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EVALUATION OF IMMUNOLOGICAL MARKERS FOR THE DIAGNOSIS OF ACTIVE AND LATENT TUBERCULOSIS

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Evaluation of immunological markers for the diagnosis of active and latent tuberculosis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Tuberculosis (TB) is the single deadliest infectious disease in the world and around one fourth of the global population is estimated to be latently infected with *Mycobacterium tuberculosis* (Mtb). The World Health Organization (WHO) targets reduction of mortality by 95% and incidence rate by 90% by year 2035. This thesis aims to contribute by identifying novel biomarkers to distinguish the different stages of infection, from latency to active disease and thereby identify individuals in need of treatment. Currently used assays for latent TB are the tuberculin skin test (TST), which cross-reacts with other mycobacterial strains as well as with the *Bacillus Calmette Guérin* (BCG) vaccine, and the interferon-ɣ release assays (IGRAs) which are more specific for Mtb. However, neither assay can distinguish a previously healed from an active TB. Nor can they distinguish individuals with a recent Mtb infection with increased risk of incipient TB, from a remote and well-controlled infection. Thus, the positive predictive values for active TB are very low. There is a need for more sensitive biomarkers to identify individuals with increased risk of progression to active disease, in order to better control and prevent transmission of TB.

In the first paper FASCIA analysis with PPD and Mtb antigens was proved to be a robust assay with similar sensitivity and specificity as compared with IGRAs. The overall sensitivity for verified active TB was 86% and for latent TB 61%. FASCIA results were concordant with IGRA results in 90% of active TB cases and in 80% of individuals with LTBI. Stronger and more frequent proliferative $CD4^+$ responses were induced in patients with extra-pulmonary TB compared to pulmonary TB ($p<0.05$). FASCIA performed well in patients with moderate immunosuppression.

It was demonstrated in the second paper that cytokine levels were significantly higher after stimulation with CFP-10 and ESAT-6 in individuals with verified TB, compared to healthy controls ($p<0.005$). The chemokine IP-10 levels after stimulation with antigens CFP-10/ESAT-6 showed a significantly higher sensitivity compared to IFN-ɣ responses in individuals with active TB ($p<0.05$).

A mathematical model was developed in the third paper using clinical and epidemiological data to estimate the probabilities of recent and remote latent TB. Results from these estimations were similar to previously published data from contact screenings for house-hold contacts after exposure to smear microscopy positive patients (35%). With a cut-off at 10% of high probability of latent TB, T-SPOT.TB detected 100% of probable recent and remote infections.

A prediction model was developed in the fourth paper where the most specific markers for prediction of recent infection were early (<1 month) high proliferative $CD4^+$ responses to CFP-10 and PPD and low responses to ESAT-6 in contacts to verified pulmonary TB. Other Mtb antigens (Rec85a, Rec85b and Rv1284) were also sensitive markers of recent infection, but did not distinguish recent from remote infection.

The findings from our studies indicate that positive predictive values for incipient TB in Mtb assays can be improved and aid clinicians in targeting those in need of treatment to prevent disease and further transmission of active TB, as well as avoid unnecessary costs and adverse events.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Tuberkulos (tbc) är den enskilda infektionssjukdom som orsakar flest dödsfall i världen. 2017 insjuknade 10 miljoner människor och drygt en och en halv miljon människor avled av sjukdomen. WHOs mål för år 2035 är att den globala tuberkulosepidemin ska minskas kraftigt och en stor satsning görs för närvarande för att skapa starka strukturer för utredning och behandling av sjukdomen i alla länder. En fjärdedel av jordens befolkning uppskattas ha latent tbc, en vilande form av infektionen som inte ger symtom. Alla som är infekterade kan inte behandlas pga resursbrist, höga kostnader och lång behandlingstid (3-9 månader). Risken för att insjukna i tbc är ökad i vissa fall, men eventuell behandling bör alltid vägas mot risken för biverkningar. Uppskattningsvis kommer endast ca 10% av smittade individer att insjukna i aktiv tbc under sin livstid och beräknas då komma att infektera omkring 10 nya individer. Risken att insjukna är högst dels för immunsupprimerade patienter och förimmunkompetenta individer de närmsta 2-5 åren efter smittotillfället (2/3 av alla fall). Dessa patientgrupper bör därför behandlas förebyggande.

Diagnostik av aktiv tbc baseras i första hand på påvisning av *Mycobacterium tuberculosis* genom mikroskopi av ett upphostningsprov, vilket är en enkel metod som används i de flesta länder. Påvisning av tbc-DNA och mykobakterie-odling är känsligare metoder, men kräver avancerade laboratorier och ca 20% av alla tbc-fall är dessutom odlingsnegativa.

Diagnostik av latent tbc kan utföras med olika sorters indirekta immunologiska tester. Tuberkulintestet (PPD) är en vedertagen metod varvid tuberkulin (ett renat proteinderivat från hela bakterien) injiceras ytligt i huden. Detta test kräver specialtränad personal för att utföra och ett återbesök efter 2-3 dagar för att läsa av en eventuell svullnad i huden. Testet är känsligt, men ofta ospecifikt då det kan vara falskt positivt p.g.a. tidigare vaccination eller infektioner med andra sorters mykobakterier. Testet kan inte heller skilja mellan aktiv och latent tbc, eller mellan en ny eller tidigare genomgången smitta.

Interferon-γ release assays (IGRA) är immunologiska tester som utförs på blodprov. Dessa tester bygger på att man stimulerar blod med utvalda proteiner (antigen) från bakterien och sedan registrerar om cellerna i immunförsvaret kan känna igen bakterien och svara med att producera ett viktigt ämne för immunförsvaret, ett cytokin som kallas interferon-gamma. Dessa tester är tbc-specifika, men kan inte heller skilja mellan de olika kliniska stadierna av tbc. Ett annat problem med diagnostiken av latent infektion är att det inte finns någon pålitlig referensmetod att jämföra sina testresultat med, då bakterierna är inkapslade i en inflammationshärd i kroppen och inte kan odlas fram.

Det finns således ett stort behov av att utveckla nya tester med målet att kunna erbjuda förebyggande behandling till nysmittade patienter och på detta sätt minska fortsatt smittspridning.

Syftet med denna avhandling var att testa nya immunologiska tbc-markörer för att skilja ut de olika stadierna av tbc och på så sätt identifiera patienter med ökad risk för tbc-aktivering.

I den första studien utvecklades ett nytt test som kallas FASCIA och baseras på flödescytometri av tbc-antigen stimulerade blodprover. 161 patienter med misstänkt tbc provtogs för att jämföra de olika testernas känslighet (PPD, IGRA och FASCIA) och specificitet och jämfördes även med sedvanliga mikrobiologiska provsvar. Fördelen med FASCIA var att testet gav en mer information om immunförsvaret, då det mätte individens förmåga till förökning av vita blodkroppar, beroende på om man tidigare varit i kontakt med bakterien. Detta gjorde det möjligt att närmare studera hur immunförsvaret fungerade vid tbc-infektion och utökade möjligheten att testa nya tbc-antigen och andra cytokiner för en bättre diagnostik av latent tuberkulos. Vi fann att testens känslighet var jämförbar med IGRA och mer specifik än PPD.

I den andra studien använde vi samma patienter som i föregående studie, men mätte istället nivåerna av interferon gamma (IFN-ɣ) och 13 andra cytokiner i blod efter 3 eller 7 dagars stimulering med samma tbc-antigen. Flera nya cytokiner fungerade bra för att diagnosticera tbc, men det var bara ett av dem, interferon-gamma inducible protein 10 (IP-10), som var känsligare än IFN-ɣ och kunde detektera alla som var tbc sjuka.

En matematisk modell utvecklades i den tredje studie, för att uppskatta sannolikheten i % för om en individ var nysmittad och/eller bar på en gammal tbc-smitta. 160 patienter som nyligen exponerats för lung-tbc provtogs och besvarade ett omfattande formulär med frågor rörande ålder, kön, BCG-vaccination, ursprung, tidigare tbc och sjukhus- eller fängelsevistelse. Dessa data användes för att predicera risken för tidigare tbc-smitta. Uppgifter om relation till smittkällan, närkontakt, exponeringstid och indexpatientens smittsamhet användes för att predicera risken för nysmitta. Modellen var tänkt att användas som referensmetod för att utvärdera de immunologiska testernas förmåga att skilja mellan de olika stadierna av tbc-infektion. Resultaten visade att PPD- och IGRA-resultaten överensstämde väl med tidigare studier i förhållande till hög eller låg sannolikhet för latent tbc hos den enskilde.

Samma patientgrupp användes sedan till den fjärde studien för att utvärdera flera nya tbc-antigen med FASCIA-metoden. Antalet tbc-specifika vita blodkroppar hos varje enskild individ mättes efter stimulering med flera tidigare kända samt även nya antigen, för att sedan jämföras med den uppskattade sannolikheten för ny eller tidigare tbc-smitta.Resultaten visade att om en patient provtogs i nära anslutning till det senaste smittotillfället (<1 månad) så syntes tydliga skillnader i cellnivåer efter stimulering med vissa antigen, vilket gjorde att individer med hög sannolikhet för att vara nyligen smittade kunde urskiljas från de som hade låg sannolikhet.

Dessa resultat skulle kunna öka precisionen för diagnostik av latent tbc med målet att identifiera nysmittade individer med högre risk för tbc-aktivering och ett behov av förebyggande behandling. På så vis kan smittspridningen av tbc minskas och onödiga kostnader och biverkningar av tbc-läkemedel kan undvikas.

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I. Borgström E, Andersen P, Andersson L, Julander I, Källenius G, Maeurer M, Norrby M, Rosenkrands I, Tecleab T, Bruchfeld J, Gaines H
Detection of proliferative responses to ESAT-6 and CFP-10 by FASCIA assay for diagnosis of Mycobacterium tuberculosis infection
Journal of Immunological Methods, 370 (2011) 55-64.

II. Borgström E, Andersen P, Atterfelt F, Julander I, Källenius G, Maeurer M, Rosenkrands I, Widfeldt M, Bruchfeld J, Gaines H
Immune responses to ESAT-6 and CFP-10 by FASCIA and multiplex technology for diagnosis of M. tuberculosis infection; IP-10 is a promising marker
PlosOne, 2012;7(11).

III. Fröberg G, Wahren Borgström E, Chryssanthou E, Correia-Neves M, Källenius G, Bruchfeld J
A mathematical model to estimate the probability of recent and remote latent tuberculosis
Submitted manuscript.

IV. Wahren Borgström E, Fröberg G, Correia-Neves M, Atterfelt F, Bellbrant J, Szulkin R, Chryssanthou E, Ängeby K, Tecleab T, Ruhwald M, Andersen P, Källenius B, Bruchfeld J
Evaluation of proliferative $CD4^+$ T cell responses to *Mycobacterium tuberculosis* antigens as predictive markers for recent infection in contacts to pulmonary tuberculosis.
Submitted manuscript.

CONTENTS

LIST OF ABBREVIATIONS

Ag	antigen
APC	antigen presenting cell
ARI	annual risk of infection
AUC	area under the curve
BCG	Bacille Calmette Guérin
C	concentration
CI	confidence interval
CD	cluster of differentiation
CDC	Centers for Disease Control and prevention
CFP-10	culture filtrate protein 10
CV%	coefficient of variation
CT-scan	computerized tomography scan
CXR	chest X-ray
Dn^+	*Mycobacterium tuberculosis* infected droplet nuclei
DOT	directly observed therapy
E	elimination
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
Elispot	enzyme-linked immunosorbent spot
EMB	ethambutol
EPTB	extra pulmonary tuberculosis
ESAT-6	early secretory antigenic target 6
ESX-1	early secretory antigenic target 6 system 1
FACS	fluorescence activated cell sorting
FASCIA	flow cytometric assay for specific cell-mediated immune response in activated whole blood
GM-CSF	granulocyte macrophage colony stimulating factor
HIV	human immunodeficiency virus
IFN-ɣ	interferon gamma
IL	interleukin
INH	isoniazid
IGRA	interferon gamma release assay

IP-10	interferon gamma inducible protein 10
IQR	interquartile range
K	infective constant
KI	Karolinska Institutet
LTBI	latent tuberculosis infection
MHC	major histocompatibility complex
MIP-1β	macrophage inflammatory protein 1 beta
Mtb	*Mycobacterium tuberculosis*
NPV	negative predictive value
OR	odds ratio
P	probability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
PPV	positive predictive value
PTB	pulmonary tuberculosis
PZA	pyrazinamide
QFT	QuantiFERON-TB Gold
QFT-Plus	QuantiFERON-TB Gold Plus
RFLP	restriction fragment length polymorphism
RIF	rifampicin
RPMI	Roswell Park Memorial Institute medium
S	saturation
SM	smear microscopy
SSI	Statens Serum Institut
TB	tuberculosis
TNF-α	tumour necrosis factor alpha
TST	tuberculin skin test
V	volume
WHO	World Health Organization

1 INTRODUCTION

1.1 HISTORICAL OVERVIEW

Tuberculosis (TB) is an ancient and deadly disease, which originates from a progenitor for all strains of mycobacteria millions of years ago in East Africa (1). Since then, the bacteria have been present causing both endemic infections and epidemics spreading globally.

The first pathological and microbiological traces of TB disease were found with polymerase chain reaction (PCR) for *Mycobacterium tuberculosis* (Mtb) from lesions in bone tissue (osteitis) in fossils from Turkey of Homo erectus from 500 000 BC (2).

Pulmonary disease is the most frequent presentation of TB (Figure 1). The first PCR verified evidence of pulmonary TB (PTB) was found in Egyptian mummies from 1 500-2 000 BC. The remains of the mummies showed histopathological evidence of TB, such as pleural scarring and adhesions in the lungs. Osteitis was also found in these mummies e.g. spinal TB (TB spondylitis) (3).

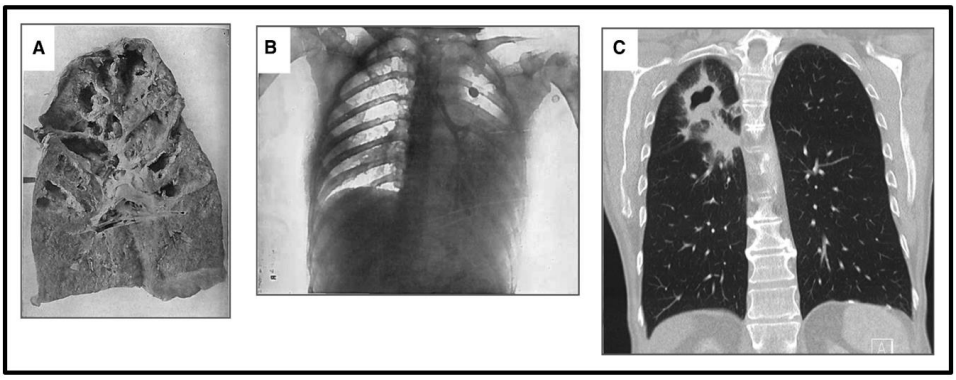

Figure 1. Pulmonary tuberculosis. A) Lung autopsy showing a cavern (top) and miliary tuberculosis. B) Chest X-ray (CXR) with contrast shows a cavity in upper left lobe (dark spot) and bronchiectasis. C) CT-scan of lungs showing pneumonic infiltrates and a cavitating lesion in the upper right lobe.

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(The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society)

Archeologic findings in Europe from the middle ages have confirmed the occurrence of TB at that time. In the 17th and18th centuries TB incidence rose in parallel with the increasing population and did not start to decline until the 20th century. The explanation for the decreasing prevalence numbers is not fully understood, but is thought to be an effect of improving socioeconomic standards and herd immunity (5). Sanitary institutions were used as centres to care for TB patients with fresh air and nourishment. Induced lung collapse (pneumothorax) or lobe collapse were alternative treatments at that time and could be

effective in about half of the patients, but was associated with a high mortality rate of around 25% from the procedure (Figure 2) (4).

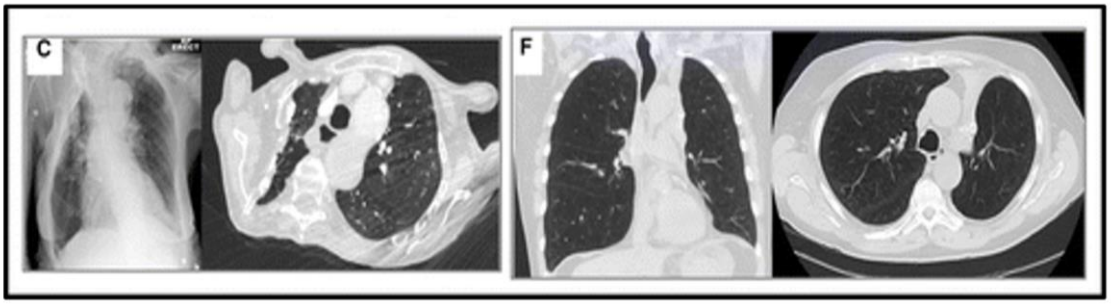

Figure 2. CXR and CT-scans of lungs status post-TB infection. C) Right-sided thoracoplasty. F) Induced collapse (pneumothorax) of left upper lobe after insertion of endobronchial valve.

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PTB has been described in various ways such as ”consumption” or ”the white plague” in the literature, for example in romanticized ways in The Magic Mountain by Thomas Mann and in poems by John Keats (6). Other artistic ways of presenting the disease was by painting, e.g. as Edvard Munchs famous painting ”The sick child” representing his sister dying from TB (front page) and in music, as for example in Puccini´s is opera La Bohème where the heroine Mimi dies a tragic death in TB.

The modern history of human TB started with Jean-Antoine Villemin who infected rabbits with pus from a tuberculous cavity and Robert Koch, who discovered the infectious agents *Vibrio cholera*, *Bacillus anthracis* and Mtb in 1882, the latter for which he received the Nobel prize in 1905 (7). Robert Koch also prepared a mixture of Mtb strains, which he called tuberculin and used as an intradermal injection initially intended to cure the disease (8). However, the treatment aroused a scandal due to serious adverse events and had no effect against disease. The purified protein derivative (PPD), a sterile solution of protein fractions precipitated from a filtrate of tubercle bacilli was developed by Florence Seibert in 1924 to be used for diagnostic purposes (9).

Other important discoveries followed during the 20th century, such as the development of the Bacille Calmette-Guerin (BCG) vaccine in 1921 from the Pasteur Institute, which originated from an attenuated strain of *Mycobacterium bovis* (10). The use of the vaccine spread widely across the world and has been distributed to millions of people until today. However, the protection against TB has been difficult to estimate and has been disputed through the years. According to a meta-analysis of 40 heterogeneous vaccine studies, the protective efficacy varied between 0-80% (11). Results from this analysis differed due to many issues, such as

different diagnostic measures used, the handling of the vaccine, malnutrition in the population and high or low endemic settings. A more recent systematic review of vaccine trials (n=24) compared BCG vaccinated to unvaccinated individuals including all ages (12). A higher protective efficacy was found for disseminated (miliary/meningeal) TB as compared to PTB (92% and 84%, respectively) in young children and in neonates (90% and 60%, respectively) if they were tuberculin skin tested (TST) negative before vaccination. There was also evidence of higher protective efficacy in trials further away from the equator where the incidence of environmental mycobacteria was lower. The authors thus concluded that sensitization to environmental mycobacteria could interfere with vaccine efficacy.
The Public Health Agency of Sweden recommend BCG vaccination in children (<18 years) from families originating from areas where the TB incidence exceeds 25/100 000 cases per year (13).

From the 1940s chest X-ray (CXR) was used to screen for PTB, first in soldiers and then in the general population in Europe. UNICEF and the Danish Red Cross started control programs with TST and administered a subsequent BCG vaccination if the individual test was negative.

Waksman and Schatz discovered streptomycin in 1944 which together with para-amino-salicylic acid (PAS) were the first effective drugs used to treat TB. PAS was developed by a Swedish professor in biochemistry, Jörgen Lehmann (14). Waksman was awarded the Nobel prize in 1952 for his discovery (5). When the drugs were used as monotherapies, resistance developed within 3 months. However, it was found that with a combination of Streptomycin and PAS no resistance developed (15), even though adverse effects were common (16). The discovery of other combination therapies including isoniazid (INH) (1952), pyrazinamide (PZA) (1962), ethambutol (EMB) (1962) and rifampicin (RIF) (1969), led to better tolerated, shorter regimens and are all still included in today's first line treatment options of TB (17).

1.2 EPIDEMIOLOGY AND ELIMINATION

TB is the single deadliest infectious disease with the highest mortality globally, affecting 10 million people in 2017 and killing 1.3 million with 300 000 deaths among TB and HIV co-infected patients (18).

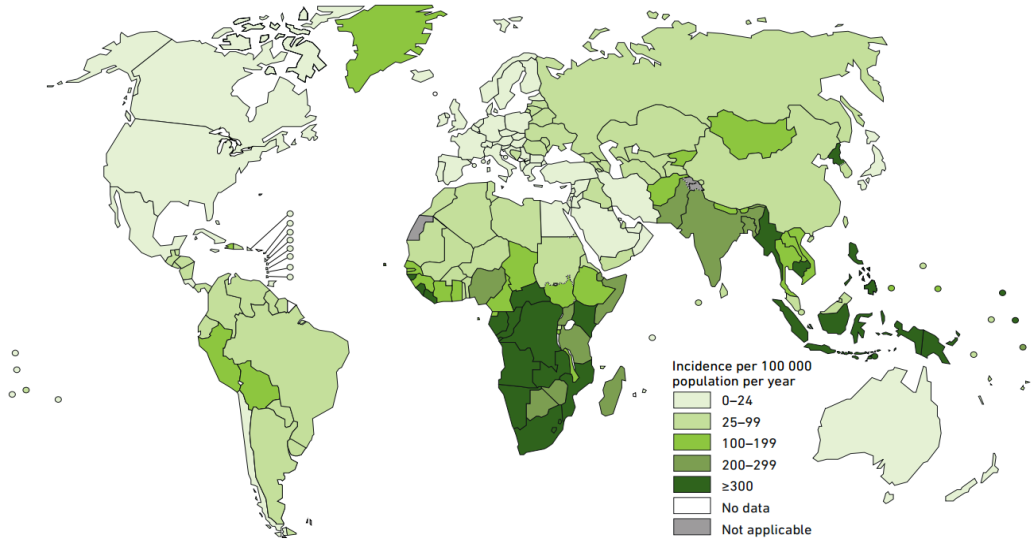

Figure 3. Estimated TB incidence rates, 2017.

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The World Health Organization (WHO) plays an important role in directing global strategies for TB diagnosis, treatment and prevention. The huge toll of TB on human life prompted WHO to declare TB a "global emergency" in 1993, the STOP TB strategy in 2000 and the END TB strategy in 2015. These are frameworks for how to strengthen health care and the economic and social situation in nations affected by endemic TB. By the year of 2035 WHO aims for a 95% reduction in TB mortality and a 90% reduction in the incidence rate. Efforts to improve TB prevention, care and research are important, as well as education and protection of human rights (18). The frameworks should be adapted to each country and the development of guidelines and implementation of strategies should include all levels of society, such as governments, civil society organizations, communities, local hospitals and health care centres. Catastrophic costs (>10% of average annual income) (20) for all families affected by TB should be avoided.

Treatment success of drug sensitive TB should be above 90% according to WHO, but in a meta-analysis of 34 studies from Ethiopia it was found to be lower, 86% (51-95%) (21). Old age, HIV infection, reinfection and treatment in rural areas were factors associated with a poor outcome. Directly observed therapy (DOT) has been an effective way to strengthen adherence to therapy and is currently used in many countries. This method is implemented by health care worker surveillance of the intake of the drug in person or with new digital techniques (22). The rapid development of multi-drug resistant TB or extensively drug

resistant strains and the close link to the HIV pandemic often lead to increased mortality rates due to less effective treatment regimens (23) (24). The lack of an efficient TB vaccine and cheap, specific and sensitive point-of-care diagnostic methods for TB are other obstacles.

In Sweden, a TB low-endemic country, the Public Health Agency reported 734 cases of active TB in 2016 and 533 cases in 2017 (25). Around 90% percent of cases were migrants from TB high-endemic countries.

Around one fourth of the global population is estimated to be latently infected by Mtb, according to studies performed with TST (26). In this stage the bacterial burden is too low to be cultured and there is no reference test for diagnosing the infection. Around 5-15% of Mtb infected individuals develop TB during life, with the highest incidence within 2-5 years and recent infections are thus a reservoir of potential TB cases (27). Remote infections more seldom progress to disease, but the relative risk of developing TB is increased among certain groups (Table 1) (28). Preventive treatment with either INH and/or RIF is recommended by WHO in recently infected and in certain risk groups (26). A meta-analysis compared 13 studies of patients diagnosed with LTBI and treated with INH, to placebo. Treated patients showed a lower odds ratio (OR) at 0.64 (confidence interval (CI) 95%) (0.48 – 0.83) for subsequent active TB after treatment (29). In Europe, the incidence is lower than in many other parts of the world and the disease is concentrated in populations at the lower end of the socio-economic scale. Recent European Center for Disease prevention and Control (ECDC) recommendations therefore include specific efforts for strengthening TB prevention and control among these groups, such as mobile units and outreach teams for active case finding, screening and treating people (30). Target groups for screening are homeless people, high-risk drug and alcohol users, people in prison and vulnerable migrant populations excluded from health and social care services.

The American Centers for Disease control and prevention (CDC) recommend testing and treating of household contacts, children <5 years, HIV infected, patients on immunosuppressive treatment, with silicosis and detection of an abnormal CXR consistent with untreated prior TB (31). WHO guidelines on LTBI are more specific and recommend testing and treating of LTBI as above, but also in HIV infected individuals irrespective of grade of immunosuppression and antiretroviral treatment including pregnant women living with HIV, before initiating anti-tumour necrosis factor alpha (anti-TNF-α) treatment, during dialysis and in organ or hematologic transplantations. WHO also recommends testing and treatment of LTBI to be considered in low-endemic countries for prisoners, health-care workers, migrants from TB high-endemic countries, homeless people and illegal drug users (26). An interferon-γ release assay (IGRA), TST or a combination of these assays should be used to test for LTBI.

Table 1. Conditions increasing the risk for progression of TB and odds ratio (OR*) or relative risk (RR**) modified according to Erkens et al (28).

Condition	OR* or RR**
HIV and positive TST	50-100
AIDS	110-170
Organ transplantation	20-74
Anti-TNF-α treatment	1.5-17
Corticosteroids >15 mg/d, >2-4 weeks	4.9
Hematological malignancy	16
Carcinoma of head, neck or lung	2.5-6.3
Gastrectomy	2.5
Jejunoileal bypass	27-63
Silicosis	30
Chronic renal failure/hemodialysis	10-25
Diabetes Mellitus	2-3.6
Smoking	2-3
Excessive alcohol use	3
Underweight	2-2.6
Age <5	2-5

Screening for active and latent TB in Sweden is recommended for contacts to PTB, in migrants from TB high-incidence regions, in HIV infected individuals and in other immunosuppressive conditions due to the increased risk of LTBI reactivation, such as prior to treatment with biologic drugs (TNF-α receptor inhibitors) for autoimmune diseases (32) and in pregnancy (33). For asylum seekers a voluntary health examination is offered, but uptake of this screening strategy varies greatly in Swedish counties and both active TB and preventable TB cases arising from recent LTBI may be missed (13).

1.3 TRANSMISSION

Transmission of TB is airborne and infection starts with an individual inhaling the small droplets (<5 µm) in an aerosol produced by an individual with active PTB coughing, sneezing, laughing or singing (34). The bacilli are inhaled and start to replicate within the alveoli (35). The probability of becoming infected upon exposure to Mtb is mainly exogenous in nature, while the probability of developing active disease after infection is mainly endogenous in nature (36). If PTB disease develops in the new host and Mtb is detected in sputum, the circle of transmission is completed (Figure 4). Patients with merely extra pulmonary foci, such as in lymph nodes, skeleton, kidneys and brain, are not contagious. Each patient with smear microscopy (SM) positive PTB on average infects 10 individuals per year. Around 5-15% of the infected individuals develop the disease in their lifetime (14) (37).

Consequently, screening to identify Mtb infected or symptomatic individuals with active TB is an important measure in TB control in low-endemic countries (26).

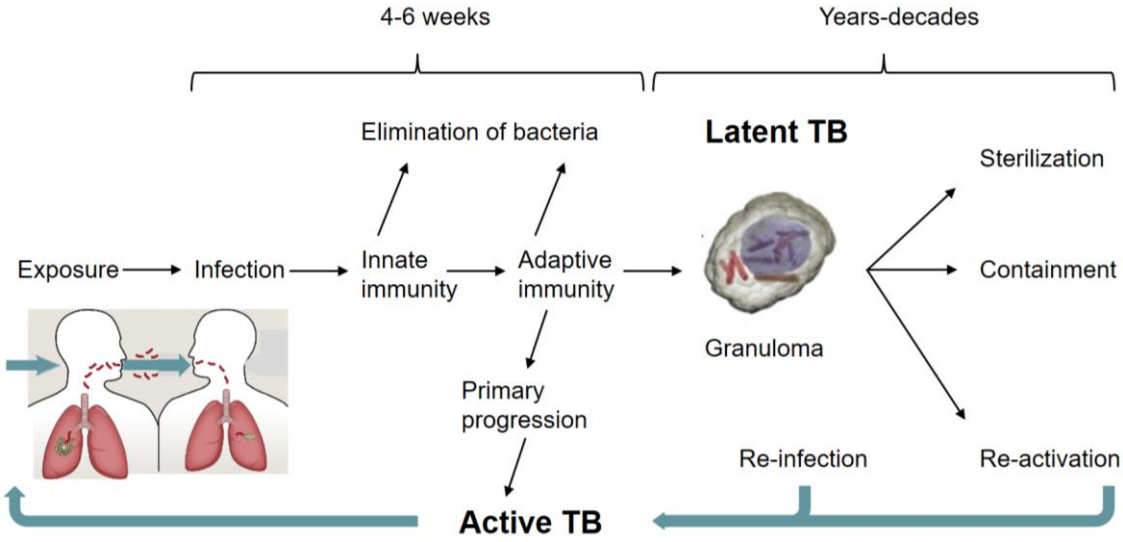

Figure 4. The natural history of TB infection.

1.4 THE PATHOGEN

The initial stage of Mtb infection is called a primary infection (14). This is a non-contagious, often asymptomatic stage, but can present with fever, erythema nodosum and polyarthritis (38). Mtb is at this stage localized in a granuloma in the lung together with enlarged hilar lymph nodes, the so called primary complex. In about 90% of infected individuals, a balance between bacilli and immune responses will develop causing LTBI (Table 2). After some time, the granuloma may involute to a fibrotic lesion, sometimes with calcification and can then be visible on a CXR (39).

When homeostasis is not reached the infection may proceed directly to disease, so called primary progressive active TB. Endogenous reactivation of LTBI after 1-2 years of homeostasis causes so called post primary TB (39). The long asymptomatic stage of early TB

disease, during which Mtb multiplies in the host, is called incipient TB (which occurs prior to clinical presentation of active disease) (18).

Table 2. Case definition by WHO (26).

Latent tuberculosis	A state of persistent immune response to prior-acquired Mtb antigens without signs of clinically manifested TB.
Tuberculosis suspect	Symptoms suggestive of TB (cough, shortness of breath, chest pain, hemoptysis) and/or constitutional symptoms (loss of appetite, weight loss, fever, night sweats, and fatigue).
Case of tuberculosis	A definite case of TB (defined below) or one in which a health worker has diagnosed TB and has decided to treat the patient with a full course of TB treatment.
Definite case of tuberculosis	A patient with Mtb identified either by culture, molecular line probe assay or a pulmonary case with one or more sputum smear examinations positive for acid-fast bacilli.

The group of *Mycobacteria* is a genus of *Actinomycetes* where the Mtb complex causes human and mammalian TB *(*Mtb*, Mycobacterium bovis* and *Mycobacterium africanum*) but the most common cause of human infection is Mtb (14). Mycobacteria are also related to *Corynebacterium* and *Nocardia* (40). Mycobacteria are aerobic gram positive rod shaped organisms with a complex cell envelope rich in carbohydrates and lipids consisting of long-chain mycolic acids, a highly branched arabinogalactan polysaccharide and a network of peptidoglycans (41). An outer membrane contains inert waxes and glycolipids and an outermost capsule is composed of polysaccharides and proteins (42). The outer wall also contains peptidoglycans with polysaccharides attached to fatty (mycolic) acids. The outer wall makes the organism acid-fast, i.e. resistant to decolourisation with acidic alcohol (Figure 5).

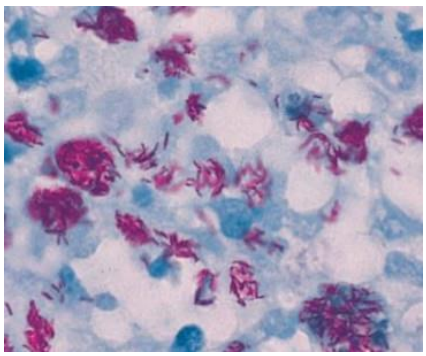

Figure 5. *Mycobacterium tuberculosis* in a sputum smear (43).

The sputum smear is stained for acid-fast bacteria with red colour (Ziehl–Neelsen stain).

The ESAT-6 secretion system (ESX-1) is a protein complex in the cell wall which forms a pore in the Mtb cell envelope for secretion of proteins and host–pathogen interactions (44, 45). This complex secretes among others two antigens; culture filtrate protein 10 (CFP-10) and early secretory antigenic target 6 (ESAT-6) which are of importance for Mtb virulence and cell membrane lysis (46).

Mtb are ingested by dendritic cells, neutrophil granulocytes and alveolar macrophages initiating an inflammatory process by secreted pro-inflammatory chemokines and cytokines (47, 48). The immune response either eliminates the bacteria, controls them within a granuloma (LTBI) or is unable to control further growth with multiplying bacteria.

The antigen presenting dendritic cells carry Mtb to the draining mediastinal lymph nodes which primes naïve T- and B-lymphocyte proliferation (49). Lymphocytes and macrophages then migrate to infected pulmonary tissue at the site of infection. Here the slowly replicating bacteria are surrounded by foamy macrophages, epithelioid cells and multinucleated giant cells. The lymphocytes surround these cells, all together forming the granuloma (Figure 6) (50).

Thus, both innate and acquired immunity mount important immune responses against Mtb. The granuloma can persist for decades in the body without progression, but the immunological balance between the invading organism and the immune system is a complex process and can easily be disturbed by immunosuppression, such as HIV infection, thus increasing the risk for TB activation (51).

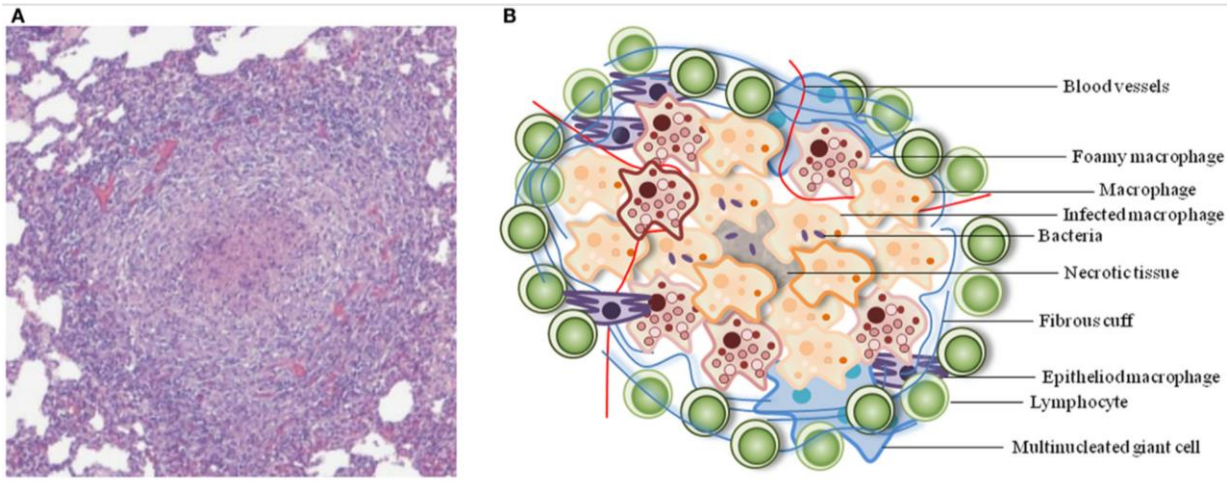

Figure 6. Typical architecture of a TB granuloma.

A) Granuloma with central necrosis and septa starting to surround the granuloma. Histologic sample stained by haematoxylin-eosin from a minipig model (52).
B) Schematic of the cellular constituents of a TB granuloma.

Reprinted with permission from Frontiers of Immunology (53).

1.5 CLINICAL FEATURES OF ACTIVE TB

Post primary TB typically affects the upper lobes of the lungs with infiltration or cavity formation, but can in extra pulmonary TB (EPTB) affect many other sites in the body as well, most often in the lymph nodes on the neck or in the hili or cause osteitis (14). Uro-genital TB is an example of a late manifestation (54). Haematogenous dissemination of Mtb can cause miliary TB, spread throughout the body, for example infect the bone-marrow or cause TB meningitis. The latter examples are severe forms of the disease and are often seen in young children or immunosuppressed individuals (55).

Mortality from TB is high, if it is not correctly treated. Studies from the early 20th century found a mortality rate of about 70% in people with sputum microscopy positive (SM+) PTB. Among sputum microscopy negative (SM-) cases this figure was around 20% (56).

1.6 HOST IMMUNE RESPONSES

1.6.1 Innate immunity

Once Mtb comes into contact with dendritic or alveolar macrophages, microbe-associated molecular patterns in the Mtb cell wall are recognized by pattern recognition receptors (Toll-like receptors and C-type lectins) on these cells (57).

Different pattern recognition receptors recognize distinct molecules of Mtb and these interactions trigger phagocytose of the invading bacilli and a pro-inflammatory response that is supposed to control the infection (58).

Cytokines and antimicrobial factors are produced which act together in preventing Mtb replication by complement activation, neutrophil- and macrophage induction (59).

Important cytokines in Mtb infection induce fever (IL-1β and IL-6), activate macrophages and form granulomas (TNF-α), attract neutrophils (IL-17) and NK-cells (IL-18) (60).

However, Mtb engulfed by macrophages can efficiently avoid cell-mediated immune activation and survives by several advanced mechanisms, e.g. by blocking phagosome maturation and fusion with the lysosome (47).

1.6.2 Adaptive immunity

When the antigen presenting cells (APCs) present Mtb to $CD4^+$ T cells, IL-12 and IFN-γ secretion drive T cell differentiation towards a T-helper type 1 (Th1) inflammatory response. Other cytokines, such as TNF-α, IL-2 and GM-CSF are then produced by both $CD8^+$ and $CD4^+$ T cells and promote further inflammation (61-63). IL-22 is a cytokine which has been previously underestimated in mycobacterial immunity. It derives from both innate and adapted immune cells and is important in local immune responses against mycobacteria by activating lung epithelial cells (64). There are also important cytokines, such as IL-10 and TGF-β which are anti-inflammatory and control the balance in the immune system to prevent excessive tissue damage.

An example of the importance of cytokine-induction in mycobacterial immunity is an inherited disorder, Mendelian susceptibility to mycobacterial infections, where the IFN-γ-IL-12 pathway is defective, leading to severe mycobacterial infections (65).

1.7 MTB ANTIGENS

During recent years the sequencing of the mycobacterial genomes revealed Mtb as the original mycobacterial strain, preceding the development of *M. bovis*. When Mtb was transmitted to cattle, certain genes were lost, such as the region-of-difference 1 and 11 (RD-1, RD-11) and new, less virulent strains evolved (66). Mtb transcripts from the RD regions include the previously mentioned ESAT-6, CFP-10 and also the TB antigen 7.7 (TB 7.7).

These proteins are absent in attenuated *M. bovis* BCG vaccine strains and also absent from most environmental mycobacteria, except for *M. kansasii, M. szulgai, M. marinum* and *M. riyadhense*. ESAT-6 and CFP-10 form a stable complex as they are secreted from Mtb (67). In contact with a macrophage they dephosphorylate a proteome which upregulates toxic hydrogen peroxide production (H_2O_2) for the oxidative burst. The lack of H_2O_2 thus allows live bacilli to reside inside macrophages (66). These antigens stimulate specific cell-mediated immune responses in Mtb infected persons, measurable as release of IFN-γ (68). ESAT-6 has also been shown to have dual functions inducing both a long-term inflammatory response and a T cell deactivation by down-regulating MHC class I molecules (69).

The antigen 85 family derives from 3 different genes encoding Ag85a-c. Ag85a (Rv3804c) and Ag85b (Rv1886c) are immunoprotective antigens found in high levels inside the phagosomes of infected macrophages (70). Here they act by hindering the maturation of phagolysosome enabling the survival of intracellular Mtb. The antigens have also been studied as potential biomarkers for LTBI (71). These antigens induce cell mediated immunity and have been tested together with either CFP-10 and ESAT-6 as new vaccine candidates in several vaccine studies (72-74). Ag85b induces strong humoral and cell-mediated immunity in mice, when inserted into *M. bovis* BCG (75) and protects against air-borne Mtb transmission when used as a booster vaccine in already BCG vaccinated guinea-pigs (76).

Rv0287 and TB10.4 (Rv0288) also belong to the ESX-1 group and play important roles in pathogenesis of Mtb (77). Ag85b and Rv0288 induced $CD4^+$ and $CD8^+$ specific responses in African children, as detected by flow cytometry (78). Vaccine studies have also been performed in cell cultures and mouse models combining Rv0288 with either CFP-10 or ESAT-6 (79, 80). Rv2710 (equal to RNA polymerase sigma factor B) is induced under stress and nutrient starvation (81) and upregulates expression of several genes, of which the protein-products often are parts of the cell wall (82).

Hypoxia in the granuloma induces a transcription factor encoded in the mycobacterial genome called the dormancy survival regulator (dosR) (Rv3133c), which can alter gene expression and activate a large repertoire of at least 48 “survival genes” (83). Latency associated antigens are the protein-products of intracellular Mtb bacilli adapting to a reduced level of oxygen, low pH and high levels of nitric oxide and carbon monoxide (84). These antigens are proteins involved in lowering the Mtb replication rate. A number of them are inducers of cell mediated immune reactivity in Mtb infected patient blood samples. Rv1284 and Rv2659c are examples of these antigens studied by Andersen and co-workers. The antigens induced IFN-ɣ responses which fluctuated over time in individuals with suspected LTBI (QFT positive), but these responses were not associated with a less risk of TB within 2-3 years (85). Rv2659c was also recently studied in a Chinese population and induced significantly higher IFN-ɣ secretion in subjects with suspected LTBI, compared to BCG-vaccinated controls and individuals with active TB (86). The latency associated antigens Rv2031 and Rv1733 have potential as immunodiagnostic markers for LTBI (87, 88). Goletti and co-workers found that Rv2628 gave a stronger IFN-γ response in patients with remote

TB infection, compared to the recently infected individuals (89). Rv2626c was found by Pena and co-workers to induce IFN-γ production in patients without evidence of active TB (90).

However, several pitfalls have been described in studies of the performance of these antigens as diagnostic tests for LTBI, the lack of a reference assay for the diagnosis of LTBI being of main importance. A problem, not yet investigated in latency associated antigens, is a possible cross-reactivity with BCG-strains and environmental mycobacteria.

1.8 DIAGNOSIS

1.8.1 Diagnostic methods of LTBI

The current diagnostic methods of LTBI are based on immune reactivity to Mtb antigens. The TST is performed by intradermal injection of PPD tuberculin (Figure 7) (9). The immune system of the Mtb infected individual reacts with a cellular delayed type of hypersensitivity that causes a swelling at the site of antigen injection. The size of the swelling in mm is then read after 48-72 h. Swellings of a certain size of >5 mm or 10 mm are considered positive, with the lower level used in immunosuppressed patients, who cannot mount strong immune responses.

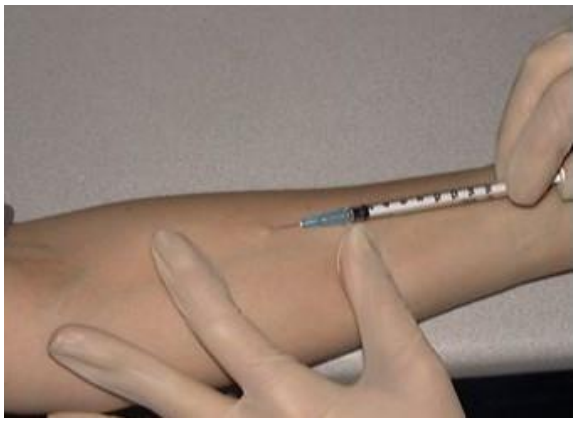

Figure 7. The tuberculin skin test (TST).

The TST has several problems and disadvantages such as borderline results of 6-9 mm which can be explained by non-specific reactions to environmental mycobacteria, immunosuppression or BCG vaccination. The latter in particular causes false positive responses if vaccination is performed after 2 years of age or after revaccination (91). Poor sensitivity is seen especially in children and immunosuppressed patients.

The subjective nature of both administering the tuberculin injection and then measuring the swelling on the skin, leads to variability. Individuals also must return two or three days later to have the test read, which hampers compliance.

More specific tests for detection of Mtb immune response have been developed, measuring IFN-γ release by leucocytes after short-term incubation with different immunogenic Mtb antigens. These assays are performed in vitro from whole blood and are called IGRAs. (92).

In 2001, the QuantiFERON-TB test (QFT) (Cellestis Limited, Carnegie, Victoria, Australia) became the first commercially available IGRA measuring IFN-γ by enzyme-linked immunosorbent assay (ELISA), using short term incubation with PPD (93). In 2005, the company improved QFT by replacing PPD with synthetically produced peptides from the RD regions 1 and 11; ESAT-6, CFP-10 and TB7.7. The test was renamed the QuantiFERON-TB Gold test (QFT-G) and was later developed to QuantiFERON Gold In-tube (QFT-GIT) (Qiagen), which is now the most widely used and evaluated assay for LTBI (92). Whole blood is collected into three tubes, one of them containing Mtb antigens, one PHA (positive control) and one negative control tube without stimulant. The control tubes discriminate negative responses from anergy or false positive responses.

QFT-GIT was more recently developed into QFT-Plus with an additional antigen tube containing unknown peptides stimulating both $CD4^+$ (tube 1 and 2) and $CD8^+$ (tube 2) T cells. TB7.7 was removed from the assay in this format and this makes comparisons with the previous format more difficult (94). QFT-Plus has been described to be of advantage compared to QFT-GIT in patients with immunosuppression, e.g. in individuals planned for solid organ transplant (95). Two small studies showed that $CD8^+$ T cell responses were strong in recent TB exposure; Barcellini and co-workers also evaluated the test in 129 TB exposed patients, showing a stronger concordance with recent TB exposure than for QFT (96). Another smaller study detected higher $CD8^+/CD69^+$/IFN-ɣ+ T cells in a few individuals out of 14 recently exposed individuals, after stimulation with QFT antigens compared to patients with active TB and BCG vaccinated healthy controls (97).

As for the role of $CD8^+$ T cells in active TB, two combined immunological measures, Mtb specific single $CD4^+$ T cells producing high TNF-α and the detection of high Mtb specific $CD8^+$ responses, were tools which distinguished active TB from LTBI (98). Another small study, showed stronger IFN-γ responses in the additional tube 2 from QFT-Plus in patients with active TB, compared to LTBI, but few comparative studies have been performed and a variability around cut-off may be expected as in QFT-GIT (99).

T-SPOT.TB is a commercial enzyme-linked immunospot (ELISPOT) assay (Oxford Immunotec) based on short-term incubation and detection of lymphocyte derived IFN-γ responses to ESAT-6 and CFP-10. The test is used in particular for LTBI testing in immunosuppressed patients and small children (13).

The drawbacks of the IGRAs as with the TST are that they cannot differentiate between active disease, remote or recent LTBI or a mere memory of previous infection (100, 101). There is also a risk of false negative results in immunocompromised patients with indeterminate responses with negative reactions in the positive control tube (102). Another concern with IGRAs is inconsistency with conversions and reversions in patients who are

tested and retested. Sources of variability can be classified as pre analytical (shaking of tubes, amount of blood), analytical (incubation duration), post analytical (analytical error), manufacturing defects or immunological differences in patients (103, 104). Results close to cut-off levels in these tests are often the most variable. The current recommendation from several authorities is therefore to retest individuals with borderline results (103, 105).

The value of IGRA tests and TST in LTBI to predict risk of progression to TB has been previously studied in both high and low endemic settings (106). Results show low positive predictive values (PPV) for both TST 1.4-3.3%, QFN 1.5-14.3% and T-SPOT.TB 3.3-10% in studies with varying observation times (1-5 years). The large variations in these studies probably depend on different follow up times, defined study groups (healthy adolescents, patients with silicosis, house hold contacts or large cohorts) and the numbers included in the studies (107). QFT PPVs correlated well with the corresponding values for TST, in contact-screening in a high TB burden setting (43). Another large meta-analysis for risk of TB activation within two years showed TB activation for TST and QFT at 1.5% and 2.7% respectively for all included, but in high-risk groups 2.4% and 6.8% respectively (108). When high QFT results (>4 IU/mL) were evaluated for PPVs of incipient TB, the results have been equally poor at 2.5% in general and 2.9% in patients with any medical risk factor (109). These low PPVs demand high numbers needed to treat (NNT) to prevent one case of active TB. On the other hand, negative predictive values (NPV) are very high (>99%), keeping in mind that indeterminate and false negative results are more common in immunosuppressive conditions (110, 111), with risk of missing patients that might benefit from preventive treatment.

The IGRA tests are highly specific of Mtb infection tested in cohorts of healthy blood donors and children with respiratory infections or non-mycobacterial lymph node infections, at 98-100% and these results are better than for TST (88%) (112). In immunosuppressed patients, both T-SPOT.TB and QFT have been shown to give a higher rate of positive reactions than with the TST, which has been confirmed in several studies of for instance HIV infected or pregnant individuals (113-115). The agreement between the different test results in people living with HIV is low and both TST and IGRAs are important as parts of a risk assessment (116). Negative test results however, in people living with HIV, do not rule out LTBI (26).

The currently used assays for LTBI are insufficient to target individuals with LTBI with a higher risk of progression to active TB (94). Neither for those with a recently acquired Mtb infection (within 2-5 years), nor for remotely infected individuals (>5 years ago) is it possible to distinguish an individual with incipient TB, from those with immunological control of the infection.

Sensitive biomarkers are needed to identify those at increased risk of TB activation. More specific tests for recent LTBI are also urgently needed, as well as easier point-of-care tests to eliminate TB globally (94, 117). An ideal test of incipient TB, such as progression of primary disease or reactivation of LTBI would likely differentiate the various stages from LTBI to

active disease (118). Such biomarkers would be of great value to target at risk individuals for preventive treatment, particularly in a TB control program in low or middle endemic settings.

1.8.2 Diagnostic methods of active TB

The cheapest and most commonly used diagnostic method worldwide for detection of PTB is microscopy for acid-fast bacilli in a stained sputum smear, with around 50% sensitivity of culture verified Mtb at most (119). It detects 5 000-10 000 bacilli/ml sputum (120). PCR for Mtb is another quick method and more sensitive than SM. Cultures on solid media (Löwenstein Jensen) or liquid cultures (BACTEC MGIT TB) are the most sensitive methods to diagnose TB with >80% sensitivity and >98% specificity and thus, serve as reference tests (119). These cultures detect very low concentrations of 10-100 bacilli/ml sputum. The liquid cultures are more rapid (mean 14 versus 24 days), more expensive and more sensitive (96%) than cultures on solid media (72%) (121, 122).

The WHO also recommends rapid and sensitive molecular tests after screening for symptoms of TB (18) but so far the only recommended point of care test for TB diagnosis is the Xpert MTB/RIF assay (Cepheid, USA). This test is widely used and provides results in less than 2 h, including resistance mutations for RIF. The test procedure requires a Gene Xpert platform which is more expensive, but has a higher sensitivity of 89% (range 58-100%) compared to direct microscopy and a specificity of 99% according to a Cochrane analysis of 22 studies (123, 124). Even though the initial cost for laboratory equipment for the assay was higher than for microscopy, it was cost-effective due to higher sensitivity and thus, a lower transmission of TB by the index case (125).

The reference method for detecting resistance mutations globally is phenotypic sensitivity testing in a culture (126) and several molecular assays are now widely used to detect multidrug resistant TB in low-income settings. PCR testing and molecular line probe hybridization assays on SM+ smears are performed directly, or after Mtb culture. There are other assays for RIF resistance detection (INNO-LiPA Rif.TB assay, Innogenetics NV, Gent, Belgium), for RIF, INH, EMB (Genotype MTBDRPlus, Hain Lifescience, GmbH, Nehren, Germany) and for second-line drugs (Genotype MTBDRsl); fluoroquinolone, streptomycin, amikacin and capreomycin resistance (127).

The INNO-LiPA Rif.TB assay has a sensitivity for RIF resistance detection of around 87% when used after DNA extraction from SM+ samples (128). The Genotype MTBDRsl assay has a higher sensitivity for RIF (99%), INH (82%) and fluoroquinolones (90%) but much lower for EMB, capreomycin and amikacin on a sputum sample. The specificity for Genotype MTBDRsl was 90-100% (129).

In active TB the use of radiological methods such as CXR, computerized tomography scan (CT-scan) or magnetic resonance imaging are additional tools. However, radiological methods are expensive and not commonly available in low-income settings. In EPTB fine needle biopsies from affected organs can be performed followed by PCR, Mtb culture and

histopathology if available (14). A diagnostic obstacle is to confirm TB diagnosis in children and immunosuppressed individuals due to often paucibacillary infections and there is a need for invasive techniques for sampling (130).

1.8.3 New diagnostic methods

A new diagnostic method for LTBI is a skin test developed with ESAT-6 and CFP-10 antigens instead of tuberculin. This combination gives a higher specificity (94, 131) than the ordinary TST in individuals with suspected LTBI.

Rapid point-of-care assays for active TB are also being further developed such as the detection of the Mtb induced interferon-inducible protein-10 (IP-10) in dried blood-spots from patients with active TB (132-134). Another rapid test is being further developed, based on detection of lipoarabinomannan, a carbohydrate antigen from Mtb, present in the urine of patients with active TB (135).

Transcriptomics is an area using genome-wide gene analyses, measured as RNA expression, to identify biomarkers. Zak et al identified a whole blood signature of 16 genes which could predict the risk of progression of Mtb infection to active disease. The sensitivity was 66% to detect progression and the specificity was 81% (136).

Another study of whole blood from patients with active TB, LTBI and pneumonia used 119 RNA transcripts for microarrays (137). The results showed a high sensitivity in distinguishing active TB from LTBI or pneumonia (97%) with this platform, but with a lower sensitivity in the HIV positive and African patients (85-90%). Specificity was around 78% for all groups except for LTBI, where it was 40%. The high sensitivity for TB could make the test useful for active TB, even though there are other tests which are easier to perform in this group.

A method to detect breath associated diagnostic metabolites by liquid chromatography has been tested in a pilot study of individuals with TB symptoms (n=50), where TB diagnosis was microbiologically verified (n=32). This method identified 23 mostly hydrocarbon molecules as biomarkers for TB. Detection of these molecules in patients with TB showed a sensitivity of 100% and a specificity of 60% (138).

Another method using chromatography is a new immunochromatographic strip containing antibodies to CFP-10 and ESAT-6 labelled with colloidal gold particles. This assay detects TB at low cost, is sensitive (100%) and rapid (15 min), but has to date only been tested in 38 Mtb culture positive individuals (139).

2 AIMS OF THE PRESENT STUDIES

I. To improve the diagnosis and differentiation of active TB and LTBI. The FASCIA (flow cytometric analysis of cell-mediated immunity in activated whole blood) was established for TB antigens in a cohort of patients with suspected TB by comparing the results with TST and IGRAs.

II. To distinguish the different stages of TB disease by evaluating cytokine profiles and study the balance between the induction and suppression of immune response signaling that appears to be of importance for the clinical outcome of Mtb infection.

III. To develop a mathematical model to estimate the probability of recent and remote LTBI in individuals exposed to PTB and compare these estimations to their TST and IGRA results.

IV. To evaluate $CD4^+$ T cell proliferative responses as immunological biomarkers in different stages of Mtb infection; Remote LTBI, Recent LTBI, TB disease and TB negative individuals as predicted by the mathematical model, to target those with highest risk of progression e.g. recent LTBI for preventive treatment.

3 PATIENTS AND METHODS

3.1 SETTINGS

Two prospective clinical cohort studies were performed in a TB low-endemic setting (TB incidence <100/100 000) between 2006-2012 at the Karolinska University Hospital in Sweden.

3.2 PATIENTS

3.2.1 Study populations and case definitions (Papers I and II)

Individuals with strong enough suspicion of active TB to send a sample to the mycobacterial laboratory were consecutively enrolled after written consent (n=179). For 18 individuals, samples were either missing or contaminated and a few missing medical records and a duplicate inclusion of one patient (Figure 8). Exclusion criterion was already initiated TB treatment (>1 week). Venous blood samples were collected and data regarding gender, origin, age, previous mycobacterial infection, BCG vaccination, previous or current TST results if known, clinical symptoms and immunosuppression were collected in a questionnaire. Results from routine diagnostic investigation were collected in a database and samples were sent to the former Swedish Institute for Infectious Disease Control for further analyses.

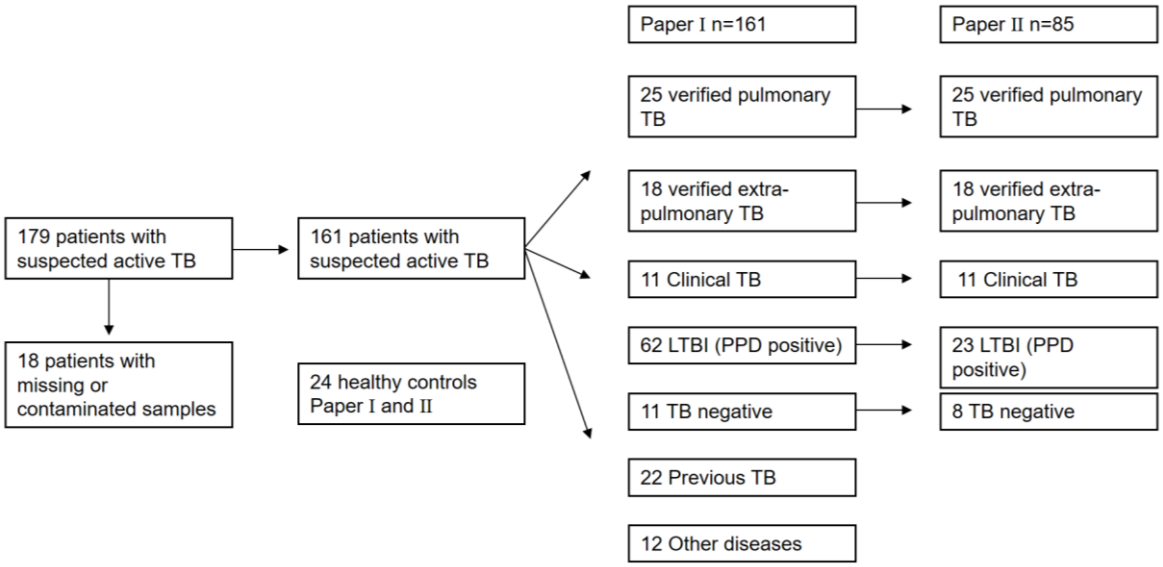

Figure 8. Flowchart for enrolment and final diagnosis of patients in papers I and II.

Health care students (n=21) at Karolinska Institutet (KI) and laboratory personnel (n=3) were enrolled as negative controls (n=24). Inclusion criteria were no TB exposure, no previous TB history, no stay in a TB endemic country (outside Western Europe, Australia and North America) for >3 months, not having worked more than a year in a prison, hospital or with

migrants and no previous environmental mycobacterial infection. The exclusion criterion was TST ≥6 mm.

TB diagnosis was verified in all cases by clinical symptoms combined with either microbiological verification by microscopy, Mtb PCR and Mtb culture in airway samples and/or with a histopathological typical picture with granulomatous inflammation of lymph nodes at the Karolinska University Hospital.

Clinical TB was defined as suggestive clinical symptoms, typical radiology and response to TB treatment, but with no microbiologic or histopathologic verification of TB.

Latent TB in these two studies was defined as previous exposure to TB and a positive TST and with no clinical sign of active TB.

Previous TB was defined as a history of TB disease and/or radiological findings indicating a past and cured infection.

TB negative was defined among contacts as having no previous or recent exposure to TB and a negative TST.

Other diagnoses were individuals that did not fit into any of the other groups.

3.2.2 Study populations and case definitions (Papers III, IV and preliminary results)

In these studies, PTB index cases (n=40) and their recently exposed contacts (n=162), as well as negative (n=24) and positive controls (n=18) were enrolled.

Contacts were consecutively recruited after verbal and written informed consent at the TB centre of the Division of Infectious Diseases at the Karolinska University Hospital, Stockholm, Sweden. Household contacts were enrolled at <1 month after last possible exposure to the index case. Non-household contacts were enrolled at 2 months after last possible exposure according to contact tracing routines at the time of the study (13). Two of the already included contacts were diagnosed with active TB and were not included in the results section (Figure 9).

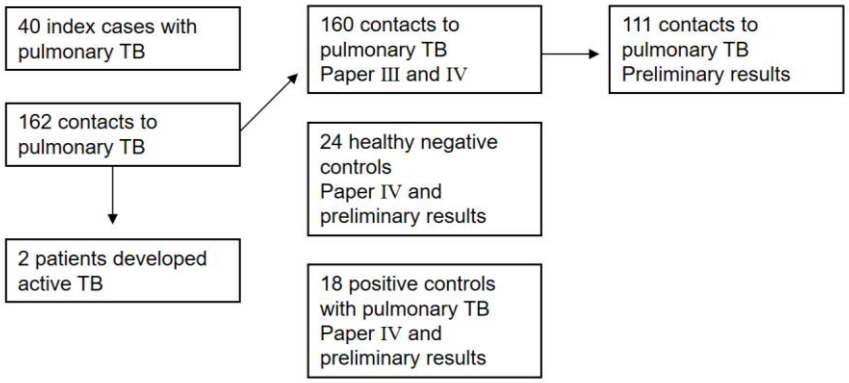

Figure 9. Flowchart for enrolment of patients in papers III, IV and for preliminary results.

Patients with microbiologically verified PTB were included as positive controls and young, health care students from Karolinska Institutet were included as negative controls. A TST was performed and blood samples were collected once for control groups and at the following time points <1 month, 1-3 months, 4-12 months after the last possible TB exposure for the contacts. The samples were sent to the former Swedish Institute for Infectious Disease Control for analyses. Results from routine diagnostic investigation (SM, PCR and culture) were collected in a database.

Contacts: Individuals from the same household or persons who were exposed indoor, at an office, school, hostel or healthcare institution.

Index cases and positive controls: PTB diagnosis was microbiologically verified in all cases by SM, Mtb PCR or mycobacterial culture.

Negative controls: Young health care students (n=24) at Karolinska Institutet (KI), with no known risk of TB exposure (as presented in papers I and II). The exclusion criterion was TST ≥6 mm. A few of the negative controls (9/24) were previously BCG-vaccinated.

3.3 CHEST X-RAYS (PAPERS I-IV)

Two radiologists read the CXR images independently of each other, according to the routines in clinical practice at the Karolinska University Hospital. Findings suggestive of active TB were apical involvement, localized fibronodular foci, cavities with acinonodular foci, miliary pattern, hilar or mediastinal adenopathy with or without pleural effusion. Calcified nodules were suggestive of previously healed TB.

3.4 LABORATORY PROCEDURES

3.4.1 Detection of Mtb (Papers I-IV)

Mtb culture and/or PCR for Mtb were performed at the Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden. Clinical specimens (sputum, gastric lavage, bronchoalveolar lavage or biopsies) were decontaminated and digested by N-acetyl-L-cysteine-sodium hydroxide (MycoPrep, BD, USA) and neutralized by phosphate buffer. After centrifugation, SM for acid fast bacteria was performed from the pellet by auramine-fluorescence staining and graded from 0-3 by a trained microbiologist. To increase accuracy for bacterial counts for papers III and IV, the mean number of bacilli/µl of sputum was estimated from repeated sputum samples. In those patients where sputum smears were discarded, an estimation of the colony forming units (CFU) of Mtb per ml specimen obtained on Loewenstein-Jensen cultures were used.

The rest of the pellet was resuspended in phosphate buffer and inoculated in MGIT tubes for incubation in the BACTEC 960 MGIT system (BD, Sparks, MD, USA) for 42 days and on conventional Loewenstein-Jensen media for 7 weeks. Positive growth was confirmed by acid-fast staining and microscopy and species identification was performed with reversed hybridization (HAIN Lifescience, Nehren, Germany).

PCR for the Mtb complex DNA was performed from clinical specimens using the Cobas® TaqMan® MTB test (Roche, Branchburg, NJ, USA).

HIV tests were performed at the Department of Clinical Microbiology, Karolinska Hospital, with detection of both viral antigens and antibodies, using Architect HIV Combo (Abbott Scandinavia AB, Stockholm, Sweden).

3.4.2 Tuberculosis skin test (Papers I-IV)

The TST was performed at the Division of Infectious Diseases, Karolinska University Hospital and at the former Wasa Vaccination. Two units of PPD (Tuberculin PPD RT23, Statens Serum Institut, Copenhagen, Denmark) were injected intradermally in the forearm and the induration was read after 48-72 h.

3.4.3 Interferon-gamma release assays (Papers I-IV)

Commercial IGRA tests QFT-GIT (ELISA method) and T-SPOT.TB (Elispot method) were performed in all patients at the former Swedish Institute for Infectious Disease Control, in all patients as previously described according to the manufacturer´s instructions.

3.4.4 FASCIA (Papers I, II and IV)

Flow-cytometric assay of specific cell-mediated immune response in activated whole blood (FASCIA) was developed as an in-house method at the former Swedish Institute for

Infectious Disease Control to study immune responses in certain infectious diseases such as varicella zoster, candida, cytomegalovirus and HIV (140-142). Whole blood was diluted 1:8 in Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco/BRL, UK) supplemented with 10 000 IU/ml penicillin (Gibco/BRL), 10 000 µg /ml streptomycin (Invitrogen, Stockholm, Sweden), and glutamax (RPMI medium). 400 µl of the diluted blood and 100 µl of antigen or medium only were added to 12x75 mm polystyrene round-bottom tubes with caps (Falcon 2058, Becton Dickinson Labware, NJ) and incubated at 37°C in a humidified atmosphere (5% CO2) with specific Mtb antigens for 7 days with medium (negative control), PHA (positive control), PPD and specific TB antigens ESAT-6 and CFP-10 for a fluorescence-activated cell sorting analysis (FACS) (Papers I and IV).

After incubation, the tubes were centrifuged at 300 x g and the supernatants from day 3 and 7 were removed and kept at -80°C until required for analysis of cytokine concentrations. Results were presented as numbers of $CD4^+$ lymphoblasts generated /µl blood.

The pellet was stained with anti-CD3 Fitc and anti-CD4 PerCP antibodies (Becton Dickinson Immunocytometry Systems (BDIS), Stockholm, Sweden). A lysing solution (1.0 ml Pharmlyse, BDIS) was added for 5 min at room temperature, followed by centrifugation, removal of the supernatants, washing with phosphate buffered saline (PBS) and resuspension in 450 µl PBS with 5% paraformaldehyde.

Samples were stored in the dark at +4°C for ≤4 h and thereafter analysed on a FACScalibur (BDIS) using CellQuest software (BDIS). The instrument was calibrated to acquire 60 ± 6µl per minute and set for four-colour analysis using FACSComp software (BDIS) in conjunction with CaliBRITE (BDIS). Ten per cent of the sample was acquired and saved as list-mode data for analysis. At first, large lymphoblasts were identified by their size and light-scatter characteristics. By finding the proliferated cells, further analyses with specific $CD4^+$ antibodies were possible. The cells were enumerated and the results were evaluated by positive and negative control samples.

Antigens from SSI (Statens Serum Institut) was received as peptide pools of ESAT-6 and CFP-10.

In paper IV we added several new Mtb antigens to the above mentioned; Rec85a and b, Rv0287, Rv0288, Rv2710, Rv1120c, Rv251c and the latency associated antigens Rv1284 and Rv2659c.

Optimal concentrations of Mtb antigens were determined before the study by testing samples from TB positive and TB negative controls and concentrations between 1-5 µg/ml per peptide were chosen for all antigens.

Healthy controls were used to set the cut-off for proliferative responses for each antigen by using the median result plus 3 standard deviations (papers I and II) or by using the 0.9^{th} percentile (paper IV).

3.4.5 Cytokine and chemokine detection by multiplex technology (Paper II and preliminary results)

Bio-Plex cytokine reagent kits were purchased from Bio-Rad Laboratories, Hercules, CA, USA. A method from the former Swedish Institute for Infectious Disease Control was used for identifying detailed cytokine profiles of the cell-mediated immune responses by using multiplex analyses in the supernatants from the FASCIA whole blood cultures (143). Two time-points (3 and 7 days) were used to detect peak levels for each tested cytokine (142). IL-2, IP-10, TNF-α and IFN-α were analysed at day 3 and IFN-ɣ, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, GM-CSF and MIP-1β were analysed at day 7.

Samples were added to microtiter plates, together with positive and negative controls. The assay uses fluorescent beads in multiple colours, with each particular set of beads coated with different monoclonal antibodies. Thereby several cytokines could be analysed simultaneously in a single test-well. The sample was read by two laser beams detecting both an antigen-antibody mediated reaction as well as the colour of the bead. The cytokines measured were selected to mirror the different cells important in TB immunity, such as granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage inflammatory protein-1 beta, (MIP-1β) and interferon-alpha (IFN-α) which activate both macrophages and $CD4^+$ T-cells and play a key role in initiating mycobacterial immunity (144) (145). Other markers were from T-cell subsets known to be of importance in TB immunity, such as Th1 cells (IFN-ɣ, TNF-α, interleukin-2 (IL-2), IP-10, IL-12, IL-6) and Th17 cells (IL-17), Th2 cells (IL-4, IL-5, IL-13) and regulatory T cells (IL-10).

Cytokines were measured using a multiplex assay; xMAP technology (Luminex Corporation). The assay was performed according to the manufacturer's instructions with minor modifications. Kit cytokine standard was diluted in RPMI medium supplemented with 10% volume of the serum standard diluent and was used as assay blank and to dilute the in-house control. 96-well microtitre filter bottom plates were used and washes were done by vacuum filtration.

Incubations were performed at room temperature on a shaker: 50 ul of mixed beads were added to the pre-wet wells and washed twice. 50 ul of standard, in-house control or sample was added to the plate and incubated for 1h. Samples were added undiluted. After the subsequent washing 25 ml of detection antibody mixture was added to the wells and the plate was incubated for 30 min and then washed. 50 ul of streptavidin-PE was added and incubated for 10 min. The plate was washed and the beads were re-suspended in 125 ml assay buffer. Assay readings were performed using the Luminex 100 platform (Bio-Rad Laboratories) with Bio-Plex manager software (Bio-Rad version 5.0) at low target reporter calibration. Sample size was set to 50 ul and data acquisition was 50 beads per region.

Standard curves were analysed using a Five- Parameter Logistic (5PL) regression curve fitting (Bio-plex software). Sample concentrations were interpolated from the standard curves. To validate the assay, an in-house control containing aliquots of PHA- stimulated whole blood culture supernatant was used. This control was used to evaluate the inter-assay

variability throughout the series of runs. The in-house control was used both undiluted and diluted 1:25. With the undiluted control the coefficient of variation (CV) was below 27% for all cytokines within standard range. CV for control diluted 1:25 was <11% for all cytokines within standard range, except for MIP-1β which had a CV of 54.7%. Un-stimulated cytokine responses were subtracted from antigen stimulated results.

Cut-offs were set high to obtain 100% specificity in this material. The highest values among the TB negative controls plus 0.1 pg/ml were used for each biomarker. Patients with microbiologically verified TB disease were used as positive controls and healthy subjects with no exposure to TB were used as negative controls. The cut-off level for each cytokine was set at the highest cytokine level in this group plus 0.1 pg/ml to reach 100% specificity. They were also used to calculate the assay's sensitivity and specificity for the diagnosis of Mtb infection.

3.4.6 LTBI probability estimations for recent and remote infection (Paper III and IV)

The individual probability (%) of recent and remote LTBI was calculated according to a novel mathematical model.

The probability of being remotely infected with Mtb was based on the annual risk of TB infection (ARI) in country of origin, in Sweden and during travels, staying in a high-risk environment (hospital, refugee camp or prison) and previously known exposure to contagious PTB. The probability of being recently infected with Mtb was based on contagiousness of each index patient (Mtb concentration in sputum), saturation of droplet nuclei in air, volume of distribution and elimination through ventilation and precipitation.

The relative probability of remote and recent LTBI (0-100%) among contacts was used as a dichotomized outcome (high vs low probability) in a prediction model for the respective antigens.

The probability (P) of being previously infected with Mtb in each country of origin (P_{origin}) was estimated per decade, using WHO data (WHO 2017) (WHO 2015) on Annual risk of infection (ARI) = ATBI incidence/Dye´s constant (Dye, Scheele et al. 1999, Dye 2008, Dye, Bassili et al. 2008) and calculated as presented below; Dye´s constant was adjusted due to improved clinical management and improved TB treatment and follow-up over time (Kompala, Shenoi et al. 2013).

$$P_{origin} = (1 - \left(\left(1 - \left(\frac{P_{origin-1990}}{100}\right)\right) \times \left(1 - \left(\frac{P_{origin\ 1990-2000}}{100}\right)\right) \times \left(1 - \left(\frac{P_{origin\ 2000-}}{100}\right)\right)\right)) \times 100$$

The number of years in each decade was calculated from birth to immigration to Sweden.

The added probability of TB exposure (P_{expo}) was estimated according to published data (Grzybowski, Barnett et al. 1975) e.g. close contact to SM+ 35%, SM- 10% while in a casual contact to SM+ 10%, SM- 2%.

Calculation of the total individual likelihood of remote LTBI was performed as follows:

$$P_{remote} = 1 - ((1 - P_{origin}) \times (1 - P_{sweden}) \times (1 - P_{travel}) \times (1 - P_{high}) \times (1 - P_{expo}))$$

The probability of being recently infected with Mtb was based on contagiousness of each index patient, Mtb concentration in sputum (C_{Mtb}), number of Mtb infected droplet nuclei produced/minute (P_{Dn}), inspiration volume (V_i), elimination through ventilation (E_v)/minute, elimination through precipitation (E_p)/minute, saturation (S) of droplet nuclei in air, volume of distribution (V_d) and the infective constant (K) which was validated according to previously published data on risk for transmission due to unknown parameters, such as the number of Mtb needed to establish an infection.

The probability of having inhaled an Mtb bacillus (P_i) per breath was calculated as follows;

$$P_i = \frac{C_{Mtb} \times P_{Dn} \times S \times V_i \times K}{E_v \times E_p \times V_d}$$

The probability of having been recently infected by Mtb was calculated by calculating an estimated number of total breaths during the exposure time:

$$P_{recent} = 1 - (1 - P_i)^n$$

3.4.7 The prediction model for recent and remote LTBI (Paper IV)

The Mtb specific $CD4^+$ proliferative responses for each antigen and for all tested contacts were used from time points <1 month, 1-3 months and >4 months in a prediction model and compared to previously calculated individual probabilities (0-100%) of recent and remote LTBI, according to a mathematical model (Paper III). A dichotomized outcome (high vs low probability) were used for the following outcomes: (i) high vs low probability of recent LTBI; (ii) high vs low probability of remote LTBI; and (iii) individuals with both high recent but low remote vs high remote and low recent probability for LTBI. Cut-offs at 10, 20 and 40% were tested and the use of a 20% cut-off resulted in groups of similar size, which was important for statistic calculations.

3.5 STATISTICAL ANALYSIS

Analyses were conducted using SAS version 9.1 (Paper I), version 9.2 (Paper II) and Sigma-plot version 13, Systat Software (papers III and IV). Heat mapping was performed with the statistical program Rv.2.14, using the package g-plots. The prediction model was developed using the statistical software R (paper IV). Statistical significance was defined as $p \leq 0.05$ and correlations >0.7 were considered well correlated.

Comparisons of paired test results from TST, QFT, T-SPOT.TB and FASCIA (Paper I) and comparisons of cytokines (Paper II) in terms of positive/negative results were made by head-to-head comparisons with McNemar`s test.

The correlation between the numerical values for the TST in millimetres and the FASCIA proliferative response after tuberculin stimulation was investigated using Spearman's rho (Spearman's rank correlation coefficient) (Paper I) and for comparison of cellular responses to cytokine levels (Paper II).

Comparisons of the proliferative responses for Mtb antigens in FASCIA were performed within and between all groups and subgroups of patients in the study with Wilcoxon's rank sum test for non-parametric data and Wilcoxon's signed rank test for paired samples when appropriate (Papers I, II and IV).

The Kruskal Wallis test for multiple nonparametric data was performed for the comparison of cytokine levels in all subgroups and Wilcoxon's rank sum test was used for finding where the differences occurred (Paper II). Comparison of calculated probabilities of recent and remote LTBI in between defined groups were calculated by Kruskal-Wallis and Dunn´s pair-wise test as post-hoc analysis (Paper III) and for differences in proliferative responses between IGRA groups (Paper IV).

Ninety-five percent confidence levels for shift in median values were calculated using Hodges Lehman estimation. Confidence levels were calculated using Fisher's Z-transform (Paper II).

Heat mapping of cytokine results was performed with the statistical program Rv.2.14, using the package g-plots. Actual cytokine levels were logged, and then measured at times higher than cut-off. The quota for each cytokine was then sorted by proliferative responses in each row and marked with a colour-code to create the heat map image (Paper II).

In paper IV the prediction model was created based on $CD4^+$ proliferative responses compared with probabilities of recent and remote LTBI. For this purpose, a backward stepwise logistic regression with Akaike information criterion (AIC) selection was used. To avoid over-fitting, 1000 bootstrap samples were used to select the optimal set of predictors and to assess the prediction accuracy, measured as the mean of the area under curve (AUC) (95% CI) of the out-of-bag sample (referred to as apparent prediction accuracy by Steyerberg et al) (146). The AUC measured the probability that a randomly selected pair of a case e.g. (high recent) and a control (low recent) was correctly classified by the prediction model.

3.6 ETHICAL CONSIDERATIONS

The studies were approved by the Regional Ethics Committee in Stockholm with ethical permits: 2006/357-31/3, 2008/1119-32 (Papers I and II) and 2008/1208-3173, 2009/1387-32 (papers III and IV). All patients were enrolled consecutively after verbal and written consent. The studies have not had any impact on the care of the patients.

All samples were coded and analysed in a blinded fashion. Data was collected in databases which are registered at the Karolinska University Hospital and at the former Swedish Institute for Infectious Disease Control, Stockholm.

4 RESULTS

4.1 PAPER I

Active TB was detected in 54/161 (34%) of the study patients examined for suspected TB. The patients were divided into groups according to clinical and microbiological data; PTB (n=25), EPTB (n=18), Clinical TB (n=11), Previous TB (n=22), LTBI (n=62), TB negative (n=11) and Other causes (n=12).

The FASCIA test was established for detection of proliferative $CD4^+$ T-cell responses after specific stimulations with PPD and specific Mtb antigens CFP-10 and ESAT-6 in all patients and in 21 non TB-exposed controls. The $CD4^+$ responses from PHA and medium tubes were used to determine if the results were accepted (95%) or indeterminate (5%). Cut-off levels for $CD4^+$ responses were set by using the median results plus 3 standard deviations for the healthy controls.

The overall sensitivity for verified active TB was 86% and for LTBI 61% and the overall specificity was 91%, when compared to the TB negative group and 100% when compared to non-exposed healthy controls. The FASCIA results for all groups were concordant with TST for 79% of the individuals. The FASCIA results for active TB were concordant with IGRA tests in 90% when indeterminate results were excluded and in 80% of individuals with LTBI. Patients with culture positive PTB were significantly more often positive in FASCIA than culture negative patients ($p<0.01$). FASCIA sensitivity was highest in the group of patients with EPTB (100%), compared to PTB (88%) and patients with EPTB also displayed significantly higher proliferative $CD4^+$ responses than patients with PTB in response to CFP-10 stimulation ($p<0.05$). FASCIA performed well in patients with moderate immunosuppression such as pregnancy, diabetes mellitus, rheumatism and renal insufficiency, but was indeterminate (PHA control failure) in two patients with severe immunosuppression (AIDS diagnosis and leukaemia).

4.2 PAPER II

Cytokine levels for IFN-γ, TNF-α, IL-2, IP-10, MIP-1β, IL-6 and GM-CSF were significantly higher after stimulation with CFP-10 and ESAT-6 in individuals with verified active TB (PTB and EPTB) compared to healthy controls ($p<0.005$) and for IL-13 after stimulation with ESAT-6 only ($p<0.0001$). These cytokine levels were also well correlated to proliferative $CD4^+$ responses at lower levels (correlation coefficient 0.76- 0.95 (95% CI)). With higher proliferative $CD4^+$ responses lower levels of cytokines were produced per cell. Cytokine responses in patients with verified TB were more frequent than in patients with LTBI and levels were significantly higher for IFN-γ, IP-10, MIP-1β, IL-2, TNF-α, IL-6 and GM-CSF after stimulation with ESAT-6 and CFP-10 ($p<0.01$), compared to individuals with LTBI and for IL-13 after stimulation with ESAT-6 ($p<0.005$).

The chemokine IP-10 detected all verified TB patients (PTB and EPTB) (n=43), including immunosuppressed individuals (n=9), after stimulation with ESAT-6 and 93% after stimulation with CFP-10. The sensitivity of IP-10 (100%) for verified TB was significantly higher than for IFN-γ (84%) (p = 0.023).

IP-10 was also positive in 87% of the LTBI group after stimulation with ESAT-6.

4.3 PAPER III

Index cases: 31 (74%) were coughing, 24 (57%) were SM+ and mean time from the symptoms begun to start of treatment was ≈3 months (range 1-26 months).

Contacts: Out of 162 individuals exposed to PTB, two developed active TB and were excluded from further analyses. The 160 TB contacts origins were; 70 (44%) individuals from Sweden, 10 (6%) individuals from Europe/America and 80 (50%) individuals from Africa/Asia. Previous TB exposure was found in 26 (16%) individuals and 26 (16%) individuals had previously been treated partly or fully for LTBI. 130 (81%) individuals were BCG vaccinated at least once.

Preventive treatment with INH and/or RIF was given to 36 out of 70 individuals classified as LTBI and the rest were followed with clinical check-ups and CXR.

134 (84%) individuals were classified as close contacts (>8h contact with SM+ or >48h contact with SM- index case) and 26 (16%) as casual contacts (147).

The results from the mathematical model previously described was used to classify the 160 contacts into 4 different groups according to calculated level of probability of recent and remote LTBI. Two cut-offs were tested;

a) Recent LTBI; ≥10% probability of recent and <10% probability of remote LTBI,

b) Remote LTBI; <10% probability of recent and ≥10% probability of remote LTBI,

c) Recent and remote LTBI; ≥ 10% probability of recent and ≥ 10% probability of remote LTBI,

d) Low probability of LTBI; <10% probability of recent and <10% probability of remote LTBI.

The same classification was performed with a cut-off at 50% for high/low probability of recent and remote LTBI.

The estimated probability of remote LTBI in correlation to the contacts´ origins were 16% for Europe/America and 38% for Africa/Asia.

The probability of recent LTBI among close contacts to SM+ index cases was 35%. Sensitivity, specificity and PPV for high probability of recent and/or remote LTBI to distinguish a TST positive result were in line with previously published models.

Contacts were also classified into 5 groups according to immunological test results during follow-up:

a) negative response (TST <6 mm, QFT <0.2 IU/ml, T-SPOT.TB ESAT-6 and CFP-10 <5 spots) at least two months after exposure and over the 12-month follow-up if available,

b) borderline response (maximum TST 6-9 mm, QFT 0.2-0.7 IU/ml, T-SPOT.TB ESAT-6 and CFP-10 5-7 spots) over the 12 month follow up,

c) positive response (TST ≥10 mm, QFT >0.7 IU/ml, T-SPOT.TB ESAT-6 and CFP-10 >7 spots) from ≤1 month and over the 12-month follow-up if available,

d) conversion (TST/IGRA negative to positive or borderline to positive with TST ≥10 mm increase, QFT ≥0.5 IU/ml increase or T-SPOT.TB ≥3 spot increase over a follow-up period of 12 months),

e) reversion (TST/IGRA positive to negative or positive to borderline with TST ≥10mm decrease, QFT ≥0.5 IU/ml decrease or T-SPOT.TB ≥3 spot decrease over a follow-up period of 12 months).

Concordant responses were highest between QFT and ESAT-6 (by T-SPOT.TB) (95%), QFT and CFP-10 (by T-SPOT.TB) (94%) and ESAT-6 and CFP-10 (94%), while concordant response was lower between TST and QFT (79%), TST and ESAT-6 (77%) as well as TST and CFP-10 (73%).

With a 10% cut-off for high probability of recent and/or remote LTBI sensitivity to detect a positive result or conversion in T-SPOT.TB was 100%, and the specificity was 35%. The corresponding sensitivity was 85% and specificity 40%, when compared with QFT. PPV for this cut-off level was 47% when compared with positive TST results and around 20% for the probability estimations compared to IGRAs.

With a probability estimation cut-off at 50%, sensitivity was generally below 70% in comparison with TST and IGRAs, specificity around 70% for all assays, while PPV was highest when compared with TST at 58%.

In figure 10 the different assays are compared to the probability estimations for recent and remote LTBI.

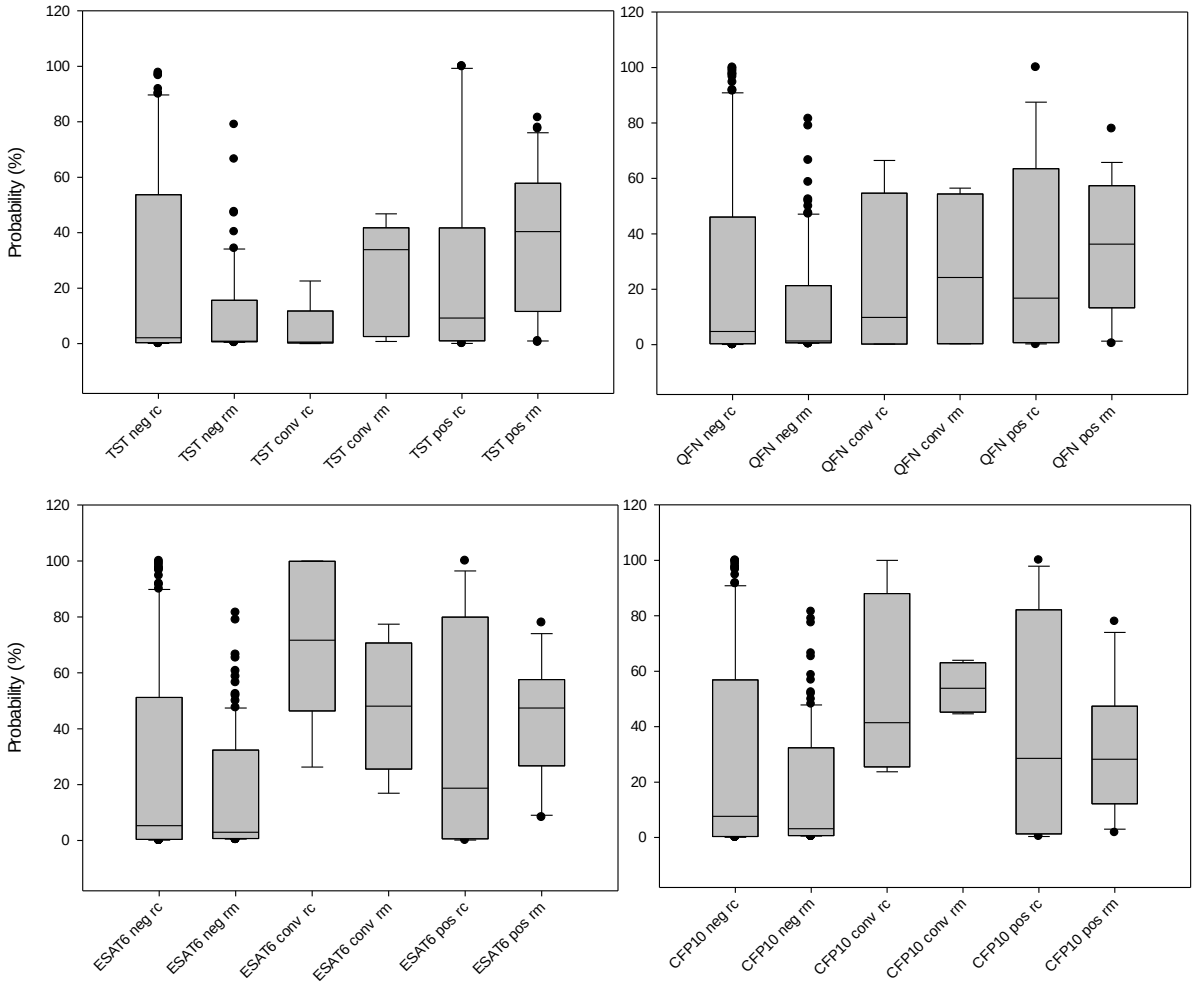

Figure 10. Probability (%) of recent (rc) and remote (rm) LTBI in TST and IGRA groups (148). TST, QFT, T-spot.TB/ESAT-6 and T-spot.TB/CFP-10 response groups were defined as; neg = negative test result at >2 month and onwards, conv = conversion from a negative to a positive result during follow-up and pos = positive test result from ≤ 1 month and no following reversion. Boxplots include the median value and the 10th, 25th and 75th and 90th percentiles.

4.4 PAPER IV

Out of 160 individuals exposed to active PTB, 46% originated from high-endemic countries and 81% were preciously BCG vaccinated. None of the contacts were severely immunosuppressed (defined as HIV positive or on treatment with immunosuppressive drugs). 9/24 negative controls were also BCG-vaccinated.

$CD4^+$ proliferative responses were measured in 160 contacts exposed to active PTB and compared with positive or negative TST result. $CD4^+$ levels for TST positive contacts were significantly higher than in TST negative contacts in all antigen stimulations, except for three (Rv2659c, Rv1120c and Rv251c).

$CD4^+$ levels were also compared in between IGRA positive, borderline, reversions and IGRA negative contacts, as well as with negative and positive control groups. Antigen Rv2710 induced strong non-specific $CD4^+$ levels in >25% of the non-vaccinated healthy controls and antigens Rv0287, Rv2710, Rv251c, Rv1120c, Rv2659c gave weak $CD4^+$ responses in IGRA groups and these markers were excluded from further analyses with the prediction model. PPD and six Mtb antigens CFP-10, ESAT-6, Rec85a and b, Rv0288 and Rv1284 were thereby identified, in which high $CD4^+$ levels were sensitive and specific enough for Mtb, to be further studied as markers for probability of recent and remote LTBI.

$CD4^+$ levels to Rec85b declined in PTB patients compared to IGRA positive contacts and BCG vaccinated negative controls.

With the results from FASCIA $CD4^+$ levels and the 20% cut-off for high probabilities of recent and/or remote infection for each individual, prediction modelling was performed at <1 month, 1-3 months and >4 months after last exposure. For contacts at ≤1 month after last possible exposure, the optimal combination of antigens was CFP-10, ESAT-6 and PPD in the prediction model of high probability (>20%) of recent LTBI (AUC 0.69, 95% CI).

In a small group of individuals Rec85a predicted high (>10%) probability of recent /low probability of remote LTBI (n=7), compared to high probability of remote/low recent LTBI (n=18), AUC=0.73 (95% CI).

None of the predictions at later time-points could distinguish neither recent nor remote Mtb infection in the groups with high probability of Mtb infection (AUC <0.65, CI 95%).

When looking closer at $CD4^+$ levels at <1 month we observed significantly higher $CD4^+$ response to CFP-10 and PPD and a lower $CD4^+$ response to ESAT-6, which could be the explanation for the AUC result obtained (Figure 11). The results from Rec85a stimulations was similar with PPD and CCFP-10 responses in line with the AUC observed in the model. High proliferative CD4+ responses to Rv1284, Rec85a and b in individuals with high probability of recent infection, compared to low probability was also observed ($p<0.05$).

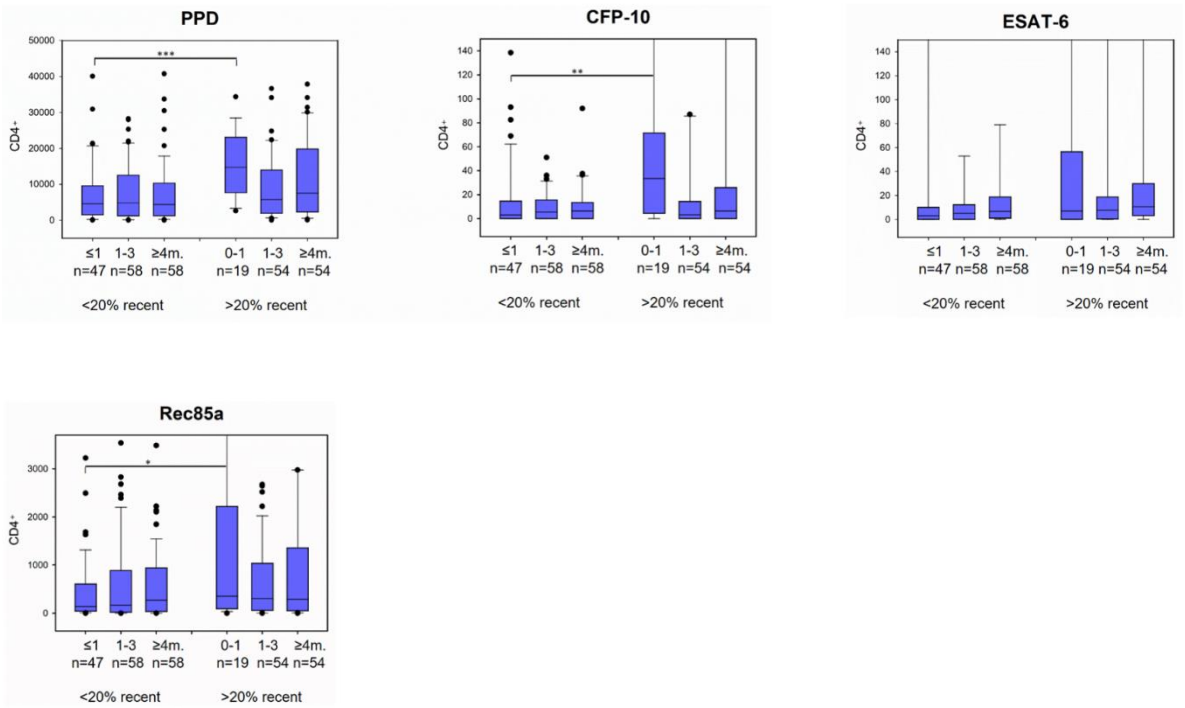

Figure 11. Proliferative $CD4^+$ responses over time after stimulation with PPD and Mtb antigens from the prediction model.

4.5 MANUSCRIPT IN PREPARATION

In an ongoing study we have further analysed the cytokines induced in a few of the individuals exposed to PTB and present preliminary results below (149).

Background: The aim was to evaluate 7 different cytokines/chemokines as biomarkers after stimulation with PPD and 8 Mtb antigens, with a prediction model estimating the likelihood of recent and remote latent tuberculosis (LTBI). The cytokines were included to investigate macrophage stimulation, differentiation of T-cells into Th1 pro-inflammatory responses or Th17 responses in individuals exposed to PTB.

Material and methods: 111/160 individuals from the previously presented cohort exposed to microbiologically verified PTB patients were tested by TST, QFT, T-SPOT.TB and cytokine/chemokine analysis. Multiplex assay was performed from the supernatant after whole blood stimulation with specific Mtb antigens in contacts at <1 month (n= 28) and/or 1-3 months (n=110) after last exposure. The cytokine responses to antigens PPD, CFP-10, ESAT-6, Rv0287, Rv0288, Rv1284, Rv2710, Rec85a and Rec85b were measured at peak levels after 3 days (IFN-ɣ, IP-10, IL-2,) and 7 days (MIP-1β, GM-CSF, Il-17, TNF-α). Data from a previous analysis concerning the likelihood of recent and remote LTBI was used for prediction modelling (Paper III) (148).

Patients with microbiologically confirmed TB were enrolled as positive controls (n=18) and young healthy students with no exposure to TB (n=24) were used as negative controls, to validate results and to determine a cut-off level for the cytokines. The prediction model

selected was Lasso regression, a regularized regression model which handles correlated data well.

Preliminary results showed a high sensitivity for secretion of IL-2, IP-10 and IFN-ɣ after Mtb antigen activation of leukocytes from individuals who were TST and/or IGRA positive, IP-10 was the most sensitive marker.

In the Lasso prediction model one set of 11 biomarkers predicted high recent/low remote probability (n=18) versus high remote/low recent (n=22) probability with a cut-off at 20%. The AUC was 0.79 (95% CI). The predictive markers for recent infection were; high levels of IL-2 to antigen Rv1284, high levels of IP-10, TNF-α and MIP-1β to antigen Rec85a and high levels of MIP-1β to PPD, Rec85a and Rv0288, combined with low responses of IL-2 to ESAT-6, low responses of IFN-ɣ to Rec85a and Rv1284 and low IL-17 and MIP-1β responses to Rv1284.

Rec85b induced high IFN-ɣ responses in IGRA positive contacts, but very low responses in patients with active TB.

5 DISCUSSION

WHO aims for a substantial global reduction of TB incidence and mortality in year 2035. In order to reach these goals active case-finding and contact-tracing around index cases is important, in order to detect the recently infected who are at considerably increased risk of incipient TB. Remote Mtb infections give rise to a lower number of TB cases, since the majority of TB cases develop within 2 years after exposure and the global burden of disease is therefore mainly associated with recent infections (150).

Current tests, the TST and IGRAs are unable to distinguish between recent and remote infections and thus, have low predictability for active TB.

Better diagnostic methods are thus wanted for, with higher prediction capacity for incipient TB in the recently infected and those with other risks for reactivation, such as immunosuppression. So-called point of care tests should be customized for use in different kinds of environments, be rapid and easy to perform and also be affordable in a global perspective (118).

The objective of these studies was to improve immunological diagnostic methods, with the specific aim to distinguish the different stages of Mtb infection; active TB, recent or remote LTBI, or a mere immunological memory of infection. LTBI and the granulomatous environment was previously believed to consist of dormant bacilli within the phagolysosome of the macrophages, but recent studies have shown that a dynamic process takes place here, with replicating bacilli constantly challenging the immune system (151).

For this purpose, two different cohorts of patients were enrolled; individuals with suspected active TB (papers I and II) and individuals recently exposed to active PTB (papers III and IV), together with positive (microbiologically verified TB) and negative (healthy TST negative non-exposed students) controls.

FASCIA was shown to be a robust assay with similar sensitivity and specificity for active TB and LTBI as the IGRA tests (papers I and IV), but it could not, as for the IGRAs (63), distinguish active TB from LTBI. Compared to TST testing FASCIA detected a lower number (61%) of presumed LTBI not due to lower sensitivity, but because of a higher specificity (92). Long-term stimulations with CFP-10 and ESAT-6 did not increase the sensitivity in TB/LTBI diagnosis, as compared to the over-night incubations performed in IGRAs. However, the possibility to test multiple Mtb antigens and evaluate other sorts cytokine secretions for sensitivity of LTBI with FASCIA, is of advantage compared to the use of existing commercial IGRAs.

The sensitivity of the FASCIA using CFP-10 and ESAT-6 for microbiologically verified active TB was high (86%), as well as the specificity (100%), with the non-TB exposed controls as reference. Patients with microbiologically verified TB showed higher proliferative responses than those with a clinical diagnosis, probably explained by incorrect TB diagnoses in the latter group.

Patients with EPTB were more frequently positive with FASCIA and showed higher proliferative responses than patients with PTB. These results could be due to more severe disease in PTB patients, with subsequent anergy and/or redistribution of $CD4^+$ cells to the lungs (152).

Cytokine analyses of supernatants detected differences in levels for IFN-ɣ, IP-10, IL-2, TNF-α, IL-6, MIP1-β and GM-CSF between active and latent TB, with IP-10 as the most sensitive biomarker in both entities (Paper II and preliminary results). IP-10 responses were also detectable in all immunosuppressed individuals (n=9). These cytokine/chemokine results demonstrate the importance of multifunctional $CD4^+$ Th-1 cell responses (IFN-ɣ, IP-10, IL-2, TNF-α, IL-6) and macrophage activity (MIP1-β, GM-CSF) in the host immune defence against Mtb (153) (58).

IP-10 results were more sensitive and specific (Paper II) compared to most previous studies and were similar to results from another study, where non-stimulated serum IP-10 was compared to IP-10 from QFT supernatants (154). Results in that study showed high sensitivity (88%) and specificity (91%) of serum-IP-10 for TB compared to healthy controls. IP-10 levels from QFT supernatants were >10-fold higher than the non-stimulated samples and reached a sensitivity of 98% and specificity 88%. Sensitivity and specificity for LTBI were 46% and 91%, respectively. Neither IP-10 test could differ between active TB and LTBI. IP-10 responses were not studied in patients with inflammatory conditions, making the specificity levels uncertain.

IP-10 is a chemokine seen in many different Th-1 inflammatory diseases and is not specific for TB. It is expressed in lymphoid organs and in many other cells including epitheloid cells and may aid in effector T-cell generation as well as attracts lymphocytes to the site of infection (155).

A TB-Net multi-centre study suggests a combination of QFT results and IP-10 analyses from the supernatant to diagnose active TB with increased sensitivity from 84 and 81%, respectively, to 87% (156). A significant difference for IP-10 levels was seen between a TB group and a control group with TB-suspects but who were actually TB negative and had other diagnoses ($p<0.0001$).

Other studies showed IP-10 sensitivities of 83% and 81% in active TB by short-term stimulation and multiplex assay (156, 157) and 75% by IP-10 ELISA in HIV+ patients with active TB (158). Another study of IP-10 performance in TB patients by enzyme immunoassay (EIA) after overnight incubation with CFP-10, ESAT-6 and TB7.7 showed a 92.5% sensitivity, but specificity was poor (48%), compared to healthy adults (159).

After publication of our study, IP-10 has been further studied in sera from TB patients without previous antigen stimulation, for example in a multiplex platform with 74 biomarkers (160). Here, sensitivity for TB was 86% and specificity 73%. Levels of IP-10 decreased significantly during treatment.

In a study with 7 days of incubation with ESAT-6 and CFP-10, IP-10 was measured by multiplex assay with 73% sensitivity and 53% specificity in TB patients (161). It is likely that IP-10 results differ depending on incubation time, since IP-10 levels peak at day 3 after antigen stimulation (143), which was the time point chosen in our study. Another approach to the problem with incubation time was overcome by measuring IP-10 mRNA by reverse transcription PCR (133). mRNA levels were shown to peak at 8 hours, as compared to IP-10 protein which continued to rise during the whole incubation time (48 h).

IP-10 has also been suggested as an alternative marker for Mtb infection in QFT-Plus TB1 (CFP-10 and ESAT-6) supernatants, showing higher levels in individuals with LTBI (defined as a positive QFT), compared to active TB ($p=0.01$) and healthy controls ($p<0.0001$) (162). These results differ as compared with our studies, where the same antigens induced significantly stronger responses of IP-10 in active TB compared to LTBI ($p<0.0001$). The contradictory results demonstrate the importance of a correct LTBI diagnosis and is a reminder of the lack of a reference method for test evaluation in LTBI.

The mathematical model to estimate the individual probability of recent and remote LTBI in individuals exposed to PTB was developed to be used as a reference method for LTBI. The estimations were based on clinical and epidemiological data and independent from results of immunological testing. In this model the probability of remote LTBI was similar to previously published data concerning origin from Europe/America and Africa/Asia (27) (163). A higher probability of remote LTBI was seen among TST positive contacts. Another important finding supporting the validity of the model was that estimated probabilities of recent LTBI in close contacts to direct microscopy positive PTB cases was 35%, which is promising for the model since similar finding have been indicated by previously published data (37) (147).

Even though the different estimations were thorough and based on previously published data, several approximations had to be made and probabilities of recent or remote LTBI had to be interpreted with caution. It was difficult to assess when symptoms started exactly, the number of droplets produced in relation to activity and cough, estimate the time and place spent together with index as well as the extent of ventilation. Other obstacles for evaluating the estimated probabilities of recent or remote LTBI are the individual host immune responses. Even though an individual inhales Mtb bacilli, early clearance of the infection by the innate immune responses could hinder the development of a specific adaptive and measurable immunity (164). The bacilli could also be eradicated by lymphocytes before an adaptive immune response develops, as suggested in a recent review (165). Nevertheless, at present there is no better reference method and this model is as meticulous and thorough as possible.

$CD4^+$ T cell proliferative responses to PPD and 11 Mtb stimulations were evaluated as immunological markers in different stages of Mtb infection (Paper IV), with the specific aim to identify those with highest risk of progression i.e. recent LTBI for preventive treatment. PPD and six Mtb antigens CFP-10, ESAT-6, Rec85a and b, Rv0288 and Rv1284 were

identified in which high proliferative $CD4^+$ responses were sensitive and specific enough to be further studied as markers in a prediction model.

For contacts at ≤1 month after last possible exposure, the optimal combination of antigens was CFP-10, ESAT-6 and PPD in the prediction model of high probability (>20%) of recent LTBI. When looking closer at these contacts median responses to PPD and CFP-10 were high at this time-point and responses to ESAT-6 were significantly lower. According to the prediction model 8 individuals were found to have levels of $CD4^+$ T cells above cut-off to PPD and CFP-10 and ESAT-6 responses below cut-off.

22 individuals were IGRA positive and 7 were IGRA converters. If all 29 of these contacts hypothetically were given preventive treatment instead of 8 identified individuals according to the prediction model, PPV would increase from ≈3% to ≈11% and the NNT would decrease from 33 to 9 contacts, in order to prevent one case of active TB.

At >1 month after last exposure the differences between groups had vanished and none of the biomarkers could distinguish neither recent nor remote LTBI. These results could hypothetically be due to an increased immunological control of Mtb, with protective responses to ESAT-6 diminishing the risk of incipient TB, but the early immunological responses have to be further studied in larger cohorts.

The early proliferative $CD4^+$ responses to CFP-10 and PPD in our cohort correspond to a study where recently exposed young children, with a rapid progression to active TB in 63/214 (29%), were tested with IFN-γ response IGRA in supernatants after 4 days of stimulation (166). Immune reactivity to CFP-10/PPD was measured and was significantly higher in individuals with incipient TB than in individuals without disease progression.

In our study high $CD4^+$ responses to Rec85a and b and Rv1284 were also sensitive markers of recent infection but could not distinguish recent from remote infection. Rec85b also induced stronger $CD4^+$ proliferative responses in IGRA positive contacts and BCG vaccinated negative controls, compared to patients with active PTB, but the clinical significance for protective immunity of these responses is uncertain. The results are similar to those from a study performed in Honduran TB patients (167), where heavily TB exposed healthy health care workers had stronger immune responses (IFN-γ and IL-17) compared to PTB patients after in vitro stimulation for 7 days with antigens Rec85a and Rec85b in whole blood. The decline of $CD4^+$ levels to Rec85b in active TB could be explained by anergy or non-responsiveness, when Mtb are actively multiplying in the lungs, or by a possible redistribution of effector $CD4^+$ lymphocytes from peripheral blood to the site of infection (152).

The reactivity induced by Rv0287, Rv1284, Rv2710, Rec85 a and b in BCG vaccinated healthy controls could probably be explained by immunological cross-reactivity to the BCG-strains, since several of the antigens used in our study are also present in the genome of *M. bovis* (Rec85a and b, Rv0287, Rv0288, Rv0251c, Rv1120c, Rv1284, Rv2710) (168).

In a small group of individuals Rec85a predicted high (>10%) probability of recent /low probability of remote LTBI compared to high probability of remote/low recent LTBI. However, the number of individuals is too small to suggest that this is a biomarker that could distinguish recent from remote LTBI and the results should be interpreted with caution.

In our preliminary results from the prediction model for high probability of recent LTBI, high levels of IL-2, IP-10, TNF-α and MIP1-β to either of the antigens Rv1284, Rec85a, Rv0288 and PPD were induced. An interesting finding was also the low IFN-ɣ secretion responses to Rec85a and Rv1284 antigens as possible biomarkers for high compared to low probability of recent Mtb infection. Results obtained with Lasso regression would be difficult to use as a clinical tool per se, but they describe the complex host immune response to Mtb and indicate important biomarkers such as IL-2, IP-10, TNF-α and MIP-1β for future research. Furthermore, the prediction model could only be performed at 1-3 months, due to a low number of enrolled individuals at <1 month after exposure.

We used non-vaccinated healthy individuals as negative controls to determine cut-offs for the proliferative responses to the respective antigens. A majority of our contacts were BCG vaccinated and mainly from TB high endemic countries, and in many European countries the epidemiological setting is similar, with TB transmission occurring mainly among migrants with a high BCG coverage. Thus, if these antigens were to be used, the cut-offs would have to be determined by the response in healthy BCG vaccinated individuals, choosing a high specificity for the proliferative responses at the expense of lost sensitivity. These markers could be more interesting to study further in populations where BCG coverage is low.

The main limitation of this study was the low number of individuals in some of the groups, due to clinical routines at that time, thereby probably missing some antigens that might be of interest. For example, we chose to include Rec85a in the prediction model since it showed relatively high responses in spite of the fact that there were no significant differences between any groups. Yet it proved to be one of the more interesting antigens in the prediction model. Two confounding factors were previous BCG-vaccination, suspected previously healed or treated infection with either Mtb or other environmental mycobacteria. Repeated TST testing could also elicit unwanted immune reactivity in other ways healthy individuals.

Future perspectives: Early high $CD4^+$ responses to CFP-10 and PPD and low responses to ESAT-6 may be used as biomarkers to improve PPV for recent infection and thus increased risk of incipient TB.

A whole blood based test capable of detecting recent TB could be developed measuring IP-10 and IFN-ɣ levels after separate long-term stimulations for PPD, CFP-10 and ESAT-6.

Markers identified are of interest to study in low TB endemic countries where BCG coverage is low. Further studies of promising antigens to differentiate recent LTBI from remote LTBI are needed to explore and validate Mtb immune responses in recently exposed contacts.

6 CONCLUSIONS

- FASCIA was confirmed to be a robust method for further studies of new Mtb antigens and immune markers such as cytokines and chemokines from the supernatants after specific Mtb stimulations. The method is comparative to the IGRAs in terms of sensitivity and specificity, but with an expanded possibility of further studies with different antigens.
- Cytokine detection by multiplex technology in FASCIA supernatants could identify immune responses with a higher sensitivity for correct diagnosis in the different stages of Mtb infection, compared to commercial IGRAs.
- A mathematical model was developed to estimate the probabilities of recent and remote LTBI based on clinical and epidemiological data. Results from the estimations were in line with previously published data from contact screenings, indicating the model could be a useful tool as a reference method for recent and remote LTBI.
- With a prediction model based on probability estimations of recent and/or remote Mtb infection, the most specific prediction of recent infection was an early high proliferative $CD4^+$ response to antigen CFP-10 and PPD and a low early response to antigen ESAT-6. These findings might further enhance the PPV of LTBI assays recent infection and as such, identify individuals exposed to PTB with a higher indication for preventive treatment. Antigens Rec85a and Rv1284 were also sensitive markers of recent infection, but they did not sufficiently distinguish between recent and remote infection in this study. $CD4^+$ responses and/or high cytokine levels to antigen Rec85b might indicate protective Mtb immunity.
- The findings from our studies show that PPVs for increased risk of progression to TB in LTBI assays can be improved. These results can aid clinicians in targeting those in need of treatment to prevent disease and further transmission of active TB in the society, as well as avoid unnecessary costs and adverse events.

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