneezing; keeping his plates and cutlery to himself; sleeping alone." Date: World War 1 (French Home Front)

Author: Bureaux de la Tuberculose Croix-Rouge Américaine

Results regarding demographical, epidemiological, general clinical characteristics, the whole set of TB data, and TB cavitary lesions' macroscopic features are gathered in Study II (Chapter 5). Results from Sub-Study A are presented below, belonging to the article named "Evaluation of the immune response in tuberculosis patients from Georgia before and after receiving therapeutic surgery" and submitted in Frontiers in Immunology journal and adapted to be presented in this thesis. The full original article is included as Annex II. Data files used for the results obtaining are openly available at Mendeley Data (DOI: http://dx.doi.org/10.17632/knhvdbjv3r.1).

6.1 Immune responses according to patient characteristics

The values for circulating immune markers measured in plasma and urine are presented in **Table 14**, where differences of pre-surgical and post-surgical concentrations are assessed. Pro-inflammatory IL-6 and IL-8 were the only cytokines found significantly increased at hospital discharge, both in plasma and urine, although the median change was modest. Anti-inflammatory IL-4 and IL-10 in plasma also experienced a post-surgery statistically significant rise, especially in the IL-4 case (30.01 vs. 42.29 pg/mL). The innate immunity circulant marker CD5L was also found as the only biomarker decreased after surgery, whereas sCD14 in plasma and urine experienced a prominent significant change between the two time points. Finally, CRP had the most meaningful rise (2468 vs. 22473 ng/mL).

Sex, toxic habits, drug sensitivity, capacity to convert sputum culture, and presence of *Mtb* smear in surgical specimens were compared presurgically in **Figure 11** according to both plasma and urine biomarkers concentrations. Pre-surgery plasma IFN-γ and IL-4 and urine IL-8 concentrations were found to be significantly higher in females than in males, especially considering the IFN-γ performance and IL-8. Alcohol users demonstrated having lower levels of the several measured analytes, reaching statistical significance for plasma IFN-γ, IL-4, MMP-9, and urine IL-8, whereas urine sCD14 was increased.

Table 14. Median concentrations values and ranges measured for each analyte and time point. Statistics: Wilcoxon Matched-Pairs signed rank test; no significant differences (ns).

Analytes	Pre-Surgery	Post-Surgery	p-value
Plasma			
IP-10 (pg/mL)	85.64 (10.38-430.5)	80.36 (23.77-377.4)	ns
IFN-γ (pg/mL)	15.33 (6.63-34.87)	15.75 (5.87-27.13)	ns
TNF-α (pg/mL)	3.595 (1.49-7.8)	3.785 (1.78-12.29)	ns
IL-1ß (pg/mL)	1.76 (0.51-3.96)	1.725 (0.51-4.45)	ns
IL-2 (pg/mL)	1.28 (0.47-4.88)	1.92 (0.65-4.05)	ns
IL-6 (pg/mL)	1.765 (0.33-4.14)	2.8 (0.29-28.38)	<0.0001
IL-8 (pg/mL)	1.335 (0.36-20.31)	2.03 (0.61-17.08)	0.0015
IL-12p70 (pg/mL)	3.1 (0.44-8)	3.255 (0.55-8.6)	ns
IL-17a (pg/mL)	9.045 (3.43-25.65)	10.45 (3.09-20.82)	ns
IL-4 (pg/mL)	30.01 (8.89-175.3)	42.29 (20.27-145.3)	0.0008
IL-10 (pg/mL)	7.01 (0.11-24.3)	8.05 (1.46-19.02)	0.0168
sCD14 (ng/mL)	1869 (917.7-3686)	2345 (1165-3584)	0.0008
CD5L (ng/mL)	1433 (586.1-6942)	1296 (419.9-8963)	0.0459
MMP-8 (ng/mL)	82.77 (15.09-195.7)	67.19 (10.02-529.6)	ns
MMP-9 (ng/mL)	1854 (715-6797)	2800 (902-14054)	ns
CRP (ng/mL)	2648 (6.46-21738)	22473 (1412-54662)	<0.0001
Urine			
IP-10 (pg/mL)	11.78 (0.01-83.41)	19.54 (0.12-86.85)	ns
IL-2 (pg/mL)	1.155 (0.03-4.69)	1.365 (0.05-2.81)	ns
IL-6 (pg/mL)	1.98 (0.02-12.95)	6.22 (0.19-188.1)	<0.0001
IL-8 (pg/mL)	4.29 (0.01-124.1)	10.22 (0.09-720.1)	0.0161
sCD14 (ng/mL)	18.87 (4.59-34.94)	28.27 (2.89-33.13)	<0.0001

Smokers also exhibited lower plasma MMP-9 and CRP and urine IL-8 but higher pro-inflammatory IL-6. Pre-surgery MMP-9 levels were significantly higher in MDR/XDR-TB patients, although lower CD14 plasma levels. Plasma IL-8 levels were significantly higher in fast converters, whereas MMP-8, MMP-9, and CRP were higher in slow converters, despite were not found significant. Finally, Urine sCD14 levels were significantly higher in patients who exhibited a positive AFB in the resected lesion.

Next, these variables were individually analyzed comparing both time points (**Table 15**). IL-6 both in plasma and urine had a remarkable significant increase in most of the explored features. These changes are especially meaningful in males, non-alcohol users, MDR/XDR-TB and fast sputum converters patients, and finally subjects having a +AFB in tissue.

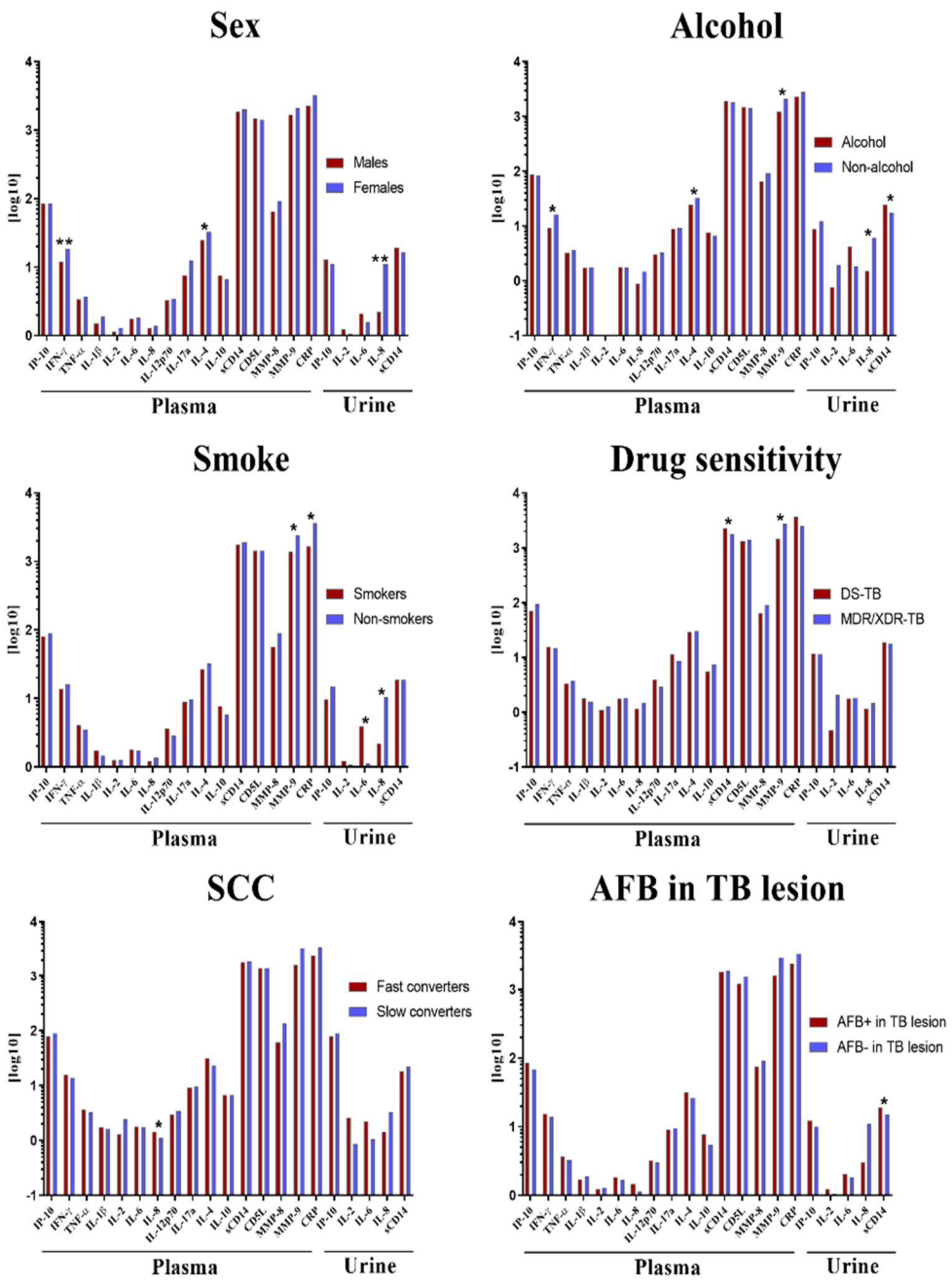


Figure 11. **Systemic biomarkers in plasma and urine before therapeutic surgery.** Pre-surgery biomarker levels according to several patient characteristics are presented on a log10-scale. Statistics: Mann-Whitney Test. (*) p-value <0.05; (**) p-value <0.001.

Table 15. Plasma and urine significant p-values (medians at recruitment and discharge moment) between pre-surgery and post-surgery measurements. Statistics: Wilcoxon Matched-Pairs signed rank test; no significant differences (ns).

Analytes	Males	Females	Alcohol users	Non-users	Smokers	Non-smokers	DS-TB	MDR/XDR-TB	Fast Converters	Slow Converters	+AFB	-AFB
	Pre vs Post	Pre vs Post	Pre vs Post	Pre vs Post	Pre vs Post	Pre vs Post	Pre vs Post	Pre vs Post	Pre vs Post	Pre vs Post	Pre vs Post	Pre vs Post
Plasma												
IP-10	ns	0.0239 (86.21-73.19)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-6	0.0002 (1.75-3.30)	0.0127 (1.84-2.77)	0.0313 (1.78-2.49)	0.0002 (1.75-2.83)	0.0012 (1.78-3.87)	0.0029 (1.75-2.49)	0.0215 (1.75-2.67)	0.0002 (1.84-2.8)	0.0009 (1.77-2.8)	0.0117 (1.75-2.49)	<0.0001 (1.84-3.21)	ns
IL-8	0.0136 (1.28-2.31)	ns	ns	0.0052 (1.47-1.97)	ns	0.0063 (1.36-1.97)	0.0006 (1.155-2.205)	ns	0.0089 (1.44-2.165)	ns	0.0136 (1.47-1.97)	ns
IL-4	0.0033 (24.78-33.05)	ns	0.0078 (24.78-34.11)	0.0167 (32.11-46.08)	ns	0.0028 (32.11-46.57)	0.0214 (28.91-46.07)	ns	0.0103 (31.65-40.93)	ns	0.0274 (31.74-42.66)	0.0068 (26.51-41.92)
IL-10	ns	ns	ns	ns	ns	ns	ns	ns	0.0073 (6.7-7.69)	ns	ns	ns
sCD14	0.0001 (1836-2380)	ns	0.0078 (1925-2443)	0.0091 (1806-2214)	0.0001 (1762-2380)	ns	0.0290 (2299-2600)	0.0179 (1759-2092)	0.0048 (1838-2355)	ns	0.0002 (1836-2443)	ns
CD5L	ns	ns	ns	ns	ns	0.0173 (1433-1274)	ns	ns	ns	0.0186 (1433-1274)	ns	0.0137 (1499-1352)
MMP-9	0.0160 (1672-3016)	ns	0.0078 (1201-2686)	ns	ns	ns	ns	ns	0.0407 (1654-2739)	ns	ns	ns
CRP	<0.0001 (2256-26015)	<0.0001 (3239-20947)	0.0078 (2256-17697)	<0.0001 (2793-26689)	<0.0001 (1659-21782)	<0.0001 (3635-26689)	<0.0001 (3729-2241)	<0.0001 (2559-24258)	<0.0001 (2405-21156)	0.0020 (3411-26689)	<0.0001 (2471-25473)	0.0156 (3411-20518)
Urine												
IL-6	0.0371 (2.09-3.95)	0.0006 (1.58-10.86)	ns	0.0001 (1.84-4.42)	ns	0.0003 (1.13-7.46)	ns	<0.0001 (2.035-6.22)	0.0007 (2.24-6.235)	ns	0.0004 (2.035-6.9)	0.0469 (1.84-6.22)
IL-8	0.015 (2.22-7.36)	ns	0.0391 (1.51-4.06)	ns	0.0342 (2.22-5.96)	ns	ns	ns	ns	ns	0.0096 (3.04-10.1)	ns
sCD14	0.0082 (18.97-28.68)	<0.0001 (16.72-27.86)	ns	<0.0001 (17.63-28.63)	ns	<0.0001 (18.48-28.81)	0.0182 (19.02-25.05)	<0.0001 (17.56-28.69)	0.0003 (18.25-27.25)	0.0049 (21.8-28.81)	0.0004 (19.06-28.63)	0.0029 (14.87-27.68)

IL-4 exhibited being different in concentrations between events, underlying the differences encountered in males, alcohol users, non-smokers, and negative AFB subjects. sCD14 demonstrated having prominent significant differences in almost all of the variables and considering both body biomarkers sources, highlighting its involvement during the analyzed timeline.

6.2 Biomarkers analysis according to severity traits

Showing symptoms, being relapsed patients, having an MDR/XDR-TB, being a slow responder, presenting lesions bigger than 36 mm, and the presence of fresh necrosis, TB lung cavities, and the bacilli detection in the resected lesion were considered signals of severity or suggesting a worse prognosis. All results were therefore analyzed according to these factors, and the correlations between them explored (**Figure 12**).

Plasma MMP-8 was the biomarker correlating with most of the severity traits under interrogation. It positively correlated with the presence of TB symptoms (as did the presence of infiltrates in the CXR), and especially with relapse and TB cavitation. Co-infection with HCV, presence of infiltrates in the CXR, and having DS-TB also correlated with relapse. Time from diagnosis to treatment, need for corticosteroids, and plasma sCD14 (negatively) and MMP-9 levels correlated with MDR-TB. The BMI and plasma MMP-8 levels pre-surgery correlated positively with being a slow responder, IL-2 plasma levels, and IP-10 urine, being the most meaningful association, presurgically positively correlated to lesion size larger than 36 mm. Being female, smoker, and alcohol user and urine sCD14 levels correlated positively with AFB positivity in biopsies. Plasma MMP-9 levels pre-surgery inversely correlated with AFB positivity. Our findings also suggest that time from diagnosis to treatment was important for obtaining a positive *Mtb* culture in lesion samples.

Next, the post-/pre-surgery ratio concentrations were calculated and analyzed according to its association with the severity traits (**Figure 13**). TNF-α, IL-1ß, IL-2, IL-8, and IL-17a ratios were found negatively correlated to MDR/XDR-TB patients. On the other hand, IFN-γ, IL-4, and IL-8 increased more after surgery in slow than in rapid-converters, and IL-10 was almost nine-fold higher post-surgery

in relapse patients. CRP, IFN-γ, and IL-4 levels post-surgery increased less in patients with lesions presenting fresh necrosis. IP-10 urine levels post-surgery were 210-times higher than pre-surgery levels and were 763-times higher in TB patients showing symptoms.

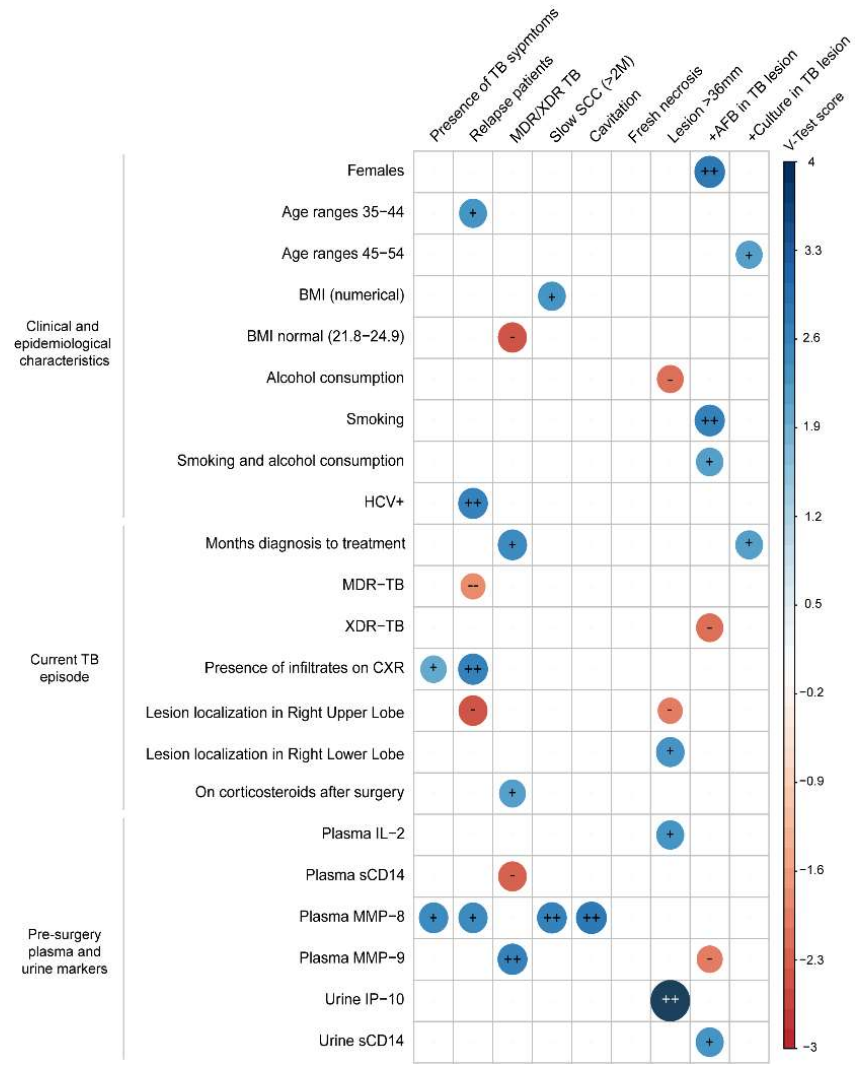


Figure 12. Correlation plot of pre-surgery biomarkers levels vs. TB severity factors. Statistics: results are expressed in terms of v-tests, which corresponds to the standard normal deviate with equal p-value as the measured correlation, finding the strongest association bigger in dot size; being blue colored with positive association while an inverse association in red. P-values are represented as (+): positive correlation, p-value <0.05; (++): positive correlation p-value <0.01; (-): negative correlation, p-value <0.05; (--): negative correlation, p-value <0.01.

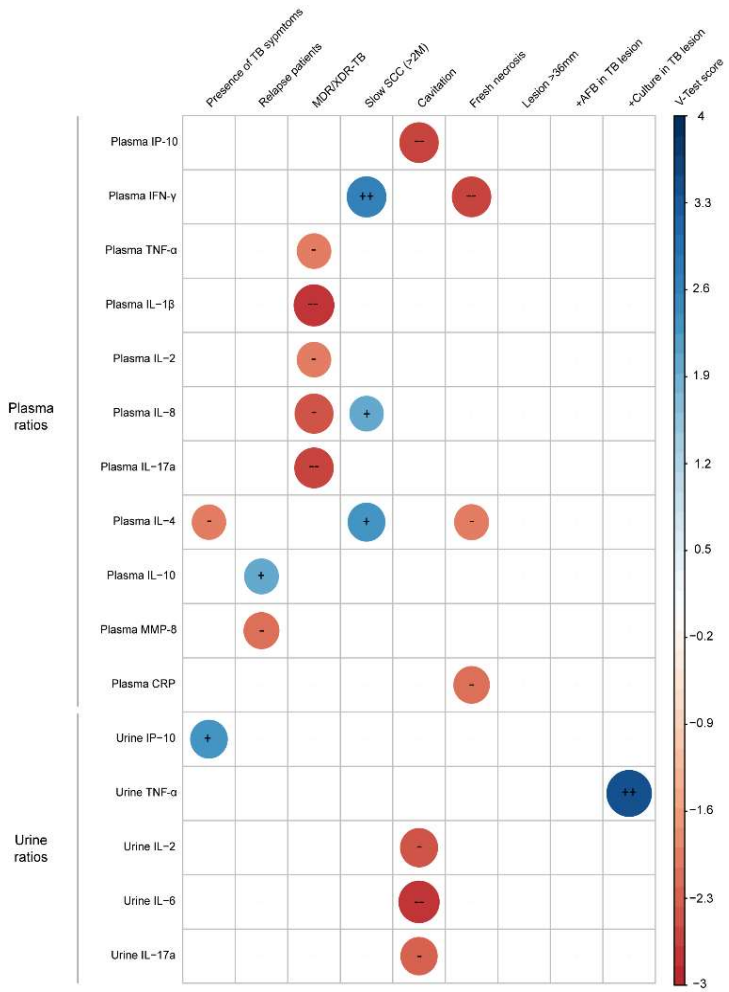


Figure 13. Correlation plot for the statistically significant ratios of post-/pre-surgery biomarkers levels vs. TB severity forms. Statistics: results are expressed in terms of v-tests, which corresponds to the standard normal deviate with equal p-value as the measured correlation, finding the strongest association bigger in dot size; being blue colored with positive association while an inverse association in red. P-values are represented as (+): positive correlation, p-value <0.05; (++): positive correlation p-value <0.01; (-): negative correlation, p-value <0.05; (--): negative correlation, p-value <0.01.

6.3 Predicting who needs surgery

In order to predict which patients should or shouldn't undergo surgery, we tried to model the worst-case scenario following the leave-one-out strategy. Considering a composite outcome including positivity for AFB staining, presence of fresh necrosis, cavitation, or a size >36 mm which may become a potential indicator of surgery, the results suggested that all patients should undergo surgery. We then modeled the

ability of plasma MMP-8, MMP-9, sCD14, and CD5L to predict having a TB lesion with fresh necrosis, with a poor result (AUC=0.4). The ability to predict AFB positivity, considering being a female, smoker, plasma MMP-9, and urine sCD14 levels, was studied next. The AUC was 0.74 (**Figure 14**), with being female and the urine sCD14 level being the most important factors.

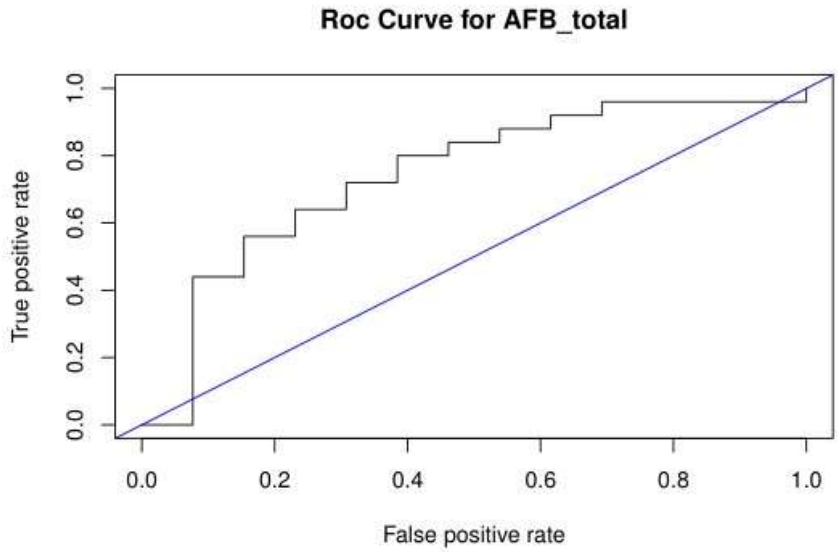


Figure 14. **Receiver Operator Characteristics curve to model the capacity to predict AFB positivity**. Being a woman, smoker, and having sCD14 levels in urine and MMP-9 in plasma were considered as potential risk factors to have a positive AFB.

7 | SUB-STUDY B RESULTS

Description: “Tuberculosis screening through the chest X-Ray in a London (England) factory during the World War 2”. Year 1943

Author: Ministry of Information Photo Division Photographer (British Home Front 1939-1945, British War Work 1939-1945)

Results regarding demographical, epidemiological, general clinical characteristics, the whole set of TB data, and TB cavitary lesions' macroscopic features are gathered in Study II (Chapter 5). Nonetheless, to better characterize the Discovery Set, we itemized in **Table 16**, according to sex, the main features of the pool of samples that underwent RNA-seq and will be evaluated in this sub-study.

Table 16. Demographic, clinical, TB-related and TB lesions characterization at the time of surgery of the Discovery Set. Comparisons refer to males vs. females. Statistics: [a] Student's t-test, [b] Mann-Whitney U-test, [c] Fisher's exact test. *statistically significant differences.

Variable	Category	Overall	Male	Female	p-value
N		N= 14	n= 7	n= 7	
Clinical and epidemiological characteristics					
Age (mean (SD))		34.36 (12.02)	37 (15.56)	31 (7.39)	0.4387 [a]
BMI (median [range])		23.55 [19.1-32]	23.4 [21.5-31-7]	23.7 [19.1-32]	0.6841 [b]
Smoker (%)	Yes	4 (28.57)	4 (57.14)	0 (0)	0.0699 [c]
	No	10 (71.43)	3 (42.86)	7 (100)	
Alcohol (%)	Yes	5 (35.71)	5 (71.43)	0 (0)	0.0210* [c]
	No	9 (64.29)	2 (28.57)	7 (100)	
Comorbidities (HCV, HBV, Diabetes) (%)	Yes	6 (42.86)	3 (42.86)	3 (42.86)	1 [c]
	No	8 (57.14)	4 (57.14)	4 (57.14)	
Characteristics of current TB episode					
Patient history (%)	Relapse	5 (37.71)	2 (28.57)	3 (42.86)	1 [c]
	New patient	9 (64.29)	5 (71.43)	4 (57.14)	
Drug Sensitivity (%)	DS-TB	7 (50)	3 (42.86)	3 (42.86)	1 [c]
	MDR/XDR-TB	7 (50)	4 (57.14)	4 (57.14)	
Fast (≤2 months) and slow (>2 months) time to sputum culture conversion	Fast converters	8 (57.14)	3 (42.86)	5 (71.43)	0.5921 [c]
	Slow converters	6 (42.86)	4 (57.14)	2 (28.57)	
Multiple lesions in the CXR	Yes (≥2)	4 (28.57)	2 (28.57)	2 (28.57)	1 [c]
	No (<2)	10 (71.43)	5 (71.43)	5 (71.43)	
Macroscopic and microbiological characteristics of the TB lesions					
Size of resected lesions (mm) (median [range])		30 [29-42]	30 [30-35]	30 [29-42]	0.4371 [b]
Necrosis (%)	Fresh	13 (92.86)	6 (85.71)	7 (100)	1 [c]
	Not fresh necrosis	1 (7.14)	1 (14.29)	0 (0)	
Granuloma biopsy AFB by patient (%)	positive	10 (71.43%)	6 (85.71%)	4 (57.14%)	0.5594 [c]
	negative	4 (28.57%)	1 (14.29%)	3 (42.86%	
Granuloma biopsy culture conversion by patient (%)	positive	1 (17.14)	1 (14.29)	0 (0)	1 [c]
	negative	13 (92.86)	6 (85.71)	7 (100)	

7.1 Differential expression analysis of the human TB granuloma compartments

In order to characterize the differences in terms of gene expression of the different compartments composing the inner structure of the granuloma, the three TB lesions compartments counts (Center of the lesion, Internal wall, and External wall) were compared to the healthy parenchyma samples counts as a control of no-disease (non-affected) correlator. The Discovery Set RNA-seq dataset was used to perform differential expression analysis between the different compartments of the granuloma and the Healthy parenchyma as baseline. The comparison between the Center of the lesion and the Healthy parenchyma revealed 6811 Significantly Differentially Expressed (SDE) genes; the Internal wall of the lesion and the Healthy parenchyma showed 4744 SDE genes; finally, the External wall of the lesion and the Healthy parenchyma comparison a total of 908 SDE genes (**Table 17**). These results exhibited that the TB granuloma tissue differentiation is greater from the center and it is reduced as closer to the edge of the TB lesions.

The overlap in terms of SDE genes in the three comparisons described above was assessed. In total, 753 SDE genes were found expressed by all three compartments, while 2974 were unique to the Center of the lesion, 865 unique to the Internal wall and 38 unique to the External wall. **Figure 15** reflect the numbers of SDE genes in each comparison and the outcome of the homologue gene overlapping. Next, the three granuloma zones were compared individually to each other, revealing no SDE genes between adjacent compartments. Even though, when the Center of the lesion and the External wall were compared, 770 transcripts were SDE, which was something expected considering the differential amount of unique SDE in each area.

7.2 Spatial transcriptomics of the human tuberculosis lesion

The individual analysis of each granuloma part suggested that several genes are not uniquely expressed as well as they do in a different grade throughout the TB lesion compartments. To explore this hypothesis, DESeq2 allowed interrogating the counts belonging to the Center of the lesion + the Internal wall + the External wall

in a linear additive manner to express distance from the center, revealing the spatial expression from the inner parts towards the edge of the surgically removed TB granuloma.

Table 17. Top 10 SDE genes per granuloma compartment comparison to healthy lung parenchyma ranked by the p-adjusted value (padj). Base Mean (baseMean) refers to the per gene average of the raw count's internal normalization taken from the samples under analysis. The padj is the Benjamini-Hochberg adjusted p-value. Genes were considered significant when the padj value was equal or less than 0.05. The log2 fold-Change (log2FC) is the logarithm, in basis 2, of the fold-change between the two factors under comparison. The values refer to up or down-regulations of genes in samples belonging to factor level 1.

Top 10 SDE ranked gene	baseMean	padj	log2FC
Center vs Healthy parenchyma (a total of 6811 SDE genes)			
MMP13	11,60	1,51E-08	6,89
NR1H3	450,54	3,13E-08	1,96
RASSF4	1331,88	6,24E-08	2,08
GBP5	1623,98	8,04E-08	3,07
KEL	22,32	1,35E-07	2,95
SLAMF7	812,64	1,62E-07	2,95
ACP2	530,22	1,62E-07	1,26
CD72	128,39	1,62E-07	2,76
IL2RG	476,86	1,62E-07	1,44
HLA-A	17429,64	1,62E-07	1,01
Internal wall vs Healthy parenchyma (a total of 4744 SDE genes)			
RN7SL396P	76,18	7,88E-08	1,59
IGKV1OR2-108	34,66	1,56E-07	4,00
MAP4K1	224,45	0,00000609	1,41
U2	13,15	0,00000785	2,39
CARMIL2	115,75	0,0000125	1,77
ZC3H12D	166,70	0,0000125	1,94
FCMR	207,54	0,0000134	2,00
SEPT1	156,94	0,0000134	1,62
KEL	22,32	0,0000134	2,31
CD22	343,78	0,0000142	1,65
External wall vs Healthy parenchyma (a total of 908 SDE genes)			
ADRA2A	147,23	0,00056172	1,48
KEL	22,32	0,00056172	2,10
RP11-345J4.6	71,98	0,00056172	1,22
CRB2	13,59	0,00065501	2,72
CCL19	441,68	0,00065501	2,82
FAM159A	41,14	0,00081017	1,83
MMP3	34,54	0,00084829	5,37
LTB	172,29	0,00084829	1,88
LTF	728,99	0,00088601	2,61
KIAA1191	772,52	0,00088601	-0,25

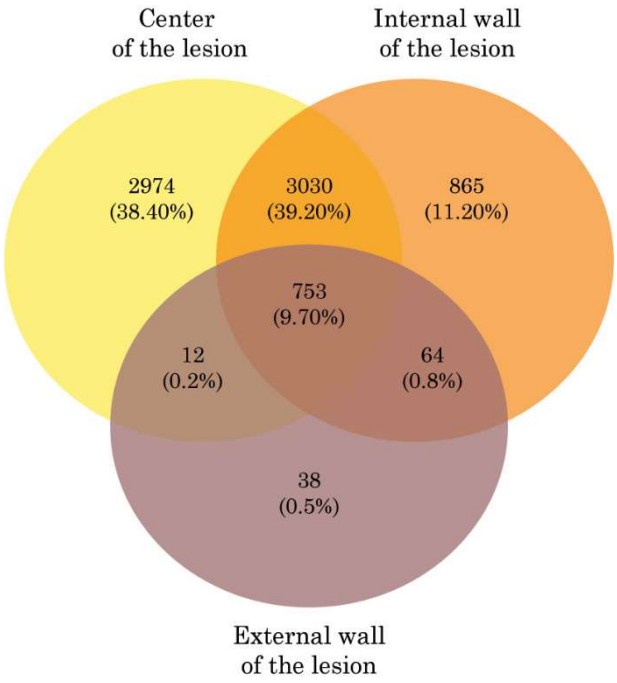


Figure 15. Venn diagram of the human TB granuloma compartments. The SDE genes intersection exploration from the Center of the lesion, the Internal wall, and the External wall were gathered together revealing uniquely expressed genes by area as well as homologous genes among the three parts.

The analysis showed 600 SDE genes indicating a meaningful role within the lesion structure. The main purpose of the analysis relies on the different gradient of expression, which is indicated by the log2FC data. Negative log2FC are related to a significant gene expression being important in the Center of the lesion, whereas positive values are mainly found expressed at the edge of the TB lesion, hence to the External wall. In consequence, log2FC values closer to 0 are those who are expressing a meaningful activity closer to the Internal wall area (**Table 18**).

7.3 Identification of the human tuberculosis signature

The main aim of Sub-Study B was to characterize the transcriptome of the human tuberculosis granuloma. After the inner characterization of the granuloma compartments, the counts belonging to Center of the lesion + the Internal wall + the External wall samples from the Discovery Set were pooled constituting a unique entity (Granuloma), as the three parts together belong to the SH-TBL Cohort

participants' TB granuloma. In total, counts from 35 samples composing the human TB granuloma were compared to the 13 pooled Healthy parenchyma samples' counts.

Table 18. Top 20 SDE genes derived from the spatial expression analysis. baseMean refers to the per gene average of the raw count's internal normalization taken from the samples under analysis. The padj is the Benjamini-Hochberg adjusted p-value. Genes were considered significant when the padj value was equal or less than 0.05. The log2FC is the logarithm, in basis 2, of the fold-change between the two factors under comparison. The values refer to up or down-regulations of genes in a linear additive manner to express distance from the center of the lesion. Log2FC values closer to 0 refer to gene mainly found expressed at the Internal wall, whereas negative and positives values refer to genes mainly expressed at the center and edge of the TB lesions, respectively.

Top 20 SDE ranked gene	baseMean	padj	log2FC
P2RX7	512,38	0,0022035	-1,27
GNPTAB	1638,12	0,0022035	-0,42
LHFPL2	1533,99	0,0022035	-1,07
FAM71A	25,85	0,0022035	3,76
HPSE	123,67	0,0022035	-1,07
C1QB	4095,35	0,0022035	-1,40
SLC6A9	105,74	0,0022035	0,77
HLA-DQA1	5657,91	0,0022035	-1,20
PDCD1LG2	274,01	0,0022035	-1,39
FCGR3A	2172,14	0,0026475	-1,35
CTSB	14258,77	0,0029803	-1,61
DFNA5	148,07	0,0030458	-1,35
CTSL	1217,72	0,0030458	-1,28
C1QA	3822,27	0,0030458	-1,32
NUPR1	766,23	0,0030458	-0,88
SUCNR1	97,07	0,0030458	-1,07
CD84	1528,13	0,0035921	-1,18
CTSZ	3227,45	0,0035921	-1,20
LACTB	356,85	0,0035921	-0,98
DNASE2	548,00	0,0035921	-0,99

The product of the differential expression analysis allowed us to generate the human TB granuloma signature, being composed of 6056 SDE genes. In the following heatmap (**Figure 16**) is pictured the top SDE 40 genes according to the granuloma biopsies parts.

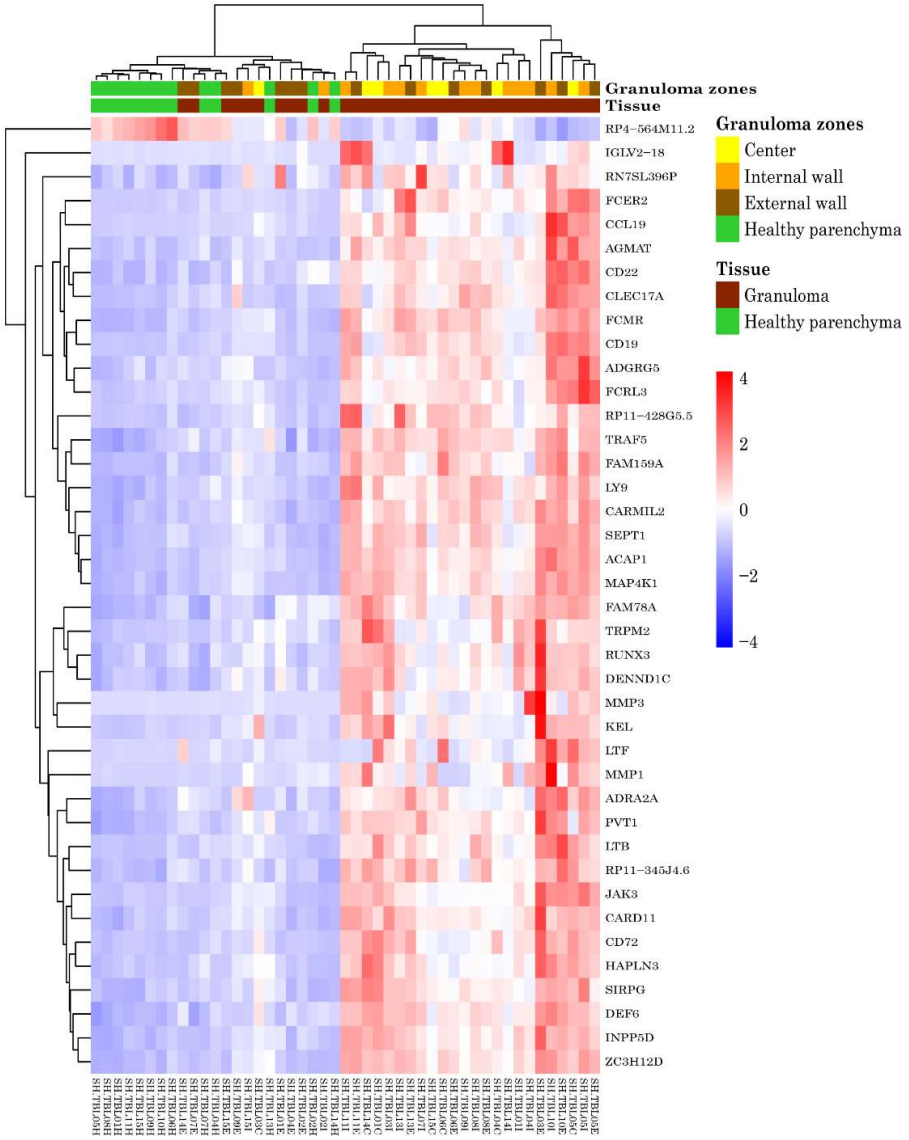


Figure 16. Top 40 SDE genes heatmap belonging to the 6056-gene human TB granuloma signature. A 6056-gene signature was generated from the Discovery Set after comparing the human TB granuloma counts (Center of the lesion + the Internal wall + the External wall pooled counts) to the healthy parenchyma expression. Hierarchical clustering revealed two defined clusters, dividing the granuloma parts and healthy control. Seven external wall samples are clustering among the healthy lung parenchyma samples, because as explained, this part gathers less unique SDE gens from its control, and therefore shares a similar molecular profile. For visualization purposes, the patient 11 Center sample was taken out.

Hierarchical clustering showed how the molecular profiles from the samples belonging to the granuloma and healthy control tissue to cluster independently. Up

to seven samples belonging to the E clustered among the H parts, thus exhibiting a similar expression profile which is explained given the few SDE between these two anatomically adjacent lung tissue parts. A posterior differential analysis performed upon the granuloma counts and the pulmonary nodulus revealed no SDE genes between the two tissues. On the other hand, a secondary analysis comparing the nodulus counts against the healthy parenchyma counts set involved a total of 175 SDE genes. The gene intersection measure between the 6056-gene human TB granuloma signature and the 175-genes nodulus showed that 100% of SDE nodulus genes are also significantly expressed in the TB lesions (**Figure 17**).

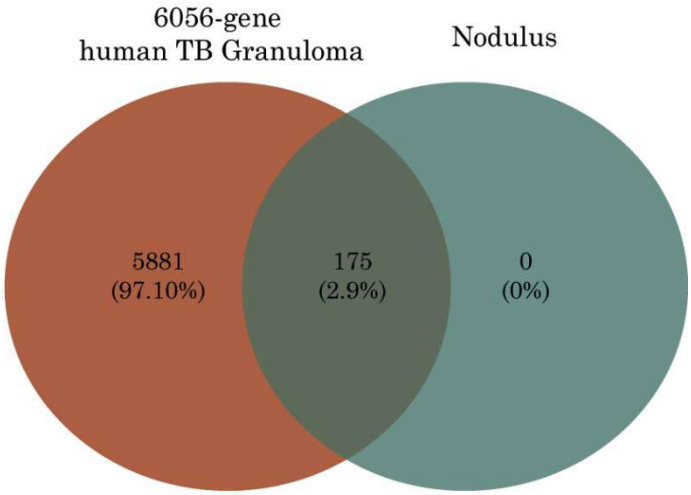


Figure 17. Venn diagram between the 6056-gene human TB granuloma signature and the nodulus 175 SDE genes. The gene intersection exploration indicated that the 175 nodulus genes are also SDE in the granuloma signature, therefore indicating that the SDE pulmonary nodulus genes are not unique.

7.4 Sex differences in the tuberculosis granuloma

As sex may play an important role in terms of disease symptomatology and prognosis, as exposed in the previous studies, we wanted to explore if differences between our Discovery Set males and females are translated into a differential gene expression in the TB lesion. Granuloma counts between men and women were compared through DESeq2. The differential expression analysis outcome revealed that 3184 genes were SDE. As being an obvious outcome of such kind of comparison, among the SDE genes, there was a strong presence of genes encoded from both X

and Y chromosomes. This was interpreted as a potential interference for the analysis. Therefore, to overcome such limitation, we generated a list belonging to X and Y chromosomes of the known genes that are encoded in these chromosomes from the ENSEMBL repository package in R. A posterior gene intersection analysis allowed to clear 153 genes to this interference, hence resulting in a total of 3031 SDE. Among the top SDE genes, we spotted a strong presence of genes encoding for the cytochrome's family genes, as well as transcripts related to the detoxification metabolism, such as CYP1A1, ALDH3A1, AHRR, etc, being located at the top of the SDE genes ranked list. This suggested that the RNA-seq very sensitively captured the systemic alcohol effect at a local level, as the alcohol use among the male participants in the SH-TBL was quite prominent. Discovery Set's male participants, five of the participants declared regular alcohol use, but two who did not, whereas none of the female patients declared being an alcohol user. Differences between men and women regarding alcohol use were found statistically significant (**Table 16**). The DESeq2 differential expression algorithm allows clearing potential interferences by including the variable in the analysis. So, to overcome the alcohol effect, the sex analysis algorithm included the patients declaring alcohol use. As a result, and after once again clearing the genes from X and Y chromosomes, a meaningful SDE gene reduction was found regarding the posterior analysis, resulting in a total of 303 SDE genes (**Figure 18**). Males and females clustered independently, suggesting that males and females exhibit a different expression profile within the human TB granuloma.

7.5 Tuberculosis granuloma expression according to worsening factors

One of the main characteristics of this cohort is that all the participants required therapeutic surgery to cope with persistent TB lesions in radiological explorations despite successful microbiological sterilization. Under this precept, patients exhibited a variety of features that potentially brought them to receive thoracic surgery (time to SCC, *Mtb* strain, necrosis type…).

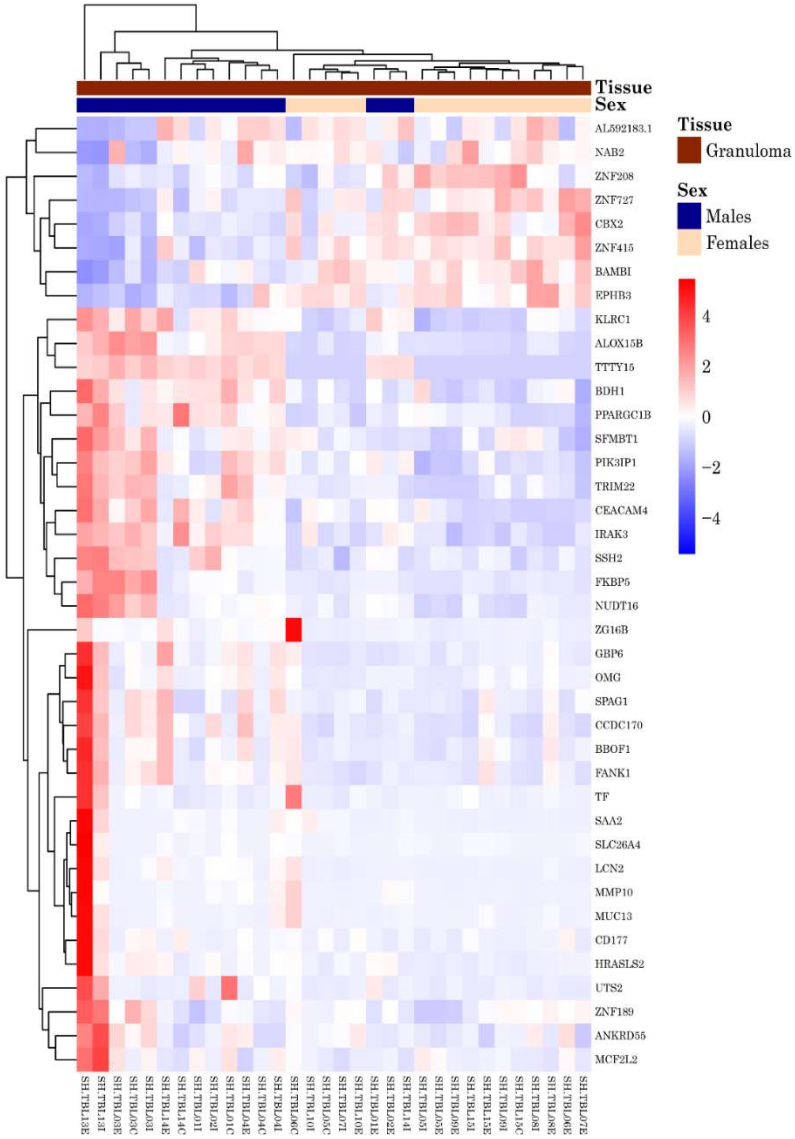


Figure 18. **Top 40 SDE transcripts heatmap belonging to the human TB granuloma sex comparison.** Genes encoding from the X and Y chromosomes were cleared. The potential alcohol use effect was corrected through the analysis algorithm. Except by three male samples, hierarchical clustering from granuloma samples belonging to males and females differently clustered in the heatmap dendrogram, exhibiting a different human TB lesion expression profile.

We hypothesized that this range of poor prognosis indicators may have a link within the TB granuloma host's expression and could allow us to distinguish which genes are related to them. To pursue such objective, we studied in the Discovery Set the differential expression regarding the differences in the time to sputum conversion,

patients having a sensitive *Mtb* strain against MDR/XDR-TB, and the presence of fresh necrosis within the TB lesion. To do so, we compared the TB granuloma expression between the two groups according to potential bad prognosis characteristics. Unfortunately, the type of necrosis presence analysis failed because 12 patients out of 13 had fresh necrosis whereas only a patient had the absence of fresh necrosis in the Discovery Set, as we considered that the resultant data from a single "absence of fresh necrosis" patient comparison could not be entirely trustworthy. Next, the DS-TB vs. MDR/XDR-TB individuals analysis revealed that 23 genes were SDE.

Finally, to analyze the time to SCC, the Discovery Set participants were divided, according to clinical records, into fast converters (n=20 pooled Center of the lesion + Internal wall + External wall sample's counts), considering those individuals converting the sputum culture in less or equal to two months, and the slow converters (n= 14 pooled Center of the lesion + Internal wall + External wall sample's counts), the group getting more than two months to exhibit culture sterilization. The differential expression analysis found a total of 1103 SDE genes between the fast and slow responders according to the TB granuloma expression (**Figure 19**). The clustering analysis showed independent expression profiles between the fast and slow converters, albeit four samples from two Fast SCC subjects clustered independently, probably motivated by a higher degree of expression of the exhibited genes, since no clinical feature was meaningfully different from the rest of the Fast SCC group.

7.6 Whole blood expression analysis

As plasma and urine samples were obtained both before and after surgery, an additional PAXgene tube for the whole blood collection in both timepoints was included too. The main goal was to test if we were able to "mirror" the TB granuloma lesion expression in whole blood. This is, to find significant genes that are found in blood and could point towards the presence of a TB lesion, and/or a specific poor prognosis feature highlighted in the TB lesion.

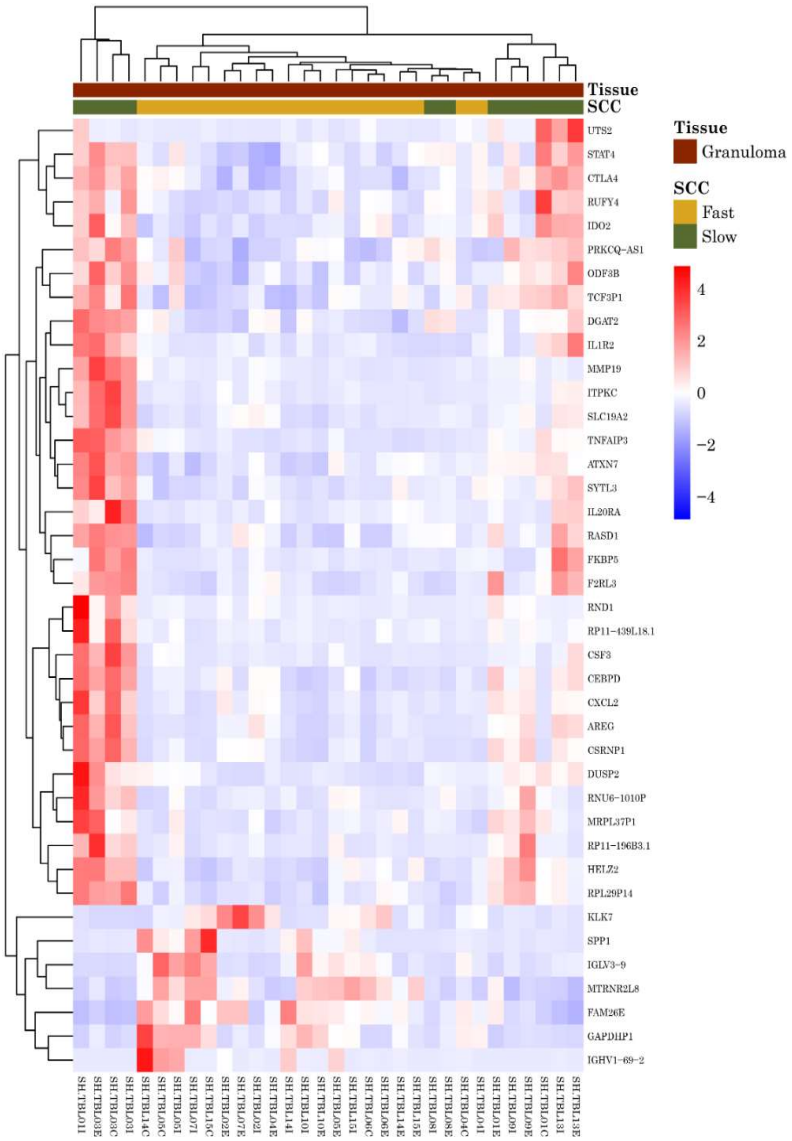


Figure 19. **Top 40 SDE transcripts heatmap belonging to the human TB granuloma sputum culture comparison.** Hierarchical clustering from the Fast SCC and Slow SCC clustered independently as shown in the dendrogram. Nonetheless, four samples from two rapid converters subjects were found clustering differently from the rest of the group, as suggested by greater gene expression as shown by the log2FC scale.

First, we wanted to explore whole blood differences in terms of gene expression between samples collected before surgery (pre-surgery) and at the hospital discharge moment (post-surgery), which exhibited 202 SDE genes. Then we wanted to compare again the expression differences between DS-TB to MDR/XDR-TB

patients before receiving surgery, this time in whole blood, but only four genes appeared as SDE (MYOM2 up-regulated in DS-TB, whereas RP11-1102P22.2, CNR2, and EIF1B-AS1 were down-regulated). We were also interested in assessing the host role in the time to SCC capacity not only in tissue but in whole blood. Fast and slow converters were again compared, and the analysis showed that 241 genes were SDE. Following this analysis, we assessed the gene intersection between the SDE genes product of both the time to SCC comparison in tissue and whole blood. This revealed that up to 18 SDE genes were commonly expressed in both study sources (**Figure 20**), and pointing out that some genes have a role locally but also, they are systemically detectable.

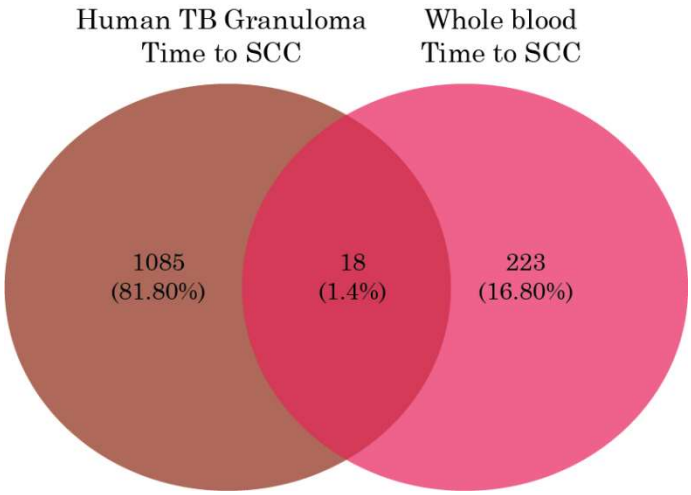


Figure 20. Venn diagram between the time to SCC in the TB granuloma and whole blood. In total, 18 SDE genes are expressed in both comparisons.

7.7 Identification of dysregulated gene sets in the tuberculosis granuloma lesion and pre-surgical whole blood

The GSEA analysis allowed us to explore which gene sets are strongly affected by the disease and which set of genes are enriched within each of the studied phenotypes in this sub-study.

First, we wanted to assess the gene enrichment in the 6056-gene human TB granuloma signature, revealing 21 gene sets were significant in the TB granuloma lesion phenotype (**Table 19**, **Figure 21**), as indicated by the False Discovery Rate

<25% (FDR), whereas 17 gene sets showed an inverse correlation. According to the Enrichment Score (ES), the gene set that had the most significant correlation with the TB granuloma signature was for the allograft rejection (ES 0.47) (**Table 20**), as well as when individual granuloma zones were analyzed (**Table 21**), followed by the interferon-gamma response (ES 0.43) and the inflammatory response (ES 0.37), whereas among the down-regulated gene sets the main three were for the protein secretion, oxidative phosphorylation, and adipogenesis (ES 27,29 and 53 respectively).

Table 19. Gene set enrichment analysis results from the 6056-gene human TB signature. Pre-ranked SDE genes from the 6056-gene human TB signature were submitted to the GSEA. Top-ranked gene sets ranked according to ES. Negative ES and NES indicate a negative correlation with the gene set enriched in phenotype. Gene sets were considered significant if <FDR 25% and the nominal p-value was <0.05. NS = no significant.

Gene Set Rank	Gene Set	Gene Set size	Enriched Score (ES)	Normalized Enriched Score (NES)
Up-Regulated in 6056-gene human TB granuloma signature				
1	Allograft rejection	118	0.47	6.07
2	Interferon-gamma response	95	0.43	4.80
3	Inflammatory response	96	0.37	4.19
4	TNF-α signaling via NF-κβ	75	0.36	3.66
5	Epithelial-mesenchymal transition	79	0.32	3.32
6	E2F targets	79	0.30	3.07
7	G2/M checkpoint	93	0.28	3.07
8	KRAS signaling up	92	0.23	2.56
9	MYC targets	40	0.35	2.55
10	IL-6 JAK/STAT3 signaling	42	0.33	2.54
11	Complement	82	0.21	2.32
12	Interferon-alpha response	34	0.33	2.31
Down-Regulated in 6056-gene human TB granuloma signature				
1	Protein secretion	27	-0.45	-2.75
2	Oxidative phosphorylation	29	-0.38	-2.37
3	Adipogenesis	53	-0.27	-2.27
4	TGF-β signaling	19	-0.40	-2.07
5	Heme metabolism	51	-0.24	-2.03

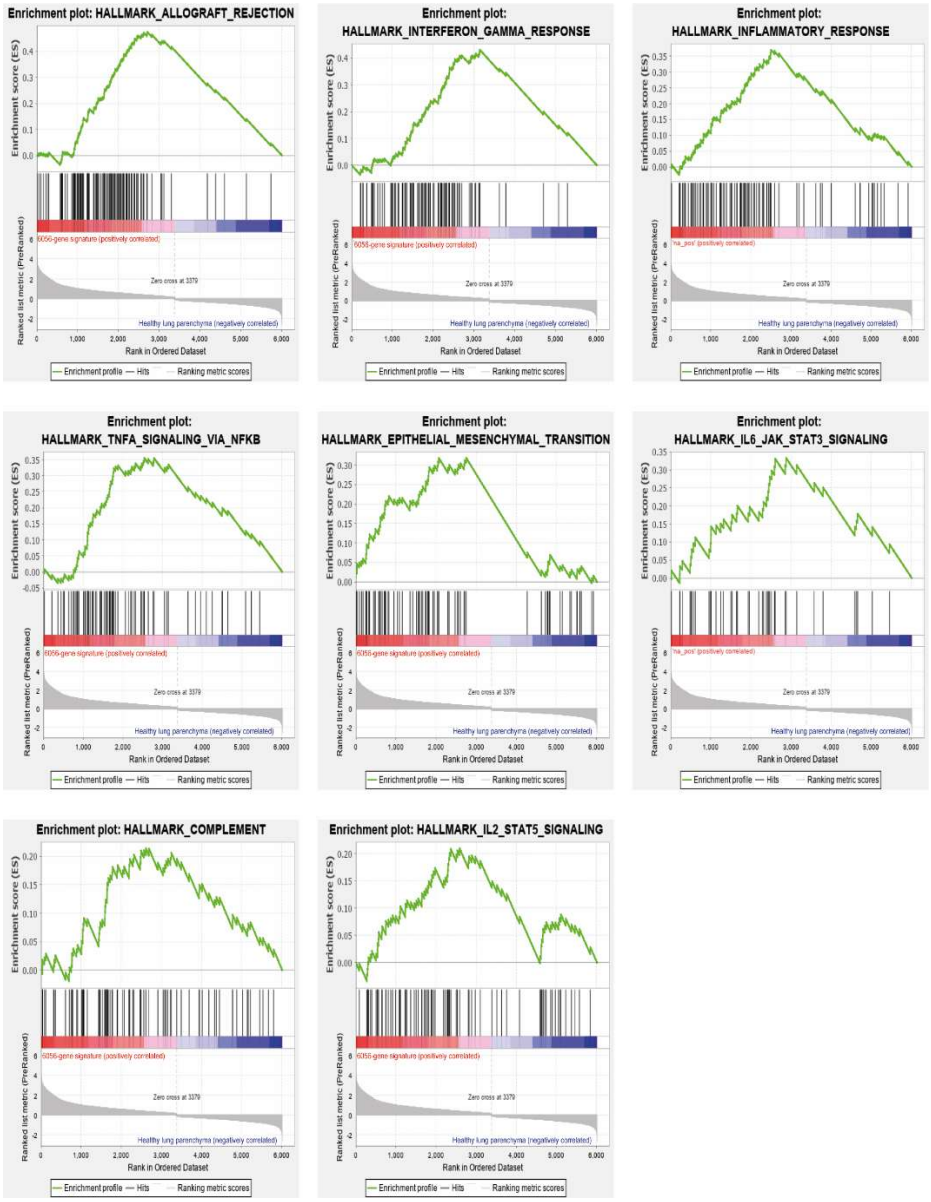


Figure 21. Visualization of the Enrichment plots relative to the 6056-genes human TB granuloma signature. The GSEA analysis revealed 21 gene sets being positively correlated with the 6056-gene signature phenotype, according to the ES. For visualization purposes, only enrichment plots related to allograft rejection, inflammation, tissue remodeling, and the complement system are included. Gene sets were considered significant if <FDR 25% and the nominal p-value was <0.05

Table 20. Enriched gene list in Allograft Rejection in the 6056-gene signature. The rank metric score is the signal to noise ratio for each gene used to position the gene in the ranked list. The Running ES is the enrichment score for this set at this point in the ranked list of genes.

Rank	Gene	Rank in 6056-gene list	Rank metric score	Running ES	Rank	Gene	Rank in 6056-gene list	Rank metric score	Running ES
1	CXCL13	19	3428	0.0053	55	HLA-DMB	1506	0.754	0.2198
2	CCL19	54	3002	0.0080	56	CAPG	1557	0.731	0.2198
3	MMP9	98	2759	0.0091	57	CTSS	1600	0.715	0.2212
4	CD79A	159	2460	0.0074	58	PRKCB	1628	0.705	0.2251
5	CXCL9	226	2238	0.0047	59	NME1	1650	0.695	0.2300
6	IRF4	277	2090	0.0047	60	NLRP3	1651	0.694	0.2385
7	LTB	291	2057	0.0110	61	LCP2	1656	0.690	0.2463
8	IL2RA	566	1401	-0.0270	62	CCR1	1664	0.687	0.2535
9	CD7	593	1358	-0.0229	63	HLA-DOA	1699	0.674	0.2563
10	HLA-DOB	601	1350	-0.0157	64	LY86	1710	0.669	0.2630
11	TIMP1	612	1340	-0.0089	65	PTPRC	1735	0.660	0.2674
12	SIT1	617	1324	-0.0011	66	CCL5	1770	0.647	0.2701
13	CD28	627	1317	0.0059	67	GBP2	1787	0.642	0.2759
14	MAP4K1	630	1315	0.0140	68	CD86	1795	0.639	0.2832
15	IFNG	688	1255	0.0128	69	HIF1A	1814	0.636	0.2886
16	THY1	745	1206	0.0118	70	CD74	1827	0.632	0.2951
17	CCL4	859	1109	0.0011	71	TLR1	1839	0.626	0.3017
18	LCK	885	1087	0.0053	72	LY75	1855	0.619	0.3076
19	ZAP70	904	1074	0.0107	73	NCF4	1882	0.608	0.3117
20	TRAT1	914	1066	0.0177	74	GPR65	1900	0.603	0.3172
21	IL10	915	1066	0.0262	75	CSK	1916	0.598	0.3232
22	TLR6	932	1053	0.0319	76	STAB1	1918	0.597	0.3315
23	CXCR3	933	1052	0.0404	77	CCR2	1961	0.585	0.3328
24	FYB1	940	1049	0.0479	78	ITGAL	1985	0.579	0.3374
25	STAT1	959	1038	0.0533	79	CD40	2000	0.573	0.3435
26	IL2RG	994	1012	0.0560	80	HLA-G	2018	0.568	0.3491
27	IRF8	995	1012	0.0645	81	TRAF2	2043	0.558	0.3535
28	IL1B	1005	1006	0.0714	82	CDKN2A	2047	0.558	0.3615
29	CD3G	1021	0.997	0.0773	83	ICOSLG	2066	0.553	0.3669
30	ITK	1027	0.993	0.0850	84	HLA-DRA	2094	0.544	0.3708
31	GZMB	1055	0.975	0.0889	85	HLA-A	2123	0.531	0.3745
32	CD40LG	1070	0.966	0.0950	86	CD4	2129	0.529	0.3821
33	IL2RB	1077	0.963	0.1024	87	CD80	2138	0.525	0.3893
34	FCGR2B	1088	0.959	0.1092	88	ST8SIA4	2187	0.510	0.3896
35	CD3E	1104	0.947	0.1151	89	IFNGR2	2200	0.504	0.3960
36	CD3D	1123	0.936	0.1206	90	IRF7	2213	0.499	0.4025
37	ACHE	1132	0.929	0.1277	91	STAT4	2238	0.492	0.4069
38	IL12RB1	1133	0.928	0.1361	92	CD8B	2264	0.484	0.4111
39	ITGB2	1145	0.924	0.1428	93	HLA-DMA	2275	0.480	0.4179
40	LIF	1231	0.882	0.1368	94	PSMB10	2316	0.467	0.4196
41	TNF	1247	0.876	0.1427	95	RIPK2	2318	0.467	0.4279
42	CCR5	1250	0.874	0.1509	96	WARS	2339	0.461	0.4330
43	CCL2	1253	0.871	0.1590	97	PTPN6	2382	0.447	0.4343
44	HLA-DQA1	1257	0.869	0.1670	98	HDAC9	2394	0.440	0.4409
45	CD2	1272	0.862	0.1731	99	TAPBP	2398	0.439	0.4489
46	CD96	1292	0.851	0.1783	100	IL18	2449	0.425	0.4489
47	CCL22	1394	0.799	0.1697	101	IFNAR2	2454	0.424	0.4567
48	HCLS1	1396	0.798	0.1780	102	LYN	2485	0.417	0.4601
49	IGSF6	1406	0.794	0.1849	103	CD1D	2549	0.396	0.4579
50	GZMA	1449	0.781	0.1863	104	IL27RA	2570	0.389	0.4629
51	WAS	1459	0.774	0.1932	105	TGFB1	2606	0.380	0.4655
52	TAP1	1464	0.772	0.2010	106	TAP2	2625	0.371	0.4709
53	CD247	1485	0.763	0.2061	107	IL15	2699	0.352	0.4670
54	SOCS1	1505	0.754	0.2114	108	EIF5A	2709	0.349	0.4739

Table 21. Gene set enrichment analysis of the specific granuloma biopsies parts from the human TB granuloma lesion. Pre-ranked SDE genes from the product of the center, internal wall, and external wall (individually) vs. healthy lung parenchyma comparison were submitted to GSEA. Top-ranked gene sets ranked according to ES. Top-ranked gene sets ranked according to ES. Negative ES and NES indicate a negative correlation with the gene set enriched in phenotype. Gene sets were considered significant if <FDR 25% and the nominal p-value was <0.05. NS = no significant.

Gene Set Rank	Gene Set	Gene Set size	Enriched Score (ES)	Normalized Enriched Score (NES)
Up-Regulated in the Center of the lesion				
1	Allograft rejection	131	0.52	7.01
2	Interferon-gamma response	116	0.48	6.03
3	E2F targets	81	0.44	4.07
4	Inflammatory response	112	0.37	4.55
5	TNF-α signaling via NF-κβ	84	0.39	4.11
Up-Regulated in the Internal Wall of the lesion				
1	Allograft rejection	95	0.46	5.15
2	Interferon-gamma response	72	0.41	4.09
3	Inflammatory response	75	0.35	3.48
4	Epithelial-mesenchymal transition	66	0.32	3.13
5	TNF-α signaling via NF-κβ	53	0.34	2.98
Up-Regulated in the External Wall of the lesion				
1	Allograft rejection (NS)	32	0.22	1.44
2	IL-2 STAT5 Signaling (NS)	17	0.18	0.87

We were also interested in the gene set enrichment from the two main poor prognosis outcomes studied in this sub-study. We first attempted to check differences between the drug-sensitivity in both tissue and blood upon the Discovery cohort, but GSEA failed as the SDE gene list input was not long enough that rejected the analysis. Next, we compared the fast converters from the slow converters, revealing only two gene sets were significantly enriched among the fast sputum converters (ES 0.50 for the epithelial to mesenchymal transition and 0.51 for the myogenesis). Differently, we found a total of 11 gene sets correlated to the slow converters group, highlighting the first enriched in score was the TNF-α signaling via the NF-κβ (ES -0.52), followed by ultraviolet radiation response (ES -0.51) and the interferon-gamma response (ES -0.39). On the contrary, in whole blood, the TNF-α signaling via the NF-κβ gene set was correlating among the fast converters (ES 0.22), although the FDR signaled that none of the output gene sets were significant. The slow converters group exhibited 7 gene sets significantly enriched, being both the interferon-alpha and beta response the third and fourth

most enriched gene sets, respectively. The resulting enriched gene sets from the above-mentioned comparisons are summarized in **Table 22**.

Table 22. Gene set enrichment analysis results from the time to SCC in granuloma and pre-surgical whole blood. Pre-ranked SDE genes from the product of Fast SCC vs Slow SCC in granuloma (1103 genes list) and whole blood (241 genes list) were submitted to GSEA. Top-ranked gene sets ranked according to ES. Negative ES and NES indicate a negative correlation with the gene set enriched in phenotype. Gene sets were considered significant if <FDR 25% and the nominal p-value was <0.05. NS = no significant.

Gene Set Rank	Gene Set	Gene Set size	Enriched Score (ES)	Normalized Enriched Score (NES)
Up-Regulated in GRANULOMA 1103-gene Fast vs Slow time to SCC				
1	Epithelial-mesenchymal transition	37	0.50	3.50
2	Myogenesis	15	0.51	2.37
Down-Regulated in GRANULOMA 1103-gene Fast vs Slow time to SCC				
1	TNF-α signaling via NF-κβ	60	-0.52	-4.64
2	Ultra violet radiation response up	19	-0.51	-2.70
3	Interferon-gamma response	32	-0.39	-2.59
4	Inflammatory response	26	-0.41	-2.42
5	Apoptosis	22	-0.40	-2.17
6	Hypoxia	22	-0.37	-2.10
7	IL-2 STAT5 signaling	22	-0.37	-2.07
8	Interferon alpha response	17	-0.39	-1.92
Up-Regulated in WHOLE BLOOD 241-gene Fast vs Slow time to SCC				
1	TNF-α signaling via NF-κβ (NS)	21	0.22	1.26
2	KRAS signaling up (NS)	17	0.21	1.02
Down-Regulated in WHOLE BLOOD 241-gene Fast vs Slow time to SCC				
1	Heme metabolism	67	-0.48	-4.57
2	G2/M checkpoint	22	-0.49	-2.71
3	Interferon-alpha response	25	-0.43	-2.50
4	Interferon-gamma response	45	-0.26	-2.10
5	Estrogen response late	21	-0.38	-2.09

7.8 Genes of interest for validation rationale

As the Discovery Set has limited sample size in terms of total patients (n=14), we used the rest of the SH-TBL Cohort participants as the Validation Set (n=24). To assess in gene expression terms if the findings on the Discovery Set were homologous with the Validation Set, we down-selected from the Discovery Set the genes of interest that better reproduce both the gene expression behavior and the phenotypes that characterize the SH-TBL Cohort. In order to accomplish such purpose, we considered that the genes of interest required of having an important

role not only in terms of a statistical rationale but also being involved with the encountered significant gene sets from each comparison analysis.

From the gene intersection interrogating the 6056-gene human TB granuloma signature, MMP3, LTB, and LTF genes, belonging to the epithelial-mesenchymal transition, the IL-2 STAT5 signaling, and the complement system, respectively, were chosen as genes of interest.

Following the same approach for the time to sputum culture conversion analysis, SPP1, DUSP2, CSF3, and TNFAIP3 were selected too, belonging respectively, to the epithelial-mesenchymal transition, the TNF-α signaling via NF-κβ, the inflammatory response, and both hypoxia, interferon-gamma and TNF-α signaling for the TNFAIP3 transcript. Finally, regarding the genes of interest to be validated in whole blood, none of the enriched genes matched with the main 20 SDE transcripts in the time to culture conversion analysis.

Next, to complete the genes of interest list, following a purely statistical rationale, we selected the SDE genes that had a log2FC equal or superior to +1/-1 as well as a baseMean greater than 50, from the top 20 SDE transcripts. From the 6056-gene human TB granuloma signature, we selected MAP4K1, FCMR, and CCL19. As the GSEA analysis failed among the fast and slow time to SCC comparison in whole blood, the rationale was entirely statistic. We picked-up ANKRD9, AE000661.37, CEACAM1, GFI1B, and HLA-DRB5. CEACAM1, GFI1B, and HLA-DRB5 were exclusively detected in whole blood. Regarding the genes of interest from the granuloma spatial analysis, the rationale behind this analysis was to choose those genes that its log2FC represents the entire gradient of expression, covering the genes mainly expressed in C towards E. This was traduced into selecting the seven first SDE transcripts: P2RX7, LHFPL2, C1QB, SLC6A9, GNPTAB, FAM71A, HPSE. The product of the gene interaction between the time to sputum culture comparison in the human TB granuloma and whole blood exhibited 18 genes in common. From those, only IDO1 gathered the statistical rationale to be added to the genes of interest to be validated, so was later included in the whole blood panel for validation.

Finally, we were also interested in assessing the genes encoding for the circulant biomarkers potentially related to poor prognosis features found significant in Sub-Study A, namely IL2RG, CD14, CXCL10, CD5L, MMP8, and MMP9 as they potentially proved being related to the worsening phenotype of the SH-TBL Cohort. IL2RG (as the IL-2 receptor), CD14, CXCL10, CD5L, and MMP9 genes, but MMP8 that was not detected, were found statistically significant upregulated in the 6056-gene human TB granuloma signature, although none of them were expressed in the comparisons made from the whole blood samples set. In any case, all seven genes were added in both tissue and blood panels to be later validated upon the Validation Set (**Figure 22**).

To sum up, after the total-RNA obtaining, patients were divided into the Discovery Set (14 patients) and the Validation Set (26 patients). RNA-seq was performed on the Discovery Set, and after the bioinformatical analysis, three signatures were obtained from the tissue derived counts, and another one from the presurgical whole blood. Following a statistical and biological rationale, we down-selected the genes better including the main pathophysiological features of the Discovery Set and the top-ranked genes from each signature. Additionally, we checked the genes intersecting between the Fast vs Slow time to SCC in both biological sources and we included IDO1, considering the statistical rationale. The genes of interest derived from tissue will be validated in all tissue samples from the Validation Set, whereas the genes of interest derived from whole blood will be validated in all pre-surgical samples from the Validation Set. Finally, we also included, from Sub-Study A, the genes encoding for the main proteins associated to several poor prognosis features. These genes will be validated in both tissue and whole blood samples from the Validation Set. 23 genes were included in the tissue panel whereas 12 to the whole blood. Regarding the housekeeping (HK) genes, seven genes were chosen to HK in the tissue validation panel whereas eight in the whole blood panel. The work pipeline summarizing the genes of interest selection is gathered in **Figure 23**. The data regarding the genes of interest expression is summarized in **Table 23**.

The results regarding the nCounter (nanoString) validation of the genes of interest expression upon the Validation Set are not included as were not available at the moment of the delivery of the present thesis.

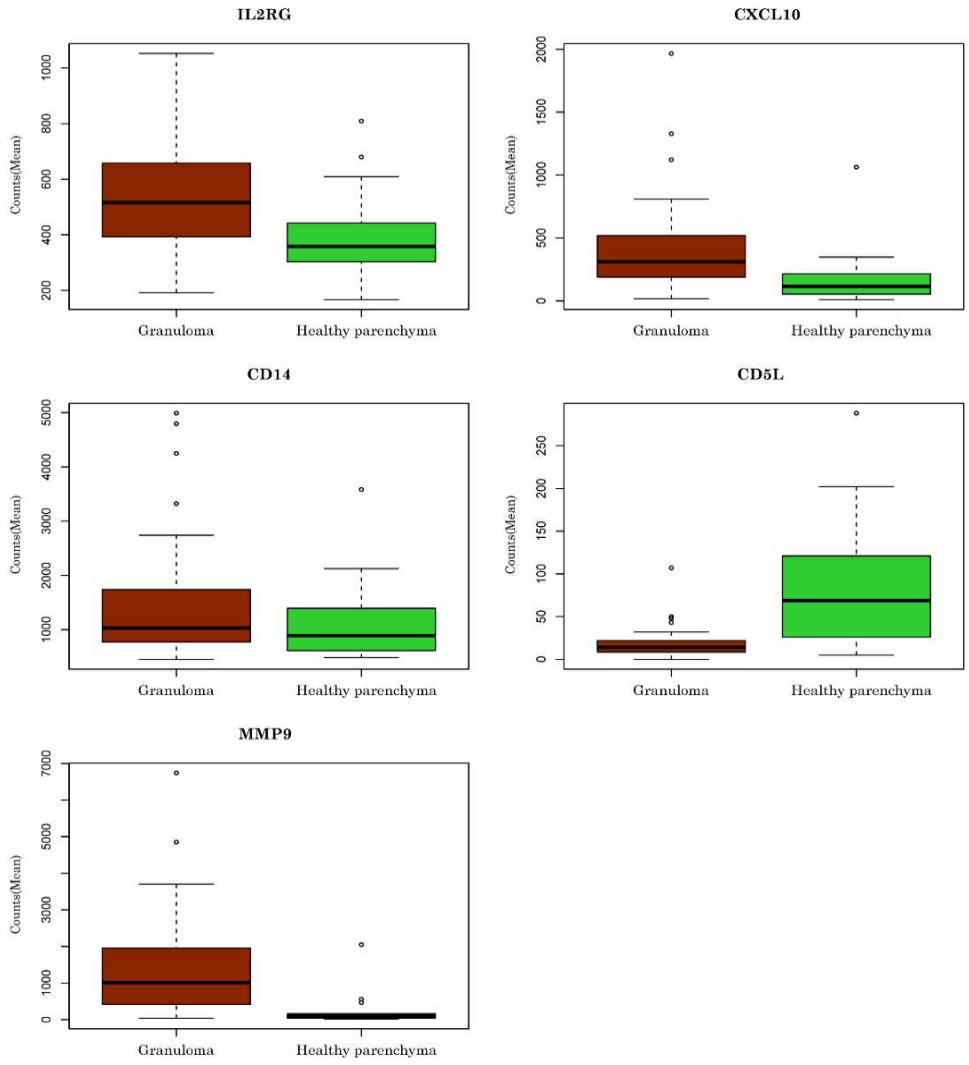


Figure 22. Normalized mean counts from the SDE in the 6056-gene human TB granuloma signature encoding for the cytokines, chemokines, and proteins related to pathophysiological features in Sub-Study A. MMP8 gene was not plotted as was not found SDE.

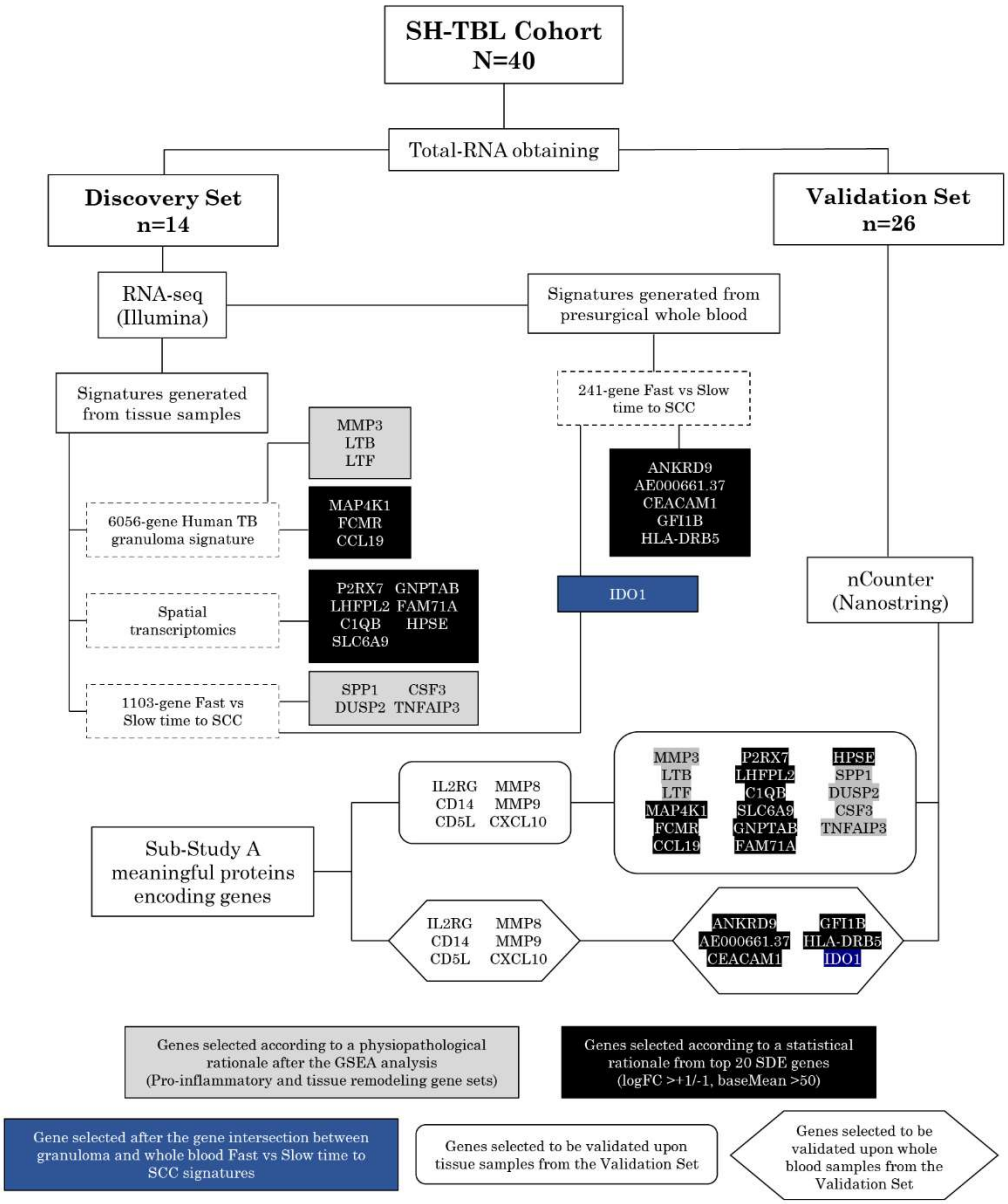


Figure 23. Work pipeline for the genes of interest selection and posterior validation upon the Validation Set through nCounter by Nanostring.

Table 23. Summary table including the genes of interest expression in the tissue and whole blood panels to be validated upon the Validation Set. Genes of interest were included following a pathophysiological and a statistical rationale. All SDE selected genes had a log2FC ≥+1/-1. Non-SDE (NS) and non-detected (ND) genes were chosen based on the results of Sub-Study A circulant proteins.

Gene of interest	baseMean	Log2FC	Gene of interest	baseMean	Log2FC
Tissue Panel			**Presurgical Whole blood Panel**		
MMP3	34,54	6,259	ANKRD9	101,27	-1,65
LTB	172,29	2,057	AE000661.37	85,98	-1,23
LTF	728,99	2,810	CEACAM1	257,77	-1,58
SPP1	3324,04	4,17	GFI1B	71,56	-1,34
DUSP2	163,41	-1,23	HLA-DRB5	873,93	2,12
CSF3	79,31	-2,98	IDO1	187,30	-1,99
TNFAIP3	2052,66	-1,47	IL2RG	NS	
MAP4K1	224,45	1,31	CXCL10	NS	
FCMR	207,54	1,90	CD14	NS	
CCL19	441,68	3,00	CD5L	NS	
P2RX7	512,381	-1,27	MMP8	NS	
LHFPL2	1638,12	-0,42	MMP9	NS	
C1QB	4095,35	-1,40			
SLC6A9	105,74	0,77			
GNPTAB	1638,12	-0,41			
FAM71A	25,85	3,76			
HPSE	123,67	-1,07			
IL2RG	476,86	1,01			
CXCL10	335,74	1,50			
CD14	1290,89	1,00			
CD5L	29,34	-1,38			
MMP8	ND				
MMP9	1108,27	2,76			
Housekeeping genes in Tissue Panel			**Housekeeping genes in presurgical Whole blood Panel**		
ACTB			ACTB		
RPL10 (huPO)			RPL10 (huPO)		
DECR1			DECR1		
PLXNB2			EPC1		
ACTG1			LARP1		
PCBP1			GNAS		
ADCY3			ASH1L		
			BCAS3		

7.9 Gene intersection analysis with predecessor human TB lesions signatures

The first reference of this study of a prior generated TB granuloma signature was the one obtained by Kim *et al.* from LCM in caseous human TB lesions through microarray (GEO accession number GSE20050). We obtained the publicly available

differentially expressed genes list between the caseous human pulmonary TB lesions and the healthy lung parenchyma. As a product of an Affymetrix microarray, gene IDs use a different nomenclature from most of RNA-seq experiments. For an efficient intersection analysis with our TB granuloma signature, we used the DAVID's Gene ID Conversion Tool (version 6.8) to convert the probe set IDs into ENSEMBL gene IDs. The product of the ID conversion turned into 5841 SDE genes identified by an ENSEMBL ID.

Next, we checked the total number of overlapping genes with the detected genes in our TB granuloma lesion vs. healthy lung parenchyma comparison, revealing a total of 5622 genes in common. A total of 1681 SDE genes were found to be overlapping between both signatures, showing consistency in the direction of expression in 1166 genes (69.36%), being MMP1, MCHR1, and SPP1 the top three ranked genes according to our log2FC. Finally, we searched among the overlapped genes if any of the genes of interest were present, finding LTB, P2RX7, LHFPL2, C1QB, HPSE, SPP1, DUSP2, TNFAIP3, MMP9, CD14, and CXCL10 upregulated in both signatures, especially highlighting the expression of these last two genes, ranking among the first 40 most upregulated SDE genes. Interestingly, none of the genes of interest derived from our whole blood samples was present among the overlapped genes.

7.10 Concordant and discordant gene expression between the 6056-gene TB granuloma signature and an independent cohort

We finally sought to assess the similarity and dissimilarity in the expression of the orthologous genes between our human TB granuloma 6056-gene signature and an independent cohort. To such end, we used a publicly available data set from another TB cohort in which the ATT response is evaluated in whole blood, namely the Catalysis Treatment Response Cohort (CTRC) (GEO accession number GSE89403). Metadata and unnormalized whole blood raw counts generated after RNA-seq were acquired. DESeq2 was employed to compare the differential gene expression between the samples obtained at diagnosis moment (DX) (n=71 whole blood

samples) and after treatment completion at week 24 (n=73 whole blood samples) among the sub-group of microbiologically cured patients, according to WHO terms. Probable and possibly cured patient's samples were discarded for the analysis. In total, 9728 genes were found SDE. Next, the gene intersection analysis revealed that 2782 genes were orthologous between the granuloma signature and the SDE genes from the CTRC cured patients (**Figure 24.A**). To validate the concordance and discordance in terms of expression direction between each pair of orthologous genes we used the disco.score, enabling to calculate the similarity and dissimilarity between both compared data sets (**Figure 24.B**). A total of 1596 orthologous genes (57.35%) demonstrated having a positive similarity between the TB granuloma signature and the cured patients when they were diagnosed with TB. On the contrary, 42.65% of the genes had a negative disco.score, hence representing the fraction of discordant genes (**Figure 24.C**). Next, we checked which of the genes of interest, both those detected in tissue and whole blood, were also detected in the whole blood of the CTRC samples, confirming that 22 genes were also expressed, but MAP4K1, MMP3, CCL19, FAM71A, CSF3, CD5L, and AE000661.37. Finally, we traced which of the overlapped genes were also belonging to the genes of interest, finding concordance (in descendant order) in LTF, MMP9, LHFPL2, HPSE, CD14, CXCL10, IL2RG, P2RX7 and a discordant expression of SPP1, FCMR, DUSP2 and ANKRD9 (in ascendant order) (**Figure 24.D**). According to these results, we hypothesized that the concordant set of genes between the TB granuloma signature and the samples of cured patients obtained at diagnosis suggested that these genes could become or be part of a gene signature indicative of disease if were detected at the onset of the TB episode. On the contrary, the gene pairs exhibiting a discordance may be pointing to a TB lesion persistence after ATT, as the inverse log2FC of the cured TB patients at diagnosis would turn into a concordance of expression between the samples of cured patients taken at week 24 and the 6056-gene human TB granuloma signature. To assess this hypothesis, we compared the fraction of the not microbiologically cured patients' samples from the CTRC at diagnosis and week 24 (n=7 whole blood samples), but the DESeq2 outcome only provided 3 SDE downregulated genes at diagnosis, hence being unable to explore this way such hypothesis.

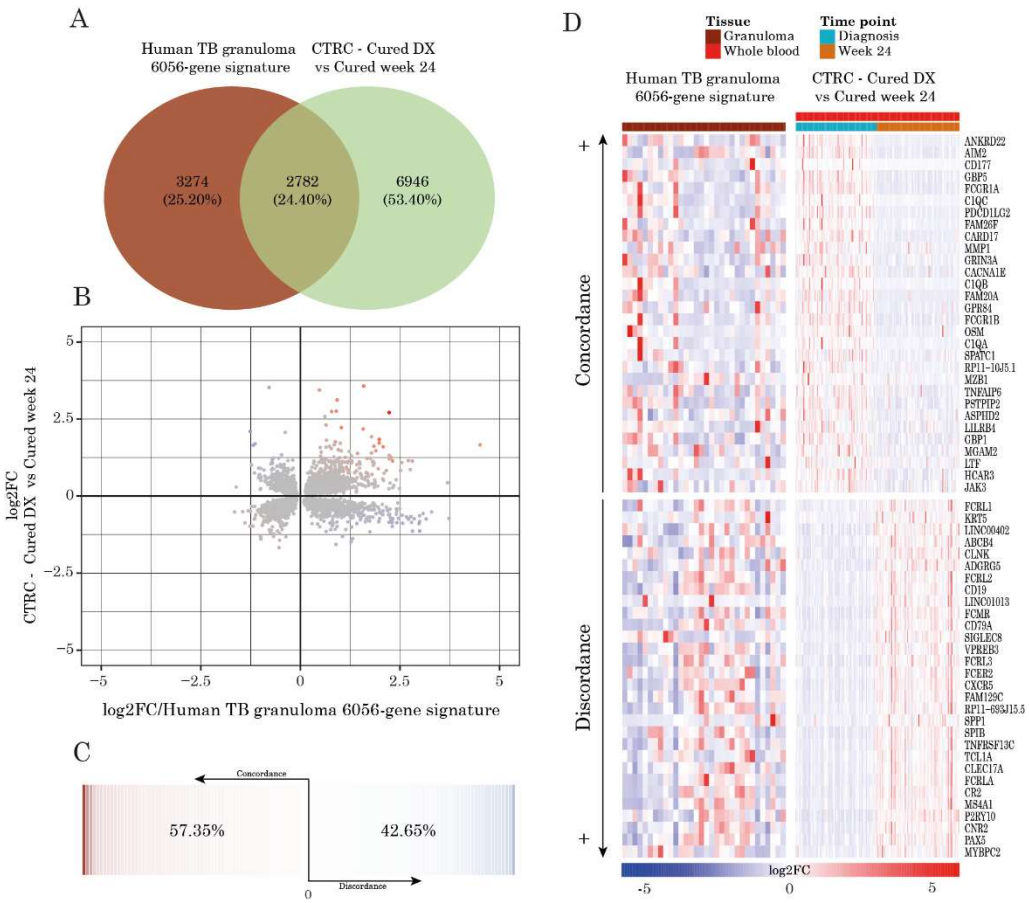
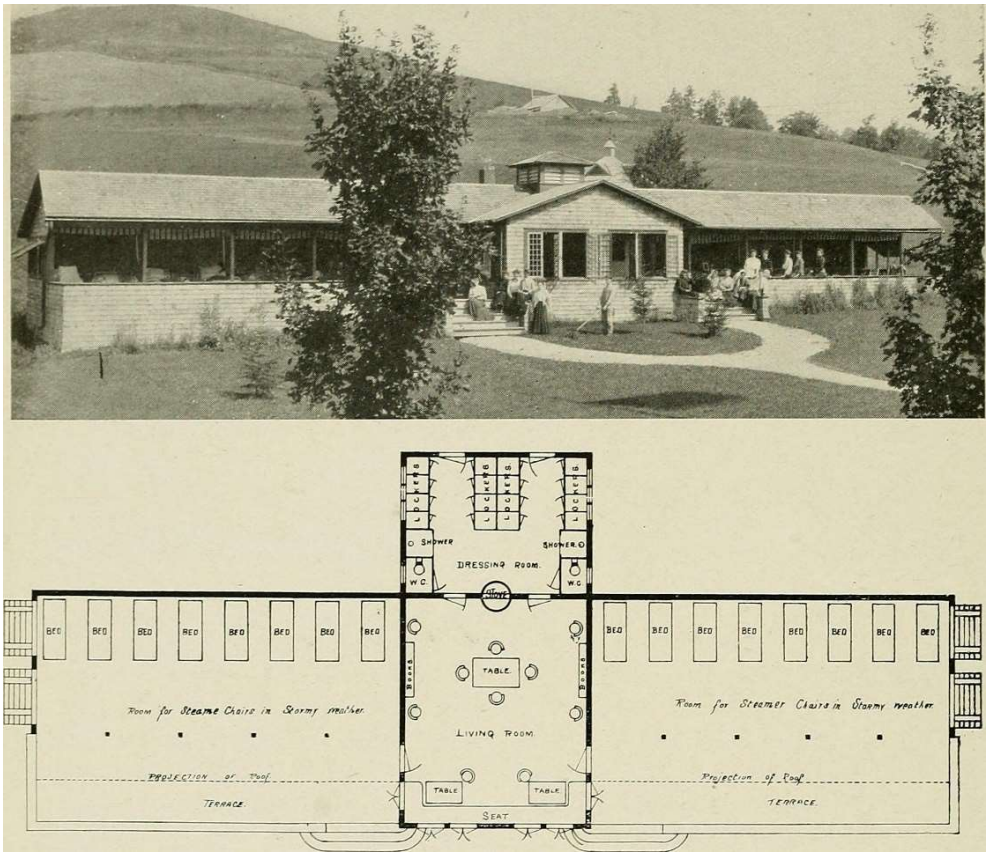


Figure 24. Analysis of the concordance and discordance of the human TB granuloma 6056-gene signature and the CTRC. (A) 2782 SDE overlapped genes were found between both data sets, although being from different cohorts and different tissue sources. (B) The spatial distribution of the overlapped genes according to the log2FC between the granuloma signature and the CTRC cured patient's whole blood samples at diagnosis (DX) and after treatment completion at week 24. Increasing intensity of the red color indicates a higher disco.score and therefore a similar pattern of expression of the gene pairs. Increasing the intensity of the blue color indicates a negative disco.score the more dissimilar the pattern of expression is. (C) More gene pairs having a concordant expression than a discordant expression were found. (D) Heatmap of the top 30 concordant gene pairs (in descendant order) and the top 30 discordant gene pairs (in descendant order) in both data sets, including C1QB, LTF, FCMR, and SPP1 from the validation genes set. Among the genes showing a discordant expression, we excluded the gene pairs having a negative log2FC for the TB lesion expression.

8 | DISCUSSION



Description: "Tuberculosis hospital and sanatorium construction blueprint, *No. 73.- Loomis Sanatorium, Liberty, N.Y. Original Improved Lean-To. Designed by Dr. Herbert Maxon King. View of Front elevation and Floor plan. Capacity, 16 patients. Cost, $1,830."* The year 1911.

Author: Thomas Spees Carrington, National Association for the Study and Prevention of Tuberculosis (U.S.)

TB is a worldwide health problem that has taken thousands of human lives for centuries. Nowadays, WHO programs such as the End TB Strategy, have focused on ending the TB epidemic by the end of 2035, reducing the deaths and the incidence rates to almost incidental cases. One of the main priorities in the TB research during this decade is to improve current TB patient management tools as well as to find new clinical correlators and biomarkers that could predict the disease outcome at the very beginning of the TB diagnosis. To do so, current strategies have focused on studying the TB disease at several stages along with patients representing the variety of the disease severity specter, but only a few of them are including TB patients with a poor resolution status requiring therapeutic surgery. To our knowledge, the SH-TBL project, from which is based this doctoral thesis, is the first of this kind into studying the clinical features to potentially associated biomarkers among patients that underwent therapeutic surgery from their PTB.

What makes the SH-TBL project unique is that a macroscopic description of the removed surgical TB lesions was possible as well to unravel, for the first time to our knowledge, the transcriptome from fresh TB granuloma lesion biopsies involving both DS-TB and MMDR/XDR-TB strains. The kind of necrosis was found as an important characteristic that may influence the prognosis of the patient, but very importantly, the persistence of *Mtb* in surgical specimens was detected in Study I and II. Individual factors as sex and toxic habits were identified as potential characteristics related to the evolution of the disease. We also identified several biomarkers correlating with macroscopic features of the lesions, the involved *Mtb* strain, and factors considered of poor prognosis. Finally, the results are suggesting an immunosuppressive effect induced by the persistence of the TB lesions, after evaluating the post-surgical biomarkers and the genotypic characterization of the 6056-gene human TB granuloma signature. In conclusion, the main goal of the project was achieved, as was possible to characterize the immune status of the SH-TBL Cohort, to determine the role of several clinical features at this stage of the disease, the microbiological status, the link of circulant biomarkers with potential severity factors of the SH-TBL Cohort as well as to determine the transcriptome of the human TB lesion and transcriptional signatures with important clinical

relevance that led to building the genes of interest platform including new TB putative biomarkers.

From an overall point of view, the project was conceivable because surgery is still in use, for instance in our cohorts' origin country, Georgia. Therapeutic surgery for PTB was the only way of treatment for decades until the appearance of the antibiotics, which led to progressively be abandoned (but reserved for selected cases) by most parts of the old European countries. Nevertheless, nowadays therapeutic surgery is still routinely in use especially in high MDR-TB burden settings like Georgia and other former Soviet Union countries. According to official guidelines, TB surgery is indicated to treat individuals under an emergency, such as irreversible death unless surgery is carried out because of severe hemoptysis, or because of elective indications, considering a poor prognosis or complications and sequelae from the PTB. The main indication is reserved for complicated MDR/XDR-TB cases, though (54).

8.1 Study I discussion

As part of the first approach of this doctoral thesis, Study I main goal was to identify which traits have the PTB patient that may influence their prognosis and led them to receive therapeutic surgery. A total of 137 patients that were treated for TB in the NCTLD were retrospectively included in the Study I cohort if they required therapeutic surgery, from the years 2014 to late 2015. First, we observed that men requiring surgery were outnumbering women, something already observed (61,178,179) as well as were older, considering that men in the 137 patients cohort declared having more toxic habits and had more comorbidities. Importantly, men had more pleural involvement and post ATT complications, suggesting that these reasons indicated to receive surgery during the TB episode earlier than women, as pointed out by the days between the TB diagnosis and the therapeutic surgery.

It can be spotted the polarization of poorer clinical features towards women, as after stratifying men between the 43 years old dividing line, women still received surgery at a younger age, likewise, the MDR/XDR-TB women gathered higher numbers of time to the SCC beyond the 2-month standard as well as the presence of fresh necrosis in the removed TB lesions. This data may be indicating that the course of

the disease could be worse among women, especially if they are suffering an MDR/XDR-TB, although men having a greater TB incidence. These findings made hypothesize that sex is displaying an important role in TB pathogenesis, especially highlighting the median age among women (28 years old), which is also an important factor for the TB progression during the reproductive years (180). Although being a generalized thought, there is an important bias under this idea, as socio-cultural aspects play an important role in many settings, for instance, the access to healthcare or the misuse of toxic agents. Even though, sex steroid hormones have been also added to support this hypothesis as they could be related to the host protection as well as to the disease susceptibility (7,181). This is also indicated in a study conducted among postmenopausal women, where the mean estrogen levels were significantly above the patients having TB from the non-TB control (182).

Study I results regarding the MDR/XDR-TB group were very much in line with the official guidelines and other published studies showing the need for receiving surgery (54,56,183). Albeit in the 137 patient cohort the MDR/XDR-TB represented the 40% of cases that underwent therapeutic surgery, they exhibited a statistically significant delay from the DS-TB into converting the sputum culture after two months under ATT. It is supported that alcohol intake may induce a significant delay in converting the culture (184,185). Some others have proposed the time to SCC status at six months rather than at two, as a better correlator of ATT success (69,186). Anatomically, a potential causality behind may be given by the architecture of the MDR/XDR-TB patient's lesions as they may develop a thicker fibrous wall in cavitary lesions, which obstructs the antibiotic penetration to the center, where bacilli are mostly gathered and hence sterilization may not occur (187,188). In either case, it is realistic to consider surgery as an extended therapeutic tool to cure MDR/XDR-TB patients, as have been proven cavitary lesions can harbor countless *Mtb* colonies which the host's immune system would not be able to control (189).

On the other hand, the DS-TB patients were 56% of the cohort representation, as the main recorded indication to receive PTB surgery among the DS-TB subjects was because of the persistence of TB lesions in the CXR, despite proving a good

adherence to ATT. When this is evaluated in detail, the official WHO guideline as well as the national guidelines from Georgia, include several conditions during the TB episode that may indicate the therapeutic surgery regardless of the *Mtb* strain, namely individuals at high risk to relapse; an anatomically localized presence of a TB lesion; no improvement in radiological and/or microbiological terms during the first three to four months under ATT; detectable changes in lungs and adjacent tissues because of the development of fibrotic structures; and tuberculomas bigger than three centimeters (54). Socio-economic structures in the former Soviet Union countries with high TB burden make it difficult to diagnose and follow-up patients as there is a late referral for medical attention. Hence, diagnostic delays and lost to follow-up patients increase the chances to develop complicated TB episodes, with extensive cavernous lesions and other irreversible morphological changes that compromise the chemotherapy effect regardless of the drug-sensitivity, being these patients therefore much more susceptible to receive surgery than any other patient located in western Europe and other high-income countries.

To date, the bulk of published articles are focused on the surgery upon MDR/XDR TB patients, so it was difficult to find data correlating our DS-TB set (59,60,179). Even though, the macroscopic characteristics of the removed lesions can add more evidence on the therapeutic surgery necessity. First, in the SH-TBL project, we divided the lesions considering the presence of fresh necrosis, dry necrosis, or the combination of both. This classification refers to the exudative and proliferative lesions: fresh necrosis is related to poorer clinical features, with an exudative component tending to liquefaction, high neutrophilic condensations, and therefore with a high bacillary load. On the other hand, dry necrosis is linked to better outcomes and prognosis, as the lesion is soon controlled by fibrosis and calcification thanks to an important role from epithelioid cells and fibroblasts, as well as the localization within the lobe (22). The presence of both necrosis types was considered an intermediate phase.

In Study I we found a high percentage of DS-TB cases (70.59%) having fresh necrosis, as well as among the MDR/XDR-TB (72.54%). Hence, a high rate of the overall removed TB lesions showed fresh necrosis, which was later highlighted when was stratified by sex, showing how MDR/XDR-TB women had a remarkable

proportion of this necrosis and therefore supporting the idea that women tend to evolve worse from the disease.

The main strength of this study was to confirm that surgical samples still had both the presence of bacilli in AFB smears and more importantly alive *Mtb* grown in culture. This does add more evidence in the feasibility to perform surgery upon the DS-TB: they had a meaningful presence of fresh necrosis (21.43%), the 56% had a positive AFB from surgical biopsies together with seven individuals having a positive culture (14%). The proportion of DS-TB relapsed patients in comparison to the MDR/XDR-TB was huge (26.47% and 3.77%, respectively). As expected, the MDR/XDR-TB set gathered the major number of positive cases regarding the surgical specimens' positivity (65.96% in AFB, 23.40% in culture) from the DS-TB. Although most parts of the MDR-TB and XDR-TB were treated longer than the standard time with second-line chemotherapy, they still had TB cavitary lesions, plus almost all of them had necrosis (94.44%) being mainly fresh necrosis; as well as patients were older men and women. These results were found similar to a previously published article (178).

The presented results regarding the positivity from the granuloma biopsies are always including only those patients exhibiting persistent lesions in the CXR. Moreover, 98.04% of the DS-TB and the 100% of the MDR/XDR-TB having either cavities or tuberculomas were considered as microbiologically cured according to the WHO definitions for both *Mtb* strains at the end of the ATT. Although finding positive AFB from tissue smears do not imply the presence of viable bacilli, the 18% of the cohort that was considered as cured had later a positive culture from surgical specimens, including two cases in which the AFB was negative. This is especially frightening among women (23.68%), and patients having fresh necrosis (21.43%).

For us, this is the most important finding in Study I, especially among the DS-TB subjects, as puts under interrogation either the clearance effect of the anti-TB chemotherapy or the currently available drug-regimens. After long months under chemotherapy and a consequent reduction of the bacterial load, a part of the bacillar population can silently persist in the host's organism. In the present context, it is conceivable that the *Mtb* can be transformed into L-forms or small-grained forms,

initially characterized for its low virulence but with the innate capacity to the reversion to the original virulent properties (190–193). The only currently validated and standardized biomarker to assess the curing of the patients is to achieve a negative sputum culture accompanied with a favorable clinical picture at the beginning of treatment in the DS-TB patients (71), yet our results are proving that sterilization did not occur despite the DS-TB patients' fraction followed treatment correctly.

In conclusion, these results allowed us to identify the clinical-epidemiological factors as well as the macroscopic and microbiological characteristics of the TB lesions that could be related to the poor disease evolution. These severity traits therefore may become part of the signals that would eventually endorse to receive therapeutic surgery. In summary, the pathophysiological phenotype or severity traits of the Study I participants mainly included the sex, the toxic habits, the radiological findings, the drug-sensitivity type, the capacity to convert the sputum culture, and the macroscopic characteristics of the TB lesions, namely the presence of a determinant type of necrosis and either the presence or the viability of the bacilli found in the removed surgical TB lesion, arising as the potential inductors of the poor prognosis highlighted by these results. Last but not least, these findings are suggesting that the current standardized correlators are not reflecting what is happening in situ, a fact that we will try to sort out in Study II.

8.2 Study II discussion

After completing the Study I, we started the second objective of this thesis as it is compiled in Study II. The SH-TBL Cohort constituted the new group of 40 patients that underwent therapeutic surgery in the NCTLD and became part of Study II. In the SH-TBL cohort, the analysis of the clinical and epidemiological characteristics revealed a significant difference in the age at what surgery was performed between men and women, being the male participants older than females. This was not surprising as was already noted in Study I, and even in a cohort shorter in size, such event was happening again, highlighted by the fact that males were significantly influenced by the alcohol and smoking misuse. We considered the toxic habits as a limitation of Study II, especially regarding the alcohol use, because the

interpretation of the results must consider not only the sex as an important biological component in the TB context (7), but to add the alcohol abuse among men as an important confounding factor, as we cannot discard that alcohol may play an important role in the results. Unfortunately, the overall TB related data from the SH-TBL Cohort was not able to reproduce the same results logic as in Study I, as we suspected that the study size influenced the outcomes of the analysis. Even though, even in a cohort of a reduced size we still found the presence of the bacilli in the resected lung lesions, both in AFB smears and after culturing the TB granuloma lesion biopsies.

8.2.1 Sub-Study A discussion

Sub-Study A was designed to evaluate the capacity of the chosen set of cytokines, chemokines, and proteins belonging to the immune system and tissue remodeling to find a correlation with the SH-TBL Cohort's severity traits, being previously characterized in the Study I cohort. Furthermore, we wanted to predict which set of these circulant biomarkers as well as which recorded clinical variables may indicate the need to receive surgery and suggest the persistence of the *Mtb* in the lungs, although achieving microbiological clearance. Finally, we also aimed to evaluate the immune system status at this stage of the disease, and have a picture of the host's immune response after receiving therapeutic surgery.

First of all, we wanted to explore the pre-surgical and post-surgical differences in the set of markers concentrations to evaluate the effect of the TB lesions removal after surgery. In general, the markers assessed in this study were found significantly increased after surgery. We cannot discard that this effect was triggered by the immune response generated by the surgery. Previously, CRP and IL-6, although this last one with a modest change but significant, have been found associated with the severity of the surgery peaking 72 hours post-surgically and normalizing after seven days in uncomplicated inpatients (194). However, the positive postoperative course of the patients in the SH-TBL Cohort paired with the fact the post-surgical measurements happened, at least, more than seven days after the surgery, hence renders this scenario potentially unlikely.

If we discard the effect of the surgery, we hypothesized that the analytes measured pre-surgically could be decreased as a result of the TB granuloma acting as an attracting pole for several classes of cells and proteins, although this should further be confirmed with the histopathologic characterization of the lesions. Otherwise, the TB lesions may be inducing a systemic immunosuppressive effect in the host's organism. Although the literature is short to support this idea, yet in another publication in which MDR-TB patients were operated from their PTB we found similar results from ours, indicating an immunological boost posterior to the lesion removal as during the disease the granuloma was possibly inducing an immunosuppressive effect potentially driven by multiple cellular classes, suggesting also being responsible for a poor response to the anti-TB chemotherapy (195). In that regard, the TB granuloma lesion may be exerting a local chronic inflammation that could lead to the activation of the Myeloid-Derived Suppressor Cells (MDSCs). The MDSCs are a group of cells that in response to strong signals, like those induced by the TLR ligands after the *Mtb* infection, can induce a systemic suppression of the innate and adaptive immunity, and this response can last differently considering the nature of the activating signals. Such behavior has been recently suggested being related not only in the context of infectious diseases but to cancer and autoimmune disorders (196,197). Further studies are strongly needed to elucidate the mechanism behind the MDSCs effect, both at the systemic level and on the resected lesion, as may be key to understand the role of the immune system in TB as well as a potential target for the HDT.

The only marker found decreased after surgery was CD5L. There is no data about the CD5L levels in patients undergoing surgery for their PTB, although our pre-surgical measurements are similar to those reported by Xu *et al*. at the diagnosis moment (198). As there is no more existing data at our analysis' timepoints, we do not know what happens with CD5L during the course of the disease. We cannot discard that CD5L is somehow reflecting the disease activity but also if it can be used as a biomarker of TB but not able to reflect the ATT response or as a prognosis biomarker. Finally, another interesting circulant analyte was sCD14, showing a meaningful increase in levels both in plasma and urine measurements. Its levels were found higher compared to the described cut-offs in recent literature,

evaluating its role as a diagnostic and prognostic biomarker of TB and other infections (199). In conclusion, we suggest that these two biomarkers should be further studied in several other cohorts and multiple timepoints to unravel their involvement during the TB disease course.

Next, we assessed the whole set of markers at their pre-surgery levels according to the severity traits. When we compared men and women, in general women exhibited a greater production of the circulant biomarkers, but only IFN-γ, IL-4 and urine IL-8 were statistically increased. Such finding is correlated with current literature, suggesting women as having a more robust immune response than men against infectious disease and vaccination while being more prone to autoimmune disorders, and as stated in Study I, maybe driven by the action of sex hormones like estrogens (7,200,201). As previously declared, we considered the sex-related comparisons highly influenced by the alcohol effect, as we cannot discard that alcohol is, together with the persistence of the TB lesions or by itself, responsible of the induction of the immune suppression as seen among male participants of the SH-TBL Cohort: such findings are in line with recent literature. The decreased levels of IFN-γ, IL-4 and urine IL-8 in alcohol users may be indicating that alcohol was inducing an immune suppression. The alcohol has a potent effect on the B lymphocytes fraction, provoking a reduction over the release of IL-4 together with the reduction and even suppression of the production of the IFNs group, which therefore may compromise the host's ability to sterilize the *Mtb* presence (202). In addition, alcohol misuse is related to worse treatment outcomes, especially on a heavy use in combination with tobacco (203). Smoking is reportedly being a potent pro-inflammatory agent and an immunosuppressor depending on several epidemiological factors, negatively influencing the production of IFN-γ, the host capacity against infections and the TB outcomes (204–206). In our cohort, urine IL-6 was significantly increased among the set of smokers, which have been interpreted as the pro-inflammatory effect of tobacco (207). Furthermore, we found correlations between the smokers and patients having a positive AFB smear in the TB lesions, in a similar way if we consider that other authors had it with the sputum smear and among the slow sputum culture converters (204).

One of the major strengths of the Sub-Study A is that we were capable to evaluate the influence of the pathophysiological features of the SH-TBL cohort by their correlation with the pre-surgical circulant markers. In summary, only the plasmatic MMP-8, MMP-9 and IL-2, and urine sCD14 and IP-10 showed to correlate with the severity traits. Although the detected IL-2 levels were low, was found positively correlating with the lesion size being bigger than 36 millimeters, and especially highlighting the potent association with the IP-10 levels in urine. It is known that IL-2 is a promoter of the T-cells proliferation and has a role for the granuloma generation, and have been tested as an HDT for TB with a beneficial effect on the time to SCC but not regarding the radiographical findings (208). However, Sigal *et al.* also found significant correlations of the IL-2 receptor levels with cavities bigger than 4 centimeters measured at baseline in a 319 cohort of PTB patients (118).

IP-10 has emerged as a very promising biomarker for active TB and as a correlator of the ATT response (152,209). It is involved with the recruitment of the Th1 cells to the infection foci and the TB granuloma's maintenance tasks (210,211). Therefore, we suggest the potential use of IP-10 as a biomarker of the persistence of the TB granuloma. We further investigated other publications correlating our IP-10 urine levels, finding that our concentrations in urine were higher to the ones exhibited by Kim *et al.,* although reaching similar levels after 12 weeks under ATT in another small-sized cohort when they were cured of their PTB (152,212), so this potentially undermines the hypothesis of the granuloma persistence in benefit to reflect bigger size TB lesions. Further studies should be conducted in other cohorts in order to confirm the strength of these correlations and their clinical meaning and mechanism.

The plasmatic MMP-9 was found correlating with the MDR/XDR-TB patient's set, and very interestingly, MMP-8 was the biomarker having more positive and stronger correlations to many severity features. The MMP's are a subset of proteins that act as enzymes catalyzing the remodeling, degradation, and the posterior destruction of the extracellular matrix, hence they may be playing a key role in the lung injury (213,214). In TB, this process is intimately related to the cavity formation and closure and a final release and transmission of the *Mtb* bacilli (213). In a Peruvian cohort of PTB patients, Sathyamoorthy *et al.* tested the role of several

MMPs, finding a correlation of MMP-1 with TB symptoms, although MMP-8 did not correlate with the slow sputum culture converters nor with the persistence of the TB symptoms, in contrast to what we found (129). Additionally, Sigal *et al.* described how MMP-8 and MMP-9 are associated with the cavities bigger than 4 centimeters and the radiological extent of TB greater than the 50% at baseline, to finally consider MMP-8 as one of the most significant biomarkers associated with the disease severity (118). Finally, other studies have found associations of MMP-8 and MMP-9 with the cavitary lesions in TB patients, the destructive pattern role of MMP-8, and its decrease detected in sputum throughout the ATT, returning to normal levels (215–217). In conclusion, these findings are very much in line with our results, considering that the SH-TBL Cohort particularly had a no-resolution of TB status which lead them to receive therapeutic surgery.

Next, we explored the correlation between MMP-9 and the MDR/XDR-TB strains, finding only one manuscript suggesting that high levels of MMPs may be linked to the presence in the host's organism of drug-resistant strains (218). As we see it, such association is legitimated by the fact that drug-resistant forms are commonly associated with a greater and persistent lung injury even after ATT completion, likewise the SH-TBL Cohort (219). As in this regard, literature is extremely short, we would like to encourage the scientific community to explore this potential association with the full set of MMPs, which some of them or in combination, may become a potential biomarker of multidrug-resistance. Moreover, circulant MMPs have been repeatedly being related to lung tissue destruction and remodeling. Therefore, we also point that using all the commercially available and detectable MMPs may become indispensable to finally elucidate its role in the granuloma formation and build a solid panel of biomarkers which could potentially indicate the persistence of the TB granuloma lesions.

The rest of the correlations also provided interesting associations. The patients having a relapsed TB were strongly correlated with the presence of infiltrates in the CXR, also correlating with the patients ranging from 35 to 44 years old as well as having an HCV coinfection. As known, the persistence of cavities detected in the CXR entails a delayed response to ATT and relapse, although there is no literature regarding the infiltrates in the CXR (220). There is existing literature relating the

radiological signs to the microbiological status (221), so according to our results, MMP-8 may become a potential biomarker capable of complementing the host's radiological status. Unfortunately, none of the potential biomarkers correlated with the presence of fresh necrosis and any other clinical variables, even when we analyzed the absence of it in the tissue specimens. This was probably attributed to the high proportion of patients having fresh necrosis solely (84.62%). Consequently, recruiting a broader cohort may help to elucidate the circulant markers related to the presence or not of fresh necrosis.

Finally, we wanted to test the ability of the severity factors and the circulant biomarkers to predict which participants of the SH-TBL Cohort may need therapeutic surgery by the leave-one-out cross-validation, revealing that all patients required of surgery. In contrast, being a female, smoker, and having both levels of plasmatic MMP-9 and urine sCD14 showed the capacity to predict the presence of *Mtb* in the TB lesions, although this outcome was statistically poor. One of the main limitations of the entire Study II is that as limited granuloma biopsies samples parts were collected to be stained for AFB and to culture, we cannot discard that even more patients were both positive for AFB and tissue culture, to finally reach similar detection rates as in Study I. Therefore, all correlations observed regarding the presence of the mycobacteria should be interpreted cautiously. To better prove this limitation, biopsy samples should be taken to histopathology analysis as well. Despite this, two individuals of the SH-TBL Cohort proved to have alive bacilli in culture, and together with Study I participants as independent cohorts, gathered more evidence to challenge the WHO definitions of treatment success, as there is a subpopulation of patients in which the ATT does not achieve lung sterilization, calling into question the reliability of the sputum culture and therefore rising the urgent need of alternative biomarkers. This is not only an individual problem, as also undermines any therapeutic intervention at the public health level as well, as this fraction of TB patients may potentially relapse and become a pool of TB spreaders.

Some other limitations are present in Sub-Study A. We did not have a healthy control correlator and/or any other patients receiving lung surgery other than TB therapeutic surgery to contrast our immunological findings. Immune responses

were measured right before the surgery moment, in some cases during the treatment course and other after termination, although all participants had a microbiological conversion. Nonetheless, we can consider all analytes measured within the ranges reported by others after treatment (130,222–224), even if the scarce literature assessing immune responses after completing treatment is complex to interpret, as there is no harmonization: they measure different analytes, in different biological fluids, and at different time points. A post-discharge follow-up was not possible to collect to contrast immunological changes throughout time and confirm the immune restoration, which in settings like Georgia it is complicated to monitor former patients beyond treatment finalization if a cured status was achieved. Finally, taking only the worst cases into account or the ones that underwent surgery does introduce selection bias, so results should not be generalized to the whole TB patient's spectrum. In conclusion, we identified a platform of circulant biomarkers in plasma and urine, namely IL-2, sCD14, IP-10, CD5L, MMP-8, and MMP-9 being closely associated with the severity phenotype in the SH-TBL Cohort, which additionally provided hints on what's happening in situ, referring to persistent lung damage and a suspected immunosuppressive mechanism underlying the human TB granuloma.

8.2.2 Sub-Study B discussion

The human TB granuloma is the outcome of the *Mtb* infection. Driven by the host-pathogen interactions, this structure displays a dual function, as it is the structure protecting the host by enclosing the bacteria as well as the cause of chronic and slow aggression that jeopardize the host's survival (5). The host's fate after the *Mtb* infection is very much depending on its ability to induce a sufficient and balanced immune response capable to sterilize its presence from the lungs. Nonetheless, an impaired immune response can trigger the granuloma formation, evolving into a complex spatial-organized structure in the foci of infection. The worst forms of the granuloma include a necrotic caseating core, harboring thousands of bacilli, and inducing a potent stimulus capable to rupture the container walls and disseminate into the lung airways. The cavity formation is one of the granuloma forms that induce a worse TB course, especially if the bacilli are resistant to second-line chemotherapy (98). As concluded in Study I, these forms are likely the main reason

to drive a therapeutic surgery, as the TB granuloma extirpation permits the immune system restoration and to achieve a TB resolution status, as we are suggesting at the end of the Sub-Study A.

The main strength of the entire project is that, for the first time to our knowledge, non-FFPE fresh human TB lesions specimens were transcriptionally characterized using a high-throughput approach, revealing the NGS-derived gene signature of the human TB granulomatous lesion. Therefore, one of the main objectives of the project was accomplished. A non-despicable number of samples were included to this end, outnumbering the previous approaches in LCM-derived human granuloma specimens in microarray (166,167), although it is not the first time that fresh human TB lesions have been used in research, but not in this context (225).

All the 14 patients belonging to the Discovery Set had persistent cavitary lesions, which facilitates the understanding of the most complicated TB cases, regardless of the *Mtb* strain. All the biopsy specimens were obtained under the consideration of the surgeon, especially the three parts constituting the human TB granuloma to prove that the necrotic core of the cavitary lesions, the internal wall, and the external wall have a different expression profile. One of the main limitations of this sub-study is that we were restricted to use apparent uninvolved healthy lung parenchyma from the cohort participants as the only control for removed biopsies, considering that there is no ethical and practical point to subject healthy individuals to an invasive procedure. In that regard, we cannot discard that the infection and the TB lesions are exerting certain stimuli to the uninvolved parenchyma that could potentially bias the expression results, and even uninvolved parts at sight may be microscopically affected by the infection.

In Sub-Study B, we performed RNA-seq upon the granuloma samples (counts pool of Center of the lesion + Internal wall + External wall) from the Discovery Set, after they underwent therapeutic surgery to cope with the persistence of the TB cavities. This allowed us to generate a 6056-gene signature from the cavitary TB lesions. There is literature regarding genome-wide approaches that characterized the human TB granuloma. Kim *et al*. generated a hierarchical gene list in lipid metabolism through microarray based on the host's expression in LCM samples

from human caseous cavitary lesions. They noted an important role of ADFP, ACSL1, and PSAP genes being involved in lipid sequestration (166). Nevertheless, in our 6056-gene signature, we did not observe the same lipid metabolisms traits and we only found being SDE the PSAP gene in the TB granuloma. We related that such difference relies on the nature of the specifically chosen panel in the microarray, rather than discarding their involvement in the caseum generation. After comparing the whole expression of this data set with the 6056-gene human TB granuloma lesion signature, we found a consistency of 1166 genes in the direction of expression among the SDE overlapped genes. This indicated that both signatures share a non-despicable 69.36% of gene homology, even considering that both studies are limited by a divergent amount of tissue and conservation methods, techniques of RNA extraction, and the used wide-genome platforms. Therefore, this proved that our approach was in line with prior studies using TB lesions tissue and confirming the reliability of our technique. Using the same data set as Kim and colleagues, Subbian *et al.* demonstrated an important role in the inflammatory response in the human TB granuloma (167). The inflammatory response module was among the most important gene sets in our results, preceded by the interferon-gamma response and followed by the TNF-α signaling via the nuclear factor kappa B (NF-κß) signaling and the epithelial-mesenchymal transition gene set. The differential regulation of the inflammatory response and the genes involved in tissue damage and remodeling observed in the Subbian *et al.* study is very much in line with our findings regarding the most significantly enriched gene sets, confirming that not only the expressed genes but the pathophysiological interpretation is in agreement with our collection of TB lesions.

When we individually analyzed the compartments of the granuloma, the GSEA analysis showed also the up-regulation of many genes in response to IFN-γ in the center of the lesion and the internal cavitary wall, but in the external layer, therefore suggesting that the core of the lesion and the adjacent wall share similar biological processes. These findings are supported by the proteomic analysis of the human caseous granuloma suggested by Marakalala *et al.,* in which is proposed that the caseum of the TB lesions is enriched with TNF and IFN signaling, whereas the peripheral cell layer is free of them, thus indicating biological differences between

separated structural compartments (226). Histopathological examinations of human TB lesion in unresolved TB cases also observed a meaningful production of IFN-γ in cavitary lesions' walls (187). More studies are required upon our collection of samples individually evaluating each compartment to establish which cell populations are present in the granuloma parts and how that may influence the architecture and the inner physiology.

On the other hand, we also detected a high activity of the NF-κβ signaling in response to TNF-α. TNF-α is key to control the infection within the TB lesions, as it is critical for the granuloma formation and later maintenance. In this context, the TNF family appears as an important regulator to the NF-κβ family. The NF-κβ are a group of inducible transcription factors that play a pivotal role in the immune system, as they act upregulating the transcription of many cytokines and hence the chemoattraction of neutrophils into the infection foci (227,228). As suggested in a system biology granuloma model, an impaired NF-κβ activity can induce an unhindered bacilli growth in the TB granuloma lesions, but an excessive NF-κβ stimulus may trigger an uncontrolled local macrophage activation and enhance a local excessive pathological inflammation, in benefit of a reduction on the bacillary burden (229). These observations are suggesting that the over-regulation of this gene set may be explained by the local inflammation and hence participate with the persistence of the TB lesions in our cohort, accompanied by the decrease of the bacillary load according to sputum cultures.

The tissue remodeling processes (via the epithelial-mesenchymal transition) are also appearing as a cornerstone of our proposed TB granuloma signature, including MMP3 and MMP1 as the most important genes in this module. Subbian *et al.* also highlighted the role of the MMPs in the human TB granuloma as are strongly regulated to execute tissue damage and remodeling process. They highlighted the role of MMP1 and MMP9 genes, which paired with our findings, in which MMP1 expression was among the most important genes of this study, together with MMP9 (167). MMP-3 appears to be one of the activators of MMP-1 (230). Some studies suggest that MMP1 is upregulated by the presence of *Mtb* and exerts a key role in the lung matrix destruction, which seems to be independent of the presence of caseous necrosis (231,232). MMP-9 is one of the main inductors of the granuloma

formation, and as it has seen in mice, acts chronically recruiting monocytes and macrophages at the site of infection, hence being the necessary consequence for the TB granuloma maturation (233). Not only in tissue, but in blood MMP9 appears as an important factor in TB. Berry and colleagues' 86-transcript signature capable to distinguish TB from other inflammatory and infectious diseases also included MMP9 as a marker (158).

Dheda *et al*. conducted a similar approach as our study aiming to describe the pathways involved in different parts of cavitary lesions from failed MDR-TB subjects that underwent to surgery (234). The IPA determined that proinflammatory pathways were especially upregulated in the cavity wall, gathering the highest expression of all significant pathways from the rest of compartments, including nitric oxide production, reactive oxygen species, IL-1, IL-6, IFN-γ and NF-κß transcription activation. Alike our findings, in this study it is showed the important involvement of proinflammatory pathways, including some of our most enriched gene sets. Even though, the cavity wall was capturing more proinflammatory signals, which contrasts with our results in the center of the lesion as is this lesion part that harvested the greatest signals of proinflammatory expression in comparison of the adjacent compartments as well as more cases of positive *Mtb* cultures, which in Dheda study was the air-caseum interface. These differences in terms of spatial expression may be given either the heterogeneity of the surgical biopsies conducted by Dheda and colleagues, because of differences between the enrichment analysis and IPA, or more likely, because the different disease stages of the two cohorts, as this study included non-microbiologically resolved MDR-TB patients, and the SH-TBL Cohort also involved microbiologically negative DS-TB and XDR-TB individuals.

The first significant enriched gene set was for allograft rejection, also found in the TB lesions compartments when were individually assessed. The most plausible hypothesis about the presence of this gene set is that may represent a composite of genes involved in the chronic inflammatory state at what the TB lesions are submitted, as the product of an impaired immune response. Even after a reduction in the bacillary burden, the "protective responses" are still exerting damage to the lung and therefore this is traduced to the persistence of the TB lesions. Nonetheless,

further analyses are needed to elucidate which genes are involved in this phenotype and what connotations may have within the physiopathology of the TB granuloma lesion.

In conclusion, our results are suggesting that the human TB granuloma, even in apparent microbiological resolved cases, the in situ inflammation mainly lead by the local action of IFN-γ and the TNF-α via NF-κß activity coexist with the tissue remodeling and destruction dynamics. Thus, this activity could be interpreted as the axis of the granuloma maintenance, although this observation may be reserved for the worst TB cases. Albeit our approach is currently limited to the GSEA results perspective and deeper analysis are required, we suggest that single-cell sequencing upon the diverse cellular fractions could help to accurately identify the main participants in the granuloma preservation. In this regard, to identify involved NF-κß transcription factors could become hypothetical therapeutic targets to repress the local over-induced inflammatory response, whereas histochemistry assays would help to identify the main MMPs involved in the granuloma maintenance and become objectives to block the lung tissue degradation.

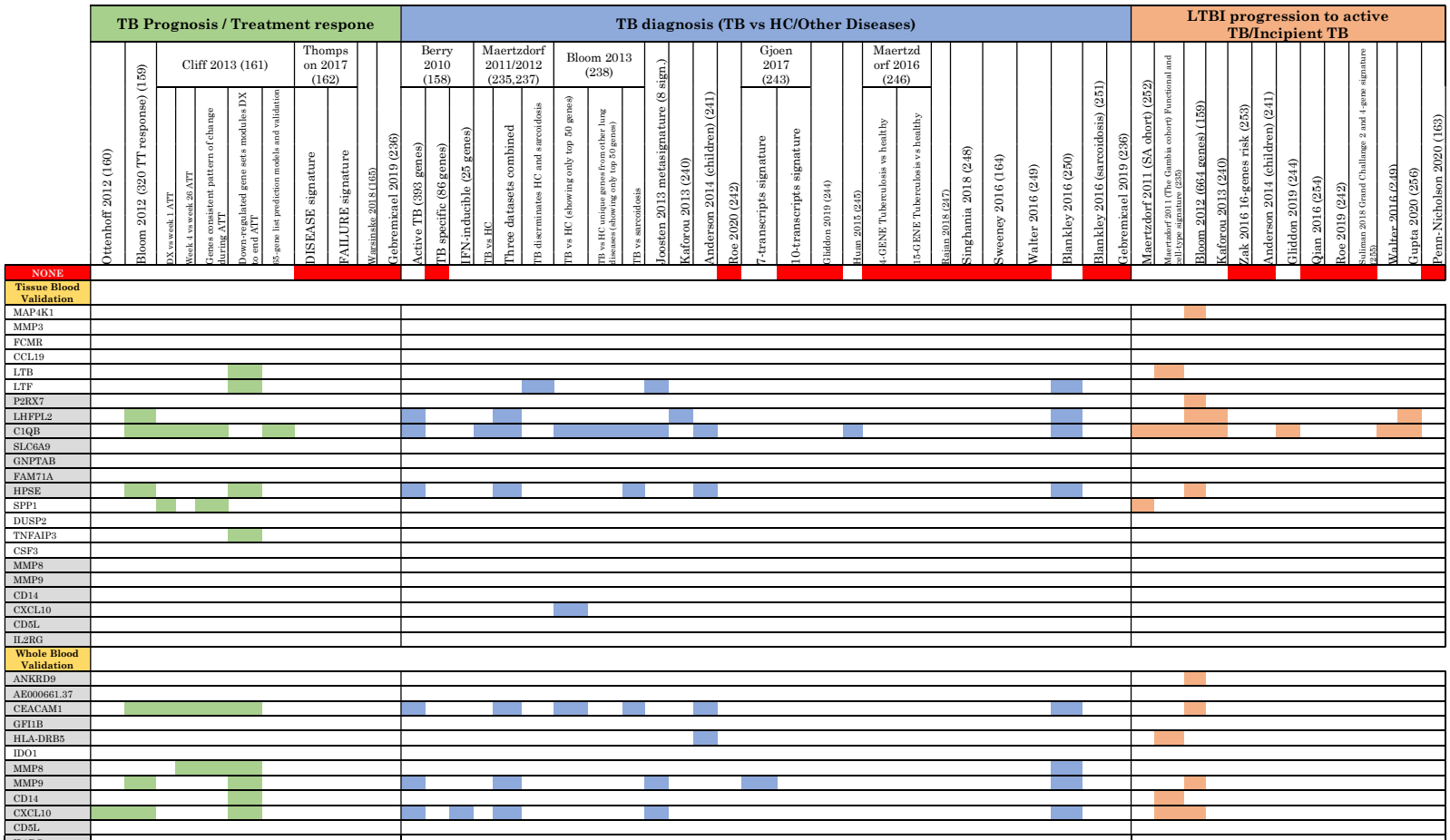
Currently, none of the public signatures created to predict the LTBI progression to active TB, diagnostic of TB, to evaluate the ATT response or predict an eventual worsening are complemented with the in situ functional expression of the human TB granuloma, as they are only reflecting the systemic behavior of the disease. In our opinion, enriching and validating these and other future signatures with the transcriptomic profile of our 6056-gene human TB signature would add additional value and be more inclusive with both the systemic and local environment of PTB. This strategy could aid the biomarkers research by providing better insights into the role of the candidate genes and help to shape the diagnostic and/or predictive performance capabilities. In that regard, one of the main objectives of this project was to find if the expression of our 6056-gene human TB granuloma signature is somewhat projected in other existing whole blood-based signatures. To this end, we concentrated the main genes representing the most important findings in this sub-study into a single gene platform, namely the genes of interest. The genes of interest were assembled including the most significant pathophysiological traits of the Discovery Set participants following both a functional and statistical rationale.

According to our results, these traits are gathering an important immunological and structural component of the human TB granuloma, and the most statistically meaningful encountered genes between the fast and the slow responders in whole blood and tissue. A secondary part of this study will involve the gene expression validation of the genes of interest upon the Validation Set participants. The validation will be performed through a multiplex analysis using the Nanostring technology, by an nCounter platform and the customized panels composed by the genes of interest. The results are not presented, as at the moment of the delivery of this thesis were not available. We expect that we will be able to reproduce the genes of interest expression from the Discovery Set in each of the samples belonging to the Validation Set.

We performed a comprehensive search on PubMed on the last 10 years of publicly available gene-based whole blood signatures built from microarray and RNA-seq platforms to evaluate the presence of our genes of interest platform, namely signatures intended for the TB diagnosis, LTBI progression, and treatment response. The results of the overlapped genes and reviewed articles' references are gathered in **Table 24**. We found the treatment response signatures having the greatest number of coincident genes with our genes of interest platform. The Complement Component 1, Q Subcomponent, Beta Polypeptide (C1QB), the Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), and CXCL10 were the genes having more intersections amongst all the reviewed public signatures.

The revision amongst LTBI progression publications, only found a couple of consistent gene-based signatures overlapping with our genes of interest, considering that the other comparisons only shared up to two genes. Maertzdorf and colleagues worked upon The Gambia cohort, and found significantly different enriched gene sets in this cohort, involving the complement system, the TLR signaling, and the cytokine-receptor interactions. Then, they contrasted these genes with the product of the differential expression between TB and LTBI participants of The Gambia cohort (235). We found a total of five genes overlapping with the highlighted genes (LTB, C1QB, HLA-DRB5, CD14, and CXCL10).

Table 24. Reviewed publicly available signatures published during the last 10 years and overlapping results with the genes of interest.



Bloom *et al.* presented a 664-gene gene list product of the differential expression between untreated active TB patients at diagnosis and asymptomatic LTBI in the South Africa 2011 cohort, emphasizing the over-representation of the IFN-signaling genes (159). Although the authors are not including the direction of the gene expression, this signature was gathering the greatest number of overlapped genes (up to nine genes, namely MAP4K1, P2RX7, C1QB, HPSE, ANKRD9, CEACAM1, MMP9, and CXCL10) among the rest of reviewed signatures. It is no surprise that we solely found two signatures based on the LTBI progression containing a consistent gene- overlap. This is, in our view, a reference that highlights the differences between the initial stages of the disease with the advanced and the aggravated state of the SHTBL Cohort, although some of these genes could indeed point to a potential initial progression of the TB lesions. In that regard, a posterior study on The Gambia cohort, the results showed the capacity of the C1Q family (and very importantly C1QB) expression in serum and RNA can distinguish the TB from the LTBI (257), suggesting its potential involvement with the lesion progression and therefore exhibiting its capacity as an early active TB progression biomarker.

Blankley *et al.* designed a 380-gene meta-signature intended to distinguish active TB individuals from any other control (250). This meta-signature was built after a modular analysis from 16 publicly available datasets. Similarly to our strategy, this design incorporated modules associated with inflammation, the interferon response, B and T-cells activity, and the lymphocyte activation. The gene intersection between our genes of interest platform and the 380-gene meta-signature showed that eight genes were overlapped (LTF, LHFPL2, C1QB, HPSE, CEACAM1, MMP8, MMP9, and CXCL10). The results showed a strong network of IFN-γ-regulated genes, which is very much in line with our findings. Yet in our study C1QB is derived from the granuloma spatial analysis and we do not know its involvement with the local physiology, here it is highlighted the role of C1QB as a consistent biomarker of TB. Cai *et al.* demonstrated the important role of the C1q family and the messenger RNA levels to diagnose PTB patients from LTBI and healthy controls in PBMC. The levels of C1qA, C1qB, C1qC were significantly increased at the onset of TB episode and declined during the ATT. In addition, they also found significantly increased levels of C1qA/B/C messenger RNA in patients having a positive AFB in PTB from

negative AFB at diagnosis, adding an extra value as a severity biomarker (258). Although we do not found any gene of the C1q family members SDE in our Fast vs Slow time to SCC comparisons in granuloma nor blood, it is needed to assess the levels of these biomarkers systemically at the SHTBL Cohort's severity disease stage, as C1q may provide useful correlations regarding the severity status of the TB patients and an invaluable link pointing towards the persistence of the TB lesions.

Finally, we found many more of the genes of interest overlapping with TB prognosis and treatment response signatures in comparison with the previously exposed studies. Accordingly, this underlines at what time point of the disease is based on our study, as our observations are made based on patients that terminated or were at late stages of chemotherapy and had a very advanced but unresolved disease status. Also found in previous Berry's work, they additionally showed a 320-transcript treatment-specific signature result from the SDE genes between active TB patients' samples at pre-treatment and after 6-month under standard chemotherapy (159). In this treatment-specific signature is noted a rapid and significant change after two weeks under ATT and onwards, suggesting that at the two months timepoint a plateau is reached in the signature's expression. The pathways analysis indicated an over-representation of the innate immune system pathways together with genes related to the TLR receptors and the complement system, hence finding similarities with our main enriched gene sets results. Up to six genes resulted in overlapping with our genes of interest platform (LHFPL2, C1QB, HPSE, CEACAM1, MMP9, and CXCL10).

A modular analysis assessing the gene expression changes from diagnosis to treatment completion by Cliff and colleagues indicated a consistent down-regulation of the modules related to B, T, and cytotoxic cells and ribosomal proteins throughout ATT (161). Despite our findings regarding the biological implications that do not reflect explicit lymphocyte and cytotoxic activity, a meaningful quantity of genes belonging to these modules intersected with our genes of interest (LTB, LTF, HPSE, TNFAIP3, CEACAM1, MMP8, MMP9, CD14, and CXCL10). CEACAM1 was one of the most recurrent genes found among the TB treatment response signatures, but there is no literature regarding CEACAM1 involvement in TB pathogenesis other

than belonging to public TB signatures. CEACAM1 can be expressed in myeloid cells, B-cells, and neutrophils, amongst other cell-fractions. It is found upregulated under pathological conditions, such as in cancer and other infectious diseases triggering an immune suppression, causality of the T-cells inhibition, and it has been proposed as a therapeutic target in immune-mediated diseases (259,260). The CEACAM interaction with T-cells may induce cytokine production suppression and its proliferation, as suggested in a meningococcal sepsis study (261). Importantly, it is also found upregulated in MDSCs in patients having severe septic shock (262). In our study, CEACAM1 was found over-expressed in the slow responders' group blood, and according to its role, it is possible that CEACAM1, alone or with the MDSCs, may participate in the orchestration of the host immune suppression, especially in the subgroup of slow responders. As we do not have more evidence other than our results, we strongly suggest that more studies with this marker are required to consolidate the link with our results either in its circulant or messenger RNA form, because in our opinion, this is a putative candidate to become part of a panel of sputum culture surrogate biomarkers.

We also tested the ability of a bioinformatic tool to explore the concordance between different data sets, as we think that this approach may be used in future studies to validate blood-based signatures and our 6056-gene signature. According to the authors, the DISCO package can compare different data sets from different species and biological sources, hence being suitable to be used for our purposes. This method was first used to identify the concordance between the murine and human transcriptional responses to the *Mtb* infection in whole-blood (176). In this project, we checked the signatures concordance of our 6056-gene signature and the CTRC, including the SDE genes derived from cured patients' samples at TB diagnosis and after 24 weeks under standard treatment, with proved microbiological clearance (162). We found 57% of consistency in the expressed genes between the 6056-gene human TB granuloma and the samples from the untreated patients at diagnosis, suggesting that this set of genes may be indicative of disease and used as diagnostic biomarkers. On the contrary, the discordant set of genes was suggestive of the persistence of the TB lesions, as were still up-regulated at week 24. Although the authors declared that this set of patients of the CTRC had sputum conversion, as

well as the SHTBL Cohort, we are missing crucial clinical data to contrast similarities suggestive of a non-resolution status from this cohort. Nonetheless, some up-regulated genes at week-24 and in the 6056-gene signature related to prognosis were found, such as SPP1, giving evidence to this idea. The SPP1 gene has already been proposed as a prognostic biomarker in patients with mycobacterial infection (263). In its soluble form, osteopontin (OPN) is recognized as a proinflammatory cytokine, promoting chemotaxis and the T-cells adhesion, as well as enhances the IFN-γ production (264,265). In other studies, OPN levels appear to significantly decrease during ATT among the fast converters group and have been related to the presence of cavities in the CXR (136,142,149). We were limited to use only the RNA-seq-derived data from the CTRC cohort, so our findings cannot be extrapolated to other settings or cohorts. Therefore, our approach requires of other signatures to validate these findings. Even though, we are providing evidence of the involvement of the SSP1 gene as a potential prognostic biomarker and a correlation with its disease involvement in its soluble form.

From an overall point of view, Sub-Study B presents several limitations. Taking only the worst cases into account or the ones that underwent surgery does introduce selection bias, so results should not be generalized to the whole TB patient's spectrum. Moreover, we only have a snapshot, and as people do not undergo thoracic surgery if they are healthy, we do not have any negative control to compare our results (this is without TB and cavitary lesions, and chemotherapy-free), and as previously stated, we cannot discard that healthy lung parenchyma may be somehow affected by the disease, yet no macroscopic abnormalities were noted. In addition, all samples belong to the same group, so contrast can not be applied. Furthermore, we did not study the isolated *Mtb* strains which could also influence the outcomes of the disease, as it has been suggested, it may induce poor outcomes given the virulence of the bacilli (266). In future studies, it will be very novel to use WGS and to assess the virulence of the *Mtb* isolates by patients and to be correlated with the differential expression found in the 6056-gene human TB granuloma signature. We failed in our attempt to find SDE between the type of necrosis in the TB lesions, as the Discovery Set was almost exclusively composed of patients having fresh necrosis. For that reason, the results could be biased for including only this

kind of necrosis related to worse outcomes, as Study I results are suggesting. Finally, and as stated in Sub-Study B, alcohol may have an important effect on the results, because as observed, statistical differences when encountered regarding alcohol use between men and women in the Discovery Set. For that reason, we cannot discard the alcohol effect could be added to our gene expression patterns.

In short, we found that some publicly available blood-based signatures had a certain grade of gene intersection with the genes of interest and the full 6056-gene signature. Thus, and although being from different biological sources, we found part of the genes of interest are also SDE in whole blood of different TB cohorts, confirming that it is possible to validate the local expression of the human TB lesions systemically and at different stages of the TB spectrum. We additionally assessed the concordance of the 6056-gene signature with an independent cohort, and our findings suggested that some genes may be indicative of the TB lesions' persistence, but additional studies are required. Our results are providing a signature that could be widely used in TB research and other granuloma diseases, and could aid in building more accurately and sensitively future biomarkers panels, reflecting not only the systemic activity but the possible persistence of the human TB granuloma. Some of the available public signatures lack a pathophysiological rationale to down-select their genes with diagnostic/prognostic capabilities, especially regarding short-sized gene signatures. In our opinion, this is an important gap in the biomarkers research as misses individual factors that are translating into differentially expressed genes within a cohort of patients, as our findings are suggesting. In that regard, our results provide different gene expression patterns in the human TB lesions, according to patients having distinct clinical-epidemiological features, like the toxic habits, the sex, and the ability to convert the sputum culture, which could be used in future stratified studies to validate particular host-responses and personalize TB patient management tools. Finally, although in this thesis we have not been able to show the results of the genes of interest nCounter validation upon the Validation Set, we positively validated most of the genes encoding for the main important circulant biomarkers from Sub-Study A. These genes are found SDE expressed at the human TB lesions, detected within the 6056-gene signature list. This approach may be useful not only

for transcriptomic-based studies but proteomics too, as could provide a wider biological picture of the candidate biomarkers role during the disease, especially regarding the human TB granuloma/systemic markers relationship.

8.3 General discussion

For the purpose to gather the most important results of this thesis, below are synthesized the main aspects previously discussed corresponding to Study I and Study II:

One of the most important findings in this project was that the persistence of *Mtb* was detected in surgical specimens, both in AFB and culture. 60.4% and 71.79% of the participants showed positive AFB in Study I and II, respectively, whereas 17.82% in Study I and the 5.12% in Study II had positive cultures. These findings exhibit that independent cohorts are still harboring bacilli, both in DS-TB and MDR/XDR-TB strains, despite demonstrating prior microbiological conversion. This event punts under interrogation the sterilizing capacity of chemotherapy and/or the drug-regimens efficiency, and supposes a public health problem as a non-despicable pool of patients are likely at risk to revert to infective and prone to relapse. We aimed to build a biomarkers panel capable to detect the *Mtb* persistence as a new viable tool capable to overcome the sputum culture limitations, considering urine sCD14 pre-surgical levels and being a female as the most important factors, followed by tobacco use and plasma MMP-9. The outcome was that the ability to predict the AFB positivity was modest. Nonetheless, we considered that these results may be limited by the technique, as small surgical biopsies were intended for the *Mtb* detection misrepresenting the TB lesion structure. So we can not discard that even more patients may be positive and hence we can not provide strong conclusions about this finding until further analysis in other studies of the SH-TBL project.

In Study I was shown that more men were requiring surgery than women in the 137 patient cohort and women had poorer clinical features, facts that were not reproduced in the Study II SH-TBL Cohort, probably induced by the cohort sizes differences. Nonetheless, we did find that in both studies women had a similar median age and were significantly receiving surgery at a younger age than men,

even being two different cohorts and different sample sizes, albeit both were derived from the same setting. Very importantly, both studies results reported a significant alcohol use together with smoking, which was found almost exclusively in both men groups. When we stratified the SH-TBL Cohort participants according to alcohol use, results from several circulant biomarkers (namely IFN-γ, IL-4, and urine IL-8) were statistically significant decreased, suggesting an immune suppression. The same biomarkers difference was also noted when men and women were compared. The alcohol use effect was presumably captured by the transcriptomic analysis of the human TB lesions when sex was compared, encountering alcohol metabolism-related genes at the top of the SDE, which had to be filtered out from the analysis. Hence, these findings pointed the fact that conclusions in Study II regarding the alcohol effect could be partially influenced by the patient's sex and likely by the persistence of the TB lesions too, so more analysis is required to elucidate the toxic agents effect in other TB cohorts and interrogate the sex-role at this stage of the disease.

From Study I was concluded that other clinical factors, added to the sex and the bacilli persistence, may influence the prognosis of the PTB patient and eventually become characteristics that may lead to receiving therapeutic surgery. Namely, the involved *Mtb* strain, the presence of fresh necrosis in TB lesions and its size, the time to convert sputum culture, and having a history of a previous TB episode. Through the SH-TBL Cohort, we analyzed the relationship of these traits with circulant biomarkers and we could obtain a list of the differentially expressed genes in some of these features. Plasma sCD14 and especially MMP-9 appeared correlating with the MDR/XDR-TB pool of patients. MMP-8 in plasma was one of the most important biomarkers, as was strongly correlating with lung cavitation, as well as with patients having classical TB symptoms and being relapsed. Importantly, relapsed patients were also correlating with the presence of infiltrates on the CXR and with HCV as comorbidity. The inability to convert sputum in less than two months correlated also with MMP-8 pre-surgical levels. Interesting findings were encountered when we analyzed total-RNA expression in TB lesions and peripheral blood. Hierarchical clustering distinguished both time to SCC groups, exhibiting a total of 1103 SDE in tissue and 241 in blood. The intersection

analysis between both gene lists showed IDO1 as a promising biomarker candidate that will be examined upon the Validation Set whole blood samples during the nCounter validation assay. Urine IP-10 was the most important biomarker positively correlating with lesions bigger than 36mm, together with IL-2 in plasma. Lesions located at the right lower lobe was a related factor, whereas those at the upper lobe negatively correlated with the median size of our collection of TB lesions' size.

We also evaluated the concentration levels between post and pre-surgical circulant biomarkers. All significant analytes levels, but CD5L, appeared decreased before receiving therapeutic surgery. These results were suggesting that PTB subjects' lesions, considering the stage and the current prognosis of the disease, could be attracting several sets of cells and proteins, and/or the persistence of the TB granuloma may be inducing a systemic immune suppression effect. When we put under the GSEA analysis the 6056-gene human TB lesion transcriptome, we observed a meaningful role of many inflammation-related gene sets (interferon-gamma response, inflammatory response, and TNF-α via NF-κß, etc). We proposed that the local pro-inflammatory hostile environment for the persistent bacilli may be linked with the systemic immune suppression. Some set of cells, such as de MDSCs, could directly or indirectly be responsible for the immune suppression, as they are expanded under similar conditions in other diseases. However, more evidence is needed to elucidate this mechanism and the role of the set of cells that the TB lesions may gather within its structure. Strikingly, the time to SCC GSEA analysis revealed that the slow responders pool of patients had significantly up-regulated the pro-inflammatory gene sets, suggesting that the inflammatory response may be not enough stimuli to complete sterilization. Nonetheless, we did not find any immune response imbalance in circulant markers after evaluating both time to SCC groups. Another important gene set derived from the TB granuloma signature was the epithelial-mesenchymal transition. Genes encoding for MMPs, namely MMP3 and MMP1, were ranking among the top SDE genes, raising the importance of the MMPs in both circulant and messenger RNA forms. Therefore, it is also highlighted the role of the tissue remodeling processes within the human TB granuloma physiopathology by the hand of the MMPs, that together with the

detected pro-inflammation phenotype could be responsible for the evolution and persistence of the TB lesions observed in the poor resolution status of the SH-TBL Cohort.

NGS allowed us to generate, for the first time to our knowledge, a 6056-gene list of the human TB lesions from fresh surgical biopsies involving both DS-TB and MDR/XDR-TB strains. This signature could be used in any context of the TB biomarkers research as current public signatures to predict the LTBI progression to TB, TB diagnosis, as well as ATT response and prognostic biomarkers, are not including the local host response to the disease. This signature could aid by providing better insights into the gene candidates and help to shape the diagnostic/prognostic capabilities. Importantly, the 6056-gene signature may also be used as a tool to validate if the proteins that are encoding can be found in its circulant form. This would provide a very appreciated knowledge about the role of the marker and its involvement with the TB lesions pathogenesis. In that regard, we found CXCL10, CD14, CD5L, MMP9, but MMP8, and IL-2 receptor IL2RG genes being SDE in the 6056-gene signature, confirming its important role not only in its protein form systemically but also within the TB lesions structure. Next nCounter validation on the Validation Set will allow us to confirm if these genes that are found in the TB lesions can also be found in whole blood.

After the differential expression analysis and the GSEA results, we aimed to gather the most important pathophysiological traits of the SH-TBL Cohort in a platform of genes in tissue and whole blood, namely the genes of interest. Following a functional and statistical rationale, we down-selected the main genes from the 6056-gene signature, the spatial analysis, and the genes derived from the time to SCC in TB lesions and blood. Additionally, we added the genes encoding for the Sub-Study A most important circulant biomarkers. We aim to validate the expression of this set of genes at the Validation Set's TB lesions and whole blood samples. We are expecting that the total expression of the genes of interest will follow the same trend as in the Discovery Set and will add more evidence on the pathophysiological features that the SH-TBL Cohort is exhibiting at this stage of the disease, which could be helpful in future TB prognostic and even diagnostic studies.

9 | CONCLUSIONS

Description: "La tuberculosi amenaça la vida i riquesa de Catalunya – tuberculosis threatens the life and wealth of Catalunya. - For the best outcome in the fight against the disease, come to the tuberculosis social assistance service, where you will find advice and aid" Year 1929.

Author: Ramon Casas i Carbó

1. Despite the whole cohort demonstrated a bacteriological conversion of sputum and were negative by smear microscopy and culture, the microbiological examination of the human tuberculosis lesions exhibited the presence of bacilli (60.4% in AFB and 17.82% in culture in Study I, and 71.79% in AFB and 5.12% in culture in Study II).
2. Host factors such as sex, toxic habits, the type of necrosis found in the tuberculosis lesions, and the size of the lesion are important features that may influence the evolution and the outcome of the disease.
3. Pre-surgery plasma IL-2 and urine IP-10 levels were positively correlated with the size of the lesion; plasma MMP-9 and sCD14 levels correlated with the MDR/XDR-TB forms; and MMP-8 was positively correlated with most of the factors considered to indicate worst outcomes.
4. The increase of the postsurgical circulating immune markers' levels may suggest either the TB granuloma acting as an attraction pole for distinct sets of cells and proteins, an immunosuppressive effect of the tuberculosis lesion, or both.
5. We generated a 6056-gene signature of the human TB signature and the list of genes of interest platform including the main pathophysiological characteristics of the Discovery Set. The transcriptomic signatures generated from this project do add more insights about future putative biomarkers role in the TB lesions physiology and could be used as a validating tool of circulant and transcriptional biosignatures.
6. The human tuberculosis lesions transcriptional profile in patients having a worsening status denotes a meaningful pro-inflammatory pattern induced by the local action of IFN-γ, the TNF-α via NF-κβ, and tissue remodeling and destruction dynamics.
7. Genes encoding the most important circulant biomarkers correlating to pathophysiological traits from Sub-Study A were found significantly differentially expressed in the 6056-gene human tuberculosis signature from Sub-Study B.

Description: "Tuberculosis patients lying on their beds in a sanatorium terrace; the advert is for a tombola in aid of tuberculosis sufferers in L'Hérault, France." Year 1933

Author: Bellevue Sanatorium (Hérault, France) (Wellcome Library reference and Iconographic Collection 659414i, Photo number: L0046138)

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11 | ANNEX I

International Journal of Infectious Diseases 56 (2017) 200–207

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

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

Review

Retrospective study of clinical and lesion characteristics of patients undergoing surgical treatment for Pulmonary Tuberculosis in Georgia

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ARTICLE INFO

Article history:
Received 7 October 2016
Received in revised form 5 December 2016
Accepted 6 December 2016

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Tuberculosis
Surgery
Gender
DS-TB
MDR/XDR-TB
Necrosis

ABSTRACT

Objectives: Our aim was to retrospectively compare clinical data and characteristics of removed lesions of the cohort of patients undergoing therapeutical surgery for their tuberculosis.
Design and methods: Demographic and epidemiological details, clinical data, data on the surgery performed, macroscopic characteristics of the TB lesions removed, and outcome were recorded retrospectively from the 137 patients who underwent therapeutical surgery for their TB in Tbilisi, Georgia during 2014 and 2015.
Results: Men represented 70% of the included patients, presented more comorbidities and underwent operation earlier in terms of days between diagnostic and surgery. Women underwent operation at younger ages, and in MDR/XDR-TB cases, showed higher percentages of sputum conversion at >2 months and of fresh necrosis in the surgical specimens, suggesting a worse evolution. Half of cases were MDR/XDR-TB cases. In spite of being considered microbiologically cured according to WHO, a non despricable percentage of cases showed viable bacilli in the surgical specimen. Even if no causality could be statistically demonstrated, differences could be encountered according to gender and drug susceptibility of the responsible strains.
Conclusions: According to our results, host factors such as gender, type of necrosis found in the lesions, size of lesions and presence of viable bacilli in the surgical specimen, should be included in future studies on therapeutical surgery of TB. As most of studies are done in MDR/XDR-TB, more data on DS-TB operated cases are needed. Our results also highlight that, in spite of achieving the microbiologically cured status, sterilization might not occur, and thus new biomarkers and new methods to evaluate the healing process of TB patients are urgently needed and radiological assays should be taken into account.

1. Introduction

According to the World Health Organization (WHO), Georgia has a high incidence of tuberculosis (TB) cases (116/100.000 habitants), with a high Multi-Drug Resistant TB (MDR-TB) burden (51% of all declared cases in 2015).[1] In countries with high incidences, therapeutical surgery is still a good option to cope with TB complications and sequelae, as well as to reduce the bacilli burden[2]; and might be essential as an adjuvant to the appropriate chemotherapy in the MDR-TB cases.[3] In this manuscript, we present a study that aimed to retrospectively compare clinical data and characteristics of removed TB lesions of a cohort of patients undergoing therapeutical surgery for their TB, in the National Center for Tuberculosis and Lung Diseases (NCTLD, Tblisi, Georgia).

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http://dx.doi.org/10.1016/j.ijid.2016.12.009

2. Materials and methods

2.1. Design

The project was reviewed and approved by the Ethics Committee IRB00007705 NCTLD Georgia #1, IORG0006411 (Tbilisi, Georgia) and the Ethics Committee of Germans Trias i Pujol Hospital (Badalona, Catalonia, Spain) to ensure compliance with all current national and European laws on clinical studies. The study was registered at the clinicaltrials.gov database with the identifier NCT02715271. The results presented here were extracted from a retrospective cohort including all patients operated per clinical routine at the NCTLD.

2.2. Data

Data were recorded retrospectively and anonymously from the 2014 and 2015 surgical notebooks in a Spreadsheet created ad-hoc, including different categories on both the patients and the lesions' characteristics. WHO definitions were used whenever possible.[4] Table 1 includes all data categories recorded, definitions and possible answers. Graph Pad Prism 6 software (La Jolla,CA, USA) was used to draw the figures. Statistical analysis was done using the independent 2 samples t-test to compare continuous variables. The associations with other categorical variables were tested with the Chi-square test or Fisher exact test. All tests were two-tailed, and p-values less than 0.05 were considered statistically significant.

3. Results

3.1. Demographic and epidemiological features

A total of 137 patients underwent therapeutical surgery for their TB at the NCTLD during the years 2014 and 2015, 96 males (69.8%) and 41 females (30.8%). Men were older (median age = 43.5 years old) than women (28 years old). 25% of men had comorbidities: Hepatitis C Virus Infection (n = 15), Diabetes

Table 1
Data recorded

Group	Category	Variables
Demographic and epidemiological data	Age (years old)	In years old
	Gender	Male/Female
	Smoking habit	on daily or almost daily basis, weekly, monthly, less than monthly, never
	Alcohol intake	on daily or almost daily basis, weekly, monthly, less than monthly, never
General clinical data	Presence of comorbidities	Diabetes mellitus, HIV infection, immunosupression other than HIV-infection, renal failure, chronic obstructive pulmonary disease (COPD) previous to TB, Hepatitis C Virus infection, hepatic cirrosis, others
Clinical data regarding the TB	history of previous treatment	new patient, relapse patient: treatment after failure, treatment after loss to follow-up patient, other previously treated patient, unknown previous TB treatment
	anatomical site of TB	Pulmonary, extrapulmonary
	drug-sensitivity	Drug Sensitive (DS-TB), Rifampicin Resistant only, Monoresistance to any other drug other than rifampicin, polydrug resistance, MultiDrug Resistant (MDR), extradrug resistance (XDR)
	date of diagnosis of the present TB episode	dd/mm/yy
	date of treatment initiation	dd/mm/yy
	number of lesions found in the chest X-ray assay	n
	bacteriology cured or not at the time of surgery according to WHO's guidelines	Yes/No
	time in negativization of culture	Recorded in months, and analyzed as always negative, negative at $\leq$2 months (M) or negative at >2 M
	time from TB diagnosis and surgery	In days
Data regarding the surgery performed	main indication for surgery	Still lesions in X-ray assays, Clinically not cured, Microbiologically not cured, Others (spontaneous pneumothorax, pulmonary haemorrhage, pleural empiema)
	number of operated lesions	n
	type of surgery performed	segmentectomy (for lesion resection or removing lung segment), lobectomy (for lung resection or removing lung lobe), Pulmonectomy (for removal of the whole lung)
	date of surgery	dd/mm/yy
	post-surgery complications	Open answer
Data on macroscopical characteristics of the TB lesions removed (surgical specimens)	lesion size	in cm, considering the maximum lenght
	type of lesion operated	tuberculoma, cavitation
	presence of necrosis	no necrosis, necrosis: moderate growth of connective tissue, alternating with unmodified lung tissue, cirrosis: complete substitution of lung tissue by the connective tissue with damage of vessels and bronchies
	type of necrosis	fresh: necrosis macroscopically looking like of a liquid consistency, dry: necrosis macroscopically looking like of a dry consistency, both: coexistence of fresh and dry necrosis
	Presence of Acid Fast Bacilli (AFB)	Yes/No
	Positivity of culture of samples in solid medium (Lowenstein-Jensen) for *M.tuberculosis*	Yes/No
Outcome	Official final outcome according to WHO definitions	Treatment completed, Treatment success, Cured, Lost of follow-up, Treatment failed, Still not known, Not evaluated, Death (if death, date of death in dd/mm/yy recorded)

mellitus (n = 5), HCV and Diabetes (n = 3), and HCV plus HIV infection (n = 1). A total of 49 patients were smokers, 48 on a daily or mostly daily basis; representing a 48.9% and 2.4% of total male and female populations, respectively. The majority of women (92.6%) declared never drinking alcohol. The alcohol intake in men increased with age. While men declaring themselves abstemious (13.5%) had a median age of 26 years old, those declaring drinking on less than monthly (33.3%), monthy (52%) or weekly basis (8.3%) had a higher median age (45.6 years old). No statistically significant differences were encountered between DS-TB and MDR/XDR-TB groups.

3.2. TB data

The 92.7% of total cases recorded were pulmonary TB exclusively (127 patients), while in 7.3% (10 patients) pleura involvement (as empyema) was found (being more frequent in males, not statistically significant). Pleura involvement was present in the 80% of MDR/XDR-TB cases.

Approximately the half of the patients included were DS-TB (56.9%), and the other half, MDR or XDR (40.1%). Mono resistance was rare. Table 2 shows the number of cases for each category, as well as the number and percentage according to gender. The percentage of men and women were very similar regardless of the drug-sensitivity of the strains involved.

The chest X-ray assay (previous to surgery) showed the presence of a single lesion (only cavities and tuberculomas considered) for most of the patients (76%), while 19.2% showed 2 lesions, and 4.8% up to three.

Most patients undergoing surgery had been considered bacteriology cured according to WHO's definitions (96.3%). No statistically significant differences according to gender or drug susceptibility were found in the non microbiologically cured group of patients.

Figure 1 shows the results of time to culture's negativization, in totals and stratified by gender and by the drug-sensitivity of the strains. As women included in this cohort were younger (28 vs 43.5 years old), we stratified the results by dividing the male cases in $\leq$43 and >43 years old, to see if the gender itself could be an influence free from the age input. Differences were encountered between females and males of $\leq$43 years old, being statistically significant for DS-TB cases ($p \leq 0.0001$). A high percentage of patients negativized the culture in $\leq$2 months (51.8%), especially if they were women or younger (median age of 33). The culture was always negative in a non despricable 31.3% of the cases; and up to 40.6% of male cases when stratified by gender. Patients with culture negative at $\leq$2 months were younger than those in whom it had always been negative (median age of 33 vs 43 years old, respectively; $p < 0.0001$). When stratified by gender, this difference was only seen in males (median age: 37 vs 47 years old; $p = 0.022$); but the overall percentage of negative cultures in females was significantly lower. Statistically significant differences in terms of negativization of culture were encountered between DS-TB and MDR/XDR-TB, both in total ($p \leq 0.0001$) and when stratifying the results by age and gender (males $\leq$43, $p = 0.0009$; males >43, $p = 0.0007$; and females, $p = 0.0169$).

Nearly half of DS-TB cases negativized the culture at $\leq$2 M (47.4%), while in MDR/XDR-TB cases, the proportion of patients with negative results at >2 M (29.0%) were increased but due to a decrease of always negative results (a 44.8% in DS-TB vs a 12.7% in MDR/XDR-TB). Males and females were different, both in DS and MDR/XDR-TB cases, even if these differences were statistically significant only in DS-TB ($p \leq 0.0001$). Negative cultures were more frequent in MDR/XDR-TB cases, especially if women or males >43 years old.

Table 2
Distribution of tuberculosis patients by drug-resistance according to WHO classification.

Drug-Resistance (WHO)	TOTAL	Males		Females	
		N	%	N	%
DS-TB	78	54	69.23	24	30.76
monoR	2	2	100	0	0
Rifampicin only	2	2	100	0	0
MDR/XDR-TB	55	38	69.09	17	30.09
MDR-TB	42	33	78.57	9	21.42
XDR-TB	13	5	38.46	8	61.53

Abbreviations: WHO: World Health Organization; DS-TB: Drug-Sensitive Tuberculosis; monoR: mono Resistant; MDR/XDR-TB: Multi Drug-Resistant/Extensively Drug-Resistant Tuberculosis.

3.3. Surgery

The main indication for surgery was the presence of lesions (cavities and tuberculomas or empyemas) in the chest X-ray assays (73.7%), both in DS (65.3%) and MDR/XDR-TB (85.4%), in spite of a good treatment adherence. In case of MDR/XDR TB, patients had had a sputum conversion but high risk of failure or relapse according to WHO's recommendations.[5] The other indications were those cases not microbiologically cured (2.9%) and a miscellania that we called the "others" group (23.3%) and which included severe complications of the TB. This group was significantly larger for males (31.2%) than for females (4.8%), something expected as men undergoing surgery wexre older and had comorbidities. Surgery for complications of TB was more frequent in DS-TB cases (32.0% vs a 12.7% in MDR/XDR-TB; ($p = 0.03$)).

Men underwent surgery earlier compared to women, according to days between diagnosis and surgery date (208.5 and 263 days, respectively; $p = 0.0037$). In DS-TB cases, there was a remarkable increase in days between diagnosis and surgery date in womens' cases vs mens' (225.5 vs 81.5; $p = 0.0002$). Statistically significant differences were found between DS-TB and MDR/XDR-TB, both in total and when stratifying by gender. Table 3 shows all these results.

No statistically significant differences were encountered between DS and MDR/XDR-TB according to age, neither in total nor stratified by gender; even if MDR/XDR-TB operated patients tended to be younger than DS-TB: 34 (28-48) vs 43 years old (26.75-50.25).

Differences between DS and MDR/XDR-TB were found in terms of history of previous treatment ($p = 0.0053$). While relapse patients were more frequent among DS-TB cases (26.47% vs 3.77% for MDR/XDR-TB), more operated MDR/XDR-TB cases were new (64.15% vs 55.88% for DS-TB), had a history of previous treatment (26.41% vs 16.18% for DS-TB), or were under treatment after lost follow-up (5.66% vs 1.47% for DS-TB).

Segmentectomy was the most frequent surgical procedure (56.9%), regardless of the gender of the patients, followed by lobectomy (30.%), pleural surgery (9.49%) and pulmonectomy (2.9%, which was performed in more MDR/XDR-patients than DS-TB: n = 3 vs n = 1).

In the majority of cases, only one lesion was removed (69.3%; vs n = 2 (17.5%), n = 3 (3.6%) and pleura (9.4%)). In the resected lung tissue, in addition to the cavity or tuberculoma, there could be conglomerates foci, a single foci, areas of fibrosis, bulls, or bronchiectasis. No statistically significant differences were encountered on number of operated lesions when stratifying the results by gender, age, or drug sensitivity.

Only 6 patients suffered post-surgery complications, 5 of them being males of a median age of 41 years old. Haemorragia was the most frequent complication (n = 3). Other complications were

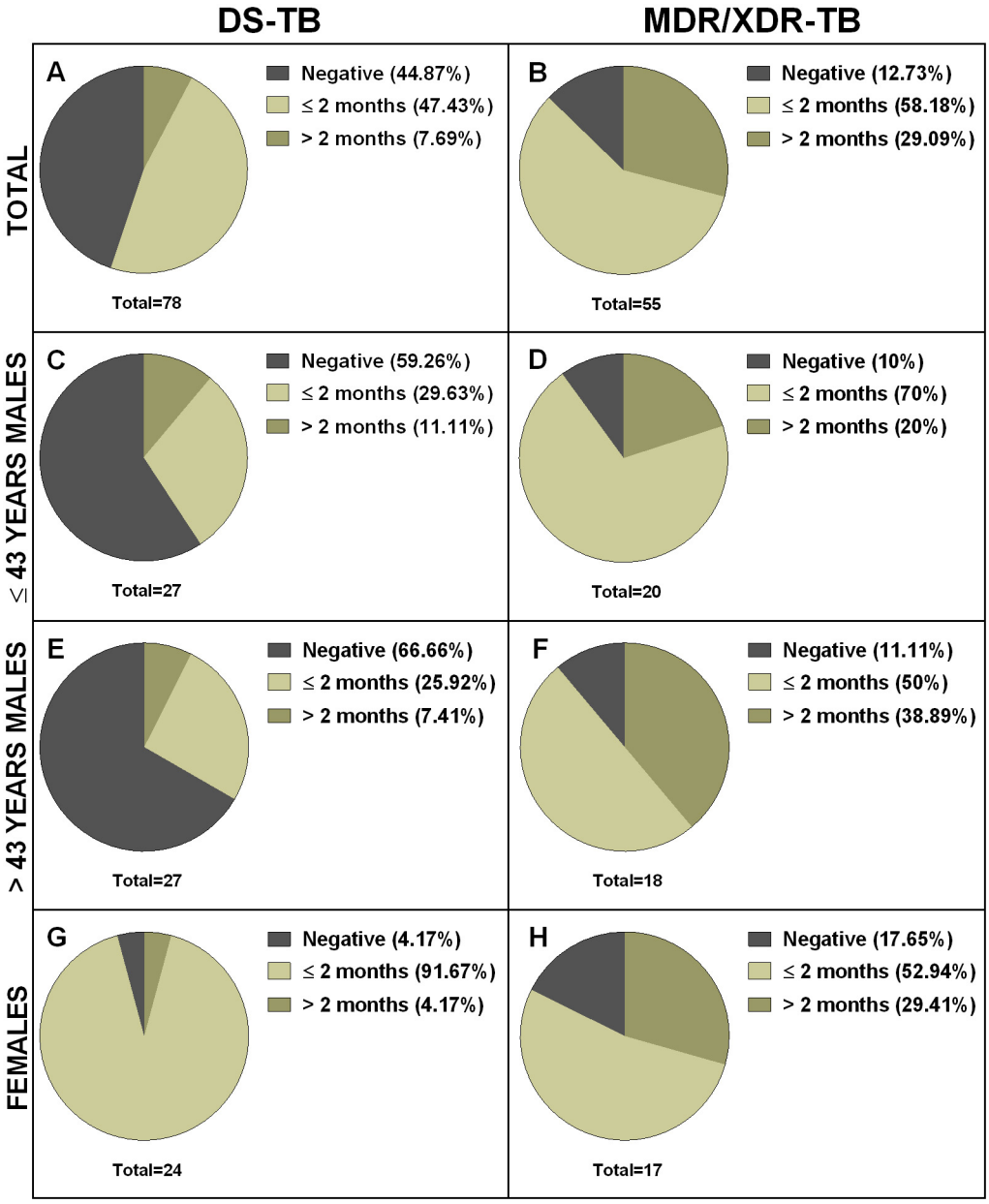

Figure 1. Time in sputum culture conversion.
Time to sputum culture conversion (always negative, less than 2 months (≤2 months) and more than 2 months (>2 months)) is represented in percentages, for DS-TB and MDR/XDR-TB cases. Total values and results stratified by gender (males (of more or less than 43 years old) and females) are graphically represented.

Table 3
Days between diagnosis and surgery date.

Drug-Resistance (WHO)	Median (25% - 75%)		
	TOTAL	Males	Females
All patients[a]	233 (100 - 252.5)	208.5 (50.5 - 344)[b]	263 (210 - 373.5)[b]
DS-TB	178.5 (38.25 - 258.75)[d]	81.5 (17.5 - 225.25)[c,e]	225.5 (192.5 - 275)[c,f]
MDR/XDR-TB	351 (233 - 455)[d]	308.5 (215.75 - 405.5)[e]	373 (266.5 - 654)[f]
MDR-TB	305.5 (232 - 399.75)	307 (225 - 400.5)	304 (237 - 511)
XDR-TB	532 (276 - 666)	454 (166 - 610)	533 (372.25 - 891.5)

Abbreviations: WHO: World Health Organization; DS-TB: Drug-Sensitive Tuberculosis; monoR: mono Resistant; MDR/XDR-TB: Multi Drug-Resistant/Extensively Drug-Resistant Tuberculosis.

[a] All patients include: mono resistant, rifampicin only resistant, DS-TB and MDR/XDR-TB patients.
[b] Statistically significant differences between males and females, $p = 0.0037$.
[c] Statistically significant differences between DS-TB Males and DS-TB females, $p = 0.0002$.
[d] Statistically significant differences between TOTAL DS-TB and TOTAL MDR/XDR-TB, $p < 0.0001$.
[e] Statistically significant differences between DS-TB males and MDR/XDR-TB males, $p < 0.0001$.
[f] Statistically significant differences between DS-TB females and MDR/XDR-TB females, $p = 0.0005$.

pneumonia plus acute respiratory failure (n = 1), broncho-pleural fístula (n = 1) and delayed unfolding of the operated lung (n = 1). Half of these patients (n = 3) were new patients and half (n = 3) were relapsed patients, having undergone treatment after failure. Half of the cases were DS-TB, and the other half, MDR (n = 2) or XDR-TB (n = 1, the female case).

3.4. TB lesions

Median size for all lesions was 2.4 cm (25%-75%: 2-2.8). No statistically significant differences in terms of lesions' size were encountered, neither according to gender, patients' age, type of necrosis (fresh, dry, both) or drug-sensitivity.

Despite the fact that out of the 137 patients 132 (96.3%) were WHO cured patients, in 123 (93.1%) there was presence of necrosis in the lesion. Necrosis was macroscopically considered fresh in 71.5% of cases, dry in 6.5%, and both fresh and dry in 21.9%. Figure 2 shows these results according to gender and sensitivity to TB drugs. Results showed statistically significant differences due to drug sensitivity in all cases but men of ≤43 years old (total, p = 0.0136; >43 males, p = 0.0418); females, p = 0.0412). Men of ≤43 years had lesions with a higher percentage of fresh necrosis when compared to women in DS-TB cases (81.8% vs 58.3%, p = 0.05) and less in MDR/XDR-cases (65% vs 86.6%, p = 0.023). Men of ≤43 years old had a higher percentage of fresh necrosis in lesions when compared to those of >43 in DS-TB cases (81.8% vs 72.7%, p = 0.014); and slightly less in MDR/XDR-TB (p = 0.0106) Figure 3.

Patients being considered bacteriology non cured according to WHO, presented a higher percentage of fresh necrosis (60%), even if the n of this group (n = 5) was too small to extract conclusions.

Fibrosis/cirrhosis was found in the 89% of lesions, with a majority of cases being fibrotic (98.3%).

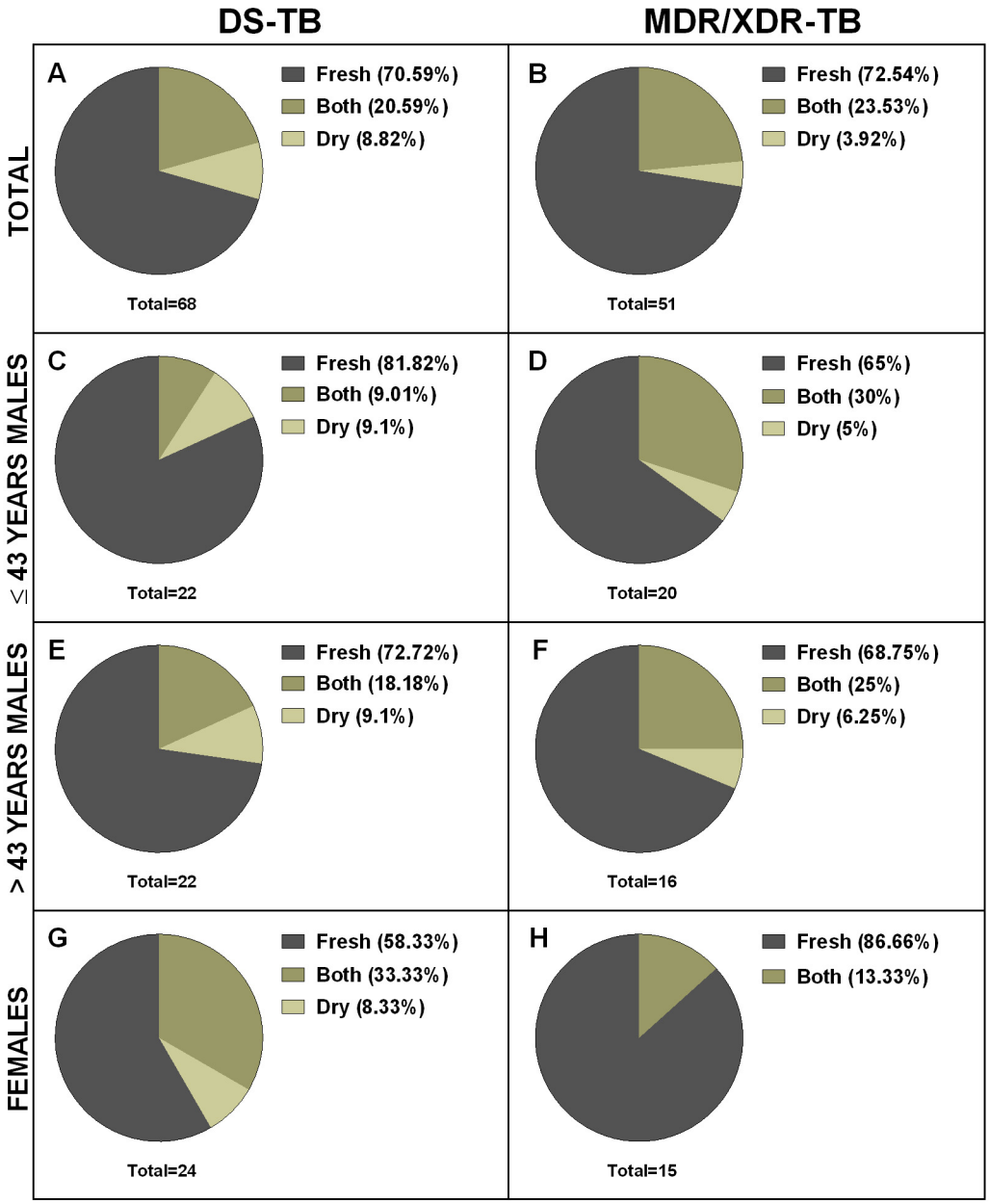

Figure 2. Necrosis type found in lesions.
Percentages of presence of the different type of necrosis (fresh, dry and both) are represented for DS-TB and MDR/XDR-TB cases. Total values and results stratified by gender (males (of more or less than 43 years old) and females) are graphically represented.

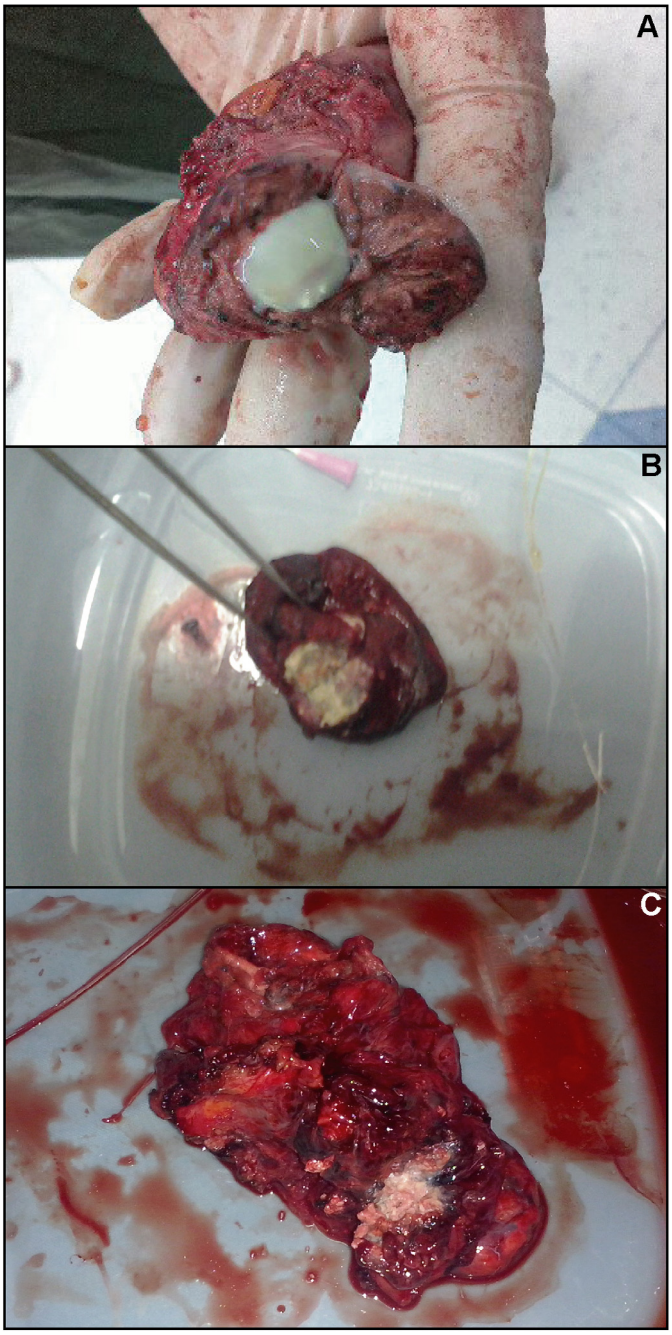

Figure 3. Types of necrosis in surgical specimens.
Macroscopic photographs of removed surgical specimens showing fresh necrosis (A), both fresh and dry necrosis (B) and dry necrosis (C).

Table 4 reports the positive results obtained for AFB stain and culture on samples from surgical specimens, among those patients operated for presence of lesions in X-Ray, with cavities or tuberculomas, and considered bacteriologically cured according to WHO's definitions. In 19 patients the microbiological study of surgical samples could not be done, either because sampling is not done during surgery due to surgical procedures (10 patients with empyema, 3 with spontaneous pneumothorax, 1 with thoracoplasty), or for technical reasons (n = 5). Even if any statistical analysis is difficult to be performed and interpreted because of the small numbers obtained, a tendency toward higher percentage of positive culture in fresh necrosis vs both fresh and dry necrosis and in those patients always having negative sputum cultures can be seen.

3.5. Outcomes

Statistically significant differences were encountered between outcomes of DS-TB when compared to those obtained for MDR/XDR-TB ($p < 0.0001$). While the percentage of cured patients was similar (30.8% for the DS-TB and 24.5% for MDR/XDR-TB) as well as those not evaluated (1.4% vs 1.8%); the categories lost to follow-up (7.3% vs 16.9%), treatment completed (47.0% vs 1.8%) and outcome still not known (8.8 vs 35.8) showed very different values. For the patients with outcome still not known, they are still on treatment. Moreover, 1.8% and 0% of failed treatment were recorded for MDR/XDR-TB and DS-TB, respectively. No statistically significant differences were encountered when stratifying the results by age or gender.

4. Discussion

Since the first recorded operation for TB, in 1726, therapeutical surgery was frequent and often essential for TB treatment, until the appearance of the chemotherapy in mid 20th century, and then was progressively abandoned. Nowadays, therapeutical surgery for TB is restricted to high TB burden countries, mostly to treat severe complications (as haemoptysis)[6] or sequelae (as important fibrosis),[7] especially if available chemotherapy options are limited (as in MDR/XDR cases),[2,5,8] and with a good overall cure rate.[2,9,10]

As in other published studies,[8,10–12] men was more frequent in our cohort. Men undergoing surgery were older than women, something understandable if we take into account that older men in our series had more comorbidities and toxic habits, more pleural involvement (secondary to the pulmonary disease), and more complications. The highest percentage of complications could also explain why men received surgery earlier compared to women (especially in the DS-TB cases), according to days between diagnosis and surgery date.

In our study, gender also seems to play a role in active TB and its course, as differences were still encountered when stratifying male cases by age and comparing female results with those for males under <43. Women underwent surgery at younger ages, and in MDR/XDR-TB cases, showed higher percentages of sputum conversion at >2 months and of fresh necrosis in the surgical specimens. This could mean that even if overall men suffer more TB, the TB course tends to be worse in women, especially if MDR/XDR. Previous literature has described higher rates of progression to disease for females during reproductive years.[13] Even if this were exclusively described for progression to disease and did not explore a worse evolution or progression within the disease state, we hypothesize that reproductive age might play a role, as women included in our study had a median age of 28 years old. The sex bias has been long debated in tuberculosis field, and even if sociocultural (access to healthcare) and behaviour (as smoking) factors have been demonstrated to have a role in it, there is increasing proof that biological factors (such as sex steroid hormones or variations at the TLR8 gene) might also have an important influence.[14,15] Our results show no statistical cause-effect relationship between gender and all the other features we recorded, but as differences are still encountered, we do think this might be due to the n included in our cohort (n = 137) is considerably high, but still low to verify the causality between factors. As cohorts of surgically treated TB patients are relatively small, we would like to encourage other scientists to use the same categories we used here, in order to be able to reproduce and even sum up the results obtained.

Our results come to be added to those of previous studies showing the need of therapeutical surgery for MDR/XDR-TB cases, which in our cohort represented nearly half of the patients included.[5,7,8,16] As expected, these cases negativized the sputum

Table 4
Percentage of AFB and culture positive in samples from surgical specimens.

	TOTAL	Lesions in X-Ray	Cavities and tuberculomas	WHO Bacteriologically Cured	Samples from surgical specimens	
					AFB +	Culture +
Characteristics according to Drug-resistance						
DS-TB	78	51 (65.38%)	51 (100%)	50 (98.04%)	28 (56%)	7 (14%)
MDR/XDR-TB	55	47 (85.45%)	47 (100%)	47 (100%)	31 (65.96%)	11 (23.40%)
Characteristics according to gender						
Males >43 years old	48	29 (60.42%)	29 (100%)	28 (96.55%)	19 (67.86%)	3 (10.71%)
Males ≤43 years old	48	34 (70.83%)	34 (100%)	34 (100%)	19 (55.88%)	6 (17.65%)
Females	41	38 (92.68%)	38 (100%)	38 (100%)	23 (60.53%)	9 (23.68%)
Characteristics according to type of necrosis						
Fresh necrosis	88	70 (79.55%)	70 (100%)	70 (100%)	41 (58.57%)	15 (21.43%)
Dry necrosis	8	6 (75%)	6 (100%)	6 (100%)	5 (83.33%)	0
Both	27	23 (85.18%)	23 (100%)	22 (95.65%)	15 (68.18%)	3 (13.64%)
Characteristics according to negativization time						
Always negative	43	15 (34.88%)	15 (100%)	14 (93.33%)	9 (64.28%)	5 (35.71%)
≤2 months	71	68 (95.77%)	68 (100%)	68 (100%)	42 (61.76%)	9 (13.23%)
>2 months	23	18 (78.26%)	18 (100%)	18 (100%)	10 (55.55%)	4 (22.22%)

Abbreviations: WHO: World Health Organization; AFB: Acid Fast Bacilli; DS-TB: Drug-Sensitive Tuberculosis; MDR/XDR-TB: Multi Drug-Resistant/Extensively Drug-Resistant Tuberculosis.

culture later than 2 months, and these results were statistically significant when compared to DS-TB cases. Patients with MDR/XDR-TB usually have either thick-walled cavitary lesions or with a destroyed lobe or lung that contains a substantial amount of bacilli, making it difficult for penetration of antibiotics.[17,18] So, pulmonary cavities harbor millions of resistant organisms and the infection in these locations cannot be controlled by the host-specific immunity and, consequently, the surgical removal of these cavities may permit cure.[19] Our data supports what led to Kurbatova et al. to propose culture conversion status at 6 months as a better correlator of treatment success.[20] In 22.2% of these cases, surgical specimens were cultured and a positive result was obtained; a lower percentage than in a previous study conducted in the same center,[21] but still high (22.2%).

More than half of the operated cases were DS-TB cases, in spite of that therapeutical surgery has been lately considered or recommended only for MDR/XDR-TB[5]; the main indication for surgery (65.38%) being the persistence of lesions in X-ray in spite of good adherence to treatment. There are many reasons confirming the legitimacy of surgical treatment of patients with pulmonary DS-TB, including a high risk of relapse, the presence of a localized lesion, the absence of any radiological and/or bacteriological improvements during the initial three to four months of chemotherapy, the irreversible morphological changes of the lungs and other respiratory organs due to the development of the fibrotic tissues during the progress of TB over the long term, or tuberculoma's size of more than three centimetres.[5] In countries of the former Soviet Union with a high prevalence of TB, there is a characteristically late referral of patients with tuberculosis (all forms) for medical attention. This results in a large number of patients with very advanced, extensive cavernous forms of a DS-TB, with strongly expressed irreversible morphological changes in the lungs, which poorly answer to medical anti-tuberculosis treatment, and therefore relatively more prone to surgical treatment than in the developed countries of Western Europe and America. Moreover, National TB guidelines of many countries of the former USSR include the persistence of cavitary lesions in X-Ray as one of the main indications for surgical treatment of DS-TB.[22,23] Our results on the presence of necrosis (87.1%) and microbiologically persistence of alive bacilli (14%) in surgical specimens of DS-TB, as well as the proportion of relapse patients (26.47% for DS-TB vs 3.77% for MDR-TB) point to the feasibility and need surgery for indicated cases among patients with DS-TB.

It is difficult to compare these results to other studies, as most literature refers only to therapeutical efficacy of surgery in MDR/XDR-TB cases, and published data on DS-TB are scarce.[2,9,11] Our results could be partly explained by the high percentage of cases in which fresh necrosis was found in DS-TB, indicating a poor resolving evolution. To our knowledge, this is the first attempt to classify operated lesions during the last years. The classification between proliferative and exudative lesions (with dry and fresh necrosis, respectively), was done years ago describing the coexistence of several type of lesions in time, some tending to fibrosis and calcification (thus with more positive outcomes) and others with a higher exudative component, tending to liquefaction and usually related to a higher bacillary load.[24] In our study, we divided the removed lesions into the presence of dry (considered as positive) or fresh necrosis (considered as negative), or the presence of both (as an intermediate phase). Even if most operated lesions showed fresh necrosis and this made it difficult to establish any statistically significant analysis, results showed differences according to gender, with a remarkable high proportion of presence of fresh necrosis in MDR/XDR-TB female cases, supporting the idea that women's lesions might evolve worse under certain conditions. Despite the fact that almost all patients with MDR-TB and XDR-TB have been treated for a long time (≥1 year) with second-line antitubercular drugs, MDR/XDR patients still have cavities; and in the absolute majority of them (94.44%), necrosis was detected in operating specimens (most of them of the fresh type). AFB stain and culture were found positive (in a 65.96% and 23.4%, respectively) in surgical specimens of those MDR/XDR-TB patients considered microbiologically cured, had cavities or tuberculomas and still had lesions in their X-ray assays. These results are similar to those previously published in the literature,[8] but still frightening.

Presence of bacilli was found in surgical specimens in an important percentage of total cases, which was higher in MDR/XDR-TB cases, older patients and female patients, and in the presence of fresh necrosis. Moreover, from the 98% of DS-TB cases presenting cavities or tuberculomas and considered bacteriology cured according to WHO definitions, a non despricable 56% presented AFB positive at samples from the surgical specimens. Even if a positive AFB stain in a biological sample does not imply the presence of viable bacilli, up to 14% of those cases considered bacteriologically cured were culture positive, including 2 cases in which the AFB stain was negative. For us, this is the most important finding in our study, and poses doubts regarding the clearance effect of anti-TB drugs. During prolonged chemotherapy and decrease of bacterial load, a part of the microbial population remains in the host's organism in the state of persistence, and as

proposed by some authors, transformated to L-forms or small-grained forms, characterized with low virulence but proven capacity for reversion and previous virulence.[25–27] The only validated biomarker now available to evaluate the curation of a patient, is to achieve a negative culture along a positive clinical evolution in a patient with a bacteriologically confirmed TB at the beginning of the treatment.[4] And yet, our results in DS-TB which followed treatment correctly, proved that no sterilization was achieved. In our opinion, our results support the idea that the correlators used are not reflecting what is happening in situ, and therefore new biomarkers are urgently needed. During the last years, different genetic profiles and other biomarkers of TB disease have been described in peripheral blood, suggesting that they could be used to establish the key of future therapeutic approaches.[28–32] As results might be different if measured in situ, surgical specimens might be a valuable tool to validate them.

In conclusion, we would like to encourage other scientists to use our categories in their studies conducted with therapeutical surgery, and to publish their results on DS-TB operated cases. Moreover, our results highlight that, in spite of achieving the microbiologically cured status, sterilization might not occur. This suggests that new studies to search biomarkers able to correlate with the in situ disease are urgently needed, and that evolution of radiological assays should be taken into account, as they are essential. Altogether, this information will help us better understand the reality of TB and will foster the development of new appropriate and tailored therapeutic options.

Funding: This work was supported by ISCIII-Subdirección General de Evaluación and Fondo-EU de Desarrollo Regional (FEDER) through Project CP13/00174 and CIBER Enfermedades Respiratorias (CB06/06/0031); and the SEPAR trough Project 2016/023.

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12 | ANNEX II

frontiers

Evaluation of the immune response in tuberculosis patients from Georgia before and after receiving therapeutic surgery

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Submitted to Journal:
Frontiers in Immunology

Specialty Section:
Microbial Immunology

Article type:
Original Research Article

Manuscript ID:
592685

Received on:
07 Aug 2020

Frontiers website link:
www.frontiersin.org

frontiers

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

CV and SV conceived the study and CV designed it. AD, AA, ZO, PB, SV, SG, KN, ET, MRS and CV collected samples and data. All authors analyzed and interpreted the data. AD and CV drafted the article. All authors revised the manuscript critically for important intellectual content and gave final approval for this version to be submitted.

Keywords

Tuberculosis, Surgery, prognosis, cavitary lesion of lung, immune response, biomarkers

Abstract

Word count: 216

Efforts to combat tuberculosis are hindered by the lack of reliable biomarkers to monitor treatment, the microbiological endpoint has limitations guiding the clinical development of new interventions, and there are no biomarkers that accurately reflect lung pathology. New biomarkers are needed to cope with these limitations following the WHO target product profile requirements. We aimed to identify the immune responses which could reflect severity features and lung pathology in tuberculosis patients receiving therapeutic surgery. 40 Georgian patients who underwent surgery for pulmonary tuberculosis after standard drug therapy were studied. Circulating immune markers were measured through ELISA and Luminex, and correlations with the lesion's characteristics were explored. Live bacilli were detected in 5% of the lesions resected from microbiologically cured patients. Differences in the levels of immune markers associated with clinical and epidemiological features were identified. Pre-surgery plasma IL-2 and urine IP-10 levels correlated to the size of the lesion; MMP-9 and sCD14 levels were associated with MDR/XDR-TB; MMP-8 correlated with most of the factors considered to indicate worst outcomes. Post-surgical immune markers increase suggests a marked reduction in the immunosuppressive effect caused by the presence of TB lesions. A correlation was found between systemic biomarkers and several clinical-epidemiological factors. These findings suggest systemic immunosuppression triggered by the persistence of TB lesions that was corrected after surgery.

Contribution to the field

The sputum culture is the only tool to evaluate the efficacy of the anti-tuberculosis antibiotics, but it has proved limitations as a surrogate treatment endpoint. Tuberculosis therapeutic surgery is a procedure reserved for complicated pulmonary tuberculosis cases, especially patients having multi-resistance to chemotherapy. Recent literature has demonstrated live bacilli in tuberculosis lesions of patients with good treatment adherence and stable sputum conversion, undermining the prognostic value of this endpoint. It is needed to find better and reliable alternatives, new biomarkers with powerful prognostic capabilities from accessible biological sources, and available at any point-of-care. This study reports immunological changes before and after surgery in patients with unresolved pulmonary TB that required surgery. These associations are examined in circulant biomarkers (blood and urine) and explores the clinical usefulness of the analyzed biomarkers. We found several biomarkers correlating with tuberculosis severity and worse prognosis features. These could be used to better monitor and management of the patient. Although more studies are needed, our findings provide insights into the role of the immune system at advanced stages of the disease, even after the removal of the tuberculosis lesions. Moreover, the project also evidenced certain immunesuppression at the systemic level that is reverted after surgery.

Funding statement

This work was supported by the Spanish Government-European Regional Development Funds (FEDER) through CV contracts [CP13/00174, CPII18/00031], MRS contract [CPII14/00021], and [PI16/01511], the "CIBER Enfermedades Respiratorias" Network (CIBERES), the "Spanish Society of Pneumology and Thoracic Surgery" (SEPAR) [16/023] and the "Agència de Gestió d'Ajuts Universitaris i de Recerca" (AGAUR) through AD contract [2017 FI_B_00797]. AA received a scholarship from the EACEA (Education, Audiovisual and Culture Executive Agency, award 2015-2323) of the European Commission.

Ethics statements

Studies involving animal subjects

Generated Statement: No animal studies are presented in this manuscript.

Studies involving human subjects

Generated Statement: The studies involving human participants were reviewed and approved by Germans Trias i Pujol Research Institute (IGTP) ethics committee (EC: PI-16-171)
National Center for Tuberculosis and Lung Diseases (NCTLD) ethics committee (IRB00007705 NCTLD Georgia #1, IORG0006411) . Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Inclusion of identifiable human data

Generated Statement: No potentially identifiable human images or data is presented in this study.

Data availability statement

Generated Statement: The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://dx.doi.org/10.17632/knhvdbjv3r.1 (Mendeley Data).

Evaluation of the immune response in tuberculosis patients from Georgia before and after receiving therapeutic surgery

Albert Despuig[1,2], Asimakis Avramopoulos[1,3], Zaira García[1], Pau Benito[1,4], Erica Téllez[5], Shota Gogishvili[6], Keti Nikolaishvili[6], Natalia Shubladze[6], Jordi Casanovas[7], Albert Obiols[7], Tomàs Aluja[7], Maria-Rosa Sarrias[5,8], Sergo Vashakidze[6], Cristina Vilaplana[1,2]*

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Keywords: tuberculosis; surgery; prognosis; cavitary lesions; immune response; biomarkers.

ABSTRACT

Efforts to combat tuberculosis are hindered by the lack of reliable biomarkers to monitor treatment, the microbiological endpoint has limitations guiding the clinical development of new interventions, and there are no biomarkers that accurately reflect lung pathology. New biomarkers are needed to cope with these limitations following the WHO target product profile requirements. We aimed to identify the immune responses which could reflect severity features and lung pathology in tuberculosis patients receiving therapeutic surgery. 40 Georgian patients who underwent surgery for pulmonary tuberculosis after standard drug therapy were studied. Circulating immune markers were measured through ELISA and Luminex, and correlations with the lesion's characteristics were explored. Live bacilli were detected in 5% of the lesions resected from microbiologically cured patients. Differences in the levels of immune markers associated with clinical and epidemiological features were identified. Pre-surgery plasma IL-2 and urine IP-10 levels correlated to the size of the lesion; MMP-9 and sCD14 levels were associated with MDR/XDR-TB; MMP-8 correlated with most of the factors considered to indicate worst outcomes. Post-surgical immune markers increase suggests a marked reduction in the immunosuppressive effect caused by the presence of TB lesions. A correlation was found between systemic biomarkers and several clinical-epidemiological factors. These findings suggest systemic immunosuppression triggered by the persistence of TB lesions that was corrected after surgery.

1. INTRODUCTION

Biomarkers remain a priority in any tuberculosis (TB) related research as they should allow treatment response to be monitored, or meaningful clinical outcomes, including relapse, to be predicted in patients with active disease (1). Several clinical endpoints have been used to measure the efficacy of TB interventions, with the sputum culture conversion (SCC) at the end of the intensive treatment being the most widely used as a surrogate endpoint in drug-susceptible TB (DS-TB) (2) and at six months for multi-drug resistant-TB (MDR-TB), even though there is insufficient evidence to support the validity of these endpoints to guide the clinical development of therapeutic interventions (3,4). Sputum remains the only sample that is tested during treatment response follow-up as well as the only way to estimate if sterilization occurs after treatment, even though it has been shown that viable bacilli can still be found in lesions after completing treatment and having a SCC (5). Other tests involving samples such as blood and urine are required in order to overcome the weaknesses of their sputum-based counterparts, such as the time required for the bacilli growing, potential cross-contaminations, and the inability to produce sputum in certain patients. The fate of the individual is best determined using the lesion pathology in the infected lung. As each lesion inside the same individual is different, and the outcome depends on local host–pathogen interactions (6), a knowledge of the lesion status could help guide therapeutic decisions and treatment research. Unfortunately, this can only be achieved using invasive procedures. However, in high TB incidence countries, and in those with a high MDR-TB burden, such as Georgia (7), therapeutic surgery is still a good option in selected cases to cope with TB complications and sequelae, as well as to reduce the bacillary burden and limit the spread of the disease (8).

As such, using a Georgian cohort of TB patients scheduled for therapeutic surgery, we aimed to identify circulating biomarkers related to the persistence of TB lesions that could indicate the severity status of these subjects. In this study, we hypothesized that the measured levels of a determined set of circulant proteins in a cohort of patients submitted to therapeutic surgery could identify which of these biomarkers related to the immune system are associated with the poor

clinical phenotype and the persistence of TB lesions in the cohort. These findings may be used as part of a potential prognosis tool or a biomarker panel and to help to endorse an eventual surgery in complicated TB cases.

2. MATERIALS AND METHODS

2.1. Study design

This manuscript is part of the Study of the Human Tuberculosis Lesions Project (SH-TBL project, registered at ClinicalTrials.gov NCT02715271), involving 40 Georgian TB patients undergoing therapeutic surgery in the National Center for Tuberculosis and Lung Diseases (NCTLD) (Tbilisi, Georgia) between May 2016 and May 2018. All patients recruited in this study received therapy according to national guidelines and at the moment of the surgery, the whole cohort had bacteriological conversion of sputum and was negative by smear microscopy and culture. The participant inclusion criteria were if patients required surgery because of persistent radiological signs of cavitary lesions in the chest CXR and computed tomography scan, according to official guidelines' surgery recommendations (8).

2.2. Data and sample collection

Clinical, microbiological, and epidemiological data from patients were anonymously, prospectively collected through an electronic Case Report Form (OpenClinica version 3.1). TB lesions were characterized macroscopically and samples were taken from different parts of the granuloma to detect the presence of bacilli by AFB and culture.

2.3. Immunological studies

Fresh urine and peripheral blood were collected the morning before surgery and at discharge to subsequently measure plasma levels of IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17a, TNF-α, MMP-8, MMP-9, IP-10, CRP, sCD14 and CD5L. Levels of all biomarkers except MMP and CD5L were also determined in urine. Biomarkers were measured using multiplex and ELISA kits (Millipore cat#HSTMAG-28SK; R&D cat#FCSTM07-2, cat#DIP100, DCRP00 and DC140; and Medical & Biological Laboratories Co. AIM/CD5L/Spa ELISA kit). All kits were used following the manufacturer's instructions.

2.4. Statistical analysis

Differences between the concentrations of biomarkers were analyzed and plotted using Graph Pad Prism 7 (La Jolla, CA, USA) applying Student's t-test, the Mann-Whitney U test, Fisher's exact test and the Wilcoxon's Matched-Pairs signed rank test for the pre- and post-surgical paired samples. Tests were two-tailed. P-values of less than 0.05 were considered to be statistically significant. Correlations were analyzed with FactoMineR through R Studio (version 3.6.2); the strength of the relationship measured by the correlation ratio (eta) for the association of binary variables with continuous ones; and by the Chi-Square statistic for the association between binary variables. The ability of combinations of selected host biomarkers and clinical/epidemiological factors to predict the features of lesions was assessed by plotting Receiver Operator Characteristics (ROC) and, because of the small sample size, applying General Discriminant Analysis with leave-one-out cross-validation.

2.5. Ethics

The project protocol and associated documents were reviewed and approved by both the ethics committee of the NCTLD (IRB00007705 NCTLD Georgia #1, IORG0006411) and the Germans Trias i Pujol Research Institute (IGTP) ethics committee (EC: PI-16-171). Written informed

consent was obtained for the collection of biological material and data from all study participants before they were enrolled.

2.6. Data sharing

Data files used for the results obtaining are openly available (DOI: http://dx.doi.org/10.17632/knhvdbjv3r.1)

3. RESULTS

3.1. Characteristics of the study cohort and their lesions

Table 1 reports the characteristics of the patients included in the cohort according to sex as a potential factor to influence the TB disease course (5). All patients had converted their sputum cultures at the time of surgery. The median range from TB diagnosis to surgery was 10 months. Surgery was performed according to indications of the official guidelines (8). The macroscopic characterization of the lesions and the presence of bacilli are presented in Table 2. Patients were classified as fast (≤2 months) or slow responders (>2 months) based on an SCC of before or at 2 months (M) or later. Larger lesions were found to be located in the right upper lobe.

A large majority (84.62%) of resected lesions presented fresh necrosis (Figure 1). Acid Fast Bacilli (AFB) were detected in tissue samples from 28 patients (71.79%) and recovered in culture from two of them (5.2%). The center of the lesion harbored the highest number of positive Mycobacterium tuberculosis (Mtb) cultures. No differences between fast and slow responders were found in terms of the presence of bacilli in tissue samples.

3.2. Immune responses according to patient characteristics

The values for circulating immune markers measured in plasma and urine are presented in Table 3. Of all the analytes measured in urine, only the Interferon gamma-induced protein 10 (IP-10), interleukin (IL)-2, IL-6, IL-8, and soluble CD14 (sCD14) were detected. Figure 2 shows the results according to the different characteristics of the cohort. Pre-surgery plasma interferon-gamma (IFN-γ) and IL-4 and urine IL-8 concentrations were found to be significantly higher in females than in males. Alcohol consumers had lower levels of several of the analytes measured, reaching statistical significance for plasma IFN-γ, IL-4, metalloproteinase (MMP)-9, and urine IL-8, whereas urine sCD14 was increased. Smokers also exhibited lower plasma MMP-9 and C Reactive Protein (CRP) and urine IL-8 but higher IL-6. Pre-surgery MMP-9 levels were significantly higher in Multi-drug resistant/Extensively drug-resistant tuberculosis (MDR/XDR-TB) patients, but lower sCD14 plasma levels. Plasma IL-8 levels were significantly higher in fast converters, whereas MMP-8, MMP-9, and CRP were higher in slow converters. Urine sCD14 levels were found significantly higher in patients who exhibited AFB in the resected lesion.

Most biomarkers levels increased post-surgery measured at discharge, the differences being statistically significant for IL-6, IL-8, sCD14, IL-4, IL-10, and CRP while CD5L decreased (Table 3). When analyzed by different sets of groups (see Supplementary Table 1), the results showed that alcohol consumers and smokers showed a significant increase in IL-6 and IL-8 in plasma and urine.

3.3. Results analysis according to severity traits

Showing symptoms, being relapsed patients, having an MDR/XDR-TB, being a slow responder, presenting lesions bigger than 36 mm, and the presence of fresh necrosis, cavitation and bacilli detection in the resected lesion were considered signals of severity or worse prognosis. All results were therefore analyzed according to these factors, and the correlations between them explored

(Figure 3). Plasma MMP-8 levels correlated positively with the presence of TB symptoms (as did the presence of infiltrates in the Chest X-Ray (CXR)), relapse, and lesion cavitation. Co-infection with Hepatitis C Virus (HCV), presence of infiltrates in the CXR, and having DS-TB also correlated with relapse. Time from diagnosis to treatment, need for corticosteroids, and plasma sCD14 and MMP-9 levels correlated with MDR/XDR-TB. The Body Mass Index (BMI) and plasma MMP-8 levels pre-surgery correlated positively with being a slow responder, and IP-10 urine levels and IL-2 plasma levels pre-surgery correlated positively with lesion size larger than 36 mm. Being female, smoker, and alcohol consumer and urine sCD14 levels correlated positively with AFB positivity in biopsies. Plasma MMP-9 levels pre-surgery inversely correlated with AFB positivity. Our findings also suggest that time from diagnosis to treatment was important for obtaining a positive Mtb culture in lesion samples.

Post-/pre-surgery ratio concentrations were calculated and analyzed according to severity traits (Figure 4). IFN-g, IL-4, and IL-8 increased more after surgery in slow than in rapid-converters, and IL-10 was almost nine-fold higher post-surgery in relapse patients. CRP, IFN-γ, and IL-4 levels post-surgery increased less in patients with lesions presenting fresh necrosis. IP-10 urine levels post-surgery were 210-times higher than pre-surgery levels and were 763-times higher in TB patients showing symptoms.

3.4. Predicting who needs surgery

In order to predict which patients should or shouldn't undergo surgery, we tried to model the worst-case scenario following a leave-one-out cross-validation methodology. Considering a composite outcome including positivity for AFB staining, presence of fresh necrosis, cavitation, or a size >36mm, the results suggested that all patients should undergo surgery. We then modeled the ability of plasma MMP-8, MMP-9, sCD14, and CD5L to predict having a TB lesion with fresh necrosis, with a poor result (Area Under the Curve (AUC)=0,4). The ability to predict AFB positivity, considering being a female, smoker, plasma MMP-9, and urine sCD14 levels, was studied next. The AUC was 0,74 (Supplementary Figure 1), with being female and the urine sCD14 level being the most important factors.

4. DISCUSSION

One of the most important findings in our study was that the bacilli were found to be present in resected lung lesions in both AFB smear samples and tissue cultures. Being female, a smoker, plasma MMP-9 and urine sCD14 levels showed a discreet ability to predict this. However, as only limited biopsy samples were collected to be stained for AFB and culture, we cannot rule out that more patients were positive, therefore any correlations observed with the presence of AFB should be taken cautiously. The bacilli recovered were proved to be alive in samples from two patients, in line with previous findings (5), thus challenging the World Health Organization's definition of treatment success, as there is a subpopulation of patients in which treatment does not achieve lung sterilization.

There are several biases in the present study. For instance, taking only the worst cases into account or those that underwent surgery introduces selection bias, therefore our findings should not be generalized to the whole spectrum of TB patients. Moreover, we only have a snapshot, and as people do not undergo thoracic surgery if they are healthy, we do not have any negative controls with which to compare our results. Similarly, immune responses were measured after treatment completion. We can consider all analytes measured to be within the ranges reported by others after treatment (9–12) even though the scarce literature assessing immune responses after completing treatment is complex to interpret given the lack of harmonization in the analytes measured, and the biological fluids and timepoints used.

Plasma CD5L levels assessed before surgery were similar to those measured in TB patients at baseline when using the same kit as Xu et al. (13). As our assessment was performed at the final time point and no literature is available in this regard, we do not know what happens to CD5L during the disease, whether our results reflect TB disease or disease activity. Plasma sCD14 levels were high compared to described cut-offs in recent literature reviews of its role as a diagnostic and prognostic marker for sepsis and other infections, including TB (14). In our opinion, these two biomarkers should be explored further in new cohort studies at different time points during the course of TB.

In general, women had higher levels of circulating markers pre-surgery, but the difference was only statistically significant for IFN-γ, IL-4 and urine IL-8. This finding is in line with the literature, which suggests that women have a more robust immune response than men to infectious diseases and upon vaccination, but are more susceptible to autoimmune diseases, probably triggered by estrogen (15,16). It should be noted that more men than women admitted to consuming alcohol and/or tobacco. This sex-related confounding factor must be interpreted as a limitation as we cannot rule out that alcohol, either in combination with the presence of a TB lesion or alone, is responsible for triggering the immune suppression seen amongst male participants. Our findings in this regard are in line with the current literature, which shows that toxic habits induce immunosuppression, as seen for IFN-γ, IL-4, and urine IL-8 in alcohol consumers and smokers. The effect of alcohol on B-cells results in reduced production of IL-4, as well as a reduction or even a suppression of IFNs production, thus reducing the chances of bacterial clearance from the lung (17). Alcohol misuse has been related to worse treatment outcomes, especially if used heavily and combined with tobacco (18). Cigarette smoke has been reported to be pro-inflammatory and immunosuppressive depending on several epidemiological factors, and to have a negative impact on IFN-γ production, the immune response to infections and TB outcomes (19–21). A smoking-related pro-inflammatory status (22) could therefore be the reason for the increased IL-6 levels found in urine. In our cohort, we also found a correlation between smoking and the persistence of bacilli in the TB lesion although, in contrast to previous reports (19), we did not find any correlation with slow sputum conversion.

One of the major strengths of this study is that a description of TB lesions was possible, thus allowing correlations to be drawn between circulating markers and lung pathology. Only plasma MMP-8, MMP-9 and IL-2, and urine sCD14 and IP-10 were found to correlate with severity traits. Although low, IL-2 levels correlated positively with lesion size. IL-2 promotes T-cell proliferation and is needed for granuloma generation, and has been proposed and tested as host-directed therapy for TB, with a beneficial effect on the SCC but not on the CXR findings (23). However, Sigal et al. also described a statistically significant association between IL-2 receptor levels and cavities larger than 4 cm at baseline in a cohort of 319 pulmonary TB patients (24).

In addition, IP-10 urine levels correlated with larger lesions. IP-10 has previously been identified as a potential biomarker for active TB and a correlator of treatment response (25,26). It is considered to be responsible for the recruitment of Th1 cells to the tubercular infection site and granuloma maintenance (27,28), therefore our results could be interpreted as IP-10 acting as a biomarker for active granulomas. The urine levels for the analytes studied here were higher than those found by Kim et al. (26) but similar to those described in a small cohort of TB patients cured within the previous 12 months (29), therefore our findings could simply reflect a larger lung injury rather than granuloma activity. Further studies should be conducted in other cohorts in order to confirm the strength of these correlations and their clinical meaning.

In this study, plasma MMP-9 levels correlated positively with MDR/XDR-TB, and MMP-8 was found to be the most important biomarker in terms of being associated with several severity traits. MMPs act as enzymes inducing destruction of the extracellular matrix, which in pulmonary TB has been related to cavity formation and the closure, release, and spread of Mtb bacilli (30). Sathyamoorthy et al. (2015) have reported a positive correlation between MMP-1 and TB symptoms, but no correlation between MMP-8 levels and sputum smear or symptoms in a cohort of Peruvian PTB patients. However, Sigal et al. (2017) described MMP-8 and MMP-9 to be associated with the presence of cavities >4 cm and the extent of TB disease to be associated with CXR >50% at baseline, therefore they considered MMP-8 to be one of the biomarkers associated with disease severity. Other studies also found MMP-9 to be associated with the volume of TB-related destruction, and MMP-8 to be associated with disease activity and impaired lung function, even after treatment completion (32), which is very much in line with what we found taking into account the unresolved TB status of our patients, which made them eligible for surgery. To the best of our knowledge, there is only one previous report suggesting a link between high MMP levels and MDR strains (33), which makes sense if we consider that MDR-TB forms are commonly associated with greater lung injury that often persists after completing treatment (34). However, given the scarcity of literature supporting the strength of this link, in our opinion, this relationship should be explored further in order to establish its validity and potential use as an MDR biomarker.

The presence of infiltrates in the CXR appears to be a good correlator for relapse, along with HCV co-infection and age ranging from 35 to 44 years. Persistent cavities in the CXR are known to be a risk factor for delayed treatment response and relapse (35), and the relationship between radiological and microbiological status has been described, although clinical interpretation must be cautious given the modest sample size (36). MMP-8 may therefore represent a promising correlator to support and complement radiological findings.

In this study, levels of immune markers increased at discharge. This could be due to an inflammatory response generated by the surgery. Both IL-6 and CRP have been found to be associated with the severity of the surgery, peaking before 72 h post-surgery and normalizing at seven days in complication-free postoperative periods (37). However, considering that only two patients reported post-surgical complications, together with the fact postoperative measurements were taken more than seven days after surgery, render this scenario unlikely. The biomarkers levels measured in pre-surgical blood could be decreased as a result of the granuloma acting as an attractant for cells and molecules, which should be confirmed by histopathological characterization of the lesions. Finally, the organized lesion may induce a general immunosuppressive effect in the body. A similar trend was noted in a study in MDR-TB patients undergoing surgery for active pulmonary TB, with the subsequent immune boost being a result of removal of the immunosuppressive effect exerted by multiple cellular compartments (38). Unresolved chronic inflammation at a local level could lead to increased pathological activation of myeloid-derived suppressor cells, with a resulting potent systemic immunosuppressive effect on innate and adaptive immunity, as suggested recently and as observed in other infections and cancer (39,40). Further studies are therefore urgently needed to elucidate the mechanism underlying this effect, both at a systemic level and on the resected lesion.

5. Abbreviations

TB	Tuberculosis
AFB	Acid Fast Bacilli
AUC	Area Under the Curve
BMI	Body Mass Index
CXR	Chest X Ray
DS-TB	Drug Susceptible Tuberculosis
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
MDR/XDR-TB	Multi-Drug Resistant/Extensively Drug-Resistant-Tuberculosis
MDR-TB	Multi-Drug Resistant-Tuberculosis
MMP	Metalloproteinase
Mtb	Mycobacterium tuberculosis
ROC	Receiver Operator Characteristics
SCC	Sputum Culture Conversion
sCD14	soluble CD14
XDR-TB	Extensively Drug-Resistant-Tuberculosis

6. Funding

This work was supported by the Spanish Government-FEDER Funds through CV contracts [CP13/00174, CPII18/00031], MRS contract [CPII14/00021], and [PI16/01511], the "CIBER Enfermedades Respiratorias" Network (CIBERES), the "Spanish Society of Pneumology and Thoracic Surgery" (SEPAR) [16/023] and the "Agència de Gestió d'Ajuts Universitaris i de Recerca" (AGAUR) through AD contract [2017 FI_B_00797]. AA received a scholarship from the EACEA (Education, Audiovisual and Culture Executive Agency, award 2015-2323) of the European Commission.

7. Acknowledgments

The authors would like to thank the patients who agreed to participate in the study and the staff from the NCTLD who helped with this project. We would also like to thank both the IGTP's cytometry platform and Eric Garcia for their technical support in acquiring the data, and Mr. Andrew Frankland, a professional English language editor who revised the manuscript to improve the grammar and readability. AA registered with the EMJMD LIVE (Erasmus+ Mundus Joint Master Degree Leading International Vaccinology Education), co-funded by the EACEA of the European Commission. The present study was granted with Spanish Government-FEDER Funds, the "CIBER Enfermedades Respiratorias" Network (CIBERES), the "Spanish Society of Pneumology and Thoracic Surgery" (SEPAR) and the "Agència de Gestió d'Ajuts Universitaris i de Recerca" (AGAUR).

8. Author Contributions Statement

CV and SV conceived the study and CV designed it. AD, AA, ZG, PB, SV, SG, KN, ET, MRS and CV collected samples and data. All authors analyzed and interpreted the data. AD and CV drafted the article. All authors revised the manuscript critically for important intellectual content and gave final approval for this version to be submitted.

9. Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

10. Data Availability Statement

The datasets regarding to individual participants that underlie the results reported in this article, after de-identification, is publicly available immediately following the publication date.

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12. FIGURES

Figure 1. **Cavitary TB lesions removed from two SH-TBL participants.** Fresh necrosis **(A)** and dry necrosis **(B)**.

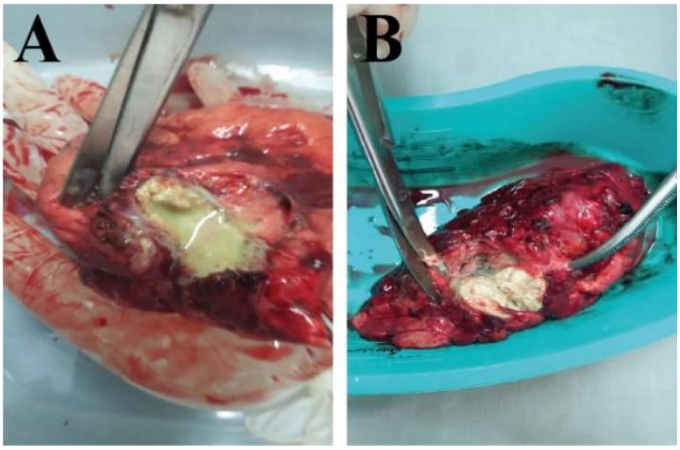


Figure 2. Systemic biomarkers in plasma and urine before therapeutic surgery. Pre-surgery biomarker levels according to several patient characteristics are presented on a log10-scale. (*) p-value <0.05; (**) p-value <0.001, Mann-Whitney Test.

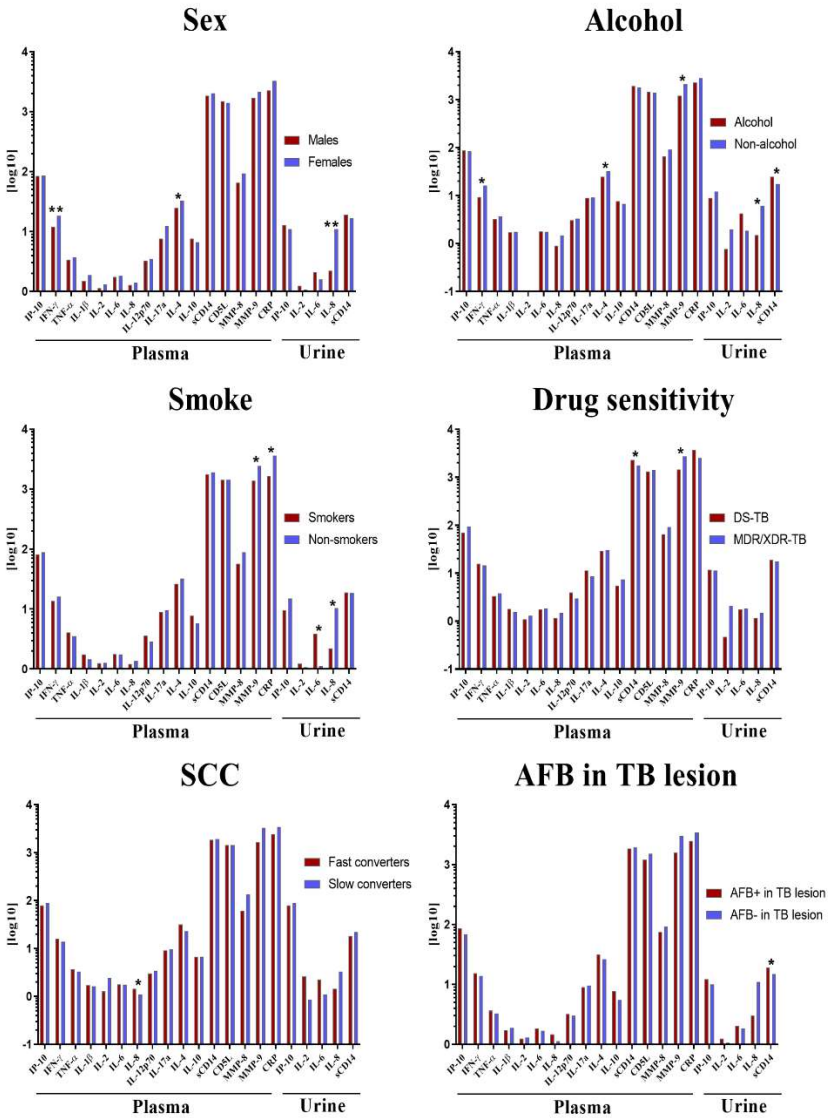


Figure 3. Correlation plot of pre-surgery biomarker levels vs. TB severity factors. Results are expressed in terms of v-tests, which corresponds to the standard normal deviate with equal p-value as the measured correlation, finding the strongest association bigger in dot size; being blue colored with positive association while an inverse association in red. P-values are represented as (+): positive correlation, p-value <0.05; (++): positive correlation p-value <0.01; (-): negative correlation, p-value <0.05; (--): negative correlation, p-value <0.01.

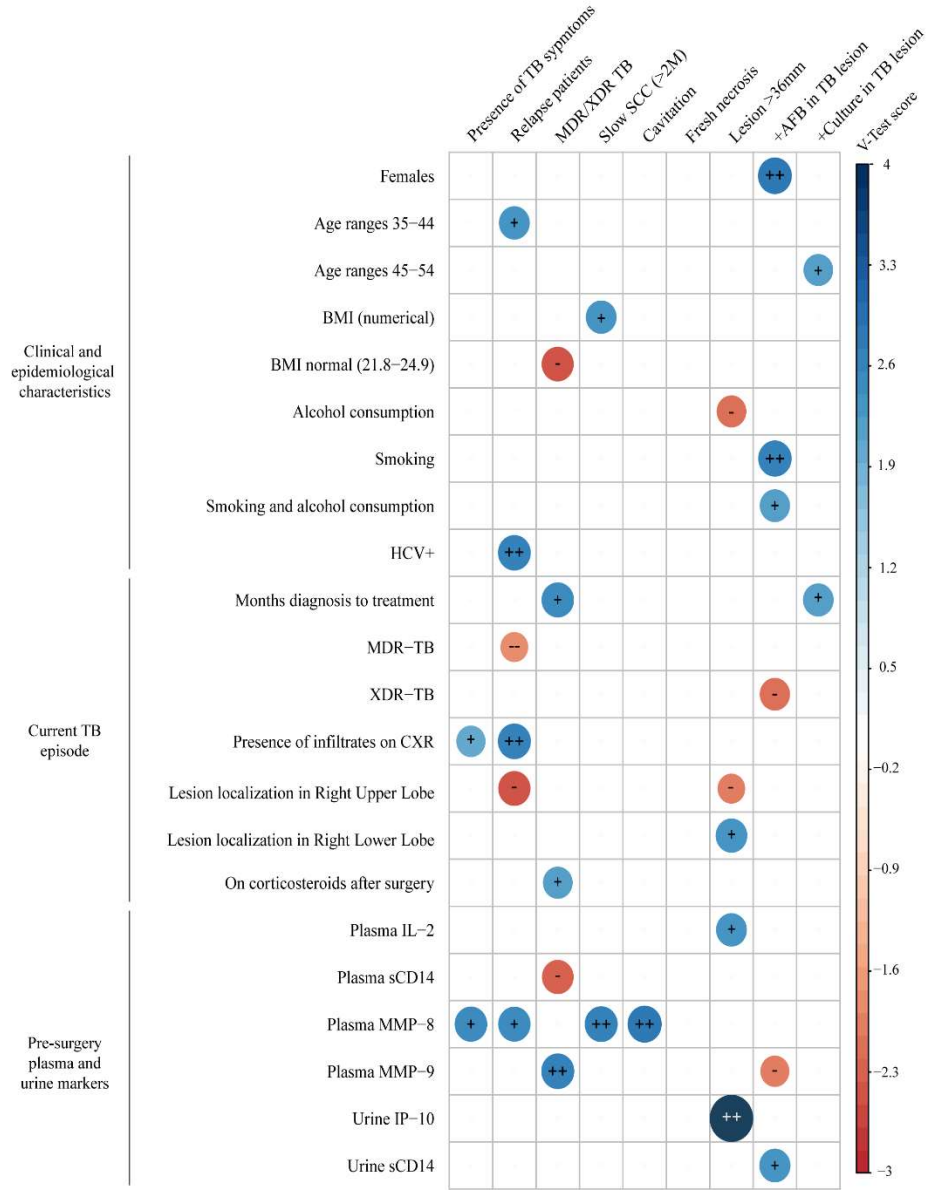
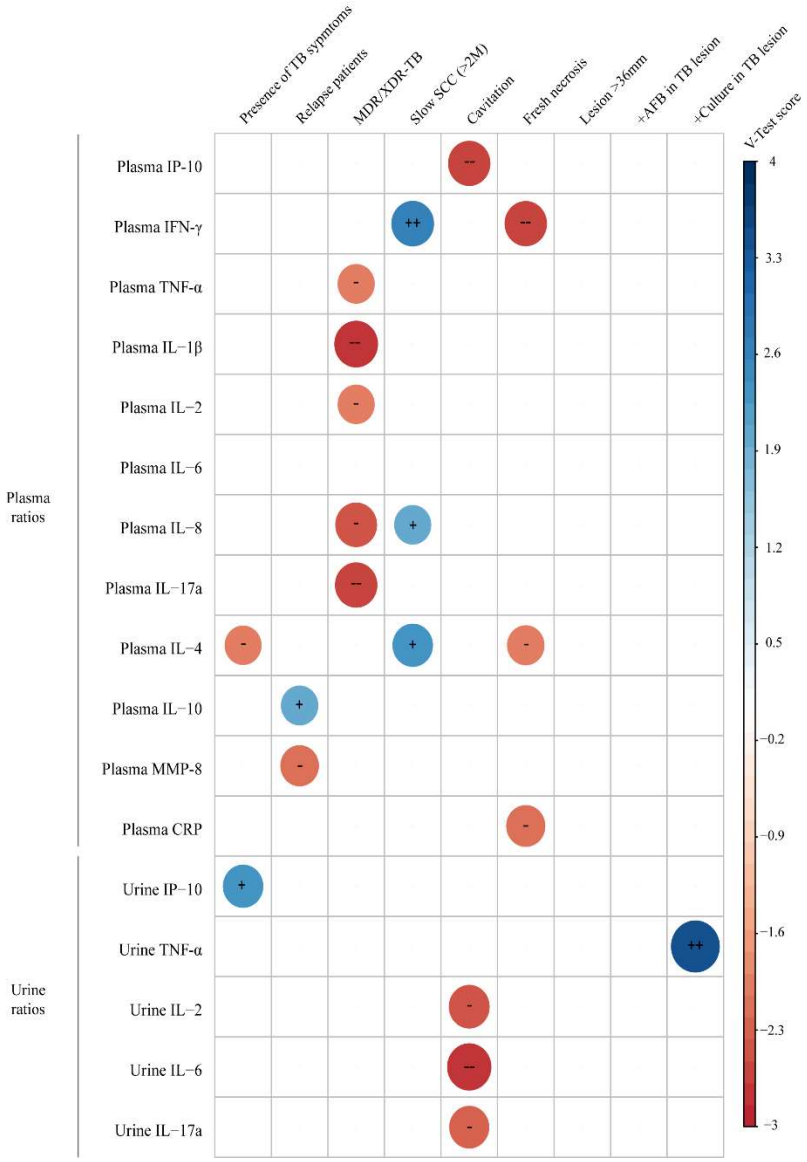


Figure 4. Correlation plot for the statistically significant ratios of post-/pre-surgery biomarker levels vs. TB severity forms. Results are expressed in terms of v-tests, which corresponds to the standard normal deviate with equal p-value as the measured correlation, finding the strongest association bigger in dot size; being blue colored with positive association while an inverse association in red. P-values are represented as (+): positive correlation, p-value <0.05; (++): positive correlation p-value <0.01; (-): negative correlation, p-value <0.05; (--): negative correlation, p-value <0.01.



13. Tables

Table 1. Demographic, clinical, and TB-related characteristics for patients at the time of surgery. Comparisons refer to males vs. females. ⸸Patient's sputum culture conversion test result was not recorded at the moment of the analysis. [a] Student's t-test, [b] Mann-Whitney U-test, [c] Fisher's exact test. *statistically significant differences.

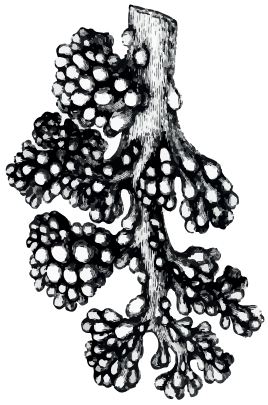
Variable	Category	Overall	Male	Female	p-value
N		N = 40	n = 19	n = 21	
Clinical and epidemiological characteristics					
Age (mean (SD))		33.45 (11.76)	37.74 (12.53)	29.57 (9.755)	0.0364* [a]
Body Mass Index (median [range])		21.5 [16.53-32]	22.5 [16.53-31.7]	20.49 [17.3-32]	0.2163 [b]
Smoker (%)	yes no	15 (37.5) 25 (62.5)	13 (68.42) 6 (31.58)	2 (9.52) 19 (90.48)	0.002* [c]
Alcohol (%)	yes no	9 (22.5) 31 (77.5)	9 (47.37) 10 (52.63)	0 (0.0) 21 (100.0)	0.003* [c]
Diabetes (%)	yes no	3 (7.5) 37 (92.5)	3 (15.79) 16 (84.21)	0 (0.0) 21 (100.0)	0.0981 [c]
HCV (%)	yes no	6 (15) 34 (85)	4 (21.05) 15 (78.95)	2 (9.52) 19 (90.48)	0.3976 [c]
HBV (%)	yes no unrecorded	2 (5) 23 (95) 11	1 (7.69) 11 (92.31)	1 (5.88) 12 (94.12)	1 [c]
Characteristics of current TB episode					
TB symptoms (%)	yes no	12 (30) 28 (70)	5 (26.32) 14 (73.68)	7 (33.33) 14 (66.67)	0.736 [c]
Months from TB diagnosis to surgery (median [range])		10 [5-60]	11 [5-53]	9 [5-60]	0.462 [b]
Drug sensitivity (%)	DS-TB MDR-TB XDR-TB MDR/XDR-TB	15 (37.5) 18 (45) 7 (17.5) 25 (62.5)	7 (36.84) 9 (47.37) 3 (15.79) 12 (63.16)	8 (38.1) 9 (42.85) 4 (19.05) 13 (61.9)	1 [c]
Patient history (%)	New patient Relapse	27 (67.5) 13 (32.5)	14 (73.68) 5 (26.32)	13 (61.9) 8 (38.1)	0.510 [c]
Months for sputum culture conversion (median [range])		2 [1-7] ⸸	2 [1-6]	2 [1-7]	0.164 [b]
Fast (≤2 months) and slow (>2 months) culture conversion	Fast converters Slow converters unrecorded⸸	28 (71.8) 11 (28.2) 1	11 (61.12) 7 (38.88)	17 (80.95) 4 (19.05)	0.2849 [c]
CXR findings					
Localization of lesions within the lung (%)	Left Upper Lobe Right Upper Lobe Left Lower Lobe Right Lower Lobe LUL + LLL	11 (27.5) 21 (52.5) 4 (10) 3 (7.5) 1 (2.5)	5 (26.31) 10 (52.63) 3 (15.79) 1 (5.26) 0 (0.0)	6 (28.57) 11 (52.38) 1 (4.76) 2 (9.52) 1 (4.76)	0.666 [c]
Multiple lesions in CXR (%)	yes (≥2) no (<2)	8 (20) 32 (80)	5 (26.31) 14 (73.68)	3 (14.28) 18 (85.71)	0.442 [c]
Presence of cavities (%)	yes no	40 (100) 0 (0.00)	19 (100) 0 (0.00)	21 (100) 0 (0.00)	1 [c]
Infiltrates (%)	yes no	8 (20) 32 (80)	4 (21.05) 15 (78.95)	4 (19.05) 17 (80.95)	1 [c]
Signs of bronchogenic dissemination (%)	yes no	6 (15) 34 (25)	1 (5.26) 18 (94.74)	5 (23.81) 16 (76.19)	0.185 [c]
Calcified granulomas (%)	yes no	15 (37.5) 25 (62.5)	7 (36.84) 12 (63.16)	8 (38.10) 13 (61.90)	1 [c]
Bronchiectasis (%)	yes no	12 (30) 28 (70)	3 (15.79) 16 (84.21)	9 (42.86) 12 (57.14)	0.088 [c]
Pleural involvement (%)	yes no	8 (20) 32 (80)	4 (21.05) 15 (78.95)	4 (19.05) 17 (53.13)	1 [c]
Days from surgery to discharge (median [range])		10 [7-91]	10 [9-21]	10 [7-91]	0.362 [b]

Table 2. Attributes of resected lesions. Comparisons refer to patients who presented microbiological conversion at two months or less against those who required more than two months to respond to treatment. ⸸Patient's sputum culture conversion test result was not recorded at the moment of the analysis. [a] Mann-Whitney U-test, [b] Fisher's exact test.

Variable	Category	Overall	Fast Responders	Slow Responders	p-value
N		N = 39⸸	n = 28	n = 11	
Size of resected lesion (mm) (median [range])		32 [22-55]	32 [22-55]	32 [22-40]	0.75 [a]
Necrosis (%)	Fresh necrosis	33 (84.62)	25 (89.29)	8 (73.73)	0.32 [b]
	Not fresh necrosis	6 (15.38)	3 (10.71)	3 (27.27)	
Cavitation (%)	Cavitation	34 (87.18)	23 (82.14)	11 (100)	0.29 [b]
	Tuberculoma	5 (12.82)	5 (17.86)	0 (0.00)	
Presence of bacilli in AFB smear					
Centre of the lesion (%)	positive	18 (48.65)	14 (50)	4 (36.36)	0.49 [b]
	negative	21 (53.85)	14 (50)	7 (63.64)	
Internal wall of the lesion (%)	positive	4 (10.26)	4 (14.29)	0 (0.00)	0.30 [b]
	negative	35 (85.75)	24 (85.71)	11 (100)	
External wall of the lesion (%)	positive	5 (12.82)	2 (7.14)	3 (27.27)	0.15 [b]
	negative	34 (87.18)	26 (92.86)	8 (72.73)	
Peripheral nodulus (%)	positive	3 (7.69)	1 (3.57)	2 (18.18)	0.18 [b]
	negative	36 (92.30)	27 (96.43)	9 (81.82)	
Complete surgical piece (Center + Internal + External wall) (%)	positive	16 (41.03)	13 (46.43)	3 (27.27)	0.47 [b]
	negative	23 (58.97)	15 (53.57)	8 (72.73)	
AFB by patient (%)	positive	28 (71.79)	21 (75)	7 (63.64)	0.69 [b]
	negative	11 (28.21)	7 (25)	4 (36.36)	
Biopsy culture positivity					
Biopsy culture conversion by patient (%)	positive	2 (5.12)	1 (3.57)	1 (9.09)	0.489 [b]
	negative	37 (94.87)	27 (96.43)	10 (90.91)	

Table 3. Median concentration values and ranges measured for each analyte and time point. No significant differences (ns), Wilcoxon Matched-Pairs signed rank test.

Analytes	Pre-Surgery	Post-Surgery	p-value
Plasma			
IP-10 (pg/mL)	85.64 (10.38-430.5)	80.36 (23.77-377.4)	ns
IFN-γ (pg/mL)	15.33 (6.63-34.87)	15.75 (5.87-27.13)	ns
TNF-α (pg/mL)	3.595 (1.49-7.8)	3.785 (1.78-12.29)	ns
IL-1β (pg/mL)	1.76 (0.51-3.96)	1.725 (0.51-4.45)	ns
IL-2 (pg/mL)	1.28 (0.47-4.88)	1.92 (0.65-4.05)	ns
IL-6 (pg/mL)	1.765 (0.33-4.14)	2.8 (0.29-28.38)	<0.0001
IL-8 (pg/mL)	1.335 (0.36-20.31)	2.03 (0.61-17.08)	0.0015
IL-12p70 (pg/mL)	3.1 (0.44-8)	3.255 (0.55-8.6)	ns
IL-17a (pg/mL)	9.045 (3.43-25.65)	10.45 (3.09-20.82)	ns
IL-4 (pg/mL)	30.01 (8.89-175.3)	42.29 (20.27-145.3)	0.0008
IL-10 (pg/mL)	7.01 (0.11-24.3)	8.05 (1.46-19.02)	0.0168
sCD14 (ng/mL)	1869 (917.7-3686)	2345 (1165-3584)	0.0008
CD5L (ng/mL)	1433 (586.1-6942)	1296 (419.9-8963)	0.0459
MMP-8 (ng/mL)	82.77 (15.09-195.7)	67.19 (10.02-529.6)	ns
MMP-9 (ng/mL)	1854 (715-6797)	2800 (902-14054)	ns
CRP (ng/mL)	2648 (6.46-21738)	22473 (1412-54662)	<0.0001
Urine			
IP-10 (pg/mL)	11.78 (0.01-83.41)	19.54 (0.12-86.85)	ns
IL-2 (pg/mL)	1.155 (0.03-4.69)	1.365 (0.05-2.81)	ns
IL-6 (pg/mL)	1.98 (0.02-12.95)	6.22 (0.19-188.1)	<0.0001
IL-8 (pg/mL)	4.29 (0.01-124.1)	10.22 (0.09-720.1)	0.0161
sCD14 (ng/mL)	18.87 (4.59-34.94)	28.27 (2.89-33.13)	<0.0001



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