The impact of the serum levels of soluble receptor for advanced glycation end products (sRAGE) and its ligand S100A12 for the course and extent of lung involvement in smear positive pulmonary tuberculosis in a cohort study in Hyderabad - India.

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ZUSAMMENFASSUNG

Die pulmonale Tuberkulose (pTbc) ist durch Lungengewebsschädigung gekennzeichnet bei der neutrophile Granulozyten (Neutrophile) eine führende Rolle bei der Pathogenese spielen. Sogenannte "**D**amage **A**ssociated **M**olecular **P**attern**s"** (DAMPs oder "Alarmine") akkumulieren während der chronischen Entzündungsreaktion und der Gewebeschädigung und initiieren eine Immunantwort. Insbesondere Neutrophilen Granulozyten sezernieren Proteasen und andere Stoffe, welche gesundes Gewebe schädigen. S100A12 wird von Neutrophilen sezerniert und induziert, nachdem es am Rezeptor für „Advanced Glycation End-products" (RAGE) gebunden hat, pro-inflammatorische Eigenschaften. Eine der „soluble cleaved RAGE" (sRAGE) Formen des RAGE, welche nach Teilung durch ADAM 10 entsteht, kann als kompeditiver Inhibitor der pro-inflammatorischen Prozessen entgegenwirken. Im Rahmen der hier beschriebenen Querschnittskohortenstudie und einem sogenannten „Self-controlled case series" Design (SCCS), wurden 119 Patienten mit pulmonaler Tuberkulose und 163 gesunden Kontrollpersonen im „Mahavir Hospital and Research Center" in Hyderabad/Indien untersucht. Der beobachtende epidemiologische Teil wurde durch in vitro Experimente erweitert, um einen Zusammenhang zwischen dem Bestehen einer pulmonalen Tuberkulose und einem Anstieg von S100A12 Konzentrationen bei gleichzeitigem Absinken der sRAGE und ADAM10 Konzentrationen im Serum zeigen/nachweisen zu können. Diese Proteine gelten somit als unabhängige Prädiktoren der Erkrankung. Die Querschnittsassoziation der pulmonalen Tuberkulose und der Serumkonzentrationen von S100A12 und sRAGE wurden im Längsschnitt bestätigt, was für einen kausalen Zusammenhang zwischen den Faktoren spricht. Zusätzlich zeigt diese Studie nicht nur, dass S100A12 die Aktivität von ADAM10 in vitro inhibiert und somit die sRAGE Konzentration regulieren kann, sondern auch, dass dieses Protein ein Prädiktor für das Ausmaß der alveolären Lungeninfiltration ist. Von uns in dieser Studie charakterisierte Veränderungen des Röntgen Thorax sind unterschiedlich assoziiert mit einem positiven Sputum Ausstrich und dem Body Mass Index (BMI), Die Studie gibt Hinweise dafür, dass die Differenz der Neutrophilen und Lymphozyten im peripheren Blut unter Miteinbeziehung des BMI- ein aussagekräfiger Marker sein könnte, um das Krankheitsvorkommen und den Schweregrad der Erkrankung besser einschätzen zu können. Abschließend kann gesagt werden, dass die Serum Level von sRAGE und S100A12 ein Indikator für das Ausmaß der Entzündung einer pulmonalen Tbc sind /sein können, wobei S100A12 ein Surrogat-Parameter für das Ausmaß der alveolären Infiltration ist und somit die durch Neutrophile verursachte Lungengewebsschädigung darstellen könnte. Die Ratio zwischen Neutrophilen und Lymphozyten im Serum von pTBC Patienten, vor allem wenn sie mit dem BMI kombiniert wird, könnte ein wichtiger Parameter zum Abschätzen des Krankheitsvorkommen und dem Ausmaß der pulmonalen Beteiligung sein.

Abstract

Pulmonary Tuberculosis (TB) is a chronic disease characterized by the development of lung tissue damage and neutrophils play a detrimental role in this process. Danger Associated Molecular Patterns (DAMP) accumulate during chronic inflammation and tissue damage triggering immune responses; although they could promote tissue repair, the recruited cells especially neutrophils, secrete proteases and other agents that damage viable tissues. S100A12 is a neutrophil derived DAMP with adjuvant and pro-inflammatory properties after binding the Receptor for Advanced Glycation End-products (RAGE). One soluble cleaved RAGE (sRAGE) form produced by the cleavage of ADAM10 acts as decoy receptor that counteracts proinflammatory processes. A cross sectional cohort and self controlled case series study was performed in Mahavir Hospital and Research Center in Hyderabad-India with 119 TB patients and 163 healthy controls. The observational epidemiological study was combined with *In vitro* experiments to demonstrate that pulmonary TB is associated to an increase in S100A12 along with a decrease in sRAGE and ADAM10 and these proteins are independent predictors of disease occurrence. The cross sectional associations of S100A12 and sRAGE with pulmonary TB were confirmed longitudinally what suggests a causal relationship with pulmonary TB. In addition, this study shows not only that S100A12 inhibits ADAM10 *in vitro* activity and might regulate sRAGE secretion but also that this protein is a predictor of the extent of alveolar lung infiltration. Moreover, in pulmonary TB specific radiographic features are differently associated with sputum smear positivity and Body Mass Index (BMI). Finally, this study provides a proof of principle for an affordable point of care marker to assess disease occurrence and severity based on the differences in neutrophils and lymphocytes in peripheral blood plus the BMI as indicator of malnutrition. In conclusion, the serum levels of sRAGE and S100A12 might be indicators of the extent of inflammation in pulmonary TB, S100A12 is a surrogate marker of the extent of alveolar infiltration and might express neutrophil related lung tissue damage, and the ratio between the absolute numbers of neutrophils and lymphocytes in the peripheral blood of TB patients in particular when combined with the BMI might serve as a useful marker to assess disease occurrence and pulmonary involvement.

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Abbreviations

TB	Tuberculosis
HIV	Human immunodeficiency virus
WHO	World health organization
MDRTB	Multi drug resistant TB
TNF- α	Tumour necrosis factor alpha
Mtb	Mycobacterium tuberculosis
MΦ	Macrophage
DC	Dendritic cell
TLR	Toll like receptor
NLR	Nucleotide-binding oligomerization domain receptor (NOD-like receptor)
CTLR	C- Type lection receptors
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
Dectin-1	C-type lectin domain family 7 member A/Dectin
CLEC4E	C-type lectin domain family 4 member E
ROI	Reactive oxygen intermediates
RNI	Reactive nitrogen intermediates
Th-1	T helper 1 cell
IFN-γ	Interferon gamma
DLN	Draining lymph node
Th-17	T helper 17 cells
Tregs	Regulatory T cells
AIDS	Acquired immunodeficiency syndrome
NK	Natural killer cell
APC	Antigen Presenting Cells
MMPs	Matrix metalloproteinases
CARD9	Caspase recruitment domain-containing protein 9
PRR	Pattern recognition receptor
BAL	Bronchoalveolar lavage
TST	Tuberculin skin test
PPD	Purified protein derivative
BCG	Bacilli Calmette–Guérin
AFB	No acid-fast bacilli
HPF	High-power field
CXR	Chest radiography
DOTS	Directly observed treatment short course
IHN	Isoniazid
RIF	Rifampin
EMB	Ethambutol
PZA	Pyrazinamide
XDRTB	Extensively drug resistant TB
DAMPs	Danger associated molecular patterns
PAMPs	Pathogen-associated molecular patterns
HMGB1	High-mobility group box 1 protein
EN-RAGE	Extracellular newly identified RAGE-binding protein
RAGE	Receptor for advanced glycation end-products
CXCR4	Chemokine CXC receptor 4

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
MAPK	Mitogen-activated protein kinase
NETs	Neutrophil extracellular traps
ROS	Reactive oxygen species
AGEs	Advanced glycation end products
MHC	Major Histocompatibility complex
ADAM	Disintegrin and metalloproteinase domain-containing protein
SNP	Single nucleotide polymorphism
COPD	Chronic obstructive pulmonary disease
BL	Bronchial lavage
CF	Cystic fibrosis
RA	Rheumatoid arthritis
OA	Osteoarthritis
JRA	Juvenile rheumatoid arthritis
BP	Blood pressure
LR	Likelihood ratio
OR	Odds ratio
TACE	Tumor necrosis factor-α-converting enzyme
WBC	White blood cell
NLB	Neutrophils-lymphocytes-BMI index
ROC	Receiver operating characteristic
AUC	Area under the curve
ALI	Acute Lung Injury
ARDS	Acute Respiratory Distress Syndrome
AD	Alzheimer disease
CSF	Cerebrospinal fluid
FEV	Force expiratory volume
CAC	Coronary artery calcium
BMI	Body mass index
IL-17	Interleukin-17
IGRA	Interferon gamma release assay
IL-12	Interleukin-12
IL-2	Interleukin-2
IL-23	Interleukin-23
PE	Phycoerythrin

CHAPTER 1: INTRODUCTION

1.1 Tuberculosis epidemiology and disease

1.1.1 Epidemiology

In 2012 there were 8.6 million cases of Tuberculosis (TB) worldwide and 1.3 million deaths (including 320 000 among HIV-positive people), these numbers highlight that TB still remains as a major global health problem (1). India and China alone accounted for (26%) and (12%) of total cases, respectively, and the worldwide case distribution in 2012 was (29%) in the South-East Asia, (27%) in the African and (19%) in the Western Pacific WHO regions (1). Therefore, India belongs to the five countries with the largest number of incident cases in 2012 (2.0 million– 2.4 million) (1). Moreover, India had 23% of the 5.7 million new and relapse cases worldwide. Similarly, this country has the largest number of TB patients with Multi Drug Resistant TB (MDRTB) over 50 000 cases (1).

TB affects the poorest and most marginalized communities around the world and is a disease highly linked to classical social determinants of poor health such as overcrowding and undernutrition. Although HIV infection became the main risk factor for TB, other important known factors associated to disease risk include: heavy alcohol consumption, smoking, diabetes, immunosuppressive drugs such as corticosteroids or Tumour Necrosis Factor α (TNF- α) antagonists and there is evidence of a genetic contribution to disease susceptibility (2). Malnutrition is a main risk factor for TB and although this association has been recognized throughout history its biological mechanisms are not known; however, it is known that malnutrition affects the cell mediated immunity which plays a principal role in the immune response to TB (3).

1.1.2 Infection and disease

M. tuberculosis (Mtb) the causal agent of TB is an aerobic, acid-fast, nonmotile, non-encapsulated, non-spore forming and intracellular bacillus (2). Human beings are one of its principal hosts within which the bacteria grows in tissues with high oxygen content such as the lungs, therefore, it affects predominantly this organ though it can cause disease in any other part of the body (2). Human infection with Mtb may cause either active disease or latent infection. In active TB there are signs and symptoms of disease and in latent infection the person has been exposed to the bacteria and is infected without developing visible signs for infection. Persons with latent TB infection are not infectious and cannot spread TB infection to others (4). Overall, without treatment, about 5 to 10% of infected persons will develop TB disease at some time in their lives, although most immunocompetent individuals either contain the infection in a latent state or may eliminate Mtb (2). Active TB occurs in two stages, either as the natural evolution of overwhelming Mtb replication following initial infection (Primary or primary-progressive TB), or resuming after a latent infection/containment of Mtb that may last many years following exposure (post-primary TB or reactivated TB) (5). Both primary and post-primary TB occurs in only a minor fraction of those at risk, as a consequence of several factors that include both innate and adaptive immune responses. Primary TB is detected in up to 20% of those exposed to Mtb and post-primary TB with reactivation of Mtb from latency occurs at a rate of 0.1–0.5% per year with an estimated 5–10% lifetime risk of developing active TB (6). Pulmonary TB has a classic clinical presentation that include chronic cough, sputum production, appetite and weight loss, fever, night sweats and hemoptysis (7), but these symptoms might also be absent in the early stages of disease (2). Extrapulmonary TB is estimated to be present in 10 to 42% of patients and its clinical features are very diverse and dependent on the infected organ or body side (7).

1.1.3 Diagnosis

The current standard methods to diagnose active TB are sputum microscopy and liquid medium culture with radiological imaging and clinical features as supplemental diagnostic aids (7).

1.1.3.1 Sputum smear microscopy

The microscopic examination of sputum smear stained with the Ziehl-Neelsen staining technique identifies individuals with active TB, and those patients with positive sputum smear test are considered the most important sources of Mtb transmission in the community, however, its sensitivity for the diagnosis of pulmonary TB is not optimal (8). This test identifies around two thirds of all adults with culture-confirmed pulmonary TB in high-prevalence countries, though this number is lower in HIV co-infected patients and in young children. The sputum smear microscopy is specific in identifying Acid-Fast Bacilli (AFB) and in some high-prevalence countries is the single available diagnostic tool. In this test the presence of Mtb bacteria in sputum is graded as follows: No AFB found in 100 High-Power Field (HPF) (negative), 1 to 9 AFB in 100 HPF (<1+), 10 to 99 AFB in 100 HPF (1+), 1 to 10 AFB per field in at least 50 HPF (2+) and more than 10 AFB per HPF in at least 20 (3+) (8). Although the sputum smear test is recommended for evaluating treatment efficacy after two months, a recent meta analysis concluded that this test has low sensitivity and specificity to predict treatment failure and relapse (9). Other studies have related the number of bacteria in sputum smear with prognosis (10, 11).

1.1.3.2 Chest radiography (CXR)

The CXR is a valuable diagnostic aid for diagnosis pulmonary TB. The CXR is assessed by trained physicians who search for shadows suggestive of TB and classify the individuals as having TB pathognomonic features such as presence of cavities or normal: it is possible to identify 82% of cases using the CXR (12). Moreover, CXR has a higher accuracy when used as single diagnostic method compared to symptom screening alone, however, the two methods have the highest sensitivity when both are used (13). The CXR is commonly used not only for TB diagnosis but also to assess disease severity, thus, several scoring systems have

been described which rate specific CXR features such as presence of cavities and percentage of lung involvement which are thought to be associated with indicators of severity like number of bacteria in sputum and sputum smear test results after two months of treatment (14).

According to the classical view the radiographic features are different in primary and post primary TB, thus, lymphadenopathy is considered characteristic of primary and the presence of cavities of post primary TB (15). The lymph nodes in the hilar and paratracheal regions are mainly affected in primary TB, while the cavitation in post primary TB affect the apical and posterior segments of the upper lobes of the lung (15). However, nowadays it is more difficult to differentiate the two disease forms based on the radiological features because they might have significant overlap (16), accordingly, recent molecular epidemiological studies have challenged this classical view by showing that the radiological features in patients with recent and remote infection are similar, thus, any difference in radiological presentation is mainly due to the diverse host immune status and response (17, 18).

The sputum smear test and the CXR have been in used for a long time and they are not effective in HIV-associated tuberculosis which could present itself as asymptomatic and subclinical TB with negative sputum smear or CXR but with positive culture. Similarly, there are special concerns on the suitability of these methods to diagnose TB in children (7).

1.1.3.3 Screening of latent TB infection

It is recommended that the screening for latent Mtb infection should be performed in those individuals that belong to a group with high prevalence of latent infection: Foreign persons born in TB endemic areas, people with high risk for disease reactivation (HIV infection, diabetes or people under immunosuppressive therapy) or in individuals who have been in close prolonged contact with TB patients (7). There are two methods to detect Mtb infection: i. the Tuberculin Skin Test (TST) measures the immune response to Mtb purified protein derivative (PPD) in the skin, but it cross reacts with non-pathogenic mycobacteria antigens and does not differentiate between real Mtb infection or simple exposure to other mycobacteria or *M. bovis*

bacilli Calmette–Guérin (BCG) vaccination (19). ii. Interferon γ (IFN-γ) release tests that measure the release of this cytokine by T cells in response to proteins only present in the Mtb complex: these tests are more specific and correlates better with Mtb exposure and infection than the TST (19). Neither the TST nor the IFN- γ release test is able to differentiate latent infection from active disease, they only establish that past exposure to Mtb produces a detectable acquire response after re-challenge with Mtb antigens (20).

1.1.4 Treatment

The sustained high TB incidence worldwide encouraged the World Health Organization (WHO) to launch a strategy known as the Directly Observed Treatment short course (DOTS) in the mid 1990s. Its core idea was to combine the provision of standarized treatment under supervision and patient support, in order to secure treatment compliance, with early case detection and diagnosis (1). The current WHO recommended TB treatment for newly diagnosed cases that are not MDRTB consist of four drugs: Isoniazid (IHN), Rifampin (RIF), Ethambutol (EMB) and Pyrazinamide (PZA) during two months of intensive treatment phase, followed by four months of IHN and RIF the continuation phase (7). This treatment is reported to achieve 90% cure rates when it is provided within national TB control programs (7). Patients are considered to be cured if they were previously registered as pulmonary smear-positive, completed treatment and had negative smear results on 2 occasions, one of which is at end of treatment (21). There are second line drugs available for patients with MDRTB which is caused by Mtb resistant to at least IHN and RIF and patients with Extensively Drug Resistant TB (XDRTB) when the bacilli are resistant to any fluoroquinolone and one injectable aminoglycosides (2).

1.1.5 TB immune response

One special feature of the immune response in TB is that although it is able to control the spread of the pathogen at the early stages, it is not sufficient to fully prevent the disease development in the long term, thus, even if proper immune responses are generated after infection, they do not eradicate the bacteria and active disease can occur after a period of clinically silent infection (22).

Lung granulomas are a hallmark feature of pulmonary TB, they are immune cell aggregates which formation is initiated following the transport of Mtb from the alveolar epithelium to the lung tissue by Macrophages (MΦ) and myeloid Dendritic Cells (DC) what results in the formation of early granulomas: the development of adaptive immunity contributes to create mature granulomas aimed at controlling Mtb spread (23). TB granulomas are characterized by a central necrotic core surrounded by layers of MΦ, epithelioid cells, multinucleated Langhans giant cells, and lymphocytes (24).

The classical view is that Mtb infection is controlled by the granulomas cellular wall and fibrotic outer layer which avoid bacterial propagation, thus, this initial suppression at the site of primary infection results in latent infection which can be seen as calcified granulomatous lesions and prevent active disease in more than 90% of cases (24). Nevertheless, the concept that TB reactivation is due to an immune system failure does not take into account the fact that transmission is a key step in Mtb life cycle (25), accordingly, a new scientific paradigm considers the TB granuloma as part of the Mtb life cycle that not only enable Mtb transmission but also restricts tissue damage to preserve host survival (26).

1.1.5.1 Innate immune response and early innate granulomas

In pulmonary TB the first contact of Mtb with immune cells after it is inhaled as respiratory droplets occurs in the distal alveoli where bacteria are ingested by alveolar MΦ. Although MΦ were considered as the main phagocytic cells for Mtb, it is now known that other cells recruited to the infected lung such as DC and neutrophils are also able to ingest the bacteria (27). This initial innate response results in the recruitment and accumulation of more phagocytic cells at the side of infection which engulf Mtb and become infected, this event not only expand the Mtb population but also establishes so called early granulomas. Mtb posses mechanisms to manipulate the intracellular environment and survive within the infected phagocytic cell. For instance, Mtb modulates the trafficking and maturation of the phagosome to escape its lysosomal mechanisms of restriction, killing and degradation (22). Furthermore, there is evidence that Mtb manipulates phagocyte cell death promoting necrosis and

averting apoptosis, indeed, apoptotic cells release fewer viable mycobacteria than necrotic cells and Mtb attenuated variants are associated with MΦ apoptosis, while virulent strains inhibit apoptosis and promote necrosis (27). Therefore unlike other infectious diseases in TB the recruitment of phagocytic cells do not limit or eliminate the bacteria, instead, it benefits the bacteria by providing new intracellular niches for growth (22). Even if innate response contributes to initial defence against Mtb, it does not control the infection in the long term or prevent active disease, however, this step in immune response is key for the development of adaptive immunity and the regulation of inflammation (27).

Phagocytic cells recognize Mtb through different pattern recognition receptors (PRRs) which detect Mtb specific cell wall components. The best characterized receptors include the toll-like receptors (TLR) 2 and 9 (27, 28), the nucleotide-binding oligomerization domain receptor or NOD-like receptor (NLR) 2 (27), the C-type lectin receptors (CTLR) Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), Dectin-1 and Mincle or CLEC4E (27). The binding of Mtb ligands to PRRs expressed on phagocytic cells activate several signal pathways generating microbicidal effectors such as Reactive Oxygen Intermediates (ROI) and Reactive Nitrogen Intermediates (RNI), as well as antimicrobial peptides to eliminate the pathogen (28). Additionally, Mtb ligands promote inflammation by the induction of the release of chemokines and pro-inflammatory cytokines in the phagocytic cells that attract more MΦ, DC and neutrophils (28). These initial events result in the production of IL-12 by DC and MΦ which leads the immune response to a T helper 1 cell (TH1) profile and the production of IFN-γ, the latter activates MΦ to trigger bacterial killing (28).

IFN-γ and TNF-α are essential in the early TB innate response because they activate MΦ to kill the bacteria (27). IFN-γ is vital for Mtb control through ROI and RNI production by MΦ and the enhancement of phagosome maturation (27) and TNF-α also activates MΦ for bacterial killing and modulates the apoptosis of infected cells (27), moreover, TNF-α production by infected alveolar MΦ drives the recruitment of neutrophils, Natural Killer (NK) T cells, CD4+ T cells and CD8+ T cells, which amplify the immune response and remodel the infection site: this process leads to the formation of the stable granuloma and is regulated by IFN-γ (25), notably, IFN-γ

deficient mice can not turn off this proinflammatory response and suffer lethal lung pathology (29). The granulomas become fully organized in the presence of activated T cells, with Mtb infected MΦ at the center encircled by a lymphocytic edge (26). Neutrophils are the first cells to arrive to the early forming granuloma followed by MΦ, with lymphocytes appearing at later stages of the process, what suggest that innate mechanisms are responsible for the early defence and necrosis, being proceeded by the acquired T cells that surround the necrotic core (24).

1.1.5.2 Adaptive immune response and mature granulomas

One characteristic of the adaptive immune response in TB is that lymphocytes are required both for immune protection and pathogenesis. For instance, in HIV infected individuals which have low CD4 cell numbers, TB appears mainly with a granulocytic infiltrate and necrosis, instead of the caseous necrosis granulomas present in non-HIV-infected subjects (30). Thus, although a weak lymphocytic response is associated with limited or no immunity, its absence restricts the transmission related caseation (30). Another important feature of TB acquire immune response is the long time needed for its development, which is due to a delay in the migration of DC from the lungs to the local lymph node where they encounter and activate CD4 T cells, what allows the bacteria to grow and expand in the lungs before they are counteracted by specific T cells (27). It has been shown that T cell activation occurs in the Draining Lymph Node (DLN) only 8-10 days after the initial contact of Mtb with DC and by the time effector activated T cells arrive to the lung after 14 days, Mtb is already within alveolar MΦ, DC and neutrophils (31, 32).

CD4 T cells are essential for Mtb control and within this set Th-1 cells are at the centre of acquire immunity; its generation is triggered by the IL-12 released from MΦ and DC after encountering Mtb and although its main cytokine is IFN-γ, they also secrete TNF-α, IL-2 and lymphotoxin (27). IFN-γ not only activates and enhances MΦ antimicrobial activity, but also it regulates IL-17 mediated inflammation by acting on nonhematopoietic cells (27), moreover, this cytokine modulates T cell apoptosis by altering the level of apoptotic signals during Mtb infection (31). CD8 T cells equally contribute to the protection against Mtb in the mice model; however, there is no much available evidence for its role in human infection (27).

Adaptive responses are relevant to the containment and control of Mtb replication by promoting the formation of mature granulomas. Within the mature granulomas the interaction of Mtb with the immune response takes places in the lymphocyte infiltration part that surround the granuloma and most probably the Mtb antigen presentation takes place in this area (24). Accordingly, the peripheral leukocyte infiltrate contains CD8+ and CD4+ T cells which encircle follicular aggregates that contain Antigen Presenting Cells (APC), B cells, and have high proliferative activity: Since the lung tissue is prone to leukocytes infiltration and organization, it might ensures that an effective local immune response is organized in the lymph node-like structural aggregates that encircle the site of Mtb infection (24).

The mature granulomas are highly vascularised and possess a fibrotic shell that establishes the borders between the different cellular layers and the outer lymphocytic infiltrate (25). The vascularisation disappears progressively in late stages generating necrosis and this process facilitates caseation, the granuloma fibrotic shield collapse and Mtb is freed to the airways ensuing transmission (25). The later process is very heterogeneous and caseation could be present even in early lesions, thus, granulomas at different developmental stages can be seen within the same individual (25), and this fact supports the emerging consensus that considers both active pulmonary TB and latent infection as a spectrum (20, 33).

1.1.6 TB associated lung tissue damage

The development of lung tissue damage is a common feature of pulmonary TB and it is mainly produced by the same mechanisms aimed at bacterial killing. Most TB symptoms are linked to a strong inflammatory response which contributes to pathology and results in disease worsening (34). Thus, it is thought that the tissue damage seen in TB is primarily a consequence of the host immune response and not a direct effect of the bacteria (35). Currently two possible mechanisms are considered, one that involves neutrophils together with IFN-γ and IL-17 and the other that mainly focus at the damaging effect on lung tissue of Matrix Metalloproteinases (MMPs).

1.1.6.1 Neutrophils, IFN-γ and IL-17

Neutrophils possess inflammatory mediators, proteases and oxidants which are released during their degranulation and generate tissue injury (36). The activity of all these molecules that include elastase, one of the most tissue destructive enzymes known (37), is tightly regulated under physiological conditions, moreover, these cells have a short lifespan and are constitutively programmed to undergo apoptosis, what limits their pro-inflammatory and tissue injury potential (36, 38). Neutrophils contribute to the early immune response against Mtb and most animal studies suggest that their recruitment to the infection site improve the outcome in the early disease stages, however, they also accumulate in later disease stages and, in the context of a poorly functioning acquired immune response, might contribute to disease exacerbation (39).

Several studies on Mtb infection in the mouse model suggest that IFN-γ has a biphasic function in TB immune response; at early stages it supports infection control but also IFN-γ prevents tissue damage in later infection stages (23). In short, mice with IFN-γ unresponsive lung epithelial and endothelial cells have high bacterial burdens and early mortality which are correlated to IL-17 overexpression and massive lung neutrophilic inflammation (40), notably, this same phenotype is observed in IFN-γ deficiency (29). Indeed, IFN-γ limits the proliferation of IL-17 producing cells by inhibiting IL-23 production what results in a decreased neutrophil migration (40). In addition, IFN-γ also inhibits CD4+ T cell production of IL-17 and directly inhibits neutrophils accumulation in the lung, besides, IFN-γ impairs neutrophil survival and in the absence of IFN-γ mice form large necrotic pulmonary lesions associated with granulocytic infiltrates, thus, this study suggests that IFN-γ has an important antiinflammatory role in TB (35).

Importantly, the neutrophilic phenotype is also associated with genetically TB susceptibility in mice, thus, the susceptible I/St strain shows high and prolonged lung neutrophil accumulation, and these cells have increased mobility, tissue influx and prolonged lifespan (41). Furthermore, neutrophils are linked to the ability of the innate response to limit damaging inflammation since the absence of CARD9, an adapter

molecule in the signaling of several PRR, results in increased inflammation and this effect is attenuated by neutrophil depletion (42).

Human studies support the findings in the mouse model; first, local neutrophilia correlates with disease severity and patients with less clinically and radiographically advanced TB have a predominance of lymphocytes in their Broncho Alveolar Lavage (BAL) and their BAL cells secrete IFN-γ (43), similarly, the lymphocyte counts in blood are increased in patients with symptoms of shorter duration but reduced in those symptomatic for more than 6 months (44), moreover, neutrophils are the major commonly infected phagocyte in human TB and most intracellular bacilli are found in these cells (45). Second, a high percentage of neutrophils and lymphopenia on the differential white blood cell count are associated with poor prognosis (46) and a high granulocyte/lymphocyte ratio is a predictor of disease occurrence (47). Third, the presence of extensive alveolar infiltrate in chest radiography is correlated with higher bacterial numbers in sputum smears (48) and the presence of a more extensive infiltrate pattern on the CXR is a clinical predictor of early mortality in hospitalized patients (49). Forth, the endothelial mediators of neutrophil migration L-selectin, E-selectin and ICAM-1 are increased in active TB (50). Accordingly, a recent study found a neutrophil-driven interferon (IFN) inducible transcript signature in human whole blood that correlates with clinical severity and supports a role for neutrophils in disease pathogenesis (51).

1.1.6.2 Matrix Metalloproteinases (MMPs)

The MMPs belong to the metzincins family of metalloendopeptidases and are involved in inflammatory and repair related processes such as cytokine activation, ligand shedding and matrix synthesis; its catalytic activity is regulated through gene expression, compartmentalization (cellular localization and accumulation), pro-enzyme (or zymogen) activation, enzyme inactivation by inhibitors and substrate availability (52). These proteases have emerged as key players in Mtb related lung tissue damage because of their ability to degrade fibrillar collagens at neutral pH (53). In pulmonary TB there is development of air filled areas in the lung known as cavities in which the parenchymal structure is entirely destroyed, this process is known as cavitation, the bacteria is able to grow freely within the cavities which are

out of reach for the immune response (54). Cavity formation involves destruction of the lung extracellular matrix and the fact that collagen can be degraded only by proteases, gives to MMPs a central role in this process (54).

A recent study provides evidence on the involvement of MMP-1 in TB lung destruction (53). This protease is able to degrade type I collagen and its expression is increased in sputum and BAL of TB patients, accordingly, Mtb infection leads to increased MMP-1 expression in the lungs of transgenic mice expressing human MMP-1 what is associated with more alveolar destruction and collagen breakdown, although the granuloma structure was not affected and there was also no increase in lung bacterial burden, therefore the authors suggested that necrosis of the granuloma and matrix destruction are separate events (53). On the other hand, MMP-9 has been shown to mediate the recruitment of MΦ to the granulomas in the zebrafish model of *M. marinum* infection. However, the fact that this protease is unable to degrade fibrillar collagens, leave MMP-1 as solid candidate for driving Mtb related tissue damage (55).

Briefly, the emerging paradigm is that MMPs have dual roles during Mtb infection both as granuloma creators and tissue destroyers: in early granuloma formation the bacteria induces MMP-9 in order to bring MΦ to the infection site what provides a niche for growth and also generate tissue remodelling, however, MMP-1 secretion at later stages produces lung matrix degradation and cavitation which leads to disease reactivation and transmission (56).

1.2 The danger model of immune response

In 1994 Polly Matzinger proposed for the first time the danger model of immune response which states that APC are activated by danger/alarm signals released from injured cells after being exposed to pathogens, toxins or mechanical damage (57). Although it was initially theoretical based, such alarm signals were revealed afterwards (58). These alarm signals termed “alarmins” or Danger Associated Molecular Patterns (DAMPs) can be constitutive or inducible, intracellular or secreted and could even be part of the extracellular matrix (58).

The preceding models of ‘self–nonself’ and PRR stated that the immune system starts a response if the potential threat was foreign (antigen-specific receptors of T and B cells) or if the potential threat was foreign and expressed Pathogen-Associated Molecular Patterns (PAMPs) (e.g., bacterial or viral PAMPs) that were recognized by PRRs such as TLR in APCs, respectively (58). The PRR and danger models can be brought together if both PAMPs and DAMPs are regarded as a common ancient set of signals that serve as signs of damage and death, thus, damage associated molecular patterns that can be pathogen specific or endogenous and serve to initiate repair processes and immunity (59).

1.2.1 Danger associated molecular patterns and sterile inflammation

In normal tissue development and repair the process of apoptosis prevents that apoptotic dying cells release their intracellular contents, because these cells are recognized and phagocytosed by professional phagocytes such as MΦ and DC (60). However, when cells die by necrosis they release their intracellular contents to the extracellular space which function as immunogenic endogenous adjuvants that activate immune cells triggering the production of inflammatory cytokines and initiate inflammation (60). Similarly, extensive cell death and deficient clearance of apoptotic cells result in secondary necrosis of dying cells and also release of intracellular DAMPs (60).

Accordingly, dead cells increase antigen-specific CD4+ and CD8+ T-cell responses when they are injected to animals (61) and can also stimulate DC to mature into immunostimulatory cells and promote their migration to the DLN (62). There are currently a number of identified DAMPs, among them, one of the best characterized are the High Mobility Group Box 1 protein (HMGB1) and S100A12 (calgranulin C) or Extracellular Newly identified RAGE-binding protein (EN-RAGE). Because DAMPs have similar adjuvant and pro-inflammatory properties to the PAMPs it is possible that they might work through the same receptors such as the TLR (63). Another cell membrane bound receptor, the Receptor for Advanced Glycation End-products (RAGE); also known as AGER, is a known ligand for HMGB1 (64) and S100A12 (65) and mediate their adjuvant and pro-inflammatory effects.

The danger model improved the understanding of some immune mechanisms such as cell death inflammation or sterile inflammation which occurs in the absence of any microorganisms as a result of trauma injury or chemically induced injury, all events that produce cell death (63). This response can neutralize injurious processes and promote tissue repair but could also cause several problems since the recruited cells to an area of inflammation especially neutrophils, actively and passively secrete proteases and other agents including DAMPs that damage the surrounding viable tissue (63). The inflammatory response to cell death plays a central role in the pathogenesis of acute and chronic diseases that involve extensive cell injury and tissue damage such as atherosclerosis, chronic obstructive lung diseases, and Alzheimer's disease among other pulmonary and vascular diseases, therefore a better understanding of its mechanisms will help the discovery of targets to design therapeutic interventions.

1.2.2 High Mobility Group Box -1 (HMGB1)

HMGB1 is a non-histone chromatin-associated protein within the nuclei of almost all eukaryotic cells where it functions as a DNA chaperone stabilizing nucleosome formation and promoting access to transcriptional factors (66). The gene coding for this protein is localized on chromosome 13q12. Its structure consists of two L-shaped DNA-binding domains termed 'HMG box' and an unstructured tail with negatively charged amino acids at its end. Although it is mainly in the nucleus, some cells might have it in the cytoplasm, on the external side of their plasma membranes, or in extracellular fluids (67). This protein plays a role in immunity when it is either passively released from necrotic cells or actively secreted by inflammatory cells, such as monocytes, MΦ, DC or NK cells and activates immune responses by its interaction with several immune receptors such as RAGE, TLR 2 and 4, chemokine CXC receptor 4 (CXCR4) and TLR-9 when combined with DNA (66).

It is thought that activation of TLRs by HMGB1 mainly occurs in myeloid cells, whereas RAGE is activated in endothelial and somatic cells (68). The activation of these receptors produces the release of proinflammatory cytokines. The interaction of HMGB1 with RAGE activates the transcription factors NF-kB and Mitogen Activated Protein Kinase (MAPK) pathways and promote cellular responses such as cell

migration, growth, differentiation, and autophagy (68). HMGB1 also induces dendritic cells maturation and migration, as well as, HMGB1 activation of DCs and monocytes produces the release of TNF-α, IL-1, IL-6, and MΦ inflammatory protein 1 (MIP-1). The protein has chemotactic activity on monocytes, MΦ, neutrophils and DCs and can promote the progression of some types of cancer cells (68). It was recently shown that mycobacterial infection is able to induce HMGB1 release *in vitro and in vivo* by MΦ and in the lungs of infected guinea pigs respectively (69).

1.2.3 S100A12 (Calgranulin C)

S100A12 is part of the S100 protein subfamily that also includes S100A8 and S100A9, they are termed calgranulins due to their calcium binding properties and their expression in cells of myeloid origin and therefore are related to innate immune functions (70). The S100A12 gene is located in the S100 gene cluster on human chromosome 1q21 and although the rodent genome does not have S100A12 (71) it is thought that rodent S100A8 might be a functional homologue (70). In humans S100A12 is constitutively expressed almost only in neutrophil granulocytes although monocytes express it at lower levels, in these cells the protein is located in the cytosol in the absence of calcium and the addition of this element produces its translocation to the membrane and cytoskeleton (70). S100A12 can be actively and passively secreted by neutrophils via a pathway that does not involve the classical Golgi route (72). A recent study identified S100A12 and S100A8/A9 among the proteins released from neutrophils in Neutrophil Extracellular Traps (NETs) (73), interestingly, it has also been shown that Mtb induces NETs production but this mechanism is not able to kill the bacteria (74). Of importance, although eosinophils in peripheral blood do not express the protein, its expression is present in these cells in the airways of asthmatic lungs (75).

S100A12 is a chemoattractant for monocytes and mast cells, with mild effects on neutrophils and none of lymphocytes (76, 77), besides, it facilitates the adhesion of neutrophils to fibrinogen and fibronectin and the adhesion of monocytes to the endothelium by up regulating the expression of the integrin receptor CD11b/CD18 (Mac-1) (76). It is suggested that by acting on mast cells S100A12 promotes vasculature activation and sustained leukocyte recruitment, mostly monocytes (75).

Notably, S100A12 is resistant to covalent modification by oxidants and since some classical chemoattractants are susceptible to oxidative inactivation, S100A12 could be important in propagating monocyte migration into chronic inflammatory lesions where ROS are produced (75).

There is controversy on the proinflammatory properties of S100A12, Hofmann et al. reported that the protein was able to induce IL-1β and TNF-α in a murine microglial cell line stimulated with bovine S100A12 (65) but Goyette et al. could not reproduce these results using human S100A12 and primary human monocytes or MΦ (78), however a recent study reported that human S100A12 was able to induce the expression of proinflamatory genes at the mRNA and protein level in human monocytes (72). Both TNF-α and LPS induce S100A12 in monocytes *In vitro* (72) and IL-6 does it in differentiated THP-1 MΦ (75).

S100A12 can bind Zn^{2+} which is needed by MMPs for functioning and thus S100A12 is able to inactivate these enzymes, S100A8 and S100A9 function in a similar way (78). Additionally, S100A12 have antimicrobial activity against Gram-negative bacteria which is enhanced by Zn^{2+} (79). Calcium-bound S100A12 activates intracellular signal cascades by binding the extracellular domain of membrane RAGE (65) and a new study demonstrated that S100A12 also binds TLR4 on human monocytes (72).

A recent study showed that the two related S100 proteins S100A8 and S100A9 are dominant within the inflammatory lung granulomas seen during active TB in human patients and in nonhuman primate models of *Mtb* infection, moreover, in the TB mouse model the exacerbated lung inflammation related to neutrophilic accumulation is dependent on these proteins which promote neutrophil accumulation by inducing production of proinflammatory chemokines and cytokines (80).

1.2.4 Receptor for Advanced Glycation End products (RAGE)

RAGE was isolated and firstly recognized as the receptor of Advanced Glycation End products (AGEs), these molecules form by the nonspecific reaction of sugars via their aldehyde functional group with an amine or a guanidine group from lysine or arginine residues of proteins or peptides: This non-enzymatic process is termed glycation and is most frequent in ageing individuals and diabetes patients (81).

RAGE is a transmembrane protein encoded in the Class III major hiotocompatibility complex on chromosome 6 and is part of the immunoglobulin superfamily (82). This multiligand receptor has 5 domains, 3 extracellular immunoglobulin-like domains (1 V domain, 2 C domains), a single transmembrane domain, and a C-terminal cytosolic tail (83). Besides AGEs, RAGE interacts with other ligands like S100/calgranulins (accumulating in chronic inflammation) in particular S100A12, amyloid-β peptide (accumulating in Alzheimer's disease), amyloid A (accumulating in systemic amyloidosis), the DNA binding protein HMGB1 and the leukocytes β2 integrin Mac-1 (83). RAGE is considered a PRR and recognizes ligands that accumulate in tissues during aging, chronic degenerative diseases, inflammation and during the host response to infection (83).

In addition to the membrane-bound full-length RAGE, various isoforms of the receptor are generated by alternative mRNA splicing and proteolytic cleavage: one splice variant of the membrane-bound form which lacks the extracellular ligand binding domain (N-truncated form, ΔN RAGE) and 2 soluble RAGEs forms exist (82). Soluble endogenous secretary RAGE (esRAGE) is produced by alternative splicing (83) and soluble cleaved (sRAGE) is produced after the cleavage of full-length membrane bound RAGE, this process has been described to be mediated by different metalloproteinases among them MMP3, MMP9, MMP13 and ADAM10 (84-87). Notably, the soluble forms of RAGE bind to the same ligands as the full length protein acting as decoy receptors that sequester circulating ligands, thus, it is thought that these forms counteract the proinflammatory processes triggered by the RAGE/ligand interaction (83). It has also been shown that RAGE splicing is tissue dependent and while full length RAGE is the prevalent form in the lung, sRAGE and not the spliced esRAGE is the predominant form in serum (85).
RAGE ligand binding is driven by electrostatic interactions between the positively charged surface of the RAGE ectodomain and negatively charged ligands. RAGE

has a positive surface charge that act as an electrostatic trap for ligands with a net negative charge, thus, RAGE activation might be influenced by the negative charge and the oligomeric nature of its ligands (81).

The binding of RAGE with any of its known ligands activates several different signaling pathways depending on the ligand, environment and cell type (82) and results in the activation of transcription factors mainly the NF-kB among others (83). RAGE expression is induced by NF-κB and sustained activation of NF-κB results in its up regulation maintaining and further strengthening the signal, converting a transient proinflammatory response into a chronic pathophysiological state (81, 83) Therefore the RAGE pathway is considered to be of particular importance in settings in which its ligands accumulate such as diabetes mellitus or Rheumatoid Arthritis (RA). Different cellular expression levels of RAGE and its competitive soluble inhibitor sRAGE might be a good candidate to modulate immune responses (83).

RAGE signaling regulates the innate immune system by mediating the migration of immune cells to the sites of infection and injury (88). Interestingly, it was recently reported that RAGE is protective during murine TB, because lung inflammation as well as lymphocyte and neutrophil numbers were increased in RAGE deficient mice after infection with live virulent Mtb, besides, these mice displayed more body weight loss and enhanced mortality (89).

1.2.4.1 Association of polymorphisms in the RAGE gene and inflammatory diseases

The RAGE gene is localized in a highly polymorphic region on chromosome 6 which also harbours important genes involved in immune response; it makes this gene candidate as potential risk or protective marker for inflammatory diseases. Accordingly, recent studies found associations of the non-synonymous RAGE Single Nucleotide Polymorphisms (SNPs) rs2070600 (Gly82Ser) located in exon 3 the ligand-binding domain of the receptor with measures of lung function (90, 91); in this variation the more common (G) allele encodes for Glycine (Gly), while the minor (A) allele encodes for Serine (Ser). In addition, this variant has been related to other inflammatory diseases such as arthritis (92), Alzheimer's disease (93, 94) and

diabetes associated microvascular dermatoses (95) moreover, this SNP has been reported to affect the serum sRAGE levels (96, 97). Other two SNPs located upstream of the gene in the promoter region rs1800625 (-429T>C / T-429C) and SNP rs1800624 (T-374A / -374T>A or A-374T / -374A>T) have been associated with the presence of type 1 diabetes (98) or type 2 diabetic complications such as insulin resistance (99) or retinopathy (100) and this SNP might affect the serum sRAGE levels (101).

1.2.5 sRAGE/esRAGE, S100A12 and HMGB-1 in inflammatory diseases

The fact that RAGE signaling through the binding of its best characterized ligands HMGB-1 and S100A12 is related to the accumulation of these proinflammatory molecules in acute and chronic diseases with extensive tissue damage, and that the two soluble forms of the receptor esRAGE and sRAGE are thought to function as decoy receptors that counteract the RAGE pathway, have risen interest in studying the expression of these proteins in such diseases with the aim to discover new drug targets or to use them as correlates of disease severity and outcome.

In Chronic Obstructive Pulmonary Disease (COPD) and asthma circulating sRAGE levels not only are significantly lower in stable COPD patients than in healthy controls, but also it is a strong predictor of the degree of airflow limitation (102). Interestingly there is a dichotomy of S100A12 and sRAGE association with COPD disease severity. S100A12 levels were significantly elevated in severe/very severe COPD patients, while sRAGE levels were significantly decreased with increasing disease severity and this observation was associated to neutrophilic inflammation (103). In addition, in another study, subjects with neutrophilic asthma or COPD had undetectable levels of sRAGE in the BAL, while levels of sRAGE in asthma/COPD without neutrophilia were similar to those in controls (104). Moreover, subjects with neutrophilic asthma had lower levels of serum sRAGE compared to those with non-neutrophilic asthma; accordingly, patients with neutrophilic COPD also had lower serum sRAGE compared to those with non-neutrophilic COPD (104).

Chronic neutrophilic inflammation is one of the causes of the progressive airway destruction in patients with Cystic Fibrosis (CF). Notably, CF patients do not have

detectable sRAGE in the airway fluids and concurrently they display high levels of S100A12, besides, RAGE expression is up-regulated on CF airway neutrophils when compared to their blood counterparts (105). CF lung biopsy specimens also reveal high expression of S100A12 by infiltrating neutrophils along with high S100A12 levels in sputum. S100A12 in serum increases significantly during acute infectious exacerbations and decreases after treatment with intravenous antibiotics (106).

Individuals with RA have decreased blood levels of sRAGE as compared with healthy controls and patients with non inflammatory joint disease. RA subjects treated with methotrexate have higher sRAGE level in the synovial fluid than patients without anti-rheumatic treatment (107). Plasma sRAGE concentration is also lower in osteoarthritis (OA) patients than in healthy controls and has an inverse correlation with disease severity (108). Indeed, in another study, the serum sRAGE level not only was lower in RA patients compared to healthy controls but also correlated negatively with disease activity and S100A12 levels which were higher in RA patients than controls (109). Also, Foell et al. reported that patients with all variants of active Juvenile Rheumatoid Arthritis (JRA) had higher S100A12 serum levels than healthy controls that correlated with several parameters of disease activity and decreased in response to different antiinflammatory therapies (110).

1.3 AIM OF THE STUDY

This study aimed to investigate the role of RAGE/sRAGE and their ligands S100A12 and HMGB1 during human pulmonary *Mtb* infections with the following specific hypothesis: i. The sRAGE, S100A12 and HMGB-1 serum levels in patients with pulmonary TB are associated with the amount of lung tissue damage and may predict the disease severity and/or progression. ii. Genetic variations within the RAGE gene may be associated with the pathogenesis and susceptibility to pulmonary TB in Hyderabad/ India.

CHAPTER 2: MATERIALS AND METHODS

2.1 Observational study

2.1.1 Study design: Cross sectional cohort and self controlled case series study.

2.1.2 Setting: The study was carried out in Mahavir hospital and research center in Hyderabad, Andhra Pradesh, India. A pilot survey was done in March 2011. The final recruitment was done from July 26 2011 to October 23 2012.

2.1.3 Participants: Criteria for inclusion as case were: newly diagnosed pulmonary sputum smear positive TB disease and treatment naïve patients who entered the DOTS program at Mahavir hospital. The diagnostic criterion for the presence of pulmonary TB was defined as the presence of one of the following: at least 2 initial sputum smear examinations positive for AFB or one sputum examination positive for AFB and radiographic abnormalities consistent with active pulmonary TB, or one sputum specimen positive for AFB and culture positive for Mtb. according to the India technical and operational guidelines for Tuberculosis control (21). Criteria for inclusion as healthy control were: absence of apparent acute or chronic pulmonary diseases or diseases of other origin and a negative history for TB disease. All healthy controls individuals were from the same geographical origin and living in Hyderabad. All subjects in the healthy control group were clinically in good health at the time of sample collection. Those individuals with signs and symptoms suggesting active pulmonary TB or a history of prior anti-TB treatment were excluded from the study. All study participants gave written informed consent before the investigations and the study was approved by the institutional ethics committee for bio-medical research Bhagwan Mahavir Medical Research Centre, Hyderabad, India.

2.1.4 End points and follow-up: All TB patients were prospectively followed up on average for 6 months from the beginning to the end of the treatment. The final end points were: death, loss to follow-up and end of therapy.

2.1.5 Variables: Demographic data, including sex, age and Body mass index (BMI) along with other relevant clinical data were recorded using the standard hospital's DOTS program questionnaire. The main potential confounders and effect modifiers were: age, sex, BMI, blood glucose, smoking, alcohol consumption, HIV infection, blood pressure, lipid profile (cholesterol, Low Density Lipoprotein (LDL) and

triglycerides), chronic kidney disease, cardiovascular disease and use of lipid lowering drugs which have been related to the risk of getting TB or to influence the levels of S100A12, sRAGE, esRAGE and HMGB-1 in serum (3, 111-113). The RAGE coding gene termed (AGER) was sequenced and the association of SNP and disease risk determined. The final treatment outcome was recorded as cured, died, failure or defaulted according to the India technical and operational guidelines for Tuberculosis control (21).

2.1.6 Data sources and measurements: Demographic data were obtained directly from patients or their relatives and healthy individuals during an interview performed by a trained researcher at initial recruitment. Smoking and alcohol consumption were self reported and assessed by including these questions according to previously described (114, 115) into the standard DOTS questionnaire. Other relevant clinical data were obtained from the hospital's TB control program records and medical staff. One peripheral blood sample was obtained after overnight fasting from healthy controls at initial recruitment and from TB patients at the start of therapy and after 2, 4 and 6 months. The serum was isolated and stored at -20°C.

2.1.6.1 Equipments

Equipment	Use	Manufacturer
MX2 digital automatic blood pressure monitor	Measurement of the blood pressure	Omron GmbH, Mannheim-Germany
Konelab 20 clinical chemistry analyzer	Measurement of lipid profile (total cholesterol, LDL cholesterol, triglycerides and HDL cholesterol) and creatinine	Thermo scientific, Rockford, Illinois- USA
Sysmex KX-21 blood analyser	Measurement of complete peripheral blood count	Sysmex, Kone-Japan
Accu-Chek Performa	Measurement of fasting blood glucose	Roche Diagnostics GmbH, Mannheim-Germany
DNA Engine, Peltier thermocycler	PCR assays	Biorad, Gurgaon-India

96-capillary 3730xl DNA Analyzer	DNA sequencing	Applied Biosystems Inc, Foster City, California-USA
Siemens Heliophos D X-rays generator	Chest radiography (CXR) analysis	Siemens, Mumbai-India

2.1.6.2 Reagents and kits

Reagent or kit	Use	Manufacturer
sRAGE ELISA kit	Measurement of serum sRAGE	R & D systems, Minneapolis, Minnesota-USA
esRAGE ELISA kit	Measurement of serum esRAGE	B-Bridge International, Tokyo-Japan
HMGB-1 ELISA kit	Measurement of serum HMGB-1	IBL international GmbH, Hamburg-Germany
S100A12/EN-RAGE ELISA kit	Measurement of serum S100A12	MBL international, Woburn, Massachusetts-USA
TNF-(α) ELISA kit	Measurement of serum TNF- α	BD biosciences, Heidelberg-Germany
IFN-(γ) ELISA kit	Measurement of serum IFN-γ	BD biosciences, Heidelberg-Germany
ADAM10 ELISA kit	Measurement of serum ADAM10	Abnova, Heidelberg-Germany
QIAamp DNA Blood Mini Kit	Isolation of peripheral blood genomic DNA	Qiagen, Hilden-Germany
GoTaq Hot Start Green Master Mix	PCR assays	Promega GmbH, Mannheim-Germany
Nuclease free water	PCR assays	Promega GmbH, Mannheim-Germany
QIAquick 96 PCR Purification Kit	PCR assays	Qiagen, Hilden-Germany

2.1.6.3 Oligonucleotides primers

Oligonucleotides primers		Product size	Manufacturer
RAGE promoter: GCAGTTCTCTCCTCACTTGTAAAC, GTTAAAGTGCTTTCTGCAGGG	AGER-Pro-F AGER-Pro-R	703bp	Eurofins Genomics, Bangalore-India
RAGE Exon 1: TGACTGTACCCAGAGGCTGG, GCAAGGCCTTTGGGAAAG	AGER-ex01-F AGER-ex01-R	657bp	Eurofins Genomics, Bangalore-India
RAGE Exon 2, 3 and 4: CTTCCAGGGAGACCAGCAATG, CCTTCACAGATACTCCTATGATGGG	AGER-ex02-F AGER-ex04-R	842bp	Eurofins Genomics, Bangalore-India
RAGE Exon 5: AATGAGAAGGGTGAGTCCTAAGG, TTGTGAGTGATCCCAGTGGC	AGER-ex05-F AGER-ex05-R	388bp	Eurofins Genomics, Bangalore-India
RAGE Exon 6 and 7: TTCTGGAAGTCTGACCCTTAGGG, GGGTGGCTGTTAGGGATAAGGC	AGER-ex06-F AGER-ex07-R	655bp	Eurofins Genomics, Bangalore-India
RAGE Exon 8 and 9: GGCAACAAAAGTGAAACTCCATCTC, TTCCTGAGGAGAAAGATTGGGG	AGER-ex08-F AGER-ex09-R	480bp	Eurofins Genomics, Bangalore-India
RAGE Exon 10: AAGCCAGACCCCTCAGACCTAG, GCATCTTCTCCCCAACTTGAGTAG	AGER-ex10-F AGER-ex10-R	503bp	Eurofins Genomics, Bangalore-India

2.1.7 Methods observational study

2.1.7.1 PCR analysis and sequencing: In all PCR assays the amplification mixture consisted of 7,5 µL of GoTaq Hot Start Green Master Mix 2x, 4,5 µL nuclease free water, 10 pmol/µL of oligonucleotides primers forward and reverse 1 µL each and 20 ng of genomic DNA template in a final volume of 16 µL. The PCR cycles (steps) were as follows: 1. 95°C 2 min., 2. 95°C 30 s, 3. 60°C 30s, 4. 72°C 1 min., 5. go to step 2: 34 times, 6. 72°C 5 min. The PCR products were cleaned up and sequenced using reverse and forward primers by Ocimum Biosolutions Pvt Ltd, (Hyderabad-India). The sequence analysis was performed using novosnp3.0.1 for Windows (University of Antwerp, Antwerp- Belgium) as allele calling algorithm. The AGER gene was

initially entirely sequenced in a pilot sub-group of 45 TB patients and 45 healthy controls and after this initial analysis only the regions of the promoter which include the AGER variants SNP ID rs1800624 and rs1800625 and the exon 3 SNP ID rs2070600 were sequenced in the rest of the study population.

2.1.7.2 Chest radiography: All patients received a posteroanterior CXR at the start of DOTS therapy, CXRs were first read by two radiologists and the final decision on their CXR specific features was reached on consensus, then a third independent radiologist assessed the CXR and the results of the first and second assessment were compared but the first was used for analysis. All radiologists were blind for clinical and laboratory data. In order to define the extent of pulmonary abnormalities, each lung was divided into two halves, an upper and a lower half, by a horizontal line drawn at the level of the upper endplate of a thoracic vertebra situated immediately below the tracheal carina. The four quadrants thus obtained were designated as follows: I, right upper quadrant (RUQ), II, right lower quadrant (RLQ), III, left upper quadrant (LUQ), and IV, left lower quadrant (LLQ). The overall extent of lung involvement, irrespective of pathology, was defined by the number of quadrants affected, i.e. from I to IV. Furthermore, alveolar space consolidation was separately graded on the basis of the four quadrant scheme. Moreover, the number of cavitations, when present, was assigned to one or more quadrants, e.g. 1/I for one cavitation in RUQ. In addition, the presence or absence of lymphadenopathy and pleural effusion has been noted, but not linked to the four quadrant scheme.

2.1.8 Study size: An initial pilot study was performed with 25 TB patients and 15 healthy controls; the required sample size was then calculated taking into account the effect size of the variables of interest and the presence of potential confounders and effect modifiers. The final sample size was 119 TB patients and 163 healthy controls.

2.2 In vitro study

2.2.1 Cell lines and culture mediums

Cell lines and mediums	Manufacturer
(SAEC) human small airway epithelial cells	Lonza, Basel-Switzerland
(NHBE) human bronchial /tracheal epithelial cells	Lonza, Basel-Switzerland
(HMVEC-L) human lung microvascular endothelial cells	Lonza, Basel-Switzerland
(THP-1) human monocytic cells	American Type Culture Collection ATCC, Manassas, Virginia-USA
(BEAS-2B) human bronchial epithelial cells	American Type Culture Collection ATCC, Manassas, Virginia-USA
(A549) adenocarcinomic human alveolar basal epithelial cells	DSMZ, Braunschweig-Germany
(SAGM™) small airway epithelial medium	Lonza, Basel-Switzerland
(BEGM™) bronchial epithelial cell medium	Lonza, Basel-Switzerland
DMEM high glucose (4,5 g/L) medium	PAA GmbH, Pasching -Austria
VascuLife® EnGS-Mv microvascular endothelial cell medium	Lifeline cell technology, Walkersville, Maryland-USA

2.2.2 Reagents and kits

Reagent or kit	Use	Manufacturer
Penicillin/ streptomycin	Cell culture	Biochrom, Cambridge
Foetal bovine serum	Cell culture	PAA GmbH, Pasching -Austria
Dulbecco´s Phosphate Buffered Saline (PBS)	Cell culture	PAA GmbH, Pasching -Austria
Ficoll-Paque plus	Isolation of PBMCs and granulocytes	GE Health Care, Munich-Germany
Dextran	Isolation of PBMCs and granulocytes	Sigma-Aldrich, Saint Louis, Missouri- USA
(MidiMACS™) separator and	Isolation of monocytes and	Miltenyibiotec GmbH, Bergisch

human CD14 micro beads	lymphocytes	Gladbach-Germamy
Amicon ultra 4 filters 30k	Concentration of supernatants	Millipore, Billerica, Massachusetts-USA
sRAGE ELISA Kit	Measurement of sRAGE in cell culture supernatants	Biovendor, Brno-Czech Republic
esRAGE ELISA kit	Measurement of esRAGE in cell culture supernatants	B-Bridge International, Tokyo-Japan
Protease activity (Pierce™) fluorescent protease assay	Positive control for protease activity	Thermo scientific, Rockford, Illinois- USA
Brij-35	ADAMs buffer	Thermo scientific, Rockford, Illinois- USA
SYBR Green master mix	PCR assay	Roche Diagnostics GmbH, Mannheim-Germany
High capacity cDNA reverse transcription Kit	cDNA reverse transcription	Applied Biosystems, Darmstadt Germany
Trizol reagent	RNA extraction	Invitrogen-life technologies, Carlsbad, California-USA
Annexin V-FITC Kit	Cell viability test	Miltenyibiotec GmbH, Bergisch Gladbach-Germamy

2.2.3 Recombinant proteins and proteases

Recombinant proteins or protease	Manufacturer
Human recombinant catalytic domain MMP1	Giotto Biotech, Firenze-Italy
Human recombinant catalytic domain MMP2	Giotto Biotech, Firenze-Italy
Human recombinant catalytic domain MMP3	Giotto Biotech, Firenze-Italy
Human recombinant catalytic domain MMP9	Giotto Biotech, Firenze-Italy
Human recombinant catalytic domain MMP13	Giotto Biotech, Firenze-Italy
Human recombinant ADAM10	R & D systems, Minneapolis, Minnesota-USA
Human recombinant TACE/ADAM17	R & D systems, Minneapolis, Minnesota-USA
Human recombinant EN-RAGE S100A12	R & D systems, Minneapolis, Minnesota-USA
GI254023X specific ADAM10 inhibitor	R & D systems, Minneapolis, Minnesota-USA
Specific ADAM10 fluorogenic peptide substrate MCA-Lys-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH2	R & D systems, Minneapolis, Minnesota-USA
Calibration standard MCA-Pro-Leu-OH	Bachem, Bubendorf- Switzerland

2.2.4 Buffers

Buffer	Preparation

MMPs buffer solution	5 mM $CaCl_2$, 0,1 mM $ZnCl_2$
ADAMs buffer solution	25 mM Tris, 2 µM $ZnCl_2$, 0,005(V/V) Brij-35, pH 9.0.

2.2.5 Antibodies

Antibody	Manufacturer
Anti-RAGE antibody clone DD/A11	Millipore, Billerica, Massachusetts-USA
Phycoerythrin (PE) goat anti-mouse IgG (H+L)	Antibodies-online GmbH, Aachen-Germany
Purified mouse IgG2a,k isotype control	BD biosciences, Heidelberg-Germany

2.2.6 Oligonucleotides primers

Oligonucleotides primers	Manufacturer
AGER FW: GACCAGGAGACACCCTGAGA	Eurofins Genomics, Ebersberg- Germany
AGER RW: CTGGGCTGAAGCTACAGGAG	
GAPDH FW: CAGCCTCAAGATCATCAGCA	Eurofins Genomics, Ebersberg- Germany
GAPDH RW: TGTGGTCATGAGTCCTTCCA	

2.2.7 Equipment

Equipment	Use	Manufacturer
Roche Light Cycler 480	PCR assay	Roche Diagnostics GmbH, Mannheim-Germany
ELISA Reader SpectraFluorPlus	ELISA	Tecan,Crailsheim-Germany
FACScan	Flow cytometry	BD biosciences, Heidelberg-Germany
CellQuest software	Flow cytometry analysis	BD biosciences, Heidelberg-Germany

2.2.7 Methods in vitro study

2.2.7.1 Isolation of PBMCs and granulocytes: PBMCs and granulocytes were isolated with Ficoll-Paque plus briefly: 30 mL of blood was collected from healthy volunteers and transferred to a Falcon tube after which 8 mL PBS and 4 mL of a solution containing 6% W/V Dextran in DMEM medium were added. The blood was let to sediment for 1 hour after which the upper phase was isolated, transferred to a

new Falcon tube, washed with PBS and centrifuged for 10 min. at 2000 rpm and 20°C. The cell pellet was resuspended in 15 mL PBS, added to a new tube containing 15 mL Ficoll and centrifuged for 35 min. at 2800 rpm and 20°C. Then the middle phase containing the PBMCs and the lower phase containing the granulocytes were collected. Monocytes and lymphocytes were separated using (MidiMACS™) separator and human CD14 micro beads following the manufacturer instructions.

2.2.7.2 RAGE RNA RT-PCR: The RNA was extracted from cell lines and primary cell cultures with the Trizol reagent following manufacture's instructions, reversed transcribed to cDNA using the high capacity cDNA reverse transcription Kit and stored at -20°C. RAGE RNA expression was assessed using indicated set of primers for RAGE and for GAPDH as housekeeping gene. All PCR assays were performed using the Roche Light Cycler 480. The amplification mixture consisted of 5 µL cDNA, 4,6 µL nuclease free water, 10 µL SYBR Green master mix and 0,2 µL of each primer at 100 pmol/µL in a final volume of 20 µL. The PCR cycles (steps) were as follows: 1. 95°C 2 min., 2. 95°C 30 s, 3. 60°C 30s, 4. 72°C 1 min., 5. go to step 2: 34 times, 6. 72°C 5 min.

2.2.7.3 Flow cytometry: 10^5 cells were treated with 10% foetal bovine serum in PBS for 15 min. to avoid non-Ag-specific binding of Abs and then stained with 1,5 µL anti-RAGE antibody clone DD/A11 for 30 min. followed by *5* µL phycoerythrin (PE) goat anti-mouse IgG (H+L) diluted 1/50 in PBS for 20 min. Purified mouse IgG2a,k was used as isotype control. Cell viability was assessed with Annexin V-FITC test. Fluorescence of stained cells was measured using a FACScan and data were analyzed using CellQuest software.

2.2.7.4 Protease experiment: THP-1 cells were seeded 5 x 10^6/mL in 6 wells plates in 2 mL and stimulated for 1 hour with each protease at two different concentrations as follows: MMP1, MMP2, MMP3, MMP9 and MMP13 at 10 and 20 ng/mL, each MMPs were prepared at the desired concentration in MMP buffer solution. ADAM10 and Tumor necrosis factor-α-Converting Enzyme (TACE)/ADAM17 at 10, and 20 ng/mL, both were prepared at the desire concentration in ADAMs buffer solution. In experiments assessing protease effect after co-stimulation with specific protease inhibitors, THP-1 cells 10^7/2mL were first stimulated 15 min with every specific

inhibitor with concentrations as follows: 10 and 25 µM GM6001 MMP inhibitor MMP2, 2 and 4 µM GI254023 for ADAM10. Supernatants were harvested and stored at -80°C, 2 x 10^5 cells were used to assess RAGE expression and cell viability, and the remaining cells were lysed with Trizol reagent and stored at -80°C for posterior RNA extraction.

2.2.7.4 ADAM10 inhibition by S100A12: ADAM10 and the specific ADAM10 fluorogenic peptide substrate MCA-Lys-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH2 were diluted at 2 ng/µL and 20 µM respectively in ADAM buffer solution, 25 µL of 2 ng/µL ADAM10 was loaded into a well of a 96 wells plate with 25 µL EN-RAGE S100A12 at 1, 6, 0,8 and 0,4 ng/µL diluted in water which was used also as control, the reaction was started by adding 50 µL of 20 µM Substrate. A serial dilution of the calibration standard MCA-Pro-Leu-OH starting at 8,8 µM was used to calculate ADAM10 specific activity. The plate was incubated at 37 °C for 30 minutes and excitation and emission were read at wavelengths of 320 nm and 405 nm (top read), respectively, in end point mode. The calculation of specific activity was performed following recombinant ADAM10 manufacture's instructions.

2.2.8 Statistical methods

Normally distributed variables were expressed as mean ± Standard Deviation (SD), not normally distributed variables as median ± Interquartile Range (IQR), and statistical differences between two groups were analyzed using two sided Student's *t* test or Mann Whitney U test, respectively. One way ANOVA with post hoc test or Kruskall Wallis and Mann Whitney U test with Bonferrini adjustment were used for normally and not normally distributed variables respectively when more than two groups were compared.

Binary logistic regression was used for adjustments for potential confounders in the cross sectional associations, for assessing predictors of disease status and to derivate the Odds Ratios (OR) for the association between RAGE genotype frequency and disease risk. Univariable logistic regression models were done and those variables found to be predictors of disease status were then analysed with hierarchical multivariable and backwards Likelihood Ratio (LR) regression models.

Hosmer-Lemeshow goodness of fit test was used as goodness of fit for model fitting assessment and VIF values were used to assess multicollinearity. The best fitting models were chosen by analysing the change in the Likelihood Ratio (LR) statistic after different variables were included or removed from the model. Cox & Snell and Nagelkerke R squares were reported to assess the proportion of variance explained by the predictors and different models.

The longitudinal data were analyzed with repeated measures ANOVA with post hoc test or with Friedman test and Wilcoxon signed rank test for normally and not normally distributed variables respectively.

Linear regression was used to analyse the relationship of sRAGE and S100A12 as dependent variables with other explanatory variables. In linear regression analysis ANOVA test was used as goodness of fit for model fitting assessment and the R^2 test to assess the proportion of variance explained by the predictors. In linear and logistic regressions, deviance and partial residuals were used to identify potential outliers influencing the models.

The association of individual CXR features and immune markers was assessed with univariable and multivariable ordinal regression analysis: the goodness of fit test was used for model fitting assessment, the amount of variation explained by the model with pseudo R^2 and the proportional odds assumption was assessed with the test of parallel lines. Proportional weighting without scale effect for alveolar infiltration was used in the comparison of hilar lymph nodes group.

Hardy–Weinberg equilibrium (HWE) for SNPs was assessed and considered to be in HWE when p>0.05 using novosnp3.0.1 for Windows (University of Antwerp). SPSS 21 (SPSS Inc., Chicago, Illinois-USA) was used for data analysis and values of p <0.05 were considered significant. Graphics were done with GraphPad Prism Software version 5.00 for Windows (GraphPad Software, San Diego California, USA) and sample size and power calculations with OpenEpi version 3.01. The strengthening the reporting of observational studies in epidemiology (STROBE) guidelines was used to guide the report of this observational study (116).

CHAPTER 3: RESULTS

3.1 sRAGE and S100A12 are differently regulated in TB patients and healthy controls.

To assess the association of sRAGE, esRAGE and the ligands of RAGE; S100A12 and HMGB1 with pulmonary TB, I carried out a cross sectional cohort study between July 2011 and October 2012 at Mahavir hospital in Hyderabad, India. During this study period 119 TB patients and 163 healthy controls were recruited. The main characteristics of the subjects are outlined in Table 1. As a group, the TB patients were younger than the healthy controls (26 ± 11 vs 32 ± 10 years; $p < 0.0001$) and had a lower BMI (16 ± 2.6 vs 24 ± 4.5 kg/m^2; $p < 0.0001$). Both groups had similar gender distribution. I included the measurements of all participants into the final statistical analysis regardless of the levels for fasting blood glucose, systolic and diastolic Blood Pressure (BP) as well as the levels for triglycerides, Low Density Lipoprotein (LDL), High Density Lipoprotein (HDL) and cholesterol that were measured in parallel. None of the participants reported history of heart disease or the current use of lipid lowering drugs (Data not shown). Consequently, after one initial comparison, I did the following set of adjustments to those variables with significant crude differences: I used the age to adjust the following parameters: BMI, a positive report of smoking, drinking as well as the systolic and diastolic BP. Malnutrition is a hallmark characteristic and risk factor for TB (3, 117) and in addition it is well documented that the BMI influences the lipid profile, the BP (111) and the creatinine levels (112), accordingly, I adjusted these variables for BMI. The levels of sRAGE and its ligand S100A12, were adjusted for age, BMI, smoking, drinking and systolic BP. This was also done for the blood levels of the proinflammatory cytokine TNF-α.

After these adjustments a higher frequency of individuals with positive smoking and drinking habits were found in the TB patient group compared to the healthy control group (0.21 ± 0.83 Vs 0.07 ± 0.28 pack -years) and (19% Vs 2%) (p 0.004 and < 0.0001) respectively (Table. 1). In addition, in the group of TB patients lower mean systolic BP (103 ± 15 Vs 123 ± 17 mm/Hg) ($p < 0.0001$) were measured. The mean serum sRAGE was lower in TB patients (0.74 ± 0.54 ng/mL) than in healthy control subjects (1.002 ± 0.56 ng/mL) (p 0.003) as shown in (Table 1). In contrast, mean levels of S100A12 and TNF-α were higher in TB patients (2.332 ± 1.631 ng/mL) and (13.4 ± 21.8 pg/mL) when compared to the healthy control group (1.090 ± 1.050

ng/mL and 6.1 ± 20.4 pg/mL; $p < 0.0001$ and 0.02 respectively). Before and after adjustment there were no differences in the blood levels of IFN-γ, HMGB-1 and esRAGE (Table 1).

These data show that the levels of S100A12, sRAGE and TNF-α are cross sectionally associated with pulmonary TB. This association was independent not only from other known risk factors for TB such as low BMI, positive habits for alcohol consumption and smoking, fasting blood glucose levels but also from parameters that are reported to influence the serum levels of S100A12 and sRAGE such as high cholesterol and triglyceride levels, elevated BMI and systolic and diastolic BP.

Table 1. Baseline characteristics, related parameters and serum levels of sRAGE and the RAGE ligands S100A12 and HMGB1 in TB patients and healthy controls.

Adjusted for: a. Age, b. BMI, c. Smoking, d. Drinking, e. Systolic BP by binary logistic regression. (SD) standard deviation, (IQR) interquartile range.

Variable	Case (n=119)	Control (n=163)	P value (Crude)	P value (Adjusted)
Age mean (SD), (Years)	26 ± 11	32 ± 10	< 0.0001	-----
Sex No. (%), male/female	58 (49)/61 (51)	82 (50)/81 (49)	0.81	-----
BMI mean (SD), (kg/m^2)	16 ± 2.6	24 ± 4.5	< 0.0001	< 0.0001 [a]
Smoking habits mean (SD), pack-year	0.21 ± 0.83	0.07 ± 0.28	0.043	0.004 [a]
Drinking habits No. (%), (yes/no)	22 (19)/97 (81)	3 (2)/160 (98)	< 0.0001	< 0.0001 [a]
Fasting blood glucose mean (SD), (mg/dL)	97 ± 30	104 ± 30	0.06	-----
Blood pressure mean (SD), (mm/Hg)				
Systolic	103 ± 15	123 ± 17	< 0.0001	< 0.0001 [a,b]
Diastolic	74 ± 10	80 ± 12	< 0.0001	0.97 [a,b]
Cholesterol mean (SD), (mg/dL)	167 ± 27	183 ± 33	< 0.0001	0.73 [b]
LDL mean (SD), (mg/dL)	104 ± 25	115 ± 25	< 0.0001	0.77 [b]
HDL mean (SD), (mg/dL)	41 ± 8.8	43 ± 33	0.37	-----
Tryglicerides mean (SD), (mg/dL)	112 ± 38	136 ± 80	0.003	0.87 [b]
Creatinine mean (SD), (mg/dL)	0.91 ± 0.13	0.96 ± 0.17	0.005	0.77 [b]
TNF-α mean (SD), (pg/mL)	13.4 ± 21.8	6.1 ± 20.4	0.004	0.02 [a,b,c,d,e]
IFN-γ median (IQR), (pg/mL)	7.0 ± 18	3.7 ± 11	0.17	-----
HMGB-1 median (IQR), (ng/mL)	3.3 ± 5.5	3.0 ± 5.6	0.67	-----
S100A12 mean (SD), (ng/mL)	2.332 ± 1.631	1.090 ± 1.050	< 0.0001	< 0.0001 [a,b,c,d,e]
sRAGE mean (SD), (ng/mL)	0.74 ± 0.54	1.002 ± 0.563	< 0.0001	0.003 [a,b,c,d,e]
esRAGE mean (SD), (ng/mL)	0.06 ± 0.15	0.07 ± 0.10	0.65	-----

3.2 sRAGE and S100A12 are independent predictors of disease status.

After the initial group comparison, I analysed if the variables that demonstrated significant cross sectional differences between cases and controls could independently predict the probability of being a TB patient or a healthy control depending on the values of these parameters. Therefore, I used the variable disease status coded as TB patient=1, healthy control=0 as dependent variable and constructed several logistic regression models. In the first analysis I integrated all variables with significant adjusted differences individually into univariable regression models. Afterwards, in order to assess which predictors remained significantly associated with the disease status when they were all included into a multivariable model, I did a hierarchical multivariable analysis using different combinations of variables in order to find the best fitting predictor model indicated by a significant change in the Likelihood Ratio (LR), its chi square test and the pseudo R square values, the final model was confirmed with a backward LR regression model.

As shown in (Table 2) the results of the univariable analysis demonstrated that the values for serum sRAGE, BMI, systolic BP, fasting blood glucose, age and positive smoking habits were negative predictors of disease status with negative regression coefficient or beta (β) whereas serum levels of S100A12, TNF-α as well as positive drinking habits were positive predictors of disease status with a positive beta β value. The regression coefficient or beta (β) indicates that for every unit decrease or increase in these variables there is a unit increase or decrease in the probability that a person is a TB patient if the regression coefficient is negative or positive, respectively. However, smoking and drinking habits as well as the S100A12 serum levels had a significant ($p < 0.05$) Hosmer and Lemeshow goodness of fit test indicating that the model is not a good fit to the data. In the univariable analysis the BMI was the best single predictor of disease status (-2Log likelihood 163.8), R^2 (Cox 0.55 and Nagelkerke 0.73) (Table 2).

Subsequently, I assessed all single predictors in a multivariable analysis and the following variables remained significantly associated with the outcome: serum levels of sRAGE, S100A12, TNF-α, as well as BMI, systolic BP and fasting blood glucose (Table 3). As shown in (Table 3) for sRAGE the beta value (β) -0.159 suggests that for every unit decrease in sRAGE there is a unit increase in the probability that a person is a TB patient (p 0.002) and the Odds Ratio (OR) or expected beta (exp β)

equal to 0.203 (95%CI 0.074 – 0.552) indicates that as sRAGE serum levels increase, the probably of having TB decreases. In contrast, for S100A12 the (β) value 0.630 and the exp β 1.873 (95%CI 1.212 – 2.891) means that for every unit increase in S100A12 there is a unit increase in the probability that a person is a TB patient (p 0.004). Consequently, as S100A12 serum levels increase, so does the probability of being a TB patient (Table 3).

Similarly TNF-α was a predictor with positive association to TB (Table 3). Every unit increase in its value augment the probability of being a TB patient β 0.020 and exp β 1.02 (95%CI 1.005 – 1.034) (p 0.032). As expected, the BMI was a predictor with negative association (β) – 0.727 and exp β 0.485 (95%CI 0.382 – 0.603) ($p < 0.0001$), thus, as the BMI increases the probability of having pulmonary TB decreases. Interestingly as shown in (Table 2), in the univariable analysis fasting blood glucose had a negative association whereas in the multivariable test (Table 3) its coefficient was positive suggesting that when all other variables in the model are held constant, the likelihood of being a TB patient increases as the fasting blood glucose levels increases (β 0.02) and exp β 1.024 (95%CI 1.012 – 1.031) ($p < 0.0001$). The systolic BP had a negative association (Table 3). In the multivariable analysis the combination of all these variables could predict better the disease status than every parameter alone (-2Log likelihood 103), R^2 (Cox 0.61 and Nagelkerke 0.83) (Table 3).

Taken together these data suggest that increased serum levels of S100A12 and decreased serum levels of sRAGE are independent predictors of disease occurrence.

Table 2. Univariable logistic regression analysis: identification of predictors of disease status.

Model 1 sRAGE constant: 0.206, -2Log likelihood 405-Chi^2 0.016, Hosmer and lemeshow test 0.64,

Predictor	β	S.E	P value	Exp β (OR)	95%CI for Exp β
Model 1					
sRAGE	-0.483	0.206	0.019	0.613	0.413 – 0.924
Model 2					
S100A12	0.681	0.108	<0.0001	1.974	1.592 – 2.443
Model 3					
TNF-α	0.019	0.007	0.010	1.024	1.005 – 1.034
Model 4					
BMI	-0.650	0.073	<0.0001	0.523	0.452 – 0.603
Model 5					
Systolic BP	-0.081	0.011	<0.0001	0.924	0.903 – 0.944
Model 6					
Fasting blood glucose	-0.009	0.005	0.081	0.992	0.979 – 1.001
Model 7					
Age	-0.051	0.012	<0.0001	0.954	0.927 – 0.973
Model 8					
Smoking	-0.566	0.319	0.076	1.763	0.943 – 3.291
Model 9					
Drinking	2.487	0.629	<0.0001	12.02	3.505 – 41.23

R^2 (Cox 0.019 and Nagelkerke 0.026). **Model 2 S100A12** constant: -1.42, -2Log likelihood 328-Chi^2 <0.0001, Hosmer and lemeshow test 0.01, R^2 (Cox 0.17 and Nagelkerke 0.23). **Model 3 TNF-α** constant: -0.48, -2Log likelihood 375-Chi^2 0.016, Hosmer and lemeshow test 0.07, R^2 (Cox 0.03 and Nagelkerke 0.04). **Model 4 BMI** constant: 12.05, -2Log likelihood 170.5-Chi^2 <0.0001, Hosmer and lemeshow test 0.88, R^2 (Cox 0.55 and Nagelkerke 0.73). **Model 5 Systolic BP** constant: 8.8, -2Log likelihood 312.1-Chi^2 <0.0001, Hosmer and lemeshow test 0.21, R^2 (Cox 0.24 and Nagelkerke 0.33). **Model 6 Fasting blood glucose** constant: 0.66, -2Log likelihood 403.8-Chi^2 0.048, Hosmer and lemeshow test 0.86, R^2 (Cox 0.012 and Nagelkerke 0.017). **Model 7 Age** constant: 1.17, -2Log likelihood 392-Chi^2 0.002, Hosmer and lemeshow test 0.82, R^2 (Cox 0.06 and Nagelkerke 0.08). **Model 8 Smoking** constant: -0.302, -2Log likelihood 405-Chi^2 0.032, Hosmer and lemeshow test 0.001, R^2 (Cox 0.01 and Nagelkerke 0.02). **Model 9 Drinking** constant: -0.494, -2Log likelihood 358-Chi^2 <0.0001, Hosmer and lemeshow test 0.001, R^2 (Cox 0.08 and Nagelkerke 0.11).

Table 3. Multivariable logistic regression analysis and identification of independent predictors of disease status

Predictor	β	S.E	P value	Exp β (OR)	95%CI for Exp β
sRAGE	-0.159	0.512	0.002	0.203	0.074 – 0.552
S100A12	0.630	0.221	0.004	1.873	1.212 – 2.891
TNF-α	0.020	0.010	0.032	1.024	1.005 – 1.034
BMI	-0.727	0.114	<0.0001	0.485	0.382 – 0.603
Systolic BP	-0.087	0.023	<0.0001	0.913	0.875 – 0.964
Fasting blood glucose	0.023	0.007	<0.0001	1.024	1.012 – 1.031

Model constant: 21.33, -2Log likelihood 103-Chi^2 <0.0001, Hosmer and lemeshow test 0.89, R^2 (Cox 0.61 and Nagelkerke 0.83).

3.3 S100A12 and sRAGE serum levels change longitudinally dependent on the duration of the antibiotic (DOTS) therapy.

To further assess if the observed cross sectional associations between sRAGE, S100A12, TNF-α, BMI, systolic BP and fasting blood glucose with pulmonary TB have a causal relationship and to rule out the confounding effect of other unknown or unmeasured factors (118) as well as to test if these variables are related to disease recovery and final treatment outcome, first I analysed if their values change over time after starting antibiotic therapy at 0, 2, 4 and 6 months by a repeated measures analysis in those subjects who had the values of these parameters available at the four time points. Second, I retrieved the final treatment outcome from the hospital records and tested if there was an association between any of the variables in the multivariable model and final outcome by a Cox regression analysis.

As shown in (Figure 1a) a significant change in the median sRAGE serum levels could be shown, it increased at the time point of 2 months (0.755 ± 0.968 ng/mL), 4 months (0.855 ± 0.619 ng/mL) and 6 months (1.117 ± 1.220 ng/mL) compared to the time point of diagnosis (0.630 ± 629 ng/mL) (p 0.008, <0.0001 and 0.005 respectively). There were no significant differences between the time points of 2, 4

and 6 months, suggesting that after an initial rise in sRAGE level between 0 and 2 months, the levels remained unchanged during the further time of therapy. In contrast, the median levels of S100A12 did not have significant changes in this first two month time interval (1.320 ± 1.352 ng/mL); instead, its levels decreased after 4 months (0.822 ± 1.196 ng/mL) compared to 0 months (1.262 ± 1.358 pg/mL; p 0.20 and <0.0001, respectively), (Figure 1b).

Similar to the trend of sRAGE, the median TNF-α demonstrated a significant change after 2 months compared to the time of diagnosis; its levels after 2 months (0.9 ± 4.9 pg/mL), 4 months (0.1 ± 13 pg/mL) and 6 months (1.5 ± 6.5 pg/mL) were significantly lower compared to the levels at 0 months (9.5 ± 31 pg/mL) (p 0.007, 0.003 and 0.006, respectively), (Figure 1c). In addition no significant differences between 2, 4 and 6 months were found. Importantly, during the treatment the mean BMI started to increase at 2 months (17.7 ± 2.8 kg/m^2) compared to 0 months (16.4 ± 2.6 kg/m^2) (p <0.0001) and further increased continuously after 4 months (18.1 ± 3.2 kg/m^2) (p <0.0001) compared to 2 months and at 6 months (18.7 ± 3.2 kg/m^2) (p 0.027), compared to 4 months (Figure 1d).

Likewise as shown in (Figure 1e) the median systolic BP increased at the time point of 2 months (108.5 ± 18.2 mm/Hg), 4 months (109 ± 18.5 mm/Hg) and 6 months (110 ± 19.2 mm/Hg) compared to the time point of diagnosis (101.5 ± 9.5 mm/Hg) (all p <0.0001) and since there were no significant differences between 2, 4 and 6 months, it suggests that after the initial rise at 2 months the systolic BP remained unchanged. In contrast, the fasting blood glucose had a tendency to increase after 2 months (96.5 ± 20.2 mg/dL) (Figure 1f).

Subsequently, the final treatment outcome retrieved from the hospital records showed that 117 patients out of the 119 recruited in the study completed the 6 months treatment. One patient defaulted treatment and did not come back to the hospital and one patient prematurely died shortly after starting therapy for unknown reasons. Two patients out of the 117 who completed treatment were diagnosed with MDR and three patients who had been previously recorded as cured returned to the hospital as relapses afterwards. The treatment cure rate was 94.1% and there was

no association between any of the variables in the multivariable model and final treatment outcome (Data not shown).

In summary these data show that the levels of sRAGE, S100A12, TNF-α, BMI and systolic BP vary over time which is dependent of the time of antibiotic therapy suggesting a causal relationship of these parameters with pulmonary TB.

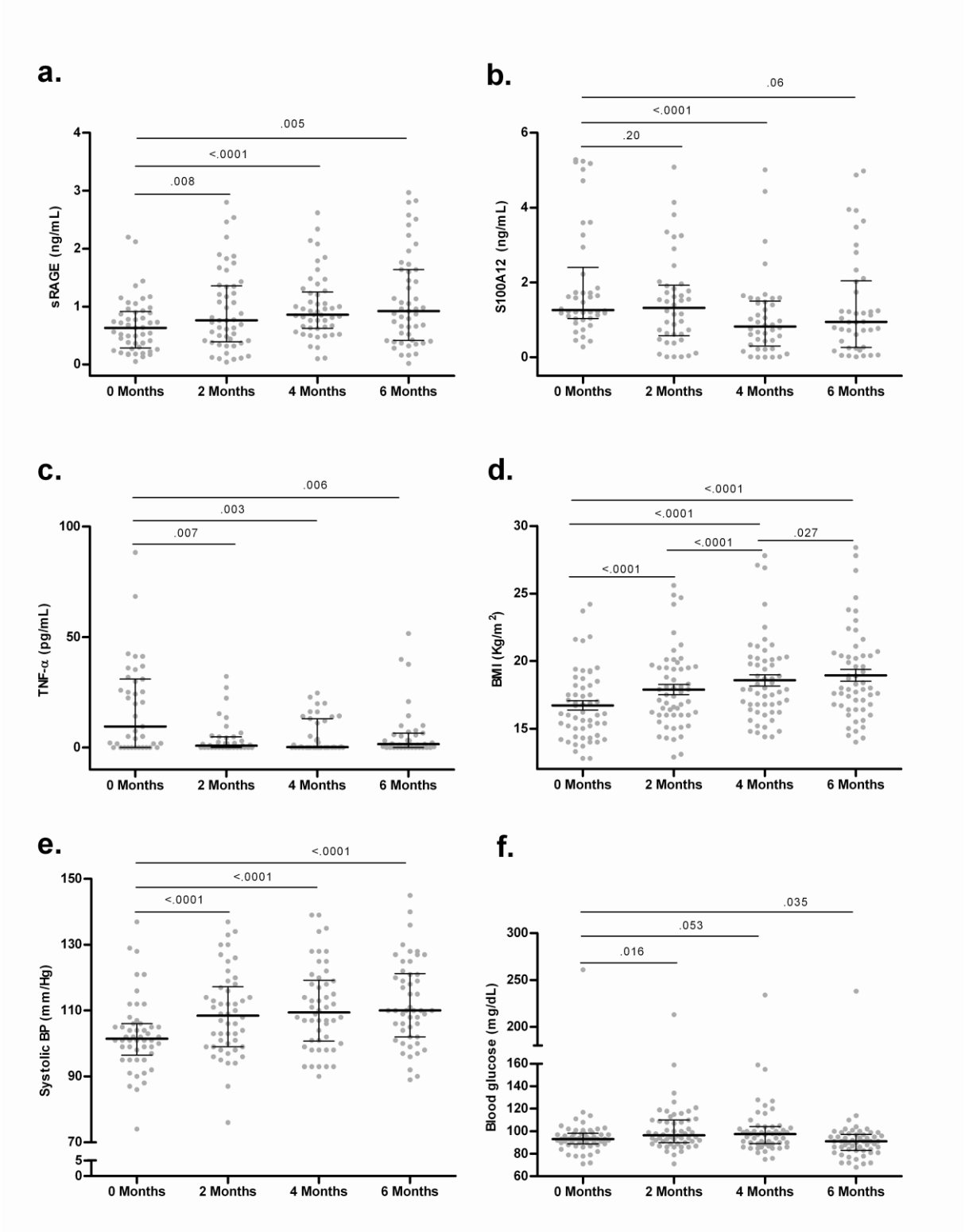

Figure 1. S100A12, sRAGE, TNF-α, BMI and systolic BP values change longitudinally during disease recovery under antibiotic (DOTS) therapy. TB patients were followed up during the antibiotic treatment on average 6 months and the values of **(a)** sRAGE, **(b)** S100A12, **(c)** TNF- α, **(d)** BMI, **(e)** systolic BP and **(f)** blood glucose were compared over time at 0, 2, 4 and 6 months in those individuals for which the four measurements were available, significance was tested by Friedman and Wilcoxon signed rank tests in

(a),(b),(c),(e) and (f) and represented as the median ± interquartile range (IQR) or with repeated measure ANOVA with post hoc test for (d) and represented as mean ± standard deviation (SD). n=50 a., b. and e., n=40 c. and d. n=55 f. individuals per time point.

3.4 Disease status, TNF-α, esRAGE and creatinine are predictors of sRAGE whereas disease status and HMGB-1 predict S100A12.

To determine the variables that are associated with sRAGE and S100A12 serum levels, I first did a correlation analysis. Subsequently, I integrated all parameters with significant correlations with sRAGE and S100A12 into a univariable linear regression followed by a multivariable backward linear regression analysis in order to identify the best combination of independent predictors for sRAGE and S100A12 serum levels.

As shown in (Table 4) in the correlation analysis sRAGE was positively correlated to BMI, creatinine, esRAGE and TNF-α. In contrast, the correlation with S100A12 was negative. However, S100A12 had a negative correlation with BMI, systolic BP, sRAGE, cholesterol, LDL and creatinine levels, however, its correlation with HMGB-1 and IFN-γ was positive.

Consequently, I assessed all variables significantly correlating with sRAGE and S100A12 serum levels by univariable linear regression. As shown in (Table 5) TNF-α, esRAGE, BMI and creatinine levels were found to be positive predictors of sRAGE levels, moreover, S100A12 and disease status were also predictors of sRAGE but with negative associations. Likewise, HMGB-1, disease status and IFN-γ serum levels were positive predictors of S100A12 while BMI, systolic BP, cholesterol, creatinine and sRAGE serum levels were negative predictors (Table 5). Importantly, the results of the univariable analysis showed that the disease status was the best predictor of sRAGE and S100A12 (R^2 0.052 and 0.17) (Table 5).

Notably, when all variables with significant correlations were assessed by multivariable regression, TNF-α, esRAGE and creatinine levels were found to be predictors of sRAGE all with positive associations (β) values 4,1, 1416 and 524 (p 0.005, < 0.0001 and 0.008) respectively. As shown in (Table 6), these data indicate that for every unit increase in TNF-α, esRAGE and creatinine levels, sRAGE

increases 4.1, 1416 and 524 units respectively. In contrast, disease status was predictor of sRAGE with negative association, (β) value -238 (p 0.001), therefore, when the variable disease status changes from healthy control to TB patient, sRAGE decreases 238 units.

Surprisingly, although in the univariable analysis S100A12 had more predictor variables, in the multivariable regression only disease status and HMGB-1 were positively associated to the S100A12 serum levels, (β) values 566 and 25 (both $p < 0.0001$), (Table 6). These data suggest that when the variable disease status changes from healthy control to TB patient, S100A12 serum levels increase 566 units. In addition, for every unit increase in HMGB-1 serum levels the value of S100A12 levels increase 25 units.

In summary I found that disease status, TNF-α, esRAGE and creatinine levels explain the variation in sRAGE levels, whereas disease status and HMGB-1levels predict the serum levels of S100A12.

Table 4. Correlation analysis of variables correlated with sRAGE and S100A12.

Variable	BMI	Fasting blood gluc.	Sys. BP	Dias. BP	Chol.	LDL	Trig.	Crea.	sRAGE	esRAGE	S100A12	HMGB-1	IFN-γ
Fasting blood gluc.	0.24 < 0.001												
Sys. BP	0.49 < 0.001	0.26 < 0.001											
Dias. BP	0.34 < 0.001	0.18 0.002	0.65 < 0.001										
Chol.	0.28 < 0.001	0.2 0.001	0.31 < 0.001	0.2 0.001									
LDL	0.26 < 0.001	0.11 0.05	0.27 < 0.001	0.16 0.005	0.89 < 0.001								
Trig.	0.17 0.003	0.21 < 0.001	0.2 0.001	0.17 0.004	0.49 < 0.001	0.19 0.001							
Crea.	0.26 < 0.001	0.07 0.19	0.11 0.06	0.08 0.15	0.24 < 0.001	0.17 0.004	0.32 < 0.001						
sRAGE	0.21 < 0.001	-0.02 0.97	0.07 0.22	-0.05 0.40	0.03 0.52	0.06 0.26	0.01 0.84	0.19 0.001					
esRAGE	0.02 0.78	0.02 0.75	-0.03 0.56	-0.06 0.27	-0.06 0.28	-0.05 0.37	-0.03 0.60	0.01 0.72	0.34 < 0.001				
S100A12	-0.24 < 0.001	-0.04 0.47	-0.15 0.01	-0.04 0.50	-0.12 0.03	-0.11 0.05	-0.09 0.16	-0.17 0.004	-0.14 0.05	-0.09 0.87			
HMGB-1	-0.05 0.36	-0.04 0.48	-0.15 0.01	-0.1 0.09	-0.09 0.12	-0.16 0.08	-0.03 0.60	-0.13 0.02	-0.05 0.42	0.06 0.32	0.14 0.01		
IFN-γ	-0.08 0.28	0.21 0.008	-0.09 0.27	-0.17 0.03	-0.08 0.30	-0.1 0.22	0.03 0.96	-0.03 0.97	0.01 0.89	0.18 0.02	0.17 0.03	0.28 < 0.001	
TNF-α	-0.11 0.05	-0.03 0.95	-0.12 0.04	-0.08 0.16	-0.15 0.009	-0.14 0.01	-0.1 0.07	-0.01 0.84	0.15 0.01	0.1 0.07	0.09 0.10	0.16 0.01	0.04 0.96

Abbreviations: Gluc. Glucose, Sys. Systolic, Dias. Diastolic, Chol. Cholesterol, Trig. Triglycerides, Crea. Creatinine. Upper value R^2, Lower value P value.

Table 5. Univariable linear regression analysis to identify predictors of sRAGE and S100A12

Predictor	β	S.E	P value	95%CI for β
sRAGE				
Disease status (Sick)	- 262	67.7	< 0.0001	-396 – -129
TNF-α	4.1	1.5	0.010	1.01 – 7.2
esRAGE	1515	250	< 0.0001	1022 – 2009
BMI	21.3	5.9	< 0.0001	9.62 – 36.1
S100A12	-0.045	0.24	0.058	-0.92 – 0.002
Creatinine	720	216	0.001	294 – 1145
S100A12				
Disease status (Sick)	1242	160	< 0.0001	926 – 1558
BMI	-63.6	15	< 0.0001	-33.3 – 93.9
Systolic BP	-11.2	4.4	0.013	-20 – -2.3
Cholesterol	-5.8	2.7	0.032	-11 – -0.51
Creatinine	-1602	549	0.004	-2683 – -521
sRAGE	-0.28	0.15	0.058	-0.58 – 0.1
HMGB-1	37.1	15.3	0.016	6.9 – 67.3
IFN-γ	6.3	2.9	0.032	0.5 - 12

Models sRAGE: TNF-α: Constant 855, ANOVA 0.01, R^2 0.024. esRAGE: Constant 788, ANOVA < 0.0001, R^2 0.11. BMI: Constant 440, ANOVA < 0.0001, R^2 0.045. S100A12: Constant 966, ANOVA 0.058, R^2 0.013. Creatinine: Constant 215, ANOVA < 0.0001, R^2 0.039.Disease status: Constant 1002, ANOVA < 0.0001, R^2 0.052. **Models S100A12:** Disease status: Constant 1090, ANOVA < 0.0001, R^2 0.17. BMI: Constant 2953, ANOVA < 0.0001, R^2 0.060. Systolic BP: Constant 2868, ANOVA 0.013, R^2 0.022. Cholesterol: Constant 2649, ANOVA 0.032, R^2 0.017. Creatinine: Constant 3129, ANOVA 0.004, R^2 0.030. sRAGE: Constant 1836, ANOVA 0.058, R^2 0.013. HMGB-1: Constant 1403, ANOVA 0.016, R^2 0.022. IFN-γ: Constant 770, ANOVA 0.032, R^2 0.031.

Table 6. Multivariable linear regression analysis to identify independent predictors of sRAGE and S100A12

Predictor	β	S.E	P value	95%CI for β
Model 1				
sRAGE				
Disease status				
(Sick)	- 238	64	< 0.0001	-364 – -112
TNF-α	4.1	1.4	0.005	1.2 – 7.0
esRAGE	1416	240	< 0.0001	942 – 1889
Creatinine	524	197	0.008	135 – 913
Model 2				
S100A12				
Disease status				
(Sick)	566	66	< 0.0001	363 – 537
HMGB-1	25	4.5	< 0.0001	17 - 34

Model 1: Disease status, BMI, creatinine, S100A12, esRAGE, TNF-α. Constant 368, ANOVA < 0.0001, R^2 0.203. **Model 2:** Disease status, BMI, systolic blood pressure, cholesterol, creatinine, sRAGE, HMGB-1, IFN-γ. Constant 462, ANOVA < 0.0001, R^2 0.46

3.5 Single nucleotide polymorphisms in the RAGE (AGER) gene show no association with disease susceptibility or sRAGE serum levels.

The association between the RAGE pathway and several inflammatory diseases as well as the differences in sRAGE serum levels have been attributed to the presence of SNPs in the RAGE gene named AGER (97, 101). Therefore, I investigated first if there was an association between the frequency of SNPs in the RAGE gene and the susceptibility for pulmonary TB. In a next step I measured if the observed difference in sRAGE levels between patients and healthy controls is dependent on the presence

of SNPs. For this, the RAGE gene was amplified and sequenced using PCR and the Sanger sequencing method.

In the first analysis I amplified and sequenced the eleven exons and the promoter region in a pilot group of 45 TB patients and 45 healthy controls (Data not shown). Subsequently, I amplified and sequenced the most relevant functional polymorphisms, those which frequency in the pilot group was > 1% or the differences between groups suggested a potential association, thus, after analysis of the pilot group, I continued the analysis with the rest of the individuals only for SNPs rs2040600 (G82S) in exon 3 and rs1800624 (374T/A) along with rs1800625 (429T/C) in the promoter region.

All markers were in Hardy–Weinberg equilibrium and had a genotyping call rate of (92.43%) and (90.13%) for SNP Rs1800624, (87.39%) and (90.13%) for SNP Rs1800625, along with (94.95%) and (99.3%) for SNP Rs2070600 in cases and controls respectively. As shown in (Table 7) there were no significant differences in the genotype frequencies and none of the SNPs showed any association with the occurrence of pulmonary TB. Next, I compared the levels of sRAGE, esRAGE, S100A12 and HMGB-1 at the time of diagnosis between the different genotype groups independent of disease status and subsequently according to genotype subgroups within cases compared to controls. Again no significant differences were found between cases and controls (Data not shown).

In order to assess the ability of this study to detect an effect in the case that it really exists taking into account the sample size used, I assessed the power of the study according to the effect sizes for every SNP and the required sample size to find a statistically significant difference. The power was 10% for SNPs rs1800624 and rs1800625 and would require 20000 and 2643 individuals per group respectively. The power for SNP rs2070600 was 30% and would require 368 individuals per group.

Thus, although in this study SNPs in the RAGE (AGER) gene did not show association with the risk for pulmonary TB or with the observed differences in sRAGE

concentration, a bigger sample size might show that SNP rs2070600 has a protective effect for the occurrence of pulmonary TB.

Table 7. SNP analysis of the RAGE SNPs rs2040600 (G82S), rs1800624 (374T/A) and rs1800625 (429T/C) in relation to disease susceptibility.

Variable	Case (n=119)	Control (n=152)	Odds Ratio (OR)	P value	95% C.I for (OR)
Rs1800624					
T/T	72 (65.5%)	97 (70.8%)	Ref.		
A/T	22 (20%)	25 (18.2%)	1.1	0.60	0.62 – 2.2
Delection/T	12 (10.9%)	12 (8.8%)	1.3	0.49	0.57 – 3.1
A/A	4 (3.6%)	2 (1.5%)	2.6	0.26	0.48 – 15
A/Delection	0	1 (1%)	------	------	------
Rs1800625					
T/T	82 (78.8%)	101 (78.3%)	Ref.		
C/T	20 (19.2%)	26 (20.2%)	0.94	0.87	0.49 – 1.8
C/C	2 (1.9%)	2(1.6%)	1.2	0.83	0.17-8.9
Rs2070600					
G/G	93 (82.3%)	118 (78.1%)	Ref.		
A/G	19 (16.8%)	30 (19.9%)	0.8	0.5	0.264 – 1.1
A/A	1 (0.9%)	3 (2%)	0.42	0.45	0.4 – 4.1

3.6 Increased sRAGE levels in the supernatants of THP1 monocytes are dependent on the presence of ADAM10 which *in vitro* activity is inhibited by S100A12.

The observational study revealed that only a small fraction of the serum sRAGE is produced by alternative splicing: the mean concentration of esRAGE was (0.06 ± 0.15 ng/mL) and the mean sRAGE (0.74 ± 0.563 ng/mL) in TB patients, with a similar tendency in the healthy controls group (Table 1). Since there was no association between any RAGE SNPs tested and the sRAGE serum levels, in a next step I investigated the cellular source of sRAGE and the mechanism of its production. Thus, I established an *in vitro* model of sRAGE secretion to study the kinetics of sRAGE levels that where found to be lower in TB patients when compared to healthy controls. Therefore, I assessed RAGE protein and RNA expression by flow cytometry and RT-PCR respectively in freshly isolated blood primary cells; monocytes, lymphocytes and granulocytes and in cultured human monocyte cell line THP-1, as

well as in pulmonary primary cells; SAEC human small airway epithelial cells, NHBE human bronchial/tracheal epithelial cells and HMVEC-L human lung endothelial cells, along with the human alveolar basal epithelial cell line A549. This analysis showed that RAGE is expressed at low levels in all tested cells at the protein and mRNA levels with no major differences (Data not shown).

Since RAGE expression was low in most analyzed cells, THP-1 cells were chosen because they grow faster and reach easily high cell numbers in culture. I seeded 10 x 10^6 cells in 2 mL medium and stimulated them for one hour with 2 concentrations of different proteases chosen because they were either related to TB for MMP1, MMP2 and MMP9 (55), or they had been shown to be able to cleave RAGE from the cell membrane and produce sRAGE for MMP3, MMP13 and ADAM10 (85, 86). Moreover, because of the observed differences in TNF-α between TB patients and healthy controls I included ADAM17 also named TACE (Tumor necrosis factor-α-converting enzyme) for its involvement in the processing of this important cytokine. After one hour incubation the supernatants were collected and assessed for the presence of sRAGE which was detected only in the supernatants of the cells stimulated with ADAM10 (Figure 2a) but not in cells stimulated with the other proteases (Data not shown). Thus, this experiment showed that ADAM10 is able to induce increased sRAGE levels in the supernatants of the cells. In order to further confirm this finding I then stimulated the cells with ADAM10 in the presence of the ADAM10 specific inhibitor GI254023. Notably, the effect of ADAM10 on sRAGE secretion was inhibited by the presence of the GI254023 inhibitor (Figure 2b). Thus, this result suggests that ADAM10 induces the cleavage of sRAGE on these cells.

In order to test if the *in vitro* findings could have implications for TB pathogenesis I analyzed the association of ADAM10 with pulmonary TB by measuring its serum concentration in one subgroup of 40 TB patients and 40 healthy controls in the recruited cohort. Interestingly, TB patients had lower ADAM10 serum levels than healthy controls (8.72 ± 1.59 Vs 10.58 ± 2.53 ng/mL) ($p < 0.0001$) and this association was still present after adjustment for the differences in BMI between the two groups (p 0.013) (Figure 2c).

ADAM10 is a disintegrin and metalloproteinase that requires Zn^{2+} for being biologically active (119), likewise, S100A12 forms complexes with this element what

not only regulates its function (75) but also thanks to this property, S100A12 is able to inhibit MMPs activity (78). Thus, in order to prove the hypothesis that S100A12 has the same effect on ADAM10, I tested ADAM10 activity in the presence of S100A12. Thus, I added ADAM10 to a specific ADAM10 fluorogenic peptide substrate in the presence of three S100A12 concentrations and measured the fluorescence emission, which is directly proportional to ADAM10 activity, after 30 minutes incubation. Strikingly, ADAM10 activity decreased with higher S100A12 concentrations (Figure 2d).

Taken together, these data suggest that ADAM10 induces the cleavage of cellular RAGE and increases the sRAGE level in the supernatants of the cells, In accordance, TB patients have lower ADAM10 serum levels than healthy controls what might explain the observed differences in sRAGE serum concentrations between the two groups. In addition, S100A12 which concentrations are higher in healthy controls than in TB patients is able to inhibit ADAM10 *in vitro* activity suggesting a potential role for S100A12 in the regulation of sRAGE secretion.

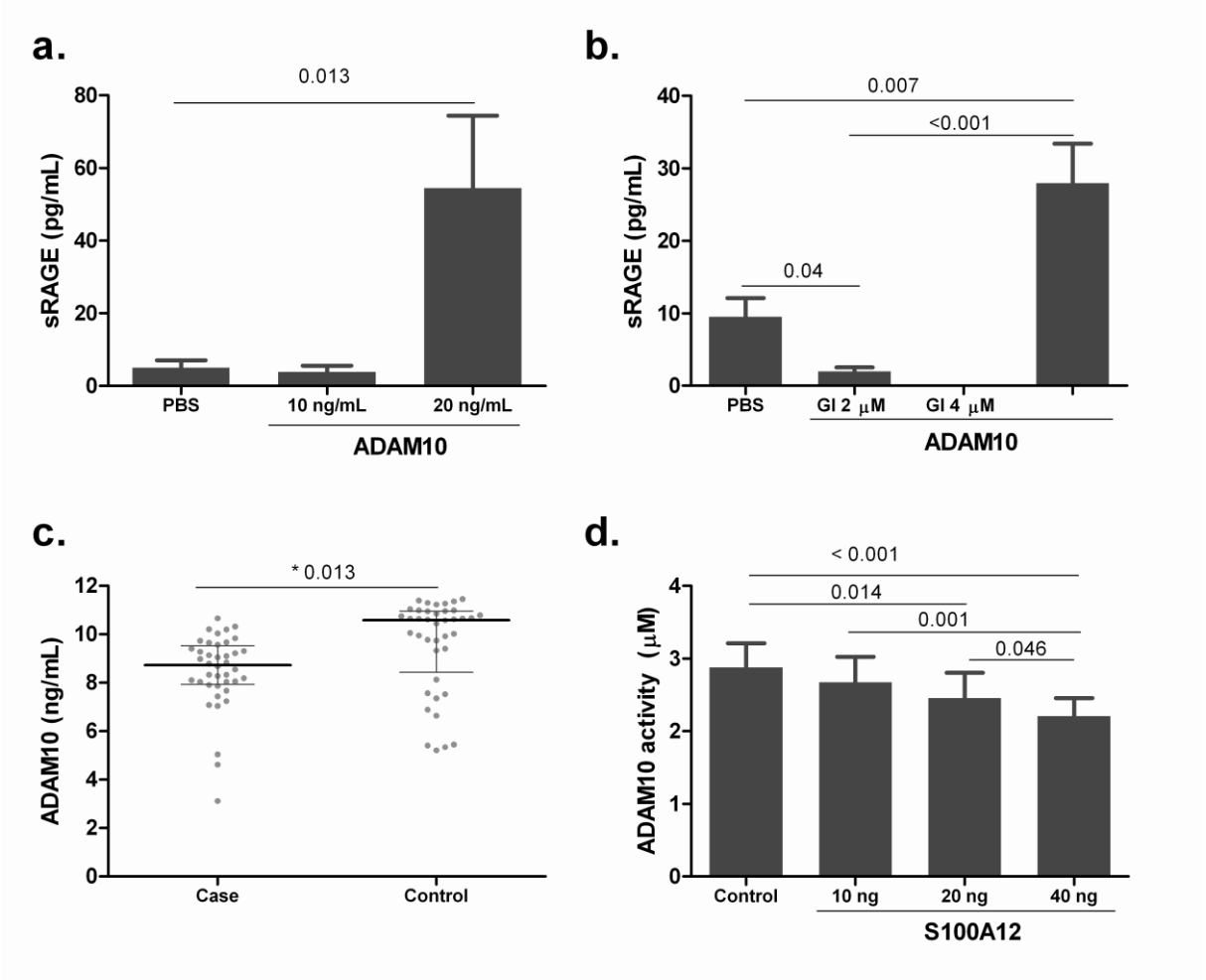

Figure 2. ADAM10 is down-regulated in TB patients and its *In vitro* activity is inhibited by S100A12: (a) THP-1 cells 10 x 10^6 were stimulated for 1 hour with 10 and 20 (ng/mL) ADAM10, the presence of sRAGE in the supernatants was assessed by ELISA. **(b)** THP-1 cells 10 x 10^6 were stimulated for 15 minutes with the ADAM10 specific inhibitor GI254023 and then for 1 hour with 20 (ng/mL) ADAM10, the presence of sRAGE in the supernatants was assessed by ELISA. **(c)** ADAM10 serum concentration was measured by ELISA in 40 TB patients and 40 healthy controls. **(d)** ADAM10 and a specific ADAM10 fluorogenic peptide substrate were diluted at 2 (ng/µL) and 20 µM respectively and co incubated with S100A12 at 1, 6, 0,8 and 0,4 (ng/µL), fluorescence emission was measured after 30 minutes incubation at 37 °C. Data are representative of at least five (a) and (b) or three (d) independent experiments and presented as the median ± IQR (a), (b) and (c) or the mean ± SD (d) and significance was tested by the Kruskall Wallis and Mann Whitney U tests or the one way ANOVA with post hoc test, respectively. *The initial significance value was adjusted for BMI by binary logistic regression in (c) and the adjusted p value is presented.

3.7 ADAM10 is a predictor for pulmonary TB and S100A12 negatively predicts ADAM10 and sRAGE serum levels.

Due to the differences in ADAM10 serum concentration between TB patients and healthy controls I assessed if this variable could be a predictor of disease occurrence. For this I first included it in a univariable logistic regression model and subsequently I tested its association with the outcome, in a multivariable stepwise logistic regression, when the variable was included together with the BMI which I could identify as the most important independent predictor and main confounder. Likewise, since the *In vitro* findings suggested a role for S100A12 in the regulation of sRAGE secretion through ADAM10 inhibition, I assessed the association between S100A12, ADAM10 and sRAGE serum levels by a linear regression analysis: I constructed three univariable linear regression models using ADAM10 and sRAGE as dependent variables and one multivariable model using sRAGE as dependent variable. Both the logistic and the linear regression analysis were performed only with the individuals in whom ADAM10 measurement was performed.

The univariable logistic regression analysis showed that ADAM10 and BMI are predictors of disease occurrence both with negative (β) values -0.389 and -0.949 respectively, and the exp β values 0.673 (95%CI 0.508 – 0.904) (p 0.008) and 0.384 (95%CI 0.251 – 0.596) (p <0.0001) for ADAM10 and BMI respectively, indicate that as the values of the two variables increase, the probably of having TB decreases (Table 8). In fact, the association of these parameters with disease occurrence remained when they were included together into a multivariable logistic regression model, exp β values 0.463 (95%CI 0.253 – 0.847) (p 0.013) and 0.346 (95%CI 0.199 – 0.587), (p <0.0001) for ADAM10 and BMI respectively (Table 8).

On the other hand, the univariable linear regression analysis revealed that S100A12 is a negative predictor of ADAM10 (β) value -0.316 (p 0.030), (Table 9). Interestingly, although S100A12 was also a negative predictor of sRAGE (β) value -0.041, this effect did not reach statistical significance (p 0.079) (Table 9). Therefore, in a next step I tested associations of sRAGE with other variables in the subgroup ADAM10 population and found that, similar to the results of the analysis with the entire study population, as shown in (Table 9) TNF-α was a positive predictor of sRAGE (β) value

0.001 (p <0.0001). Consequently, I included S100A12 and TNF-α into a multivariable model and tested the association with sRAGE. Remarkably, this model showed that S100A12 is a negative and TNF-α is a positive predictor for sRAGE serum concentrations (β) value -0.047 (p 0.043) and (β) value 4.303 (p 0.007) respectively.

Taken together, these data suggest that ADAM10 serum concentrations are decreased in patients with pulmonary TB and that this protease is an independent predictor of disease status. In addition, S100A12 is a negative predictor of ADAM10 and sRAGE serum levels. However, the association of S100A12 and sRAGE is dependent on TNF-α serum levels.

Table 8. Univariable and multivariable logistic regression analysis ADAM10 and BMI as predictors of disease occurrence.

Predictor	β	S.E	P value	Exp β (OR)	95%CI for Exp β
Model 1					
ADAM10	-0.389	0.147	0.008	0.673	0.508 – 0.904
Model 2					
BMI	-0.949	0.220	<0.0001	0.384	0.251 – 0.596
Model 3					
ADAM10	-0.770	0.308	0.013	0.463	0.253 – 0.847
BMI	-1.075	0.275	<0.0001	0.346	0.199 – 0.587

Model 1 constant: 3.53, -2Log likelihood 102.61-Chi^2 0.004, Hosmer and lemeshow test 0.001, R^2 (Cox 0.09 and Nagelkerke 0.13). **Model 2** constant: 18.3, -2Log likelihood 37.1-Chi^2 <0.0001, Hosmer and lemeshow test 0.95, R^2 (Cox 0.60 and Nagelkerke 0.80). **Model 3** constant: 27.9, -2Log likelihood 29.3-Chi^2 <0.0001, Hosmer and lemeshow test 0.95, R^2 (Cox 0.63 and Nagelkerke 0.85).

Table 9. Univariable and multivariable linear regression analysis to identify serum levels of S100A12 as predictor for ADAM10 and sRAGE serum levels.

Predictor	β	S.E	P value	95%CI for β
Model 1				
S100A12	-0.316	0.142	0.030	-0.599 – -0.032
Model 2				
S100A12	-0.041	0.023	0.079	-0.087 – 0.005
Model 3				
TNF-α	0.001	0.002	<0.0001	0.001 – 0.007
Model 4				
S100A12	-0.047	0.023	0.043	-0.093 – -0.002
TNF-α	4.303	1.577	0.007	1.197 – 7.408

Model 1 dependent variable ADAM10: S100A12 Constant 9.914 ANOVA 0.03, R^2 0.061. **Model 2 dependent variable sRAGE:** S100A12 Constant 0.966, ANOVA 0.07, R^2 0.011. **Model 3 dependent variable sRAGE:** Constant 0.861, ANOVA <0.0001, R^2 0.071. **Model 4 dependent variable sRAGE:** Constant 0.936, ANOVA 0.005, R^2 0.037.

3.8 Patients with pulmonary TB show high neutrophil and low lymphocyte counts and neutrophils are positive predictors of S100A12 serum levels.

Neutrophils are known to be the main source of the expression of S100A12 in humans (70). Several clinical studies have associated the S100A12 serum levels with neutrophil activation and neutrophilic inflammation during several chronic inflammatory diseases (110, 120). In order to analyse if the serum levels of S100A12 are associated with the differential peripheral White Blood Cell (WBC) counts in pulmonary TB, a subgroup of TB patients and healthy controls were analysed to compared first the differences in total cell numbers between the two groups and secondly to assess the association of S100A12 serum levels with the different cells populations in the peripheral blood by linear univariable and multivariable regression analysis.

As shown in (Figure 3a) I could demonstrate that the TB patients have a higher amount of total WBC in their peripheral blood than healthy controls (9400 ± 3400 Vs 7000 ± 2600 cells/mL, p <0.0001). Moreover, a closer analysis of the different cell populations revealed that in TB patients the fraction of neutrophils count (6561 ± 3510 Vs 3850 ± 1449 cells/mL) (p <0.0001), monocyte count (186 ± 74 Vs 128 ± 50 cells/mL) (p <0.0001) and eosinophil count (184 ± 123 Vs 128 ± 24 cells/mL) (p 0.030) was higher than in healthy controls (Figure 3b, c and d respectively). However, in TB patients the lymphocyte count is reduced when compared to the healthy controls (2280 ± 1229 Vs 2701 ± 1212 cells/mL, p 0.018) (Figure 3e).

Accordingly, the univariable regression analysis indicated that the WBC, neutrophils and eosinophils were positive predictors of S100A12 with (β) values 0.240, 0.265 and 9.421 (p 0.011, 0.016 and 0.012) respectively, the associations with lymphocytes and monocytes although positive did not reach statistical significance (Table 9), however, when all variables were included into a multivariable model, the neutrophil count in the peripheral blood remained as the only significant positive predictor of S100A12 serum levels (β) value 0.265 (p 0.016) (Table 9).

In summary, these results demonstrate that patients with pulmonary TB have increased neutrophil count and decreased lymphocyte count in their peripheral blood when compared to the healthy controls. Importantly I found that the neutrophil count is the only independent positive predictor of S100A12 serum levels.

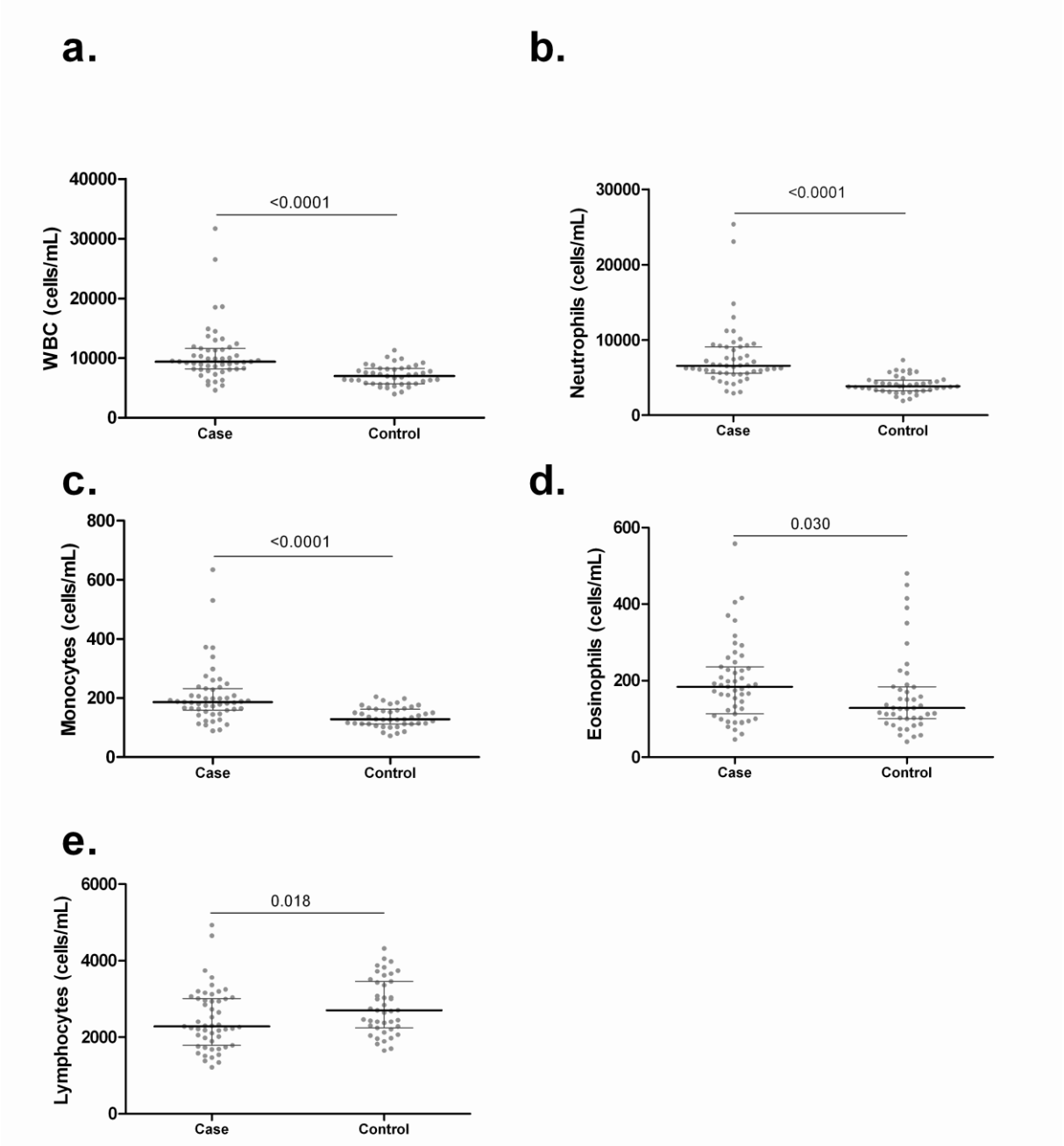

Figure 3. TB patients have increased neutrophil counts and decreased lymphocyte counts in their peripheral blood when compared to healthy controls. (a) Total WBC, **(b)** Neutrophil count, **(c)** Monocyte count, **(d)** Eosinophil count and **(e)** Lymphocyte count. Data are presented as the median ± IQR and significance was tested with Mann Whitney U tests, n=51 for cases and 43 for controls.

Table 10. Univariable and multivariable linear regression analysis to identify the predictors of S100A12 serum levels.

Predictor	β	S.E	P value	95%CI for β
Model 1				
WBC	0.240	0.078	0.011	0.068 – 0.412
Model 4				
Neutrophil count	0.265	0.094	0.016	0.059 – 0.470
Model 3				
Monocyte count	7.945	3.947	0.069	-0.742 – 16.63
Model 5				
Eosinophil count	9.421	4.126	0.012	2.541 – 16.32
Model 2				
Lymphocyte count	0.975	0.474	0.064	-0.069 – 2.019
Multivariable				
Model 6				
Neutrophil count	0.265	0.094	0.016	0.059 – 0.470

Model 1: WBC. Constant 867, ANOVA 0.011, R^2 0.41. **Model 2: Neutrophils.** Constant 1417, ANOVA 0.016, R^2 0.36. **Model 3: Monocytes.** Constant 1596, ANOVA 0.069, R^2 0.20. **Model 4: Eosinophils.** Constant 1614, ANOVA 0.012, R^2 0.40. **Model 5: Lymphocytes.** Constant 607, ANOVA 0.064, R^2 0.21. **Multivariable model 6: Neutrophils.** Constant 1417, ANOVA 0.016, R^2 0.36. Other variables in the model: Lymphocytes, monocytes, eosinophils.

3.9 Specific Chest Radiography (CXR) features of pulmonary TB have substantial inter observer level of agreement.

The serum levels of S100A12, sRAGE and HMGB-1 have been associated with disease severity in COPD and asthma by their correlation with specific measures of lung function such Force Expiratory Volume (FEV) (102, 104, 121), To analyse if there is an association of S100A12, ADAM10 and sRAGE serum levels with the

extent of pulmonary involvement of TB patients, CXRs were taken at the time of diagnosis of all patients and if possible after 6 month of antibiotic therapy. In a first step the CXRs were evaluated by two independent radiologists in Berlin as described in material and methods. I also retrieved the results of the sputum smear test at the time of diagnosis and after two months of therapy with the aim of using these values as indicators of infectivity as previously reported (11). To test the diagnostic accuracy between different radiologists I compared the level of inter rater agreement between the two radiologists and a third independent radiologist that assessed the CXRs after a brief training.

As shown in (Table 11) it was found that 93.2 % of TB patients had fresh alveolar infiltrates ranging from one to all four lung quadrants affected. In addition, 42.7 % of the patients had intrapulmonary lymph nodes and in 26.5% cavities were seen in the CXR. On average 54.2 ± 22 percentage of the lung was affected in the patients as demonstrated in (Table 11).

The analysis of the measurement of the sputum smear positivity revealed that 61,2% of the patients tested positive for visible Mtb with the extent of 2+ or 3+ (Table 11). In 7.7% of the patients less than 1+ bacteria and in 30.8% of the patients 1+ bacteria were detected in the sputum (Table 11). However as shown in (Table 11), after two month of antibiotic therapy in most of the patients 88.9% no bacteria were detected in the sputum.

As shown in (Table 12) I could demonstrate substantial agreement between radiologists at identifying most of the parameters for diagnosis and assessing the extent of lung involvement. Overall there was 73% agreement on the percentage of lung affected by the disease. There was moderate agreement for assessing the particular features of the disease, alveolar infiltration and pleural effusion as well as for the presence of fresh cavities. However, only a fair recognition for the presence of intrapulmonary lymph nodes could be demonstrated.

These data suggest that the radiographic features for the extent of pulmonary lung involvement in TB patients can be objectively assessed.

Table 11. Chest radiography (CXR) and sputum smear results

Variable	n =117
Alveolar infiltration n (%)	
No	8 (6.8)
1 quadrant	32 (27.4)
2 quadrants	51 (43.6)
3 quadrants	19 (16.2)
4 quadrants	7 (6)
Intrapulmonary lymph nodes n (%), (yes/No)	50 (42.7)/67(57.3)
Cavities n (%)	
No	86 (73.5)
1 Cavity	27 (23.1)
2 Cavities	4 (3.4)
Area of lung affected (%) mean (SD)	54.2 ± 22
Pleural effusion n (%), (yes/No)	17 (14.5)/100(85.5)
Sputum smear grade at diagnosis n (%)	
< 1+	9 (7.7)
1+	36 (30.8)
2+	32 (27.4)
3+	40 (34.2)
Sputum smear status after 2 month n (%)	
Negative	104 (88.9)
Positive	8 (6.8)
No results available	5 (4.3)

Table 12. Inter rater agreement on radiological findings.

Inter-rater agreement among categorical variables	**Kappa**	**P value**	**Prevalence and bias adjusted kappa**	**Interpretation of prevalence and bias adjusted kappa**
Alveolar infiltrates	0.421	0.005	0.720	Substantial
Intrapulmonary lymph nodes	0.285	0.060	0.348	Fair
Cavities	0.480	<0.0001	0.510	Moderate
Pleural effusion	0.448	0.002	0.636	Substantial
Agreement among continuous variables		r_c	**P value**	**95% limits of agreement (Bland and Altman)**
Total (%) of lung affected		0.734	<0.0001	-50 to 31.4%

3.10 The percentage of lung involvement is negatively associated to malnutrition.

To further assess if the CXR could be used as correlates of the extent of pulmonary involvement in TB patients I analysed the association of every individual CXR feature (Table 11) with the BMI as well as the grade of sputum smear positivity at the time of diagnosis, since these two parameters are generally considered good indicators of systemic involvement of the disease (122, 123). Initially I used the BMI as dependent variable to test which CXR feature could be a predictor of this parameter by linear regression analysis.

(Table 13) illustrates the main findings of the linear regression analysis in which I found that the extent of area of lung affected is a predictor for the BMI (β) value -0.025 (p 0.021). Thus, as more area of the lung is affected, lower values of BMI are detected in TB patients. The BMI was not associated with any of the other specific radiographic features characterized in this study.

Table 13. Univariable linear regression to investigate the association of the extent of area of lung affected as predictor of BMI

Predictor	β	S.E	P value	95%CI for β
(%) of lung affected	- 0.025	0.011	0.021	-0.046 - -0.004

Model parameters dependent variable BMI: Constant 17.73 ANOVA 0.02, R^2 0.046.

3.11 The extent of alveolar infiltration and the presence of intrapulmonary lymph nodes on the CXRs are associated with sputum smear positivity in TB patients.

Next I assessed if there is an association between the different radiographic features of pulmonary TB with the grade of sputum smear positivity. For this, I used the sputum smear as ordinal categorical variable, in which there is a clear ordering within the different categories of the variable. Thus, in addition to being able to classify a TB patient as sputum smear positive or negative, they can be assigned to one of the four smear categories <1, 1+, 2+ and 3+; e.g. one patient in a higher category has more bacteria in sputum than those in the category below. For this analysis I used ordinal univariable and multivariable regressions selecting sputum smear positivity 3+ as the reference category, thus, the test measures the probability of achieving this category.

As shown in (Table 14) the extent of alveolar infiltration and the presence of intrapulmonary lymph nodes were independently associated with the grade of sputum smear positivity, in both, the univariable as well as the multivariable analysis. For the extent of alveolar infiltration the reference category was no infiltration, thus, patients with alveolar infiltration in two quadrants of the lungs have a higher probability of having 3+ in their sputum smear compared to those with no infiltration Exp β 4.69 (95%CI 1.13-19.38, p 0.036). These results were similar for patients with a higher extent of alveolar infiltration in three quadrants Exp β 7.96 (95%CI 1.62-39.10) (p 0.011) and four quadrants of the lungs Exp β 21.46 (95%CI 2.63-175) (p 0.004) respectively. Interestingly, while the association in patients with alveolar infiltration only in the one quadrant did not reach statistical significance Exp β 4.16 (95%CI

0.96-18.03) (p 0.057), the results with the other categories suggested a trend by which the probability of having the highest amount 3+ of bacteria in the sputum smear is increased as the extent of alveolar infiltrates increases. Likewise, compared to patients with intrapulmonary lymph nodes, patients with no detectable lymph nodes have a higher probability of being in the highest sputum smear positivity 3+ category Exp β 2.06 (95%CI 1.03-4.14) (p 0.041), (Table 14).

These results indicate that the CXR features for pulmonary TB alveolar infiltration and presence of intrapulmonary lymph nodes as characterized in this study are associated with the grade of sputum smear positivity. Accordingly, patients with a higher extent of alveolar infiltration have higher degree of sputum smear positivity than those without. Similarly patients without detectable intrapulmonary lymph nodes have higher degree in sputum smear positivity compared to those with detectable intrapulmonary lymph nodes.

Table 14. Univariable and multivariable ordinal regression: alveolar infiltration and intrapulmonary lymph nodes as predictors of sputum smear positivity

Predictor	β	S.E	P value	Exp β (OR)	95%CI for Exp β (OR)
Model 1					
Alveolar infiltration					
1 quadrant	1.397	0.747	0.061	4.04	0.94 – 17.48
2 quadrants	1.516	0.722	0.036	4.55	1.11 – 18.76
3 quadrants	1.839	0.800	0.021	6.29	1.31 – 30.17
4 quadrants	3.107	1.072	0.004	22.3	2.73 – 182
Model 2					
Intrapulmonary lymph nodes	0.646	0.344	0.060	1.91	0.97 – 3.74
Model 3					
Alveolar infiltration					
1 quadrant	1.425	0.748	0.057	4.16	0.96 – 18.03
2 quadrants	1.545	0.724	0.033	4.69	1.13 – 19.38
3 quadrants	2.076	0.812	0.011	7.96	1.62 – 39.10
4 quadrants	3.066	1.071	0.004	21.46	2.63 – 175
Intrapulmonary lymph nodes	0.724	0.355	0.041	2.06	1.03 – 4.14

Dependent ordinal variable sputum smear (<1+, 1+, 2+ and 3+). Model 1 -2Log 41, Chi^2 0.041, Goodness of fit 0.97, Pseudo R^2 (Cox and snell 0.082 and Nagelkerke 0.089), test of parallel lines 0.972. **Model 2** -2Log 23, Chi^2 0.060, Goodness of fit 0.71, Pseudo R^2 (Cox and snell 0.030 and Nagelkerke 0.032), test of parallel lines 0.714. **Model 3** -2Log 69, Chi^2 0.015, Goodness of fit 0.90, Pseudo R^2 (Cox and snell 0.11 and Nagelkerke 0.12), test of parallel lines 0.965. **Reference categories:** Sputum smear: 3+, Hilar lymph nodes: Yes, Alveolar infiltration: No infiltration.

3.12 S100A12 positively predicts the extent of fresh alveolar infiltrates detected in the CXR of patients with pulmonary TB.

The previous analysis showed that individual radiographic features of pulmonary TB in the CXR of the patients served as indicators of pulmonary involvement. Therefore, I next wanted to test if there was an association between the serum levels of the following parameters: sRAGE, esRAGE, S100A12, HMGB-1, the cytokines TNF-α, IFN-γ, as well as the differential cell counts with the extent of lung involvement or any of the specific radiographic features for pulmonary TB. For this I either used the radiographic features as dependent variables in regression analysis or performed simple group comparisons as appropriate depending on the type of dependent variable.

Notably, the univariable ordinal regression revealed that the counts of the peripheral blood monocytes as well as S100A12 were positive predictors for the extent of alveolar infiltration as shown in (Table 15 models 1 and 2) Exp 1.01 (95%CI 1.00-1.01) (p 0.049) and Exp 1.37 (95%CI 1.10-1.71) (p 0.005) respectively. Moreover, the counts of the peripheral blood neutrophils was also a positive predictor, but it was on the borderline of statistical significance OR 1.18 (95%CI 1.0-1.39) (p 0.050) (Table 15 model 3). Interestingly, when the three variables were integrated into a multivariable model, S100A12 remained as the only parameter that was significantly associated with the extent of fresh alveolar infiltrates Exp β 2.60 (95%CI 1.35 – 5.00) (p 0.004), (Table 15 model 4). In this analysis the dependent variable alveolar infiltration was treated as an ordinal variable with five categories and the last category “infiltration in four quadrants” was the reference category, therefore, the test measures the probability of achieving this category.

In summary, the results of this analysis indicate that S100A12 is the sole independent predictor for the extent of fresh alveolar infiltrates in the CXR of patients with pulmonary TB and the probability of having infiltration in all four lung quadrants is higher as the serum levels of S100A12 increases.

Table 15. Univariable and multivariable ordinal regression to identify the predictors of the extent of fresh alveolar infiltrates in the CXR of patients with pulmonary TB.

Predictor	β	S.E	P value	Exp. β (OR)	95% for Exp. β (OR)
Model 1					
Neutrophils	0.165	0.084	0.050	1.18	1.00 – 1.39
Model 2					
Monocytes	0.007	0.003	0.049	1.01	1.00 – 1.01
Model 3					
S100A12	0.317	0.112	0.005	1.37	1.10 – 1.71
Model 4					
Neutrophils	-0.106	0.228	0.643	0.90	0.58 – 1.41
Monocytes	0.011	0.010	0.272	1.01	0.99 – 1.03
S100A12	0.956	0.333	0.004	2.60	1.35 – 5.00

Dependent ordinal variable alveolar infiltration (None, 1 quadrant, 2 quadrants , 3 quadrants and 4 quadrants). Model 1 -2Log 128 Chi^2 0.052, Goodness of fit 0.47, Pseudo R^2 (Cox and snell 0.075, Nagelkerke 0.081), test of parallel lines 0.516; n = 51. **Model 2** -2Log 118 Chi^2 0.051, Goodness of fit 0.66, Pseudo R^2 (Cox and snell 0.076, Nagelkerke 0.081), test of parallel lines 0.490; n=51 **Model 3** -2Log 295 Chi^2 0.005, Goodness of fit 0.79, Pseudo R^2 (Cox and snell 0.068, Nagelkerke 0.073), test of parallel lines 0.089; n = 112. **Model 4** -2Log 70 Chi^2 0.002, Goodness of fit 0.83, Pseudo R^2 (Cox and snell 0.39, Nagelkerke 0.42), test of parallel lines 0.47; n = 51. **Reference category:** Alveolar infiltration 4 quadrants.

3.13 In patients with pulmonary TB there are differences in S100A12, ADAM10 and IFN-γ serum levels dependent on the presence of detectable intrapulmonary lymph nodes in CXR.

In addition, as shown in (Figure 4 a and b) the comparison between patients with detectable intrapulmonary lymph nodes in the CXR with those without revealed that patients with intrapulmonary lymph nodes have higher serum levels of IFN-γ and ADAM10 (13.9 ± 6.15 Vs 1.3 ± 0.93 pg/mL) (p 0.007) and (9.48 ± 1.76 Vs 8.18 ± 1.24 ng/mL) (p 0.026), respectively. In contrast, these individuals had lower S100A12 serum levels (1.47 ± 2.45 Vs 2.20 ± 2.78 ng/mL) although this effect did not reach statistical significance (p 0.086), (Figure 4 c). This comparative analysis did not reveal significant differences of the serum levels of sRAGE, HMGB-1 and TNF-α with the presence of intrapulmonary lymph nodes (Data not shown). However, the fact that S100A12 is a predictor of the extent of fresh alveolar infiltrates and in the two groups with detectable intrapulmonary lymph nodes in the CXRs the individuals have different serum levels of this variable, made me hypothesize that the differences in the extent of alveolar infiltrates between individuals in the two groups could mask a difference in S100A12 serum levels. To test this hypothesis I applied weights to every

subject according to its extent of fresh alveolar infiltrates and performed new comparisons between the two groups. Interestingly, as shown in (Figure 4 b) the weighting procedure uncovered also a statistical difference in the serum levels of S100A12 (p 0.038).

Taken together, these results demonstrate that the presence of detectable intrapulmonary lymph nodes in the CXRs of TB patients is associated with increased serum levels of IFN-γ and ADAM10 but decreased serum levels of S100A12

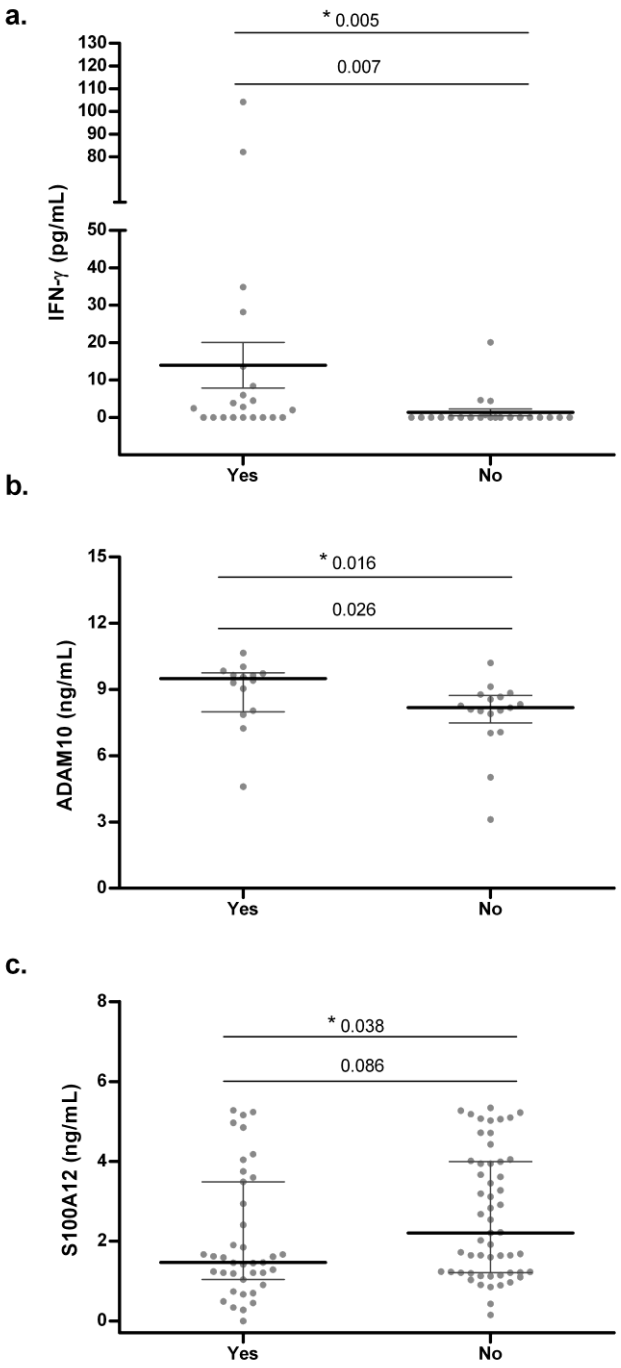

Figure 4. There are differences in the serum levels of S100A12, ADAM10 and IFN-γ, dependent on the presence of detectable intrapulmonary lymph nodes in the CXR of patients with pulmonary TB lymph. TB patients were separated into two groups according the presence or absence of intrapulmonary lymph nodes and the serum levels of **(a)** IFN-γ, **(b)** ADAM10 and **(c)** S100A12 was compared between the two groups, the data are presented as the median ± IQR and significance was tested with Mann Whitney U tests before and after proportional weighting without scale effect for alveolar infiltration *. Yes n=21 and No n=22 in a., Yes n=14 and No n=17 in b., Yes n=39 and No n=55 in c.

3.14 The neutrophil/lymphocyte count ratio combined with the BMI is a strong marker for disease occurrence of pulmonary TB.

Fresh alveolar infiltrates do consist of the infiltration of lung tissue with fluids of different composition such as granulocytes among others (124). Neutrophils has been found to be associated with early granuloma whereas the presence of lymphocytes at the infection cite has been shown to be related to the development of mature granuloma to contain and at best eliminate the pathogen (5). It has been reported by Barnes et al (46) that a specific balance between the neutrophil count and the lymphocyte counts in the blood of individuals with pulmonary TB might be important and that changes of the ratio of these two cell populations might serve as an additional indicator of the status of disease pathology. Joel Ernst has proposed that for the TB associated immune responses different stages can be separated that reciprocally might represent either processes associated with increasing inflammation due the influx of innate immune cells such as neutrophils which can be associated with a higher likelihood of tissue damage (39) or processes that are likely to control the further spread of the pathogen such as the priming of lymphocytes in the local lymph nodes after antigen presentation by macrophages and dendritic cells (22). There is evidence that lymphocytes especially T cells and neutrophils have central roles in controlling both infection and tissue damage (34). In this study I could demonstrate that the serum levels of the neutrophil derived protein S100A12 are increased in TB patients and serve as additional predictor of disease occurrence along with the extent of fresh alveolar infiltrates in the CXRs of patients with TB. Therefore I hypothesized that the ratio of neutrophil and lymphocytes counts in the peripheral blood of patients might be associated with disease occurrence and the extent of lung involvement. Consequently I calculated and compared the ratio of the neutrophil and the lymphocyte count in peripheral blood in TB patients compared to healthy controls and then assessed the accuracy of this parameter for differentiating these two groups using a Receiver Operating Characteristic (ROC) analysis.

As expected, TB cases had higher neutrophil/lymphocyte count ratio than controls (2.6 ± 1.57 Vs 1.4 ± 0.57) ($p < 0.0001$), (Figure 5a). Accordingly, the ROC analysis confirmed the importance of this parameter Area Under the Curve (AUC) 0.941 (95%C.I 0.899 – 0.983) ($p < 0.0001$), (Figure 5b). Thus, this single variable identifies

94.1% of the individuals as cases or controls. Since I could demonstrate that the BMI was the best single predictor of disease status (Table 2 model 4) I did a ROC analysis for BMI, which also confirmed the initial finding, AUC 0.936 (95%C.I 0.912 – 0.961) (p <0.0001), the data shown in (Figure 5c) demonstrate that the BMI identifies 93.6% of the individuals as cases or controls. In a next step I therefore combined the two variables in a regression model (Data not shown) to create a model of neutrophil/lymphocyte count ratio combined with the BMI. Consequently I did a ROC analysis with this model. Remarkably, the combination of these variables was able to discriminate 98.4 % of cases and controls AUC 0.984 (95%C.I 0.965 – 1.00), (p <0.0001) (Figure 5d).

In summary, these data suggest that in TB patients reciprocal levels of increased neutrophil counts and decreased lymphocyte counts can be observed that do serve as a strong marker for disease occurrence which is improved when combined with the BMI. Therefore this new neutrophil/lymphocyte count ratio together with the BMI is an interesting marker of disease occurrence.

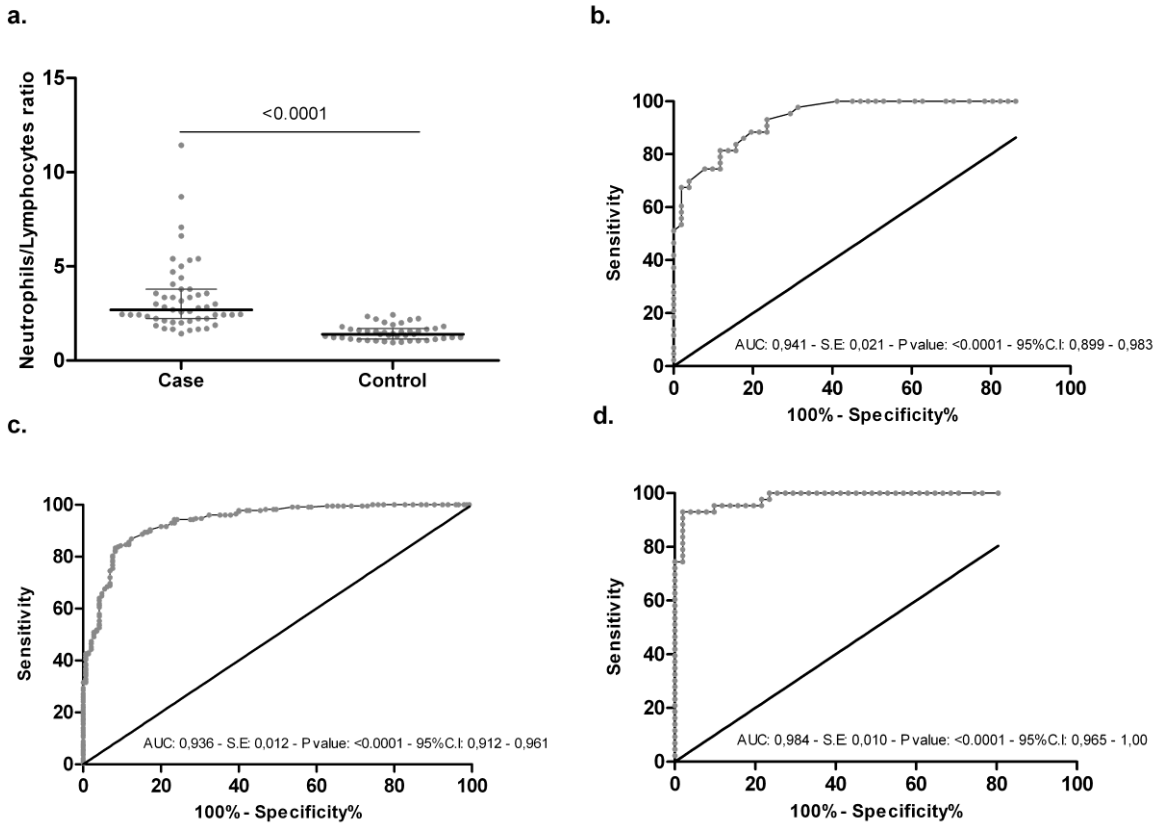

Figure 5. The neutrophil/lymphocyte count ratio combined with the BMI is a marker of disease occurrence. The total number of neutrophils in peripheral blood was divided by the total number of lymphocytes in TB patients and healthy controls **(a)**. Receiver Operating Curve (ROC) analysis for neutrophil/lymphocyte ratio **(b)**, BMI **(c)** and the regression model of neutrophils/lymphocytes ratio and BMI **(d)**. Data are presented as the median ± IQR and significance was tested with Mann Whitney U tests in (a). n=51 for cases and 43 for controls in (a), (b) and (d). n=119 for cases and 163 for controls in (c).

3.15 The neutrophil-lymphocyte count ratio is associated with the extent of lung involvement and in particular with fresh alveolar infiltrates.

To test the hypothesis that the neutrophil/lymphocyte count ratio is also associated with the extent of lung involvement seen in the CXRs of TB patients, I measured its association with the characterized parameters of lung involvement and in particular fresh alveolar infiltrates. Moreover, I also evaluated its association with the sputum smear positivity and potential infectivity by using the CXR features or the sputum smear test as dependent variables in regression analysis or by simple group comparisons as appropriate depending on the type of dependent variable.

As shown in (Table 16) I found that the neutrophil/lymphocyte count ratio is a positive predictor of the extent of lung involvement, (β) value 4.043, (p 0.047). Notably, this ratio was also a positive predictor of the extent of alveolar infiltration Exp β 1.59 (95%CI 1.14 – 2.23), (p 0.007), suggesting that as the value of this variable increases, so does the probability of having a higher extent of alveolar infiltration (Table 17). Also a higher grade of sputum smear positivity was associated with the ratio, although it did not reach statistical significance Exp β 1.37 (95%CI 0.99-1.88), (p 0.055) (Table 18). In contrast to the prediction of disease occurrence, the addition of the BMI to the ratio did not improve the ability of this parameter to predict the extent of lung involvement or fresh alveolar infiltrates (Data not shown).

In conclusion, these results demonstrate that the neutrophil/lymphocyte count ratio is a positive predictor of percentage of lung involvement and the extent of fresh alveolar infiltrates, thus, as the ratio increases, so do the percentage of lung involvement and the extent of alveolar infiltration.

Table 16. Univariable linear regression Neutrophil/Lymphocyte count ratio as predictor of percentage of lung involvement.

Predictor	β	S.E	P value	95%CI for β
Neutrophil/Lymphocyte count ratio	4.043	1.974	0.047	0.061 – 8.024

Model parameters dependent variable percentage of lung involvement: Constant 40.56 ANOVA 0.047, R^2 0.068.

Table 17. Univariable ordinal regression Neutrophil/Lymphocyte count ratio as predictor of the extent of fresh alveolar infiltrates in the CXR of TB patients.

Predictor	β	S.E	P value	Exp. β (OR)	95% for Exp. β (OR)
Neutrophil/Lymphocyte count ratio	0.466	0.172	0.007	1.59	1.14 – 2.23

Dependent ordinal variable fresh alveolar infiltrates (None, 1 quadrant, 2 quadrants, 3 quadrants and 4 quadrants). Model -2Log 107 Chi^2 0.006, Goodness of fit 0.48, Pseudo R^2 (Cox and snell 0.153, Nagelkerke 0.162), test of parallel lines 0.662. **Reference category:** alveolar infiltration 4 quadrants.

Table 18. Univariable ordinal regression Neutrophils/Lymphocytes count ratio as predictor of sputum smear positivity.

Predictor	β	S.E	P value	Exp. β (OR)	95% for Exp. β (OR)
Neutrophil/Lymphocyte count ratio	0.311	0.162	0.055	1.37	0.99 – 1.88

Dependent ordinal variable sputum smear positivity (<1+, 1+, 2+ and 3+). Model -2Log 111 Chi^2 0.039, Goodness of fit 0.62, Pseudo R^2 (Cox and snell 0.080, Nagelkerke 0.086), test of parallel lines 0.680. **Reference category:** Sputum smear: 3+.

CHAPTER 4: DISCUSSION

The alarmins S100A12, that is expressed mainly in granulocytes and HMGB-1 accumulate in settings of chronic inflammation and tissue damage triggering proinflammatory mechanisms by binding either RAGE or TLR (67, 75). The two soluble forms of RAGE, esRAGE and sRAGE function as decoy receptors that counteract the RAGE pathway by binding S100A12 and HMGB-1 (83). In fact, the serum levels of these molecules are differently regulated in acute and chronic diseases and have been therefore proposed as correlates of disease severity and outcome (67, 75). Pulmonary TB is characterized by the development of lung tissue damage which is produced by an inflammatory response with variations across individuals, that although aimed at bacterial killing contributes to pathology and disease worsening (34). In this study, I investigated the role of the soluble form of the alarmin receptor RAGE (sRAGE) and its ligands S100A12 and HMGB1 in pulmonary TB by combining observational epidemiology with *In vitro* laboratory work. The major findings of my doctoral thesis are:

1. Pulmonary TB is associated to an increase in S100A12 and a decrease in sRAGE and ADAM10.

2. The variation in S100A12 and sRAGE occur in parallel with changes in TNF-α, BMI, systolic BP and blood glucose, however, S100A12, sRAGE and ADAM10 are independent predictors of disease status.

3. The cross sectional associations of S100A12 and sRAGE with pulmonary TB were confirmed longitudinally what suggests a causal relationship with pulmonary TB.

4. SNPs in the RAGE gene show no association with sRAGE serum levels or with the risk for pulmonary TB.

5. S100A12 is a negative predictor of alveolar lung infiltration and as IFN-γ and ADAM10 correlates with the presence of detectable intrapulmonary lymph nodes measured by CXR.

6. The differences in neutrophils and lymphocytes total numbers in peripheral blood can be combined with the BMI as indicator of malnutrition to generate a Neutrophil-Lymphocyte-BMI (NLB) index for pulmonary TB as marker of disease occurrence and severity.

4.1 Regulation of sRAGE, S100A12, TNF-α and ADAM10 in pulmonary TB

I hypothesized that the serum levels of sRAGE, S100A12 and HMGB-1 in patients with pulmonary TB would be associated with the amount of lung tissue damage and may predict the disease severity and/or progression. In fact, my results demonstrate that sRAGE and S100A12 are differently regulated, the former decreased and the latter increased, in TB patients compared to healthy controls. Extensive cell injury and death are common features of chronic inflammatory diseases and alarmins accumulate during these processes triggering immune responses, and although this mechanism is important for tissue repair, it also has damaging effects on viable tissue (63). Accordingly, the serum levels of these proteins has been reported to be associated with chronic pulmonary diseases such as COPD, asthma and CF which are linked with dysregulation of the host inflammatory response and neutrophil function (102-105). The lung tissue damage in pulmonary TB is produced by inflammatory immune responses that contribute to pathology (34) and recent studies give neutrophils a central role in this process (39).

4.1.1 The pattern of decreased sRAGE and increased S100A12 as indicator of the extent of inflammation.

The soluble forms of RAGE that circulate in serum esRAGE and sRAGE bind to the same ligands as the full length protein acting as decoy receptors that sequester circulating RAGE ligands, thus, it is thought that these forms counteract the proinflammatory processes triggered by the RAGE ligand interaction (83). Indeed, sRAGE treatment attenuates RAGE activation related inflammation protecting against ischemic damage and this effect is similar to the complete abrogation of RAGE in knock out mice (125). Accordingly, sRAGE levels increase and predicts prognosis in patients with acute heart failure (126), likewise, they increase in other settings of acute injury such as in Acute Lung Injury (ALI) or Acute Respiratory

Distress Syndrome (ARDS) (127-129), correlate with disease severity (129) and predict mortality (128). On the other hand, the serum levels of the proinflammatory RAGE ligand HMGB-1 also increase in ARDS (130), acute heart failure (131) and sepsis (132). However, in contrast to acute injury, in chronic inflammatory processes involving the lungs or the vascular endothelium the levels of sRAGE are decreased like in COPD (102-104, 133), cardiovascular disease or atherosclerosis (134, 135) and RA (107, 109, 110, 120). Of note, in most of these chronic diseases, the decrease in sRAGE is accompanied by an increase in neutrophil derived S100A12 which is associated with disease severity.

The results of my thesis together with the findings of other studies might indicate that there is a distinction in the patterns of sRAGE and S100A12 expression that differ depending on the severity of the inflammatory response (Figure 6). Thus, I argue that at early stages of inflammation in settings such as sepsis or acute lung injury the expression and accumulation of RAGE ligands together with a high expression of TNF-α trigger an inflammatory response; as a result, the increase of antiinflammatory sRAGE might serve as a mechanism of the host's immune response to control inflammation and tissue damage. In my results I could observe that a higher amount of TNF-α was associated to increased sRAGE levels in patients with pulmonary TB. However, in particular in chronic inflammatory processes such as in RA, it has been shown that the sRAGE serum levels are decreased what often was observed to occur in parallel to an increase in S100A12 along with neutrophil influx and this pattern has been suggested to be an indicator of deficient inflammatory control (107, 109). The levels of S100A12 and sRAGE are related to the extent of inflammatory process which has been demonstrated in longitudinal studies such as in acute lung injury where sRAGE and S100A12 levels return to those seen in control subjects with disease recovery (110, 129), similarly, in my study the levels of sRAGE and S100A12 return to normal values after 2 and 4 months respectively of antibiotic therapy and seems to be accompanied by disease recovery since 94.1% of patients are cured at the end of treatment.

Remarkably, in contrast to S100A12, there was no significant association of HMGB-1 with pulmonary TB. I propose the following potential explanation; while S100A12 seems to be a specific marker of neutrophilic inflammation, HMGB-1 might be a

systemic marker of endothelial function, in fact, this protein is associated to vascular damage (131, 136, 137).

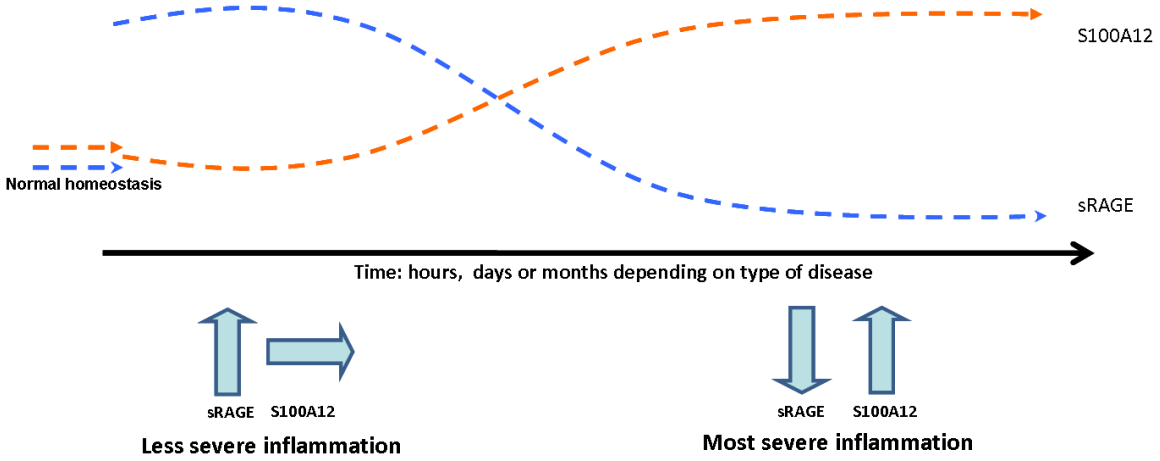

Figure 6. Patterns of sRAGE and S100A12 expression. sRAGE and S100A12 expression differs depending on the extent of inflammation, in less severe inflammation sRAGE increases while in most severe settings its levels decrease along with increase in S100A12 concentration.

4.1.2 The role of ADAM10 in sRAGE production and sRAGE-S100A12 inverse regulation.

RAGE is expressed at low levels in most tissues and it is thought that the presence of its different isoforms is tissue specific (83). Thus, although full membrane bound RAGE is principally expressed in the lungs, esRAGE and sRAGE are most often found in endothelial cells and serum respectively (83), moreover, monocytes and the monocytic cell line THP-1 have been described to express RAGE and sRAGE (85). Indeed, my results showed that soluble cleaved sRAGE and not the form produced by alternative splicing esRAGE is mainly present in the serum of TB patients and healthy controls. RAGE cleavage is mediated by different proteases and this process might also be tissue specific. Several studies that used diverse *in vitro* approaches and cell types have reported that ADAM10 and MMP9 in systems using human monocytic THP-1 and embryonic kidney HEK cells (84, 85, 87) or MMP3 along with MMP13 in alveolar epithelial cells produce RAGE cleavage (86). I assessed RAGE expression in several primary culture and cell lines and found that according to what have been reported this receptor is expressed at low levels in all cells that I tested.

However, because of previous reports and the convenience of the cell culture model my experiments were performed with the monocytic cell line THP-1. In these cells and with the approach that I used only ADAM10 was able to produce sRAGE cleavage and these results are similar to those from previous studies using THP-1 or HEK cells (85, 87). However, it is possible that each protease has a specific action site in the body and its effects on sRAGE cleavage might depend on the tissue and physiological environment.

Interestingly, several studies have observed a pattern of inverse regulation between sRAGE and S100A12, thus, a decrease in sRAGE concentration is accompanied by an increase in S100A12. Of note, this pattern has been reported only in chronic inflammatory diseases such as COPD (103), CF (105), RA (109) and diabetes (138). In fact, I found the same pattern in TB, moreover, the two proteins had a negative correlation and S100A12 was a negative predictor of sRAGE but this effect was dependent on TNF-α. The reason for sRAGE decrease is not known, Sukkar et al (104) proposed that sRAGE was degraded by neutrophil derived proteases; however, they lack solid experimental evidence to support this argument. Indeed, I assessed the association of sRAGE and total MMP activity (Data not shown) and found no significant association which should exist and be negative if the hypothesis of Sukkar et al. were true. Another possible explanation was that there is an association between the SNP rs2070600 in the binding region of the RAGE gene and sRAGE serum levels (96, 97) but such an association was not evident in my study.

In contrast, I not only showed that ADAM10 produces sRAGE cleavage *in vitro* but also that the concentration of this protease is lower in the serum of TB patients compared to healthy controls, consequently, this result might explain the decreased sRAGE in TB patients. Accordingly, Alzheimer disease is associated with decreased sRAGE serum levels (139) and it has been reported that these patients have lower levels of ADAM10 in peripheral cells and Cerebrospinal Fluid (CSF) than normal controls (140). Thus, an important new question arises from these observations; is there an association between ADAM10 and S100A12 that could explain the pattern of decreased sRAGE and increased S100A12? In fact, S100A12 is able to bind Zn^{2+} and MMPs need this element to exert their functions, therefore S100A12 is able to

inactivate these enzymes (78) and similar to MMPs, ADAM10 also need Zn^{2+} to become activated (119).

The results of this study indicate that S100A12 is able to inhibit ADAM10 activity in vitro; moreover, S100A12 is a negative predictor not only of sRAGE but also of ADAM10 concentrations in TB patients and healthy controls. Thus, in pulmonary TB the decreased sRAGE levels might be the result of both, a lower concentration of ADAM10 together with its diminished activity due to the inhibitory effect of S100A12, however, the negative association between sRAGE and S100A12 is mild, therefore, most of the effect must be due to the decreased ADAM10 levels. Of importance, I measured ADAM10 concentration but not activity and the negative association between S100A12 and ADAM10 suggests that S100A12 might influence not only ADAM10 activity but also expression. Interestingly, the decrease in sRAGE serum levels has been associated to an increase in neutrophil influx (104, 105). One alternative explanation for the lower levels of ADAM10 in TB patients is that this protease might be affected by malnutrition since it is regulated by retinoid acid receptors (119) and these receptors are influenced by the deficiency in Vitamin A seen during active TB (141).

In short, in pulmonary TB the patterns of sRAGE, S100A12 and ADAM10 resemble those seen in other inflammatory diseases and might serve as an indicator of the extent of inflammation. Thus, I found that while sRAGE and ADAM10 decrease, S100A12 increases. The levels of these proteins return to normal values after 2 and 4 months of antibiotic treatment for sRAGE and S100A12 respectively. The changes in S100A12 and sRAGE occur together with an increase in TNF-α which starts to decrease after 2 months of treatment. The decrease in sRAGE might be due to a decrease in ADAM10 concentration and activity which could be affected by neutrophil accumulation and S100A12 expression (Figure 7).

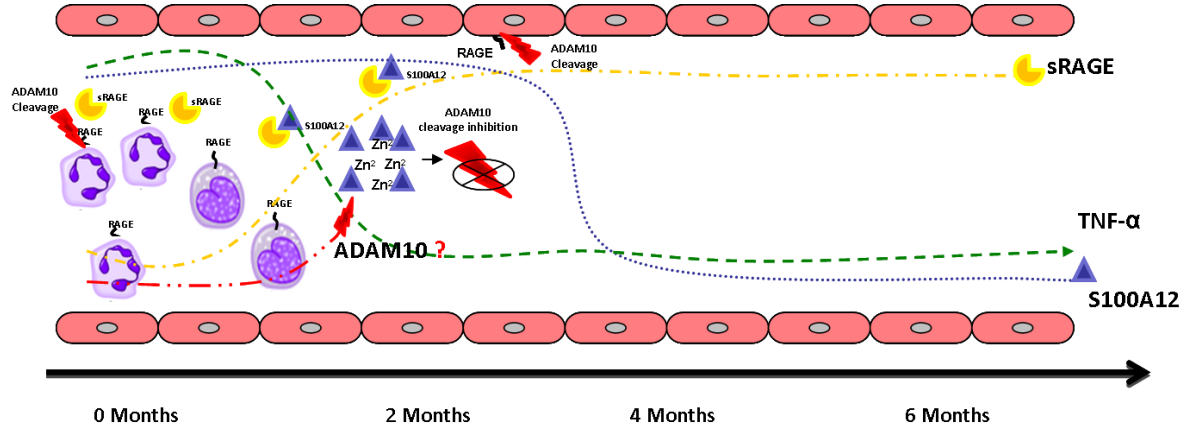

Figure 7. Proposed model of the regulation of sRAGE, S100A12, TNF-α and ADAM10 in pulmonary TB. In pulmonary TB sRAGE and ADAM10 decrease while S100A12 increase. Their levels return to normal values after 2 and 4 months of antibiotic treatment for sRAGE and S100A12 respectively. Likewise, there is an increase in TNF-α which starts to decrease after 2 months of treatment. The decrease in sRAGE might be due to a decrease in ADAM10 concentration and activity; this latter is affected by the binding of Zn^{2+} to S100A12.

4.2 In pulmonary TB there are different patterns of lung involvement that depend on the host immune response.

In order to assess the association of sRAGE, S100A12 and HMGB-1 with disease severity I first had to test if some or all radiography features could be used as indicators of disease severity. Although sRAGE and S100A12 serum levels have been associated to endothelial and lung function (104, 109, 134), these studies used well standarized measures such as FEV for the lungs (102) or the Coronary Artery Calcium (CAC) for the endothelium (135). However, I could not perform such measurements in this study because the required equipment was not available in the hospital where the study was conducted. Therefore, I tested if the CXR could be used as an indicator of severity by assessing their association with two well established measures of severity in TB; the number of bacteria in sputum smear and the BMI (8, 122).

4.2.1 In pulmonary TB specific radiographic features are differently associated with sputum smear positivity and BMI.

The CXR is a useful and sensitive diagnostic tool for pulmonary TB, however, it has poor specificity and large inter and intra reader variations (142). Different numerical scoring systems have been applied for grading the CXR in order to use it either for disease diagnosis or prognosis, this latter by its assumed correlation with severity (14, 142, 143). However, in most studies severity has been assessed by the ability of the CXR score to predict sputum smear positivity after 2 months of treatment (14, 143) and a recent meta-analysis revealed that the sputum smear examination at the end of the second month of treatment has low sensitivity and modest specificity for predicting failure and relapse (9). Accordingly, my results show that 94,1% of patients are cured after 6 months of treatment and although 8 patients 6,8% still had a positive sputum smear test at two months, most of these individuals were cured at the end of treatment.

Whereas a variety of radiological features has been related to TB (15, 144), only a few of them have been associated with sputum smear positivity and clinical signs or symptoms of this disease (46, 48). Notably, I found that specific CXR features can be characterized individually and associated with sputum smear positivity and BMI. Accordingly, I found that the percentage of lung involvement is higher in patients with lower BMI and individuals with more alveolar infiltrate and no detectable intrapulmonary lymph nodes have higher number of bacteria in sputum smear. Accordingly, a relationship between BMI and the severity of pulmonary TB determined by CXR features was reported recently (122) and a decrease in BMI is associated to an increase in the area of lung affected (14). In addition, the presence of fresh alveolar infiltrates as well as the presence of cavities in the CXR increase the probability of detecting AFB in sputum smear (48). Interestingly, histopathological studies demonstrate that the interaction of Mtb with the immune response takes places in the leukocyte infiltration part that surrounds the granuloma (24). On the other hand, these results also underline a beneficial role of intrapulmonary lymph nodes since their presence is accompanied by fewer bacteria in sputum, indeed, local lymph nodes are recognized as the main site of T cell activation (27) and a

delay in the migration of DC from the lungs to these organs is responsible for the long time needed to develop acquire immune responses in TB (31, 32).

Surprisingly, I did not find any association between sputum smear positivity and the presence of cavities, though it is thought that the presence of cavities is related not only to the number of bacteria in sputum and therefore infectivity but also with prognosis (145-147). However, most of the patients in this cohort do not have cavities and those with cavities tend to be on average older than individuals without cavities what might suggest that in younger individuals infectivity is more dependent on the presence of alveolar infiltrate than to the presence of cavities. Thus, the use of scoring systems based on the presence of cavities may not be applicable to such subjects; instead, a rating of individual specific features of the extent of lung involvement is more appropriate because every specific CXR feature might reflect different pathophysiological stages of the disease. On the other hand, although the presence of cavities is considered an indicator of post primary TB together with upper lobe infiltrates (15), it is interesting to note that although most of our patients were classified as post primary based on the presence of infiltration in the upper lobe, they did not have cavities, moreover, the presence of hilar lymph nodes is considered a feature of primary TB and a number of the patients in my cohort had detectable hilar lymph nodes in spite of having upper lobe infiltrate. Accordingly, the traditional concept of classifying TB as primary or post primary based on CXR findings has been questioned by molecular epidemiological evidence showing that CXR abnormalities in patients with recent infection are similar to those with remote infection, indicating that contrary to the classical view, primary and post primary TB can not be reliably differentiated on the basis of CXR criteria (17, 18), in addition, it has been argued that the presence of hilar lymph nodes is not specific of primary TB (148). Consequently, a recent review acknowledges that the two disease forms have significant overlap on their radiological features (16).

4.2.2 S100A12 as a surrogate marker of neutrophil related extent of pulmonary involvement in TB.

S100A12 has been associated with disease severity in chronic inflammatory pulmonary diseases. Thus, S100A12 levels are elevated in the most severe forms of

COPD and this observation is associated to neutrophilic inflammation (103) and a similar effect is seen in neutrophilic asthma (104). Likewise, CF patients display high levels of S100A12 in airways fluids and high expression of S100A12 by infiltrating neutrophils along with high S100A12 levels in sputum (105), moreover, S100A12 in serum increases significantly during acute infectious exacerbations and decreases after treatment with intravenous antibiotics (106). Remarkably, S100A8 and S100A9 two related calgranulins are present in the inflammatory lung granulomas in active human pulmonary TB and in the TB mouse model these proteins mediate neutrophilic accumulation by inducing production of proinflammatory chemokines and cytokines (35). These studies highlight a role of S100A12 and S100 proteins in neutrophil associated inflammation. Accordingly, my results not only demonstrate that neutrophils are the source of S100A12 but also that this protein is a predictor of fresh alveolar infiltration, indeed, patients with more infiltration have higher S100A12 serum levels.

Emerging evidence suggest that the TB lung tissue damage is primarily a consequence of the host immune response but not a direct effect of the bacteria (35) and neutrophils together with IFN-γ and the interleukin-17 (IL-17) are thought to play a central role in this process because these cells release potent inflammatory mediators that generate tissue injury (36). Neutrophils contribute to the early immune response in TB, however, they are thought also to be involved in disease exacerbation at later disease stages in the context of an inadequate acquired immune response (39). Consequently, in one study it was shown that a higher extent of alveolar infiltrates on CXR was associated with early mortality in hospitalized TB patients (49). The results of the study presented suggest that similar to other chronic pulmonary diseases, S100A12 can be used as a surrogate marker for the estimation of the extent of alveolar infiltration and severity in pulmonary TB and further may highlight the important role of neutrophils in TB pathogenesis.

In summary, I argue that the patterns of S100A12, ADAM10 and INF-γ are associated with the specific CXR features alveolar infiltration and the presence of intrapulmonary lymph nodes. These associations might indicate different stages of the immune response in pulmonary TB which may be related with differences in the extent of lung involvement. Thus I propose, that some individuals have a higher

extent of alveolar infiltration that may be related to neutrophilic inflammation, no detectable intrapulmonary lymph nodes and a higher extent in sputum smear positivity; in these patients the serum levels of S100A12 are likely to be elevated while the serum levels of ADAM10 and INF-γ are likely to be diminished. Whereas in other patients there is less alveolar infiltration, they are more likely to have detectable intrapulmonary lymph nodes and have a lower extent of sputum smear positivity; in these individuals S100A12 would be decreased whereas ADAM10 and INF-γ are elevated.

4.3 Association of peripheral blood neutrophil and lymphocyte counts with disease occurrence and pathology.

The important role of neutrophils in TB pathogenesis and tissue damage has been deciphered not only by animal models but also by human studies. Thus, mice that are genetically susceptibility to TB have a high and prolonged lung neutrophil accumulation along with increased mobility and lifespan of these cells (41). Furthermore, the absence of the PRR adapter molecule CARD9 produces an increased inflammation that is attenuated by neutrophil depletion what links neutrophils to the ability of the innate response to limit damaging inflammation (42). Likewise, there seems to be an association between neutrophils function and IFN-γ in TB because IFN-γ inhibits CD4+ T cell production of IL-17 and directly impedes neutrophils accumulation in the lung and in absence of IFN-γ mice form large necrotic pulmonary lesions associated with granulocytic infiltrates (35). Of importance, mice with IFN-γ unresponsive lung epithelial and endothelial cells have high bacterial burdens and early mortality which are correlated to IL-17 overexpression and lung neutrophilic inflammation (40) and this same phenotype is observed in IFN-γ deficiency (29).

In humans, while local neutrophilia correlates with disease severity, patients with less advanced TB have a predominance of lymphocytes in their bronchoalveolar lavage (BAL) (43). Remarkably, the absolute lymphocyte counts in the blood are increased in patients with symptoms of shorter duration but reduced in those symptomatic for more than 6 months (44), in addition, neutrophils are the major commonly infected phagocyte in human TB (45) and I demonstrate in this study that the neutrophil

derived protein S100A12 is increased in TB patients and is a predictor of disease occurrence and severity. Taken together, the results of other studies and those of this one might suggest that the absolute number of neutrophils and also lymphocytes in peripheral blood is associated with disease occurrence and severity, consequently, I argue that the presence of high neutrophil and low lymphocyte counts may contribute to the extent of alveolar infiltrates in the CXRs of patients with pulmonary TB.

In an attempt to provide a proof of principle that the relation of the absolute counts of neutrophils and lymphocytes in the peripheral blood are related to the extent of lung involvement, the results of my study show that TB patients have a higher neutrophil/lymphocyte count ratio than the healthy controls but also that when this ratio is combined with the BMI as indicator of malnutrition, an Index that I termed “Neutrophils-Lymphocytes-BMI” (NLB) can be generated which identifies TB patients and healthy controls and correlates with alveolar infiltration and area of lung affected. Indeed, early studies had recognized that a high percentage of neutrophils and lymphopenia on the differential white blood cell count are associated with poor prognosis (46) and Sutherland et al. had reported that a high granulocyte/lymphocyte ratio is a predictor of disease occurrence (47), however, in the latter study, the absolute granulocyte count including eosinophils was used what introduced more variance in their estimates because the association with eosinophils is not so strong what resulted in a lower sensitivity and specificity. Of note, other human studies have highlighted the association of neutrophils or alveolar infiltrates with prognosis, thus, the presence of alveolar infiltrate in CXR correlates with higher bacterial numbers in sputum smears (48) and is a clinical predictor of early mortality in hospitalized patients (49). It was recently found that a neutrophil-driven, interferon (IFN)-inducible transcript signature in human whole blood correlates with clinical severity (51). As an upshot of all these studies I introduce an affordable point of care Index that combines the number of neutrophils and lymphocytes in peripheral blood with the degree of malnutrition indicated by the BMI. This new NLB index is a simple tool to be used in low income countries such as India or those in the African continent where TB related mortality is highest.

4.4 Study limitations and proposed further research.

Although all the individuals used as controls in this study were clinically healthy at the moment of sample collection, I am not certain about their status in terms of past exposure to Mtb, thus some might be latently infected with the bacteria what could eventually affect the results. Whereas I could not afford to perform the IGRA test which is more specific for Mtb, the TST was positive in most of those who were tested and some participants refused to be tested, however, this test is not reliable in an endemic area such as India because it does not discriminate between real Mtb infection and exposure to other not tuberculous mycobacteria or from BCG vaccination; therefore, in future studies this aspect should be addressed.

On the other hand, I assessed ADAM10 and the peripheral blood cells count in a subgroup and only cross sectionally, thus, I did not have longitudinal data for these variables. In addition, in this study the follow up was done only for 6 months what does not allow to assess associations with long term outcome or lung function. Likewise, although I did not find associations between sRAGE and the extent of lung involvement, in other studies sRAGE has been associated with lung function based on the FEV, thus, future studies should assess ADAM10 and the peripheral blood cells counts longitudinally and perform FEV measurements; the latter might reveal an association between sRAGE and lung function in pulmonary TB. Moreover, although I show that S100A12 inhibits ADAM10 in vitro, I measured total ADAM10 concentration and not activity, similarly, I could not prove in the lab that S100A12 was directly inhibiting sRAGE secretion; thus, further studies should assess ADAM10 activity in clinical cohorts and test the effect of S100A12 on sRAGE secretion in vitro.

4.5 Conclusions

In summary in this study I demonstrate that in pulmonary TB the serum levels of sRAGE and ADAM10 decrease while S100A12 increases, moreover, the pattern of decreased sRAGE along with increased S100A12 might be an indicator of the extent of lung involvement. Further I conclude that ADAM10 might have a role in sRAGE secretion and the inverse regulation of sRAGE and S100A12 serum levels. In pulmonary TB specific radiographic features are differently associated with sputum smear positivity and BMI, moreover, S100A12 is a surrogate marker of the extent of

alveolar infiltration and might express neutrophil related lung tissue damage. Finally, the ratio between the absolute numbers of neutrophils and lymphocytes in the peripheral blood of TB patients in particular when combined with the BMI might serve as a useful marker to assess disease occurrence and pulmonary involvement.

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APPENDIXES

Appendix I: Informed consent form

BHAGWAN MAHAVIR HOSPITAL AND RESEARCH CENTRE
Mahavir Marg, Masab Tank, Hyderabad

INFORMED CONSENT

THE INDO-GERMAN GRK-1673 PROGRAM

Title of study: The role of Single Nucleotide Polymorphisms (SNPs) of the Toll-Like Receptor (TLR) Signaling System and the receptors RAGE, Mincle and Dectin1 for the Susceptibility and pathogenesis in India - Functional Epidemiologic Analysis.

Principle investigators: Ralf R. Schumann and Hortense Slevogt.
Institute of Microbiology and Hygiene, Charité-Universitätsmedizin Berlin.
Co-Investigators: Dr. Vijayalakshmi Valluri, Dr.Suman Latha, Prof. Niyaz Ahmed.
Bhagwan Mahavir Medical Research Centre, University of Hyderabad

PATIENT CONSENT FORM

Explanation of the condition: Tuberculosis is a multifactorical disease. With the availability of the complete genome sequence of M.tb hopes were raised about new therapies and interventions against the disease that still kills one person /minute in the world. Variations in the human gene are likely to regulate susceptibility or resistance to tuberculosis and disease progression. This necessitates discovery of new therapeutic interventions with through investigation of M.tb physiology in survival, persistence and replication inside host and a comprehensive understanding of the host factors involved in the disease process. The present study evaluates the importance of molecular pathogenesis to identify new targets, clinical and molecular epidemiology to understand the polymorphism in the target genes and design intervention strategies to come with new small inhibitor molecules, vaccine candidate and nutritional intervention.

Why am I being asked to take part in this study ?
You are requested to participate in this research study because either you have TB or you are free from TB.
Number of subjects expected to participate:

I will be one approximately----- subject in Mahavir PPM-DOTS who will participate in this study.
If you decide to be in this study your part would involve:
In providing 10ml blood sample. The blood will be collected using sterile needle.

Risks/Discomforts:

Since we are not giving any drug orally or by injection, there are no risks, as such, involved with your participation in the study. However, as we would be drawing blood using a sterile needle, there may be a little discomporft during pricking the needle. The

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discomfort is only temporary. Only experts with adequate knowledge would be collecting your blood sample.

Benefits:

The patient does not directly benefit from the study, but the study now, and that of the stored blood sample, would help in the research on TB related problems and therefore may help other patients in future.

Confidentiality:

The following procedure will be followed in an effort to keep yourpersonal information confidential in this study:
Your identity would be coded, and all date will be kept in a secured, limited access location.

Your identity will not be revealed in any publication or presentation of the results of this research. As a result of being in this study, identifiable health information about you will be used, generated, and or reported for the purpose(s) outlined in the beginning of this consent form.

As such, there is certain specific information that you need to know.
Health information is any information that can be linked to you, and that relates your past, present, or future physical or mental health or condition. For the purpose of this study, your health information means your:

- Medical history
- Results of physical examination
- Laboratory (blood, urine) tests

Your health information will be accessible for use by:

- The research team for this study
- The sponsor(s) of this study-Department of Biotechnology, Govt. of India
- The institutional Ethical Committee

Access to your health information for this reason would be for an indefinite time period. All of the individuals or groups referenced above are obligated to protect the privacy of your health information.

You have the right to revoke (remove) your consent for allowing access to your health information at any time in writing. If you revoke this consent, you may no longer participate in the research activity. Revoking your consent means that all access to your identifiable up to that point may still be used.

Consent to participate:

I have read or had read to me and understood the above information before signing this consent form I have been offered ample opportunity to ask questions and have received satisfactory answers. I hereby volunteer to take part in this study. I also consent to the investigators storing a specimen of my

Blood:

Or a component of my blood suh as

Serum:

Plasma:

DNA:

For their later use as a part of this study.

Subjects Rights:

- Your participation in this study is voluntary. You do not have to be in this study if you do not want to be.
- You have the right to change your mind and leave the study at any time without giving any reasons and without any penalty.
- Any new information that may make you change your mind about being in this study will be given to you.
- You will get a copy of this consent form to keep
- You do not waive any of your legal rights by signing this consent form.

Questions about the study or your rights as a research subject

If you have any questions about the study, you may contact:

1. Medical Officer: Dr.P.S.Raju, Bhagwan Mahavir Hospital & Research Centre, Hyderabad. 500 004. Tel-040- 23497303.

2. Principal Investigator:

If you sign below it means it means that you have read(or have had read to you) the information given in this consent form and would like to be a volunteer in this

Signature and name of investigator obtaining the consent Date & Time

Signature of Witness Date & Time

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Appendix II: Institutional ethics committee approval

Institutional Ethics Committee for Bio – Medical Research
Bhagwan Mahavir Medical Research Centre
#10-1-1, Bhagwan Marg. A.C. Guards, Hyderabad – 5000004, A.P., India
Phone: 23316057, 23497360, 23303134

Justice Bhaskar Rao
Chariman

Prof. P. P. Reddy
Member Secretary

Certificate

This is to certify that the research proposal entitled "The role of Single Nucleotide Polymorphisms (SNPs) of the Toll-Like Receptor (TLR) Signaling System and the receptors RAGE, Mincle and Dectin1 for the Susceptibility and pathogenesis in India - Functional Epidemiologic Analysis" was presented by Dr.Suman Latha, Co-investigator, Dept. of Immunology, Mahavir Hospital & Research Centre before the Institutional Ethics Committee on 11th March, 2011.

The objectives of the proposal and methodology proposed are satisfactory. The expected outcome of the project is beneficial to the study group and clinicians.

The Investigators clarified the comments made by the members of the committee.

The Investigator was advised to confine their research to the parameters mentioned in the project and not to deviate from the proposal without approval of the committee.

The committee was pleased to approve the implementation of the project at Bhagwan Mahavir Medical Research Centre subject to the review of the progress of the project from time to time.

This Certificate is issued under the seal of Institutional Ethics Committee, Bhagwan Mahavir Medical Research Centre, Hyderabad, India.

Member Secretary

Appendix III: DOTS program questionnaire

FIRST TIME VISIT

MAHAVIR PUBLIC–PRIVATE MIX DOTS

Protocol Rev.
1.) CUE
2.) Sputum pos
3.) Follow up.

PATIENTS HISTORY FORM

1. TB.NO .....
2. Religion ..... (Hindu / Muslim / Christian / Jain)
3. Occupation ..... (Student / Unemployed / Daily Wage / Monthly Wage)
4. Sp. Examination ..... (PPM /PVT / Govt) (ORT) Sc ..... Hc..... Sc.....
5. SP Culture ____ Done (+ VE / -VE / ND) S / R R INH EZ
6. Date of Registration .....
7. Category I II III
8. Disease Classification P EP
   - CODE 01=LA, 02= PLEF, 03= BONES, 04=JOINTS, 05=SPINE, 06=TB ABD, 07=GENUR, 08=PC, 09=TBM, 10=MTB, 11= OTHER

   If extra pulmonary, site (please write code).....
9. Weight WO..... W2/3..... W 4/5..... W 6/8 .....
10. Reff – by .....(PP / TBC / GOVT / OTHERS)

    a) PP code
    b) IF TBC, SOR.....
    c) Others – Family members / Old TB Patient / Neighbours

11. Personal History Part –I

a) Sex ..... (M / F)
b) Status .....(Married –pregnant / Abortion / Unmarried)
c) No of Children .....Age of last Child .....
d) No of People Living ..... No of Rooms .....
e) No of people Earning ..... Total amount / month .....
f) Habits .....(Smoking / Drinking / Tobacco / Pan)
g)Cormorbid conditions DM/HTN/HIV
h) Knowledge about TB Yes / No
i) Fuel used Fire wood / coal / kerosene / LPG Gas

Personal History Part – 2

a) Past History TB / Antibiotic ..... (Yes / No)

b) If Yes Details (Drugs/duration) _____

12. Family history

c) Relation to Pt (With Sp Code : 01- + Ve, 02- -Ve , 03 – EP):

Mother | Father | Husband | Wife | Son | Daughter | Sister | Brother | In-Laws

Code:

d) Effected person has/had ATT from _____ (PPM / Govt / Pvt)

e) Effected persons Trt Date _____ Duration (No. of Months) _____

f) Treatment outcome

13. No. of Health Providers Visited Before Maharir _____

Details _____

a) Suspicion of TB (In Weeks Only) _____

b) First seeking medical advise. (In Weeks Only) _____

c) Direct Cost

	Provider 1	Provider 2	Provider 3
1. Fee	Rs. _____	_____	_____
2. Invest	Rs. _____	_____	_____
3. Drugs	Rs. _____	_____	_____

d) Indirect Cost

4. Transport	Rs. _____	_____	_____
5. Wages	Rs. _____	_____	_____
6. Total Cost	Rs. _____	_____	_____

e) Indebtedness (SAVING /LOAN/MORTGUAGE/SALE)

14. Investigations (ESR/X-RAY / MANTOUX/BIOSY / OTHER INVT)

a) BCG Scar - (Yes /No)

b) X-Ray Findings:

1) Right-Lung (Cavitation/Infil No Cavity / Cons./PLEEF./ Normal)

2) Left-Lung (Cavitation/Infil No Cavity / Cons./PLEFF./ Normal)

15. Bronchodilators (Yes / No) _____

16. 'N' hood DOTS Center Code No _____

17. INH Chemo _____

18. Remarks (Any) _____

19. Treatment outcome (CURED/TRT COMPLETED/DIED/FAILURE/DEFAULTED)

Appendix IV: Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

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