The impact of helminth infection in patients with active tuberculosis

Ebba Abate
The thesis is dedicated to my mother and brother who have left this world too soon
Supervisors

Thomas Schön (Ass. Prof)
Department of Infectious Diseases
Kalmar County Hospital and Linköping
University
Sweden

Olle Stendahl (Prof)
Department of Clinical and Experimental
Medicine
Medical Microbiology
Linköping University
Sweden

Abraham Aseffa (MD, PhD)
Director, Armauer Hansen Research
Institute
Addis Ababa, Ethiopia

Opponent

Zvi Bentwich (Prof)
Head, Center for Infectious Diseases and
AIDS
Ben-Gurion University
Israel

Evaluation Board

Jan Ernerudh (Prof)
Department of Clinical and Experimental
Medicine
AIR/Clinical Immunology
Linköping University
Sweden

Maria Jenmalm (Prof)
Department of Clinical and Experimental
Medicine
AIR/Clinical Immunology
Linköping University
Sweden

Peter Bergman (Ass. Prof)
Department of Laboratory Medicine
Division of Clinical Microbiology
Karolinska Institute
Sweden
Financial support

This work was supported by the Swedish Research Council, the Swedish Heart and Lung Foundation, King Oscar II Jubilee Foundation, SIDA/SAREC and European and Developing Countries Clinical Trials Partnership (EDCTP), Swedish Medical Association (SLS), the Groschinsky Memorial Foundation and the Marianne and Marcus Wallenberg foundation.
Table of Contents

ABSTRACT ................................................................................................................................. 1
SUMMARY IN SWEDISH/SAMMANFATTNING PÅ SVENSKA ............................................... 3
LIST OF ORIGINAL PAPERS .................................................................................................. 5
ABBREVIATIONS ...................................................................................................................... 7
BACKGROUND ........................................................................................................................ 10
  Historical overview and global epidemiology of tuberculosis .............................................. 10
  *Mycobacterium tuberculosis* ................................................................................................. 10
  Tuberculosis .......................................................................................................................... 10
  Tuberculosis and HIV/AIDS .................................................................................................. 11
  Host susceptibility to tuberculosis ......................................................................................... 12
  Pathogenesis and host immune response to tuberculosis ...................................................... 13
    The innate immune response to *Mycobacterium tuberculosis* ......................................... 13
    The adaptive immune response during tuberculosis ............................................................. 14
    The granuloma .................................................................................................................... 15
Clinical characteristics of tuberculosis .................................................................................. 16
Diagnosis of tuberculosis ......................................................................................................... 17
Treatment of tuberculosis ......................................................................................................... 18
Drug resistance in tuberculosis ............................................................................................... 19
Tuberculosis vaccines ............................................................................................................... 20
Classification, global epidemiology and public health impact of helminths ............................ 21
The host immune response to helminths .................................................................................. 22
The role of regulatory T-cells in health and helminth infection .............................................. 22
Helminth co-infection with HIV/AIDS .................................................................................... 25
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helminths and non-infectious diseases</td>
<td>25</td>
</tr>
<tr>
<td>Impact of helminths on vaccine efficacy</td>
<td>26</td>
</tr>
<tr>
<td>Helminth co-infection with tuberculosis</td>
<td>27</td>
</tr>
<tr>
<td>AIMS</td>
<td>29</td>
</tr>
<tr>
<td>PATIENTS, MATERIALS AND METHODS</td>
<td>30</td>
</tr>
<tr>
<td>Study setting</td>
<td>30</td>
</tr>
<tr>
<td>Study participants (Paper I-V)</td>
<td>33</td>
</tr>
<tr>
<td>Clinical and laboratory patient characteristics</td>
<td>36</td>
</tr>
<tr>
<td>Baseline characteristics and the TB-score</td>
<td>36</td>
</tr>
<tr>
<td>HIV testing and CD4+ cell counts</td>
<td>37</td>
</tr>
<tr>
<td>IgE, eosinophil cell count and Quantiferon measurement</td>
<td>37</td>
</tr>
<tr>
<td>Stool examination</td>
<td>37</td>
</tr>
<tr>
<td>Sputum smear examination</td>
<td>38</td>
</tr>
<tr>
<td>Isolation of peripheral blood mononuclear cells (PBMCs)</td>
<td>38</td>
</tr>
<tr>
<td>Analysis of regulatory T-cells by flow cytometry</td>
<td>38</td>
</tr>
<tr>
<td>Analysis of IFN-γ, IL-5 and IL-10 by ELISPOT</td>
<td>39</td>
</tr>
<tr>
<td>Statistics (Paper I-V)</td>
<td>39</td>
</tr>
<tr>
<td>Ethical considerations</td>
<td>40</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSIONS (Paper I-V)</td>
<td>42</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>64</td>
</tr>
<tr>
<td>CONCLUDING REMARKS</td>
<td>65</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>68</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>82</td>
</tr>
</tbody>
</table>
ABSTRACT

The geographic distribution of helminth infection and tuberculosis (TB) overlap substantially. Experimental animal models and limited data from humans have shown that intestinal helminths could subvert the host immune response towards a T-helper 2 (Th2)-type immune response and an increased regulatory T-cell activity (Tregs). This in turn affects the host's ability to mount an effective Th1 immune-mediated protection against Mycobacterium tuberculosis. However, evidence for this hypothesis in the human setting from helminth infected TB patients is limited. This thesis primarily focuses on the immunological and clinical impact of helminth infection on pulmonary TB.

The kinetics of the Quantiferon-Gold (QFN) assay, which measures IFN-γ response to TB-specific antigens in whole blood was assessed and showed a modest decline during TB treatment to the level observed for healthy blood donors. We further assessed another clinical monitoring tool, the-TB-score, composed of clinical signs and symptoms of TB, and found an early decline two weeks after initiation of TB-treatment where a failure of decline correlated with increased mortality. Overall, the helminth co-infection rate was significantly higher in TB patients compared to healthy controls. Helminth co-infection was associated to a significantly higher rate of eosinophilia and IgE-levels in healthy controls and patients with tuberculosis. During the first weeks of anti-TB treatment, a marked decrease in the rate of helminth infection was observed in HIV co-infected compared to HIV-negative TB patients. However, helminth co-infection was more common in HIV negative than HIV positive TB patients. There was no detectable impact of helminth infection on the clinical presentation of pulmonary tuberculosis. At baseline, helminth co-infected TB patients showed an increased frequency of Tregs compared to helminth negative TB patients and healthy controls. This was accompanied by an increased rate of PPD stimulated IL-5 and spontaneous production of IL-10 by peripheral blood mononuclear cells among helminth co-infected TB patients. A placebo controlled randomized trial was conducted in order to test the hypothesis that albendazole treatment of helminth positive TB patients may improve the clinical response of TB by reducing the immunomodulatory effect of helminthes on TB immunity. A total of 140 helminth co-infected TB patients were randomized to albendazole (400 mg per os for three consecutive days) or placebo. No significant difference was observed between the albendazole and placebo group in terms of the primary outcome (TB score change...
between baseline and week 8). Among the secondary outcomes, a significant decline of peripheral eosinophil cells was observed in the albendazole treated group, but no effect on other outcome variables (changes in chest x-ray findings, IgE level and sputum smear conversion). Regarding the immunological assessment no significant difference was observed for changes in Tregs, and PPD-induced production of IFN-γ or IL-5 although a non-significant trend of a decrease in IL-10 expressing PBMCs were observed in the albendazole group.

Taken together, the burden of helminth infection was higher in TB patients than in a healthy control group. Helminth co-infection during pulmonary TB in the human setting induces an immune response characterized by increased IgE production, eosinophilia as well as increased levels of Tregs and spontaneous IL-10 production. Thus, the immunological impact of helminth infection on the outcome and risk for developing TB merits further investigation.

Testresultaten för Quantiferon-Gold (QFN), ett hellblods-baserat ELISA-test som kvantifierar den cellulära produktionen av interferon-γ (IFN-γ) efter stimulering med TB-specifika antigen, monitorerades hos TBC-patienter som genomgick behandling, och visade på en viss minskning av IFN-γ-produktion under behandling, till nivåer jämförbara med en frisk kontrollgrupp. Vi utvärderade även TB-score som är ett annat kliniskt hjälpmedel för att monitorera kliniskt förlopp vid TBC. TB-score bygger på kliniska symptom och tecken, och våra resultat visar en tydlig minskning två veckor efter påbörjad behandling där utebliven reducering av TB-score korrelerade med ökad mortalitet.

Överlag fann vi en högre prevalens av maskinfektion i hos patienter med TBC jämfört med en frisk kontrollgrupp. Hos båda grupperna var maskinfektion associerat med signifikant högre förekomst av eosinofili samt ökade nivåer av IgE. Under första behandlingsveckorna mot TBC observerade vi en tydlig minskning av andel HIV-positiva patienter med maskinfektion jämfört med TBC-patienter utan HIV. Dessutom var maskinfektion vanligare hos HIV-negativa TBC-patienter än TBC-patienter med HIV.

Vi fann ingen påverkan på den kliniska bilden vid diagnos av TBC i relation till maskinfektion. Däremot var samtliga maskinfektion och TBC associerad med en högre andel Tregs jämfört med TBC-patienter utan maskinfektion och friska individer. Likvälv fanns en ökad andel IL-5 producerande vita blodkroppar i perfiert blod (PBMCs) efter PPD-stimulering samt en ökad spontan produktion av IL-10 hos TBC-patienter med samtida maskinfektion.

I en randomiserad placebo-kontrollerad studie undersökte vi hypotesen att behandling av maskinfekterade TBC-patienter med albendazol, förbättrar svaret på TBC-behandling genom att reducera den immunomodulerande effekten av maskinfektionen på värdförsvaret mot TBC. TBC-patienter (*n*=140) med samtida maskinfektion blev randomiserade till albendazol (400 mg per os under tre dagar) eller placebo. Ingen signifikant skillnad återfanns hos de patienter som behandlades med albendazol jämfört med placebo vid gäller förändring i TB-score mellan behandlingens början och vecka 8, förändringar i lungröntgen, IgE nivåer eller
sputumkonversion. Däremot gav albendazol en signifikant minskning av eosinofila granulocyter i perifert blod.

Vad gäller den immunologiska utvärderingen återfanns inga signifikanta skillnader mellan de båda grupperna vad gäller Tregs och PPD-inducerad produktion av IFN-γ eller IL-5. Däremot fanns en trend för minskade nivåer av IL-10 producerande PBMCs hos gruppen som behandlades med albendazol.

Sammanfattningsvis fann vi en högre prevalens av maskinfektion hos TBC-patienter jämfört med kontrollgruppen, och maskinfektion hos TBC-patienter gav upphov till ett immunsvar karaktäriserat av ökade IgE-nivåer, eosinofili samt ökade nivåer av Tregs och spontan produktion av IL-10. Dessa immunologiska förändringar styrker vikten av fortsatta kliniska studier av maskinfektioners påverkan på läkningsförmåga vid aktiv TBC och risken hos exponerade att utveckla TBC.
LIST OF ORIGINAL PAPERS

This thesis is based on the following publications and manuscripts, referred to in the text by their Roman numerals:

**Paper I**

**Paper II**

**Paper III**

**Paper IV**

**Paper V**
Additional work performed during the PhD period outside the scope of this thesis


# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AFB</td>
<td>Acid fast bacilli</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AHRI</td>
<td>Armauer Hansen Research Institute</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>ART</td>
<td>Anti-retroviral therapy</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette Guérin</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSL</td>
<td>Biosafety level</td>
</tr>
<tr>
<td>CC</td>
<td>Community control</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFP</td>
<td>Culture filtrate protein</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell mediated immunity</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DOTS</td>
<td>Directly Observed Treatment, Short-course</td>
</tr>
<tr>
<td>DSMB</td>
<td>Data and safety monitoring board</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immuno sorbent assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-linked immunospot assay</td>
</tr>
<tr>
<td>ESAT</td>
<td>Early secretory antigenic target</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence Minus One</td>
</tr>
<tr>
<td>FMOH</td>
<td>Federal Ministry of Health</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forehead box P3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factors</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active anti retroviral therapy</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGRA</td>
<td>Interferon-gamma release assay</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IUTLD</td>
<td>International Union against Tuberculosis and Lung Diseases</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>Multi-drug-resistant tuberculosis</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MNL</td>
<td>Mediastinal lymphnode</td>
</tr>
<tr>
<td>MUAC</td>
<td>Mid-upper arm circumference</td>
</tr>
<tr>
<td>NKs</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse-transcriptase inhibitors</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric-oxide</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PIHCT</td>
<td>Providers initiated HIV counseling and testing</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>QFN</td>
<td>QuantiFERON</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPO</td>
<td>Research and publication office</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEA</td>
<td>Soluble egg antigen</td>
</tr>
<tr>
<td>SFU</td>
<td>Spot forming unit</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>Th</td>
<td>T- helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factors</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T-cell</td>
</tr>
<tr>
<td>TST</td>
<td>Tuberculin skin test</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>Joint United Nations Programme on HIV/AIDS</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>Extensively drug-resistant tuberculosis</td>
</tr>
</tbody>
</table>
BACKGROUND

Historical overview and global epidemiology of tuberculosis

Tuberculosis (TB) is an infectious disease caused by bacilli belonging to the Mycobacterium tuberculosis complex [1]. This complex consists of seven species including Mycobacterium tuberculosis (M. tuberculosis), M. canetti, M. africanum, M. pinnipedii, M. microti, M. caprae and M. bovis [2]. Tuberculosis has a long history and coexisted with humans since ancient times [3]. It was reported that all modern members of the M. tuberculosis complex had a common African ancestor [4].

Mycobacterium tuberculosis

Mycobacterium tuberculosis is a fastidious, slow growing, lipid-rich, rod shaped bacterium, which resists decolorization with acid-alcohol. The bacterium has a slow growth rate of 12-16 hours compared most other bacteria, which have a generation time measured in minutes [5]. The cell wall of M. tuberculosis is rich in lipids which contribute to the acid fastness and hydrophobicity. The waxy coat also contributes to the resistance to many disinfectants, common laboratory stains as well as to antibiotics [6].

Tuberculosis

Tuberculosis is predominantly a disease of the lung, with pulmonary TB accounting for about 70% of the cases. Extra-pulmonary disease sites include lymph nodes, bone, and meninges [7]. There are 22 high-burden countries, which account for approximately 80 % of the estimated number of new TB cases [1]. The consequences of TB on society are immense. Worldwide, it has been estimated based on tuberculin skin test (TST) positivity, that one person out of three is infected with M. tuberculosis [8]. In 2012, there were 8.7 million new cases of TB globally, with 1.4 million deaths in the same year, which confirms TB as a serious global health issue [1] (Figure 1).
Tuberculosis and HIV/AIDS

Tuberculosis re-emerged as a global threat in the late 1980s following the Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) pandemic [9]. Tuberculosis continues to be a leading cause of illness and death among people with HIV/AIDS in resource-poor areas of the world [10]. The yearly risk of a patient with HIV to develop TB is about 5%, which is similar to the life time risk for an immune competent patient [11]. Sub-Saharan Africa remains most severely affected, with nearly one in every 20 adults (4.9%) living with HIV, and harboring 69% of the people living with HIV worldwide [12]. Of the 8.7 million incident TB cases in 2011, 1.1 million (13%) were among people living with HIV [1].
Tuberculosis and HIV/AIDS in Ethiopia

Tuberculosis continues to be one of the major public health concerns in Ethiopia fueled by the expansion of the HIV pandemic since the 1990s. According to the 2012 WHO TB report, Ethiopia was ranked 7th among the world’s 22 high burden countries with an estimated incidence of 258 per 100,000 population, and a TB mortality of 18 per 100,000 individuals. The reported HIV prevalence in the incident TB cases was 17 per 100,000 individuals [1].

In addition to a high TB burden, Ethiopia has been seriously affected by HIV/AIDS, with an estimated 1.5 million people living with HIV [12]. The high rate of chronic malnutrition, poverty, overcrowding, in combination with a high sero-prevalence of HIV infection has created an environment making TB a very serious health problem in Ethiopia [13].

Host susceptibility to tuberculosis

The host susceptibility to TB and the clinical course of the infection depend on a complex interplay between host, bacterial as well as environmental factors, such as poverty, malnutrition, overcrowding, and exposure to other pathogens [14-16]. Several genetic factors have been shown to influence host susceptibility to TB. Polymorphisms in certain genes encoding for natural-resistance-associated macrophage protein (NRAMP1), the interleukin-1 (IL-1) gene cluster, the
vitamin D receptor and mannose-binding lectin have been associated with susceptibility to TB. The essential role of T-helper 1 (Th1) associated cytokines such as IFN-γ and IL-12 was confirmed by the increased susceptibility to mycobacterial infections in patients with mutations in genes coding for these cytokines [17,18].

**Pathogenesis and host immune response to tuberculosis**

*M. tuberculosis* is an air borne pathogen and following infection alveolar macrophages are the primary target cells for inhaled mycobacteria [19]. If the bacteria are not directly killed by the innate host response which may be the case in up to 50% of exposed individuals, the infection is controlled in part by walling it off from the rest of the uninfected lung in distinct foci known as granulomas [20]. The immune response against TB plays a fundamental role in the outcome of *M. tuberculosis* infection. *M. tuberculosis* is a pathogen capable of causing both progressive disease and latent infection [8,21]. After successful control of the primary TB infection, some bacilli may remain in a non-replicating or slowly replicating dormant state for the rest of the life of the individual. This infectious state, defined as latent TB infection, is clinically asymptomatic. Control of *M. tuberculosis* infection is mainly dependent on the success of the interaction between innate and adaptive immune response of the host.

**The innate immune response to Mycobacterium tuberculosis**

Phagocytosis of *M. tuberculosis* by macrophages represents the first major host-pathogen interaction in TB [22]. Recognition of *M. tuberculosis* by phagocytic cells is through surface receptors including Toll-like receptors (TLRs), complement receptors (CR), mannose receptors, scavenger receptors, and dendritic cell (DC)-specific intercellular-adhesion-molecule-3-grabbing non-integrin (DC-SIGN). This recognition causes activation of phagocytic cells and production of cytokines and pro-inflammatory cytokines such as TNF-α, IL-1-β, IL-6, IL-12 and IFN-γ. In relation to innate immune effector responses, vitamin D is also involved in the killing of *M. tuberculosis* through the production of the peptide cathelicidin [23].

Neutrophils are among the first cells to respond to inflammatory stimuli by migrating to the site of infection where they kill pathogens by both reactive oxygen species (ROS) and antimicrobial peptides [24]. The role of neutrophils in host immunity to TB through mechanisms including apoptosis have been previously shown [25,26]. Other cells of the host innate immune system of importance for the defense against *M. tuberculosis* include DCs, natural killer cells (NK) and
epithelial cells [27, 28]. Innate effector mechanisms against M. tuberculosis include phago-
lysosomal fusion, reactive oxygen and nitrogen intermediates and antimicrobial peptides such as
 cathelicidin induced by vitamin-D [29]. Natural killer cells may kill intracellular M. tuberculosis
by the pore-forming perforin, where the anti-mycobacterial factor granulysin binds to the
bacterial cell surface, and disrupts the membrane causing osmotic lysis of the bacteria [30].
However, this mechanism is more pronounced when M. tuberculosis specific CD8+ T-cells are
activated [31]. The major defense mechanisms within the macrophages include low pH, ROS
produced by NADPH oxidase, reactive nitrogen species (RNS) produced by the inducible nitric
oxide synthase (iNOS), iron (Fe2+) scavengers and exporters, proteases and antimicrobial
peptides such as cathelicidin [32]. Apoptosis is an important mechanism for the infected host cell
to limit replication of M. tuberculosis. Apoptosis of phagocytic cells may prevent dissemination
of infection as well as reduce the viability of intracellular mycobacteria [33].
In murine models it has been shown that nitric oxide (NO) is essential for host defense against
M. tuberculosis, but the relative importance of NO has not been fully established in humans
[34, 35]. At the site of TB infection in humans, the presence of iNOS and nitrotyrosine in
macrophages has been shown as indicators for NO production [36]. Idh et al., reported low level
NO in both HIV negative and HIV co-infected TB patients compared to blood donors and
house-hold contacts [37]. In a randomized study conducted in Gondar, Ethiopia, supplementation
of peanuts (rich in arginine) in conjunction with anti-TB drugs increased exhaled NO production
and enhanced clinical improvement during anti-TB treatment in HIV-coinfected TB patients
[38].

The adaptive immune response during tuberculosis
Following activation, dendritic cells are recruited to the lung where they transport mycobacterial
antigens to the mediastinal lymphnode (MLN). Within the MLN, antigen presenting cells (APCs)
activate antigen-specific T-cells. The infected macrophages and DCs secrete cytokines including
IL-7, IL-12, IL-15, IL-23 and TNF-α, leading to attraction of more leukocytes to the infection
site [39]. Due to the nature of TB infection, the majority of bacilli reside within an endosome and
M. tuberculosis antigens are presented mostly on major histocompatibility complex (MHC) class
II [40]. However, it has also been shown that M. tuberculosis antigens could be presented by
MHC class I pathways activating CD8+ cytoltyic T-cells which kill M. tuberculosis by the
granulysin-perforin system [31]. CD4+ T-cells may differentiate into Th1, Th2, Th17 and
regulatory T-cells (Tregs) depending up stimuli. The Th1 response primarily results in increased IFN-γ production and subsequent activation of macrophages, with killing of intracellular bacteria by phagolysosome fusion and effector mechanisms of the macrophage such as nitric oxide. In contrast, the Th2 response produces B-lymphocyte stimulating factors (IL-4, IL-5, IL-10 and IL-13), which suppress the Th1 response. Th17 cells, stimulated by IL-23, IL-6, IL-21, and low TGF-β levels, are involved in recruitment of cells of the innate immune system and Th1 cells, and secrete IL-17. Regulatory T-cells produce anti-inflammatory cytokines such as IL-10 and TGF-β, which have the capacity to suppress microbicidal mechanisms in the macrophage. The role of B cells in TB is not clear, but has been recognized as potentially protective [41]. Of relevance for the Th1/Th2 balance and the effector function of the macrophage, stimulation of the macrophage with IFN-γ, TNF-α or IL-1β will lead to a M1 phenotype, with up-regulation of iNOS, NO-production and antimicrobial activity. Stimulation with the Th2-related cytokines IL-4, IL-10 or TGF-β will generate a M2 phenotype, with increased arginase activity beneficial for tissue repair but not for bacterial killing [29].

The granuloma
The granuloma is formed through recruitment of immune cells during the innate response and consolidated by adaptive immunity, as a result of complex cytokine and chemokine signals. The formation of a granuloma is a dynamic process that begins shortly after infection and continuously evolves over time. It can be divided into three distinct phases, the “innate granuloma,” which is a loose aggregate composed primarily of recruited macrophages and neutrophils; the “immune granuloma” formed following the emergence of antigen-specific T-cells; and the “chronic granuloma,” resulting from distinct morphological changes in granuloma structure [42]. The cellular core of the granuloma contains infected macrophages, and occasional multi-nucleated giant cells surrounded by dendritic cells, neutrophils, eosinophils, T and B-cell and fibroblasts [43]. The structure and functions of the granuloma are regulated and controlled by the complex interplay between cytokines which includes IFN-γ and TNF-α. Regulatory cytokines including IL-10 and TGF-β may undermine granuloma maintenance [44]. In the case of an impaired immune response, such as during HIV-infection, the immunological balance is disturbed, which may lead to tissue damage from necrosis as a result of bacterial dissemination [45]. It is controversial whether the granuloma may actually provide a shelter for *M. tuberculosis*.
(especially during the early stages of infection) or is part of the host protective function during the later stages of the infection.

Figure 3: Schematic illustration of a TB granuloma: Infected macrophages and dendritic cells are surrounded by bystander macrophages, neutrophils, and subsets of T-lymphocytes where the cells are enclosed by a fibrotic cuff (left). *M. tuberculosis* can be contained within the granuloma for long periods, but if immune control fails as a result of HIV-infection or other factors, the bacilli start to replicate, and a necrotic granuloma core develops. The granuloma then ruptures and *M. tuberculosis* is dispersed into the airways (right).

**Clinical characteristics of tuberculosis**
The lungs are the main site of disease for pulmonary TB and are also the primary route of infection. For pulmonary TB, the universal clinical symptom is cough for more than two weeks, which is initially dry but in rare cases blood stained sputum could be produced as the disease progresses [46]. The other most common clinical manifestations after cough include fever,
malaise, fatigue, sweating, and weight loss, [46]. Upper lobe infiltrates as well as fibrosis with or without cavitation are common chest X-ray presentations in active TB [47].

**Diagnosis of TB**

The laboratory diagnostic methods used for detection and identification of TB include smear microscopy, culture and molecular techniques involving specific nucleic acid markers [48]. However, culture is still the “gold standard” for the diagnosis of active TB [49]. Smear microscopy of acid fast-bacilli (AFB) is often the only available diagnostic method in low resource countries [50]. For the diagnosis of pulmonary TB, at least two consecutive sputum specimens including one morning sputum should be collected, based on the Directly Observed Treatment Short-course drug therapy (DOTS) guidelines by the WHO recommendations [51]. Although several methods can be used to visualize acid-fast organisms, the Ziehl-Neelsen technique is the most widely used method in resource poor countries [52,53].

The culture media used for the isolation of mycobacteria include egg-based and agar-based media [49,54,55]. All isolates from cultures reported positive for mycobacteria should be identified to the level of species using either biochemical or molecular methods [56]. Recently, a nucleic-acid amplification based assay, Xpert MTB/RIF assay (Cepheid-Australia), has been introduced. The Xpert MTB/RIF assay is a fully automated molecular assay in which real-time polymerase chain reaction technology is used to simultaneously detect *M. tuberculosis* and rifampicin resistance mutations in the *rpoB* gene [57]. In 2010, WHO endorsed the roll-out of this test for the investigation of TB suspects, especially in settings with a high prevalence of HIV-associated disease and MDR-TB [58]. The assay requires minimal laboratory expertise, and results are available within 2 hours. Even though the assay enables rapid results and is less labor intensive, it has some drawbacks regarding the high cost of the instrument and cartridges. In addition, issues related with proper maintenance and services are of relevance for high endemic rural areas. Additionally, reports of false positive rifampicin resistance results merits further assessment of the assay [59].

**Immunological diagnostic tools**

A gold standard for the diagnosis of latent TB is not yet available. The century old tuberculin skin test (TST) and the more recently developed IFN-γ release assays (IGRAs) are used as screening tools for latent TB infection [60]. IGRAs are slightly more specific than TST and both
Tests have a high negative predictive value but a poor positive predictive value (2-3%) in estimating the risk for developing active disease [61].

**Tuberculin skin test (TST)**

The TST has been used for more than 100 years [62, 63] and has been widely employed to measure the prevalence of TB infection in the community as well as to select TST-negative patients for BCG vaccination. The TST is administered intradermally by injecting 0.1 mL of 5 Tuberculin Units of Purified Protein Derivative (PPD) on the dorsal surface of the forearm. After 48-72 hours, a positive reaction (usually defined as ≥ 10 mm) is indicated by induration [64]. Individuals with prior infection with *M. tuberculosis* will mount an immune response to mycobacterial proteins [62]. However, TST is associated with limitations such as low sensitivity in individuals with impaired cellular immunity as well as false positive results in individuals previously vaccinated with BCG or exposed to environmental mycobacteria [65, 66].

**Interferon-γ release assays (IGRAs)**

The IGRA assays are based on the principle that T-cells of TB sensitized individuals produce IFN-γ when they re-encounter *M. tuberculosis* antigens [67, 68]. Recent evaluations showed that IFN-γ assays that use *M. tuberculosis* RD1 antigens, such as ESAT6 and CFP10 have advantages over TST because they are specific to *M. tuberculosis* and are not affected by BCG or other *Mycobacterium* species with a few rare exceptions [69, 70]. In contact investigations, the results of IGRAs had a better correlation with measures of exposure than TST [60, 71]. Since 2005, IGRAs have increasingly been used for the detection of latent TB infection either as replacement or as adjunct to the tuberculin skin test [72].

**Treatment of tuberculosis**

The history of TB changed dramatically after the introduction of anti-mycobacterial agents. The use of anti-TB drugs started in the 1940-ies, through the discovery of streptomycin and para-aminosalicylic acid [73]. In TB-endemic areas TB-treatment is administered through DOTS programs, where patients are observed when they take their medication to ensure compliance, as non-compliance is a major contributor to the development of antibiotic resistance [74].
Anti-TB drugs are grouped into first line and second line drugs. First-line anti-TB drugs are isoniazid, rifampicin, ethambutol and pyrazinamide [75]. Other drugs such as aminoglycosides (amikacin and kanamycin), fluoroquinolones, capreomycin, para-aminosalicylic acid and thioamides (ethionamide and prothionamide) are used as second-line or alternative agents to treat TB when the bacilli are resistant to first-line drugs [75]. The current short-course treatment guideline aims for a complete elimination of active and dormant bacilli and involves two phases. During the initial phase four drugs (usually isoniazid, rifampicin, pyrazinamide and ethambutol) are given for two months [76]. The continuation phase in which fewer drugs (usually isoniazid and rifampicin) are administered for an additional 4 months, targets the killing of any remaining or dormant bacilli in order to prevent relapse. As a routine, all sputum-positive patients on DOTS should have repeated sputum specimens examined at the end of the 2nd and 6th month, and treatment outcomes are reported through the WHO guideline as cured, treatment completed, defaulted, transferred out and treatment failure [58].

**Drug resistance in tuberculosis**

Multi-drug resistant (MDR) TB is defined by resistance to at least rifampicin (RIF) and isoniazid whereas extensively drug-resistant (XDR) TB is defined as MDR-TB with additional resistance to any fluoroquinolone and to at least one of the injectable drugs (capreomycin, kanamycin and amikacin). The prevalence of MDR and XDR-TB has been increasing with alarming rate [1]. The emergence of drug resistance has posed a major challenge on the control of TB globally. Drug resistance in TB is the result of spontaneous mutation in combination with selection by poor programmatic and individual care performance [77]. The effect of anti-TB drug resistance has been around since the first anti-tuberculosis agent, streptomycin, appeared on the market as a mono-therapy [78]. The problem of increasing resistance to *M. tuberculosis* is also of significance to African countries such as Ethiopia with a high burden of disease. The recent Ethiopian national estimate of MDR-TB was 1.6% and 11.8% for new and re-treatment patients, respectively [13]. However, this is likely underestimated considering the limited TB diagnostic and identification facilities in the country and the need for a regular update of resistance survey data.
Tuberculosis vaccines

The current vaccine against TB, bacille Calmette-Guérin (BCG), is a live vaccine derived from an attenuated strain of *M. bovis*. It is an inexpensive vaccine that has been applied since decades and has a long-established safety profile. Studies have shown that BCG gives good protection against disseminated TB in young children [79]. Natural exposure to environmental mycobacteria [80,81], nutritional and socio-economic diversity, genetic differences and co-infection with other endemic infections such as helminths have been suggested as responsible for the low protection in adults [14,82-84].

Thus, there is a great need to develop a vaccine that gives better protection against TB. However, the development of new vaccines against pulmonary TB has proved to be challenging [84]. In the last ten years more than ten vaccine candidates have been developed [82]. The approach to develop a TB vaccine includes a replacement vaccine for BCG or a booster vaccine, a post-exposure vaccine or a therapeutic vaccine. A newly developed booster vaccine containing two of the *M. tuberculosis* antigens secreted in the acute phase of infection (Ag85B and ESAT-6), delayed and reduced clinical disease in cynomolgus macaques exposed to *M. tuberculosis* [85]. Another promising approach is the recombinant live vaccine, VPM1002, which is a recombinant BCG strain expressing listeriolysin of *Listeria monocytogenes*. The rationale behind this strategy is the assumption of improved release of BCG-derived antigens into the cytosol and increased apoptosis of host cells in vitro [86]. However, it appears unlikely to have a protective vaccine on the market in the coming decade [11]. The very recent phase 2b randomized placebo controlled clinical trial with MVA85A conducted in South Africa on infants previously vaccinated with BCG, did not show any protective results [87].
Helminths

Classification, global epidemiology and public health impact

Helminths, a Greek word meaning “worms”, are multicellular organisms characterized by elongated, flat or round bodies. The definitive classification is based on the external and internal morphology of egg, larval, and adult stages and helminths are primarily divided into two major phyla: platyhelminths (includes trematodes and cestodes) and round worms (nematodes) [88]. Humans are infected after ingesting eggs from contaminated soil or food, which are referred as soil transmitted helminths (*Ascaris lumbricoides* and *Trichuris trichiura*), or through active penetration of the skin by infective larval stages present in contaminated soil (hookworm) [89].

The most prevalent helminth species affecting about one-third of the world’s population living in resource poor regions are *A. lumbricoides*, *T. trichuira* and hookworm (*Necator americanus* and *Ancylostoma duodenale*) [90-92].

Infections by most soil-transmitted helminths are asymptomatic, especially in adults. In children, soil-transmitted helminth is one of the world’s most important causes of physical and intellectual growth retardation and malnutrition. Helminths induce tissue reactions, such as granuloma, and provokes intestinal obstruction or rectal prolaps, especially in children [93]. Studies have also highlighted the profound effect of soil-transmitted helminth infection on school performance and attendance and future economic productivity [94,95]. Yet, despite all these consequences, helminth infection is an area which remains largely neglected. It has been suggested that this neglect originates from three features: (i), the people most affected are the world’s most impoverished, particularly those who live on less than US$2 per day; (ii), the infections cause chronic health problems and have subtle clinical presentation; and (iii) a difficulty of quantifying the effect of soil-transmitted helminth infections on economic and educational development [89]. However, in the last decade it has become clear from revised estimates that the combined disease burden of helminth infections might be as great as that of malaria or TB [96]. Furthermore, it has been described that even asymptomatic helminth infection might increase host susceptibility, affecting other diseases like HIV/AIDS, TB and malaria [14,97-100].
The host immune response to helminths
Helminth parasites are an evolutionarily ancient and diverse group [101], but show species-independent modes of immune modulation in clinical and experimental studies. Because of such character they have been described as a “master of immunoregulation” capable of escaping the host defense system through suppression of both Th1 and Th2 associated host immunity and of establishing chronic infections [93].

Helminths induce a strong Th2-type immune responses characterized by production of cytokines such as IL-4, IL-5, IL-9, and IL-13 and increased levels of circulating IgE antibodies, eosinophils, and mast cells [93]. CD4+ Th2-lymphocytes are the central player in the helminth-induced immune response by the production of cytokines and chemokines such as CCR3. It has been shown that mice depleted of CD4+ T-cells, did not mount a protective immune response following vaccination with Schistosoma mansoni, and lacked the ability to expel the intestinal helminths [102,103]. The Th2 dominance in many infections is maintained by IL-10 and TGF-β mediated suppression of competing Th1 and Th17 cell populations [83]. This has been shown in a mouse filariasis model using L. sigmodontis where the chronic phase of infection is marked by T-cell anergy, loss of proliferative responses to parasite antigen challenge, reductions in effector cytokine levels, and elevated expression of inhibitory immune molecules and cells such as, IL-10, TGF-β and Tregs.

The role of regulatory T-cells in health and helminth infection
Regulatory T-cells are a sub-population of T-cells, which modulate the immune system in order to prevent tissue damage from pro-inflammatory responses, maintain tolerance to self-antigens, and abrogate autoimmune disease [104]. These cells can be divided in to two subsets, natural Tregs that develop in the thymus, and induced Tregs (Tr1, Th3) that arise from conventional CD4+ T-cells in the periphery, a process promoted by chronic antigen exposure [105].

Regulatory T-cells express CD25, the α chain of the IL-2 receptor. The quantitative identification of Tregs requires reliable surface markers that are selectively expressed on Tregs. The forkhead/winged helix transcription factor (Foxp3) is an important marker to identify these subsets [106], but it may be transiently expressed on activated CD4+ T-cells. CD127 is another marker expressed on Tregs which inversely correlates with Foxp3 and is considered an improvement in the definition of Tregs [107].
Expansion of both natural and induced Treg populations has been demonstrated in human helminth infections including an increased frequency of Foxp3 expressing Tregs [108]. These cells pose a more potent immune suppression in helminth infected mice than helminth uninfected mice [109,110]. Similarly, in human filarial and Ascaris infections, the presence of helminths correlate with both the production of IL-10 and TGF-β [111], and generalized T-cell hypo-responsiveness [112]. In addition, the observed epidemiological association of increased rate of helminth infection with less allergic manifestations and autoimmune inflammatory conditions in humans is linked to an attenuated immune response by Tregs [113-115].
Figure 4: A model showing the immunomodulatory effect of helminths on host immune cells. Once the antigen-presenting cells are activated by helminthic components like early secretory and soluble egg antigen (SE/SEA), they in turn activate CD4+ T-cells to induce a potent Th2-type immune response and expansion of regulatory cells such as Tregs (both natural and inducible types), and alternatively activated macrophages (M2). As a result of a skewed Th2-type response and expansion of regulatory cells, the Th1 type response is attenuated including a functional impairment of the pro-inflammatory axis.
Helminth co-infection with HIV/AIDS

Following the HIV epidemic, infection with both helminths and HIV-1 is common in resource poor areas [116]. Epidemiological cross-sectional studies show an association of helminth infection with HIV [117,118]. However, this was more common with protozoan opportunistic parasites such as Cryptosporidium parvum. There was a decline in the rate of helminth-HIV coinfection before as compared to after the era of anti-retroviral therapy in Ethiopia [117,119-122].

The immunological influences of helminths have been investigated in several human diseases including HIV and TB. Increased expression of co-receptors of HIV-1 chemokines on T-lymphocytes and monocytes obtained from helminth infected individuals has been reported [123,124]. Peripheral blood mononuclear cells (PBMCs) obtained from helminth-infected individuals show increased susceptibility to HIV-1 infection [125,126]. An increased risk of mother to child transmission of HIV was observed in pregnant women co-infected with helminths compared to controls [127]. Furthermore, a decreased CD8+ cytolytic HIV-1-specific T-cell response, and increased IL-10 production were observed in HIV patients co-infected with S. mansoni compared to those with HIV-1 infection only [128]. In a placebo controlled trial, Ascaris co-infected HIV positive patients treated with albendazole showed a significant increase in peripheral CD4+ T-cells and a decreasing trend in HIV-1 RNA level compared to the placebo group [129].

Helminths and non-infectious diseases

The difference in the prevalence of allergic diseases between urban versus rural and between economically poor and industrialized countries has launched the “hygiene hypothesis” [130]. An increased colonization with helminths may lead to a decrease in allergic and autoimmune diseases possibly by increased activation of anti-inflammatory responses induced by helminths. Studies on the interactions between helminths and allergic immune responses have shown variable results, ranging from decreased rate of allergy in helminth infected individuals [131], increased risk of allergy [132,133] and the lack of any association [134]. Most of the epidemiological and experimental studies show an important role of helminths in suppressing the occurrence and outcome of autoimmune diseases [128,135-137]. In mouse models, infection
with *S. mansoni*, inhibited the development of type1 diabetes [128]. Helminths were found to be protective in several models of inflammatory bowel disease [135,138]. Currently, *Trichuris suis* is used to treat patients with ulcerative colitis, Crohn’s disease and multiple sclerosis [136,137,139].

**Impact of helminths on vaccine efficacy**

The lack of efficient responsiveness to oral vaccines like cholera, polio and rotavirus in developing countries initiated the hypothesis that the presence of helminths in the gastrointestinal tract might have affected efficient uptake of these vaccines [140]. It was shown that *A. lumbricoides* infection impaired the immune responses to oral cholera vaccine by decreasing seroconversion [140,141]. The negative impact of helminths in the response to vaccines like BCG has also been shown in Ethiopia. In one study, BCG vaccination improved PPD-specific IFN-γ production and T-cell proliferation from PBMCs in albendazole-treated healthy subjects with intestinal helminths compared to controls [142]. It was further shown that BCG-vaccinated mice with prior *S. mansoni* infection had significantly higher TB bacillary load as measured by viable count. In addition, lower levels of IFN-γ and nitric oxide together with increased levels of IL-4 and IL-5 were observed in mice with prior *S. mansoni* infection [143].

Other studies demonstrating an attenuated vaccine response in helminth positive individuals are in agreement with the results observed for BCG vaccination [144-148]. Filarial infections decreased the responsiveness to tetanus toxoid after tetanus vaccinations [149]. In another study, a tetanus antitoxin level was measured two months after immunization with tetanus toxoid vaccine, and only 7.1% of patients infected with onchocerciasis became immunized compared to 44.5% in the control group free from onchocerciasis [150]. Such findings underline the influence of helminth infections on vaccine efficacy, and thus warranted the need to consider this issue when designing and testing new vaccines. Indeed, without deworming prior to vaccination, as it has previously been used empirically in veterinary medicine, the efficacy of vaccines could be compromised in endemic areas for helminth infection. Therefore, a better understanding of the interplay between the immune response of the host and the impact of co-infections is needed.
Helminth co-infection with tuberculosis
The geographic distributions of helminths and TB overlap substantially, increasing the likelihood of co-infection with both pathogens [119]. Both helminths and *M. tuberculosis* use several independent mechanisms to affect host immune responses and these mechanisms may interact with important consequences for the immunology and outcome of both infections [14,151,152]. Epidemiological cross-sectional and case-control studies evaluating the prevalence and association of helminth infection in active TB showed that these infections indeed coexist. Tristao-SR et al., found that a high intestinal nematode infection rate in pulmonary TB patients [153]. An increase in the prevalence of helminth co-infection in TB was reported in Gondar, Ethiopia [121,154]. Moreover, in the same setting an increased rate of helminth infection among active TB patients compared to healthy community controls was recently shown [119]. Furthermore, significantly higher frequency of intestinal helminths was reported in patients with the lepromatous and multibacillary form of leprosy than patients with paucibacillary leprosy or to a control group without leprosy [155]. Even though those epidemiological studies are unable to address the immunological aspects behind the findings, it is clear from experimental models and limited data from human infection that immunomodulation induced by helminths may affect the ability of the host to control infection and disease from *M. tuberculosis*.

The findings of many of the experimental studies are in favor of the negative impact of helminths on TB infection and risk of developing TB after exposure. The Th1 immune-mediated protection against *M. tuberculosis* is characterized by strong *M. tuberculosis*-specific Th1 responses and co-infection with helminths could modulate these immune responses through influence on the Th1/Th2 balance with increased Th2 dominance as well as increased activity of Tregs [156]. Impairment of Th1 immune response characterized by a decrease in PPD-specific IFN-γ and IL-17 responses was shown during chronic filarial infection [157]. In this context, it was reported that peripheral T-cells obtained from individuals with onchocerciasis responded poorly to *M. tuberculosis* antigens [158].

On the other hand, in contrast to the hypothesis that chronic helminth infections may increase susceptibility to and worsen the clinical course of TB infection, the recent results of a mouse model study could not detect any immunological effects of helminths during TB infection [159]. In this study the histological examinations and quantitative TB cultures demonstrate similar or reduced mycobacterial burden in helminth co-infected animals compared to those infected with
TB only where PPD-specific cellular proliferation and IFN-γ production were not suppressed in co-infected animals [159]. Additionally, in a cotton rat model with a filarial nematode (S. litmosoides), it was reported that proliferation and IFN-γ production of spleen cells in response to PPD were similar between co-infected (M. tuberculosis-S. litmosoides) than those with M. tuberculosis infection only [159]. A possible explanation for these divergent findings may lie in the fact that the impact of helminthiasis on the host response to M. tuberculosis is dependent on the type of helminth/intensity of infection, timing and the level of exposure to M. tuberculosis itself.

**Concluding remarks on the interaction between helminth infection and tuberculosis**

Taken together, the co-existence of helminth infection in areas of high TB incidence may affect the ability of the host to respond to M. tuberculosis and BCG vaccination. This may be of importance for the risk of developing TB on exposure as well as for reactivation of latent disease or the outcome of active TB. Helminth infections have a significant immunomodulatory influence on the immune system and there is experimental evidence showing that helminth clearly attenuate the Th1 immune response required for the control of M. tuberculosis infection. However, whether helminth infection indeed has an impact on the clinical outcome or immunological responses in human TB remains an open question.
AIMS

- To assess the initial tuberculin skin test results and kinetics of an interferon-γ release assay (Quantiferon) during active TB in a high endemic area.

- To prospectively investigate whether early changes in a clinical scoring system (TB-score) can predict treatment outcome in patients with pulmonary TB.

- To assess the magnitude and clinical characteristics of helminth infection in TB patients in comparison with household contacts and community controls and to evaluate its correlation with eosinophil and IgE responses.

- To investigate the immunological response and clinical outcome of helminth co-infection in patients with active TB before and after albendazole treatment.
PATIENTS, MATERIALS AND METHODS

Study setting
The patients in papers I-V were recruited from three health institutions (Gondar University Teaching Hospital, Gondar Health Center and Debark Hospital) in Ethiopia located in Gondar town and the surroundings. Gondar is located 750 km from Addis Ababa in North-West Ethiopia.

![Map of Ethiopia](image)

**Figure 5**: With a population of approximately 93 million [160], Ethiopia has the second largest population in Africa.
Figure 6: The majority of the inhabitants practiced Ethiopian Orthodox Christianity (84.2%)\cite{161}

Figure 7: The Gondar city is nicknamed "The Camelot of Africa" due to the presence of a group of royal castles (Photograph: Wikipedia, the free encyclopedia).
The Gondar University Hospital is a tertiary level teaching and referral hospital with 450 beds rendering referral health services for over 5 million inhabitants in North-West Ethiopia. The hospital provides inpatient and outpatient services, including care and treatment for TB and HIV/AIDS patients. Antiretroviral treatment (ART) for HIV has been available in Gondar hospital since March 2005. The ART regimen consisted of a combination of nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI), according to the national HIV guidelines [162]. At the end of 2012, there were close to 9470 HIV-positive adults on follow up in the Gondar ART clinic and about 6000 of these patients were starting ART while the remaining patients were on pre-ART. In 2010, a TB isolation and treatment ward, with a 28 bed capacity, was built to improve the hospital TB infection control with a certain focus on MDR-TB. The need is much greater than the present beds available, but capacity building activities are underway, including a new hospital construction with 800 beds.

The Gondar Health Center is the largest of the three health centers currently available in Gondar town. The health center provides primary health care to the population of Gondar town. It has a TB treatment center (DOTS clinic) where TB patients receive medication after TB diagnosis. Debark is a small town in northern Ethiopia located 90 kilometers north of Gondar town in the Semien Gondar Zone of the Amhara Region. Debark is the closest town to the Semien Mountains National Park. It is the largest settlement in Debark area.

All laboratory work was done in Gondar University Hospital laboratory except for TB culture which was done in the BSL3 facilities at the Armuer Hansen Research Institute (AHRI), Addis Ababa, Ethiopia. Optimization for ELISPOT and flow cytometry was done at AHRI and Linköping University, Sweden while all patient analyses were done in Gondar.
Figure 8: Outlook of the University of Gondar Hospital (left) and Debark hospital (right). Photograph: Ebba Abate

Study participants (Paper I-V)
Inclusion of TB patients and recruitment of blood donors and household contacts to TB patients took place at the TB treatment clinics (DOTS center) within the Gondar University Hospital, Ethiopia; Gondar Health Center (DOTS-polyclinic) and Debark Hospital. Only outpatients fulfilling the inclusion criteria of newly diagnosed smear positive-TB (Paper I, and III) or smear-positive and smear-negative pulmonary TB patients (Paper II, IV and V), aged between 15 to 60 years, and who provided oral and written informed consent were included. The number and distribution of participants included in the 5 papers are described in Table 1.
Table 1 Patients and control subjects included in paper I-V

<table>
<thead>
<tr>
<th>Patient code</th>
<th>n</th>
<th>Inclusion site</th>
<th>Papers (analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>24</td>
<td>GUH</td>
<td>I (QFN, PPD)</td>
</tr>
<tr>
<td>DW</td>
<td>47</td>
<td>GUH</td>
<td>I (QFN, PPD), II (TB-score, CD4 counts, final outcome), III (Helminth data, IgE, Eos)</td>
</tr>
<tr>
<td>PDW</td>
<td>65</td>
<td>GHC</td>
<td>II (TB-score, CD4+ cells count, final outcome), III (Helminth data, IgE, Eos)</td>
</tr>
<tr>
<td>TB-score</td>
<td>143</td>
<td>GUH</td>
<td>II (TB-score, CD4+ cells count, final outcome)</td>
</tr>
<tr>
<td>SN</td>
<td>107</td>
<td>GUH</td>
<td>II (TB-score, CD4+ cells count, final outcome)</td>
</tr>
<tr>
<td>ALBN</td>
<td>265</td>
<td>GUH, GHC, DH</td>
<td>IV (baseline), V (ELISPOT and Flow cytometry, n=16)</td>
</tr>
<tr>
<td>ALBP</td>
<td>140</td>
<td>GUH, GHC, DH</td>
<td>IV (baseline), V (deworming trial, follow up)</td>
</tr>
<tr>
<td>HHC</td>
<td>71</td>
<td>GUH, GHC, DH</td>
<td>III (Helminth data, IgE, Eos)</td>
</tr>
<tr>
<td>CC</td>
<td>245</td>
<td>GUH</td>
<td>I (QFN, TST, n=41), II (TB-score, CD4+ cells count, n=82), III (Helminth data, IgE, Eos, n=112)</td>
</tr>
<tr>
<td>CCw</td>
<td>61</td>
<td>GUH</td>
<td>V (negative control ELISPOT, Flow cytometry, n=56)</td>
</tr>
</tbody>
</table>

GUH= Gondar University Hospital; GH= Debark Hospital; GHC= Gondar Health Center
Eos= Eosinophil; QFN= Quantiferon; TST= Tuberculin skin test
Smear-positive TB was defined as 2 of 3 morning sputum samples positive, or one of 3 positive, with chest X-ray findings and clinical symptoms, suggestive of active pulmonary TB [13]. Smear-negative pulmonary TB was defined as symptoms suggestive of TB with 3 sputum smear samples negative for AFB, radiographic abnormalities consistent with pulmonary TB, and a lack of clinical response to one week of broad-spectrum antibiotic therapy (MOH, 2008). The exclusion criteria were patients requiring hospital admission, pregnancy, clinical signs or medical treatment indicating any concomitant chronic or infectious diseases other than TB/HIV. As control group, healthy community controls were recruited from the blood bank of Gondar University Hospital. All blood donors had passed pre-donation clinical screening to rule out any chronic illnesses and previous TB history. Moreover, all controls had a normal chest x-ray findings, a TB-score ≤ 3 and did not show any clinical signs or symptoms of clinical TB. Patients were evaluated for laboratory and clinical markers as indicated in the respective papers and as outlined in Table 1. In addition, household contacts were included (Paper III) based on the information obtained from the TB patient. A household contact was defined as a person who lives together (> 6 months) and spends more than 12 hours per day with a TB patient.

Outline of the interventional clinical study on the effect of albendazole in patients co-infected with helminths and pulmonary TB (Paper V)

The study design of Paper V was a randomized, double-blind follow-up study in which newly diagnosed pulmonary TB patients with concurrent helminth infection presenting consecutively from 1st of March 2009 to 15th of October 2012 at the DOTS clinics at the Teaching and Referral Hospital of the University of Gondar, the Gondar Health Centre and at Debark Hospital were randomized to albendazole or placebo groups after oral and written consent. Each patient received 400 mg X III per os of albendazole or placebo at two weeks after initiation of TB treatment. The primary outcome was TB-score change at week 8 compared to baseline. The secondary outcomes were sputum smear conversion after two months, changes in chest x-ray pattern from baseline to week 12, CD4 cells count, IgE and eosinophil response after 3 months as well as immunological responses such as changes in Tregs and levels of IFN-γ, IL-5 and IL-10 producing PBMCs after 3 months. Random numbers was generated by a computer and was done in a block size of eight by the Department of Epidemiology, Addis Continental Institute of Public
Health, Ethiopia. The treatment allocation was concealed in individual envelopes opened only when a patient was enrolled. Albendazole and placebo were identical and numbered by Addis pharmaceutical company, Ethiopia. Both the investigator and clinical staffs were blinded to the randomization code and substance. The code was broken after the follow-up visit of the last patient and when the statistical analysis had been performed.

**Clinical and laboratory patient characteristics**

**Baseline characteristics and determination of the TB-score**

The TB-score consists of signs and symptoms which were recorded as previously described [163]. Each variable of the TB score contributes to one point resulting in a TB-score from 0 to 13 (Table 2). Furthermore, age, sex, the presence of a BCG scars and body mass index (BMI) were registered for all study participants.

**Table 2: Parameters in the TB-score**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough</td>
<td>1</td>
</tr>
<tr>
<td>Haemoptysis</td>
<td>1</td>
</tr>
<tr>
<td>Dyspnoea</td>
<td>1</td>
</tr>
<tr>
<td>Chest pain</td>
<td>1</td>
</tr>
<tr>
<td>Night sweating</td>
<td>1</td>
</tr>
<tr>
<td>Anaemic conjunctiva:</td>
<td>1</td>
</tr>
<tr>
<td>Tachycardia (&gt;100/min):</td>
<td>1</td>
</tr>
<tr>
<td>Lung auscultation (pos):</td>
<td>1</td>
</tr>
<tr>
<td>Temperature ≥37C</td>
<td>1</td>
</tr>
<tr>
<td>BMI&lt;18:</td>
<td>1</td>
</tr>
<tr>
<td>BMI≤16:</td>
<td>1</td>
</tr>
<tr>
<td>MUAC &lt;220:</td>
<td>1</td>
</tr>
<tr>
<td>MUAC &lt;200:</td>
<td>1</td>
</tr>
</tbody>
</table>

**TB score sum**

BMI: Body Mass Index; MUAC: Middle Upper Arm Circumference
HIV testing and determination of CD4+T-cells count

Testing for HIV was done with different diagnostic test methods applied and used in Ethiopia at different periods based on the national HIV/AIDS guidelines, i.e. (i). Enzygnost Anti-HIV 1/2 Plus (Dade Behring, Germany) and confirmed with Vironistika HIV Uni-Form II Ag/Ab (Biomérieux, France) using ELISA, (ii). Rapid HIV test kits which include Determine, Capillus and Unigold, (iii). Rapid HIV test kits which include KHB, Stat pack and Unigold. CD4+T-cell counts were analyzed by using a FACSCount machine (BD, San Jose, California, USA) at the Gondar University Hospital Laboratory which is involved in continuous external and internal quality control programmes.

Determination of IgE, eosinophil cell count and Quantiferon by ELISA

Serum IgE was determined with a commercial ELISA kit (Immundiagnostik, Germany) according to the manufacturer’s instruction. The absolute eosinophil count of peripheral blood was computed in cells/mm³ from the value of total and differential white blood cell counts obtained using Cell Dyn 1800 (Abbot, USA). The quantiferon (QFN) test was done according to the manufacturer’s instructions (Cellestis, Australia). All QFN samples were collected in the morning at 09.00 am.–12.00 am. One milliliter of blood was collected into each of the three QFN blood collection tubes. At the time of sample collection and prior to incubation, the samples were mixed thoroughly by shaking the tube 10 times (5 s) to ensure that the entire inner surface of the antigen coated tube was covered with the blood. The samples were transported and directly incubated following the shaking procedure at 37 °C for 18- 22 h after which the supernatant was frozen at -20 °C for later analysis.

Stool examination

Stool samples from three consecutive days were collected from each participant and examined using direct stool microscopy and Kato-Katz techniques [164] by the same technician throughout the study. The classification into helminth positive or negative was based on the examination of all three samples together from each patient. If at least one of the samples was positive for helminth ova or larva the patient was regarded as helminth positive. One in 10 slides were randomly selected and checked again blindly by a second microscopist for quality control. The same stool sample collection and examination strategy was used at week 12 in paper V.
Sputum smear examination
Acid-fast bacilli (AFB) staining and examination was done at baseline and week 8 on morning sputum samples from three consecutive days. The AFB load in sputum smears was measured as previously described by the World Health Organization as: negative (0 AFB/100 oil fields), scanty (1-9 AFB/100 fields), 1+ (10-99 AFB/100 fields), 2++ (1-10 AFB/field) and 3 +++ (>10 AFB/field) [165]. Sputum conversion was defined as all three consecutive sputum smears turning negative for AFB at week 8.

Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood
Heparinized venous blood (25 ml) was collected in DOTS clinics and transported within one hour to the laboratory where PBMCs were directly isolated. Heparinized blood was layered on a Lymphoprep density gradient solution (Axis- Shield POC AS, Norway) with a 1:2 proportion of blood, and then centrifuged at 800g for 30 minutes at 20 ºC. The resulting interphase ring consisting of a mixture of mononuclear cells was collected and then washed twice with PBS (Sigma-Aldrich, Munich, Germany) followed by centrifugation at 250g for 10 minutes. Finally, cells were re-suspended in RPMI-1640 (Sigma-Aldrich) supplemented with 10 % sterile heat-inactivated FBS (Sigma-Aldrich) and 1% penicillin-streptomycin (Sigma-Aldrich), before counting in a Bürker chamber. Cells were stored in freezing medium containing of 10% DMSO and fetal calf serum (FCS) in -80ºC freezers until use. Cells were frozen for a maximum of 2 months period. Trypan-blue exclusion dye (Sigma-Aldrich) was used for detection of cell viability. Only cells from patients with a viability after thawing of above 75% were included.

Analysis of regulatory T-cells by flow cytometry
The isolated PBMCs were stained with fluorochrome-labeled monoclonal mouse anti-human antibodies CD4-fluorescein-isothiocyanate (FITC) (BD Biosciences, clone RPA-T4), CD25-Percp Cy5.5 (BD Biosciences, clone M-A251 ), and CD127-Alexa 647 (BD Biosciences, clone HIL-7R-M21), followed by fixation/permeabilization using cytofix/cytoperm solution (BD Biosciences) and intracellular staining with monoclonal antibodies to Foxp3-phycoerythrin (PE) (BD Biosciences, clone 236A/E7). Fluorescence minus one (FMO) controls was used for gating purposes. Cells were included in the analysis if the cell viability was 75% after thawing. Tregs were defined as the population of cells that were CD4+/CD25hi/CD127low/Foxp3+. Flow
Flow cytometry data were collected on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest acquisition software and were then analyzed using Flowjo 7.6.5 (Tree Star, USA).

**Analysis of IFN-γ, IL-5 and IL-10 by ELISPOT**

Peripheral blood mononuclear cells (250,000/well) were plated onto ester-cellulose-bottomed plates (PVDF plates, Mabtech, Solna, Sweden) coated with a capture mAb specific for human IFN-γ (1-D1K), IL-5 (TRFK5) and IL-10 (9D7) at 15µg/ml in PBS. Cells were incubated in a humidified incubator at 37°C in 5% CO2 for 24 h with PPD-antigen in duplicate using unstimulated and CD3-stimulated cells as negative and positive controls, respectively. Cells were then removed by washing five times with PBS-0.5% FCS, and then biotinylated anti-mouse IFN-γ (7-B6-1), IL-5 (5A10) and IL-10 (12G8) were added at 1 µg/ml in PBS and incubated for 2 h at room temperature. After washing five times in PBS, streptavidin-alkaline phosphatase (Mabtech) was added for 1 h at room temperature. After washing with PBS, filtered (0.45µm filter) BCIP/NPT- plus substrate (Mabtech) was added. Plates were extensively washed using tap water. The number of spots for each well was counted with automated ELISPOT reader (AID, Germany), and the results for each stimuli (PPD, negative and positive control) correspond to the mean of duplicates.

**Statistics (Paper I-V)**

In Paper I, data are presented as mean ± standard deviation (SD). To compare groups, the Student’s t-test was used for parametric data and the chi-square test for discrete variables. Kappa was calculated according to Cohen. A multiple regression model was used entering significant variables using the STATISTICA software package (StatSoft, Tulsa, USA).

In Paper II, it was estimated that to detect a minimal clinically important difference of 1.1 TB-score points of the TB patients at week 8 compared to the blood donors, 80 blood donors were needed at a power of 80% (alpha 0.05). We assumed that a sample size of 250 TB patients (including a 10% loss to follow-up) was needed in order to detect a mortality difference from the expected 8% to 18% in the patients who did not show a decrease in the TB-score of > 25% between baseline and week 2, on the assumption of a 10% overall mortality and an estimated fraction of 20% not reaching a < 25% decrease between baseline and week 2. Data are presented as the median and inter-quartile range and analyzed using Fisher’s exact test for categorical variables and Wilcoxon and Mann-Whitney tests for continuous variables. In a multiple logistic
regression model setting mortality as the dependent variable (STATISTICA software package; StatSoft, Tulsa, USA), age, sex, HIV, presence of ART treatment, and CD4+T-cell counts (< 200 cells/mm³) were included in the final analysis.

In Paper III, data are presented as median and inter-quartile range. Significance testing was done with Mann-Whitney and Wilcoxon tests for continuous data and Fisher’s exact test for discrete variables. Variables with a p<0.1 in the univariate analysis were entered into a multiple regression analysis with helminth status as the dependent variable. The regression analysis was performed using the STATISTICA software (Tusla, USA).

In Paper IV, continuous data are expressed as means with standard deviation (SD). Comparison between groups was done by Students t-test for continuous data and by chi-square test or Fisher’s exact test for categorical data. Variables with a p-level <0.1 were entered a multiple logistic regression model using the STATISTICA software package (StatSoft, Tulsa, USA). Analysis for the randomised trial (paper V) was by intention to treat. The study code was kept in a sealed envelope until all data were analyzed. Effects of deworming compared to placebo on primary and secondary outcomes were evaluated by chi-square test or Fisher’s exact test for discrete variables and Students t-test for continuous variables. Multiple regression analyses were performed adjusting for baseline. A p<0.05 was regarded as statistically significant.

The sample size for the randomized trial in paper V was calculated under the following assumptions. In our previous study [166], newly diagnosed TB patients showed a 75% decline from baseline TB-score value after two months of anti-TB treatment. Accordingly, the sample size was calculated under the assumption that 210 helminth positive TB patients would have to be included giving 80 % power at the 5 % significance level to detect a difference in treatment response of 15 % including a 5 % drop out rate.

Ethical considerations
Oral and written informed consent was obtained from all study participants. The study has received ethical clearance from the Ethics Review Board of the University of Gondar (RPO/55/37/2001; Res/05/530/01/12); and from the Medical Ethics Board at Linköping University, Sweden (M143-06, T2-08). In addition, approval has been obtained from the federal Drug Administrative and Control Authority (DACA) (02/6/22/36), Ethiopia for paper V where an independent data and safety monitoring board (DSMB) regularly reviewed the results and
adverse events and adverse reactions. All patients included were treated according to the DOTS program following the WHO adopted national TB guideline of Ethiopia. Pre-and post-test counseling was given to all study participants during HIV testing as part of the hospital/health center routine, provider initiated HIV counseling and testing (PIHCT) programme. Helminth-positive patients received anti-helminthic treatment free of charge after the completion of the study at week 12. There was no additional cost to or economic incentive for the patients participating in the study. Study participants were compensated for their transportation expenses during follow-up visits.
RESULTS AND DISCUSSIONS

The influence of TB treatment and HIV on the tuberculin skin test (TST) and IFN-γ response to *M. tuberculosis* specific antigens using T-cell based assays: IGRA (Quantiferon) and TST

Interferon-γ release assays (IGRA) are based on the cell-mediated immune response and subsequent measurement of IFN-γ production against a mixture of synthetic peptides simulating proteins present in *M. tuberculosis*: the early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) and in the case of Quantiferon (QFN) also to the antigen TB7.7. The IGRA tests are more specific than TST in particular in BCG vaccinated individuals as the target peptides in the IGRA tests are secreted by *M. tuberculosis* and *M. bovis*, but are neither present in the BCG vaccine, nor in most non-tuberculous mycobacteria. Previous studies considering the impact of anti-TB treatment on the specific IFN-γ response as measured by the QFN assay have shown controversial results in the case of both latent and active TB treatment. Indeed, some authors have described that the IFN-γ response to specific mycobacterial antigens decreased or became negative after TB treatment [70,167-170], while others have reported persistently positive or even stronger responses during and after anti-TB treatment [66,171-175]. Qualitative and quantitative changes in the IGRA response may be a candidate biomarker to monitor the response to TB drug therapy and for the evaluation of interventions. However, little is known about the kinetics of IGRAs during anti-TB treatment in relation to disease severity in high endemic areas for TB and HIV. Additionally, only a few studies have used a head-to-head comparison between TST and an IGRA test.

We conducted a small prospective, head-to-head study (Paper-I) to assess the effect of TB treatment on T-cell responses as measured by the QFN assay in newly diagnosed active TB patients in Ethiopia with the aim to use this assay as a prognostic tool for treatment response in subsequent studies (Paper IV and V). Blood donors were recruited as a healthy community control group.
The proportion of patients positive for QFN positive patients declines during the course of anti-TB treatment

In paper I, we showed a significant decline in the proportion of QFN positive patients which was more pronounced in HIV- positive patients following TB treatment (day 0 to month 7). This finding is in agreement with previous reports [168,176,177]. This has further been supported by a study assessed the decline of IFN-γ (ESAT-6 and CFP-10) using an in-house ELISPOT protocol in the Gambia [178]. HIV-positive TB patients showed a higher rate of QFN reversion (from negative to positive) than HIV-negative TB patients (p<0.05 in that study). The observed QFN conversion was attributed to recovery of the immune response during anti-retroviral therapy (ART) and may be dependent on initial CD4+ cells count levels and timing of ART treatment. The decline in the proportion of QFN positive patients observed in most studies so far, in particular in HIV-negative TB patients is most likely due to the effect of anti-TB treatment, which decreases the antigen load and subsequently attenuates the cell mediated immune activation and IFN-γ production. It was also previously described that the magnitude of the M. tuberculosis antigen specific IFN-γ T-cell response is proportional to the antigen load of the infecting organism in human and animal models [179,180].

A high clinical severity score correlated with negative TST results

The correlation of TST and the QFN assay with disease severity, treatment outcome and smear conversion rate (at 2nd month) was assessed (paper I). Multiple regression analysis showed a correlation between an initial negative TST result at baseline and a higher disease severity score (advanced TB), which was not confounded by sex or HIV. The lack of such correlation in QFN-negative individuals indicates that the severity of the disease may not affect the performance of the QFN assay to the same extent. Moreover, this observation underlines the clinical differences between the two tests during active TB and support the observation that TST reaction may be suppressed relative to the QFN response in more advanced TB. No correlation was observed between QFN results and final TB treatment outcome or sputum smear conversion rate at the end of the intensive phase of TB treatment.
The impact of HIV and CD4+ T-cells counts on the performance of the Quantiferon assay

The QFN assay showed a significantly higher rate of positive results in HIV-negative TB patients compared to HIV-positive TB patients (93.8% vs. 70.3%, n=69, p<0.05), whereas no significant difference was noted when the rate of positive TST results (≥ 10mm) were analyzed (87.9% vs. 73%). Previous studies showed that HIV co-infection could influence the QFN test since IFN-γ production might be limited by the reduced number and function of CD4+ T-cells [65]. A low CD4+ T-cells count (<200 cells/mm³) was significantly associated with TST negative results (p=0.03), whereas this association was not present for QFN-negative results. It has been previously described that HIV-infected patients are more frequently anergic to skin testing, particularly those with a CD4+ lymphocyte count lower than 100-200 cells/mm³ [181]. The correlation of negative TST result and low CD4+ T-cells count confirms the impact of immune-suppression (characterized by low CD4+T-cells count) of TB patients on the performance of TST. This finding is supported by the fact that we observed a significant correlation of negative TST results with advanced forms of TB in the same cohort. Low CD4+ T-cells counts in some studies have been found to be associated with an increase in both indeterminate and false-negative QFN results [182,183], but low CD4+ T-cells counts do not seem to account for all indeterminate and negative results in HIV-positive TB patients [184]. In agreement with this observation, we could not detect a significant correlation between negative QFN results and low CD4+ T-cell counts, including patients with CD4+ T-cells counts lower than 200 cells/mm³ in the HIV-positive/TB group. We found that even patients with very low CD4+ T-cell counts (30 cells/mm³) may present an IFN-γ response high enough to be detected in the QFN test, suggesting that the quality and function of the CD4+ T-cells is of great importance. Possibly, also other cells including CD8+ T-cells could contribute to the increased IFN-γ production in patients with low levels of CD4+ T-cells as QFN is analyzed in whole blood.

Poor concordance between QFN and TST results

The agreement of the TST and QFN tests was poor in TB patients (Kappa= 0.09) vs. healthy community controls (kappa=0.59), as described previously [182]. One reason for this difference may be that the cell mediated immune response to mycobacterial antigens differ in latent versus active TB. The highest frequency of QFN and TST positive test results was observed in TB patients with >200 CD4+ T-cells/mm³ compared to TB patients with < 200 CD4+ T-cells/mm³.
This illustrates the influence of the number of CD4+T-cells in the performance of the TST. Thus, the two tests showed fair agreement in the control group (kappa=0.59) and in this group the controls in general had higher CD4+ T-cells counts than the HIV-negative TB patients (p=0.01) in this cohort. Although the BCG vaccine coverage of community controls and TB patients in our study was low, there was no correlation between discordant results and the presence of a BCG scar. A limitation of this study was that mycobacterial culture was not done for confirmation of sputum conversion at the end of the 2nd month. As in most other follow up studies, the small sample size was one other limitation of our study as well as a not negligible loss to follow-up.

We conclude that the average rate of positive QFN tests is significantly reduced from baseline to the time of TB treatment completion resulting in levels comparable to the background level of healthy blood donors (51%). The agreement between the TST and QFN tests is poor in TB patients, including a poor correlation to disease severity. Qualitative conversion of IGRA tests from positive to negative has a very limited clinical value as a surrogate marker of treatment efficacy as it does not occur reliably in patients who completed the TB treatment. Further investigations on T-cell kinetics and functions are needed to understand the significance of persistent QFN-positive results, which do not correlate to poor treatment responses. The observation that healthy controls had a relatively high QFN level comparable with the level observed for TB patients after treatment indicates that this assay likely unsuitable for clinical monitoring or diagnosis of TB in high endemic areas.

**TB clinical scoring (TB-score): relevance to treatment monitoring**

The current WHO guidelines recommend sputum smear results for monitoring smear positive pulmonary TB patients [185,186]. For smear-negative TB patients it is recommended to use weight and clinical symptoms, which may be crude and in case of the clinical symptoms, a subjective evaluation tool for clinical studies [187,188]. The proportion of HIV co-infected TB patients is highest in Africa [1] resulting in a large proportion of smear-negative pulmonary TB patients, who cannot be monitored using smear conversion and whose treatment effect therefore is evaluated only by weight gain [58].
Clinical scoring systems have been developed and are widely used for many diseases such as the NYHA score for chronic heart failure [189,190]. Various clinical prediction tools have been proposed for TB [185,186,191,192], all depending on methods not readily available in high endemic areas and some only developed for smear positive pulmonary TB patients [192]. Studies from Guinea-Bissau in west-Africa have proposed a scoring system (TB-score) that can be applied to both smear positive and negative pulmonary TB patients. High TB-scores correlate with mortality and this scoring system is suitable for field use in highly endemic areas [163]. However, the score still needs to be evaluated with data from various geographical settings and TB manifestations as well as for the impact of HIV and CD4+ T-cells counts on its performance.

The purpose of our study (Paper II) was to prospectively investigate the kinetics of the TB-score during the course of anti-TB treatment and whether early changes in the TB-score results could predict treatment outcome in Ethiopian TB patients. Another aim was to evaluate the TB-score for use as a primary clinical outcome marker for subsequent studies described in Paper V. In this part, 250 active pulmonary TB patients and 82 blood donors (controls) were included.

The TB-score declines during the course of TB treatment and is not influenced by HIV

Pulmonary TB patients were followed at three different time points: at initiation of TB treatment (week 0), on the 2nd week and during the completion of the intensive phase of TB treatment (8th week). A highly significant and rapid decline in the median TB-score value was observed already after the 2nd week of TB treatment compared with the base line value (8 points vs. 4 points; p< 0.0001); and the rate further dropped to a lower level at the end of the intensive phase of anti-TB treatment. The rapid response already at week two coincides with the observational time point where most patients have clinically responded to anti-TB treatment. The low median TB-score value observed in TB patients after 2 months of TB treatment was still significantly higher than the median TB-score value of controls (2 points vs. 0 points; p< 0.0001). The HIV co-infection rate among TB patients was 53.6% in this study. The TB-score was not influenced by HIV infection or CD4+ T-cell counts as a similar declining pattern was observed when HIV negative and HIV positive TB patients were followed from initiation until completion of the 2 month intensive phase of TB treatment. Overall, the progressive decline of the TB-score observed during the course of TB treatment and the fact that this was not influenced by HIV infection suggests that TB-score may be an appropriate patient clinical monitoring tool during
the course of TB treatment in our Ethiopian study setting as well. So far it has been used in clinical interventional studies evaluating deworming (paper V) and vitamin D supplementation in Guinea-Bissau [193].

**TB-score correlated with mortality**

The TB-score levels correlated with mortality at week 2, moreover, mortality was associated with a failure to achieve more than a 25% decline in TB-score at week 2. This suggests that repeated clinical scoring during the initial weeks of TB treatment could be of value to identify high-risk patients, who might need hospital admission to manage adverse events. A significantly higher TB-score was observed between TB patients who died vs. patients who successfully completed the TB treatment at week 0, (8 points vs. 9 points, p< 0.04), week 2, (4 points vs. 7 points, p<0.0001) and week 8, (2 points vs. 5 points, p<0.0038). The highest mortality rate was observed in HIV positive patients (13/134) and in particular in patients with a low baseline CD4+ T-cells count. Indeed, HIV infection appears to be the dominant risk factor for mortality among Ethiopian TB patients, and clinical scoring and close monitoring should be prioritized for this group.

The TB-score can serve as a simple tool for standardized evaluation of early clinical improvement in TB patients in high endemic areas. Early monitoring of patients by assessing the TB-score in the first 2 months of treatment could facilitate the identification of patients at high risk of a poor clinical outcome. This relatively simple clinical score could provide an objective tool for healthcare workers to identify high-risk patients. The clinical evaluation can quickly be carried out without using expensive diagnostic tools and could be suitable for follow-up in clinical trials [163]. Nonetheless, although the TB-score has attained a similar level of performance in settings that share epidemiological characteristics [163], further studies are needed to assess its external validity, its acceptance and implementation by clinicians and its eventual impact to prospectively predict high-risk TB cases and making decision to hospitalize pulmonary TB patients. Moreover, the scoring system needs to be developed to improve its capacity to predict clinical outcomes, especially with regard to HIV co-infection. Furthermore, the definition of cut-off levels for practical use to pin-point high-risk patients needs to be explored in future studies, as well as how to implement the scoring algorithm at different levels
of the healthcare system. The reproducibility study conducted recently [194] revealed the need for refinement of the TB-score. It is important to select signs and symptoms showing lack of reliable inter- and intra-observer variability and the need to refine the TB-score further by omitting variables with relatively poor reproducibility.

As a limitation of the study, 24% of the TB patients in this cohort did not have survival data at the end of TB treatment. There might be undetected deaths in the defaulting group (10%). Due to lack of sputum culture facility, smear-negative TB patients were not confirmed by culture but the national WHO-based guidelines to define smear negative cases were used instead. The impact of ART in decreasing mortality in HIV-coinfected TB patients could not be assessed since the study was not primarily designed to address this issue.

In summary, we showed that early monitoring of patients with TB-score in the first weeks of treatment could facilitate the identification of patients at high risk of poor clinical outcome. The TB-score could be a useful clinical monitoring tool during TB treatment. The observation that the TB-score declines during the course of successful TB treatment and the correlation between high TB-scores and a poor clinical outcome during TB led us to use this scoring method as a clinical monitoring tool during the course of TB treatment in Paper V.

**TB-Helminth co-infection**

In sub-Saharan Africa, there is extensive epidemiological overlap between helminthic infections, TB, HIV/AIDS and malaria [99,195,196]. There are several reports showing high rate of helminth co-infection with HIV [127,130,197-199]. Nevertheless, epidemiological reports on the burden of helminth infection in active pulmonary TB patients are scarce. We therefore aimed to assess the impact of asymptomatic helminth infection among patients with active pulmonary TB in Ethiopia which is high endemic for helminth infection and TB. Newly diagnosed TB patients (n=112), house-hold contacts (n=71) and healthy community controls (n=112) were included. The burden of helminth infection in the three groups was assessed and correlated with surrogate markers of Th2-type immune response (IgE levels and eosinophilia). TB patients were followed and examined for their helminth status at baseline, and in the 2nd, 8th and 12th week after treatment initiation.
High burden of asymptomatic helminth infection among patients with active pulmonary TB

Twenty nine percent (29%) of the TB patients in paper III had concurrent helminth infection, which is in line with previous reports showing that the helminth co-infection in active TB patients was nearly twice as high as their healthy household contacts although this study reported generally higher rates [154]. A total of seven different helminthic species were identified. *Ascaris lumbricoides* was the most common parasite detected followed by hook worm infection for TB patients, house-hold contacts and healthy individuals. Although still controversial, some studies have suggested that intestinal parasites could be a risk factor for developing TB upon exposure due to their potent modulation of the host immune response. Tristao-SR *et al*. [153] and Elias, *et al*. [154] reported a higher rate of intestinal nematodes in patients with pulmonary TB than control groups, and Diniz *et al*. [155] observed a strong association between intestinal nematode infection and multibacillary leprosy. Our findings of a high helminth burden among TB patients further confirm the impact of chronic helminth infection in TB, but additional follow up studies with much larger sample sizes are needed to confirm the causal relation between helminth infection and the risk of developing of active TB.

Helminth infection correlated with eosinophilia and elevated IgE level

Large extracellular pathogens like helminths induce immune mechanisms that are of a Th2-type, characterized by an elevation of peripheral blood eosinophilia and elevated IgE accompanied by a profound increase in cytokine responses including IL-4, IL-5, IL-9, IL-13 [200,201]. Helminth products can drive Th2-type responses even in the presence of Th1 inducers. For example, when stimulated with *Schistosoma mansoni* soluble egg antigen (SEA), DCs are able to induce Th2 type responses in the presence of bacterial Th1 stimuli [202].

We assessed the peripheral eosinophil cell count and IgE level among TB patients, household contacts and healthy community controls in relation with helminth status. In healthy individuals, eosinophils make up only 2-5% of peripheral white blood cells. However, during active parasitic helminth infection the proportion of eosinophils in the blood can reach 40% [203]. Together with high IgE levels and mastocytosis, eosinophilia is considered to be one of the cardinal features of parasitic helminth infection. It was previously shown that Ethiopians have low levels of hematological and immunological markers, such as peripheral white blood cells and CD4+ T-
cells and an increased immune reactivity compared with Dutch citizens and people from other African countries [204]. Such variations could be due to differences in genetics, dietary patterns, sex, age, and altitude. Taking this into consideration, two cut off values were used in our studies for eosinophilia in this population (>500 cells/mm$^3$ and >300 cells/mm$^3$). The conventional cut of value for eosinophilia is 500 cells/mm$^3$ but this may not be appropriate for the Ethiopian population as previously described [205]. In a multivariate analysis, eosinophilia (adjusted OR: 15.2; 95% CI: 1.4–160.3, p = 0.02) and increased IgE-levels (adjusted OR: 7.6; 95% CI: 1.2–48.4, p = 0.03) were independently associated with asymptomatic helminth infection, which was not confounded by sex or HIV status. Significantly lower median eosinophil count and IgE level was observed among TB patients with concurrent helminth infection compared to helminth infected household contacts (eosinophil counts: 234 cells/mm$^3$ vs. 600 cells/mm$^3$, p= 0.005; IgE: 351 IU/L vs. 378 IU/L, p=<0.001), and community controls (Eosinophilia: 234 cells/mm$^3$ vs. 602 cells/mm$^3$, p= <0.001; IgE: 351 IU/L vs. 420 IU/L, <0.001). The elevated IgE level and eosinophilia observed in our study as a result of helminth infection was in agreement with recent studies done in Brazil and China [205,206]. Furthermore, helminths have also been described as strong inducers of IgE in infants [207,208]. It was indicated that specific IgE production significantly reduces TST reactivity in younger children. The effect of eosinophil degranulation on the killing of the helminthic parasite, Strongyloides was shown in a previous study [209,210]. Furthermore, eosinophil granule proteins can modify immune responses in L. sigmodontis infection [211]. Overall, our findings show that peripheral blood eosinophils are highly increased under the influence of helminths, and that helminths are associated with an isotype switch that leads to high serum IgE concentration [212].

**Helminth infection markedly declined in HIV-positive TB patients during TB treatment**

Interestingly, we observed a decline in the rate of helminth infection among HIV-positive TB patients during anti-TB treatment. All patients were asymptomatic and did not receive any anti-helminthic treatment during the 12 week follow-up period. There has previously not been any routine screening programme for helminth infection in the area. There was a marked decline in the rate of helminth infection among HIV-positive TB patients compared to HIV-negative TB patients. This pattern was stable among the 77 TB patients included in the analysis, where most of the helminth positive patients, 86% (66/77) had the same worm status at week 0 compared to
the follow up samples at week 2, week 8 and month 3. This observation has to our knowledge not been previously reported. Since the introduction of highly active antiretroviral therapy (HAART), mortality and morbidity from a wide variety of opportunistic viral, bacterial, fungal and parasitic infections have decreased dramatically among HIV-infected individuals in economically developed countries [213]. It is after such observation that Lucas described the scenario in his paper entitled “Missing infections in AIDS” [214].

Even though, the target of the protein inhibitors (PIs) is the HIV-1 protease, some parasites could be an unspecific target for HIV-1 PIs. Protease inhibitors was shown to exhibit an inhibitory effect against the malaria parasite [215]. Additionally, the results of in-vitro and in-vivo investigations [213,214,216-218], indicate that HIV-1 PIs have a direct effect on opportunistic parasites such as Cryptosporidium parvum, in particular aspartic protease inhibitors. Nonetheless, HIV patients in the current TB cohort did not receive PIs for HIV treatment, a category which was uncommon in the ART regimen used in Ethiopia during the time of the study. Thus, the effect of PIs documented previously could not be the reason for the decline of helminth infection observed in the HIV-coinfected TB patients. Interestingly, it has been reported recently that drugs used in HIV patients (NNRTI/NRTI and trimethoprim-sulfamethoxazole) showed an inhibitory effect against the liver stage of Plasmodium parasites [219]. There was also a report indicating an increased effect of first line drugs used for TB treatment on a protozoan parasite [220]. The same authors showed the combined effect of rifampicin, isoniazid, and ethambutol, in treating malaria in a murine model. The findings of the progressive decline in the rate of helminth infection (presence of eggs/larvae) without anti-helminthic treatment in asymptomatic helminth co-infected TB- patients merits further study. The clinical implication of helminth infection on active TB was studied further at base line (Paper IV) and follow up (Paper V) during TB treatment.

**The impact of helminth infection in pulmonary TB**

In Paper III, we showed a high burden of helminth infection among TB patients and strong correlation of eosinophilia and elevated IgE level with helminths. In a larger study cohort of 265 helminth negative TB patients and 140 helminth positive patients with TB, we observed that the magnitude of helminth infection in patients with active TB was significantly higher than the
prevalence in community controls (35% vs. 27%, p = 0.03). Interestingly, the rate of hook worm infection was significantly higher in the helminth positive community controls compared to patients with active TB where Ascaris was more prevalent (Table 3a and 3b). Taking the findings of this and other studies into account, we hypothesized that the skewed immune response caused by helminths may affect the pathogenesis of tuberculosis including the clinical presentation of TB. Thus, in paper IV we studied the clinical impact of helminth infection in patients with active TB at initiation of TB treatment. A total of 377 pulmonary TB patients were included in this paper. About one third of these patients 81% (96/119) were confirmed as sputum culture positive for M. tuberculosis and no atypical mycobacterial infection was detected.

**Influence of helminth infection on the clinical characteristics of active TB infection**

The HIV co-infection rate of TB patients in paper IV was 33% which was lower than the close to 50% rate previously reported from same area [38,116,119]. A recent report by UN-AIDS indicated that the rate of new HIV/AIDS cases is declining globally after the advent of HAART alongside with consistent HIV/AIDS control and prevention measures [12]. This is supported by the fact that 70% (77/110) of the HIV co-infected TB patients included in the current study had already started ART. The TB patients in the current cohort had low body mass index (BMI) (< 18.5 kg/m²) and mid-upper arm circumference (MUAC) (< 22 cm) indicating malnutrition [221], although no difference was observed between helminth- positive and negative TB patients included in this cohort. In addition, no significant difference was observed in the mean TB-score values between helminth- positive and helminth- negative TB patients. Comparing the individual parameters of the TB-score, the body temperature as well as the rate of tachycardia was suppressed in helminth-positive patients suggesting an attenuating effect of the chronic helminth infection on the Th1 type pro-inflammatory response and increasing Tregs and Th2 activity. However, following adjustment for HIV in a multivariate model the association with body temperature and tachycardia was no longer present. Consequently, no major differences in the clinical presentation of TB in helminth- positive versus helminth-negative patients were observed at baseline. The immunological impact of helminth co-infection confirmed as an increased rate of eosinophilia also in this study cohort may be too subtle to induce a detectable clinical difference in this sample size due to the strong pro-inflammatory background of active TB, but it may still be of importance for the outcome of disease. Such potential impacts are
addressed in the clinical follow up study for the helminth-positive patients in an interventional trial including deworming (paper V).

**Low rate of helminth infection in HIV co-infected TB patients**

It has earlier been described that parasitic infections could disturb the balance of anti-HIV immune responses and contribute to HIV replication [125,222], which could accelerate the progress of the HIV disease into AIDS [223,224]. The impaired cell-mediated immune response caused by HIV infection might also lead to a higher susceptibility to parasitic infections. The high prevalence of opportunistic parasites such as *Cryptosporidium parvum* and *Isospora belli* among HIV-positive patients is well known [116,225], but this correlation is less evident with helminths [225-229].

The major finding in paper IV among a total of 377 TB patients was a significantly lower rate of helminth infection in HIV-positive compared to HIV-negative TB patients (24% vs. 36%, \( p=0.03 \)). This is in support of our hypothesis from paper III that the HIV treatment (NNRTI and NRTI) as well as co-trimoxazole prophylaxis given to HIV-positive patients might contribute to the observed difference. To our knowledge there is no systematic evaluation of helminth infection or routine deworming in patients initiated on HAART in the study area (Ermias Diro, personal communication). It has been described earlier that since the introduction of highly active antiretroviral therapy (HAART) the mortality and morbidity for a wide variety of opportunistic viral, bacterial, fungal and parasitic infections have decreased dramatically among HIV-infected patients [213,214]. The potential indirect or direct role of ART on reducing the rate of helminth infection is supported by the fact that the helminth prevalence in the same area where we recruited the current cohort and elsewhere in Ethiopia was clearly higher before the introduction of ART in 2005 [120,122,230]. In a study done in Ethiopia in 2004, a year before the introduction of ART, it was reported that the prevalence of intestinal helminths was higher in HIV-positive patients (69%) than healthy controls (57%) [122]. In a similar area, higher helminth prevalence was reported among HIV positive patients (48%) than healthy controls (39%) before the introduction of ART in the area [230]. On the other hand, it has been described earlier that HIV might ‘protect’ against the establishment or survival of a mature nematode infection, or might lead to under-diagnosis due to reduced fecundity. This has been shown in relation to schistosomiasis, but has not yet been examined in relation to nematodes [231].
Furthermore, apart from the effect of HAART it should also be noted that a change in the health-related behavior among HIV positive TB patients could contribute for the observed difference as they received much more health care attention following the HIV diagnosis. Therefore, a well designed follow up study with larger sample size including a more complete and detailed ART regimen data, previous treatment history of anti-helminth drugs and hygienic practice among patients is needed to further explore these findings.

Our sample size as well as the clinical parameters chosen could restrict the possibility to detect subtle differences in the clinical presentation with regards to helminth infection in the background of active TB. We used three consecutive stool samples and both direct and Kato-Katz method in order to optimize the detection rate. Nevertheless, our diagnostic strategy to assess helminth infections could leave occult helminth-infected patients undetected. Different helminth species may have a variable effect on the clinical presentation and outcome and apart from Ascaris lumbricoides this could not be evaluated within the present study due to low detection rate of other parasites.

**Effects of albendazole treatment on the clinical outcome and immunological responses in patients with helminth infection and pulmonary tuberculosis: a randomized clinical trial**

Chronic helminth infections induce important immunomodulatory effects through Tregs, alternatively activated macrophages (also termed M2 macrophages) which are polarized by anti-inflammatory responses, and the production of cytokines such as IL-10 and TGF-β known to down regulate a Th1 type response [152]. It has been demonstrated that the Th1 immune-mediated protection against *M. tuberculosis* is characterized by strong *M. tuberculosis*-specific Th1 responses and that co-infection with helminths could modulate these immune responses as a result of an increased Th2 and Treg activity [14,151,156]. The findings of the experimental studies support the negative immunological impact of helminths on TB infection. Babu et al., showed an impairment of Th1 immune responses characterized by a decrease in PPD-specific IFN-γ and IL-17 production during chronic human filarial infection [157]. This observation was in agreement with a study conducted in Ethiopia which showed impairment of the Th1 response in healthy individuals with chronic helminthic infection which was reversed after anti-helminthic treatment [142]. However, apart from the results from experimental models and a few cross-sectional clinical studies from the human setting performed so far, there is a lack of clinical
studies investigating the clinical and immunological impact of helminth co-infection with TB in man.

Based on our previous findings, in Paper V we longitudinally assessed the clinical and immunological impact of helminth infection during active TB infection as an effect of deworming by albendazole. More specifically, we addressed the hypothesis that asymptomatic helminth infection may influence the clinical improvement and immunological balance in TB patients as a result of an effect on Tregs and cytokines associated with the Th1/Th2 balance. Thus, we designed a randomized placebo-controlled clinical trial where such markers were assessed before and after anti-helminthic treatment in newly diagnosed pulmonary TB patients.

**Baseline clinical and immunological characteristics**

A total of 1251 newly diagnosed pulmonary TB patients were screened for eligibility. Of these, 846 TB patients could not be recruited into the study due to a number of exclusion criteria, and 265 were helminth-negative and thus excluded from enrolment. In total, 140 eligible helminth-positive TB patients were randomized: 72 to the albendazole treatment arm and 68 to the placebo arm (Figure 9). Despite three years of enrolment of helminth-positive TB patients from three clinical sites after 2 years of preparation we were still not able to reach our target sample size (210 helminth positive TB patients).
No significant differences in the clinical baseline characteristics were observed between patients allocated to albendazole or placebo, including HIV co-infection rate and immunological variables. However, a difference in baseline peripheral CD4+ T-cells was noted between the albendazole and placebo group (407 cells/mm$^3$ ± 248 vs. 551 cells/mm$^3$ ± 348).
Effect of albendazole treatment on study outcomes

There was a highly significant decrease in helminth infection in the albendazole treated group at week 12 compared to placebo, 8% vs. 39%, p<0.001. However, a limitation of our study was a high loss to follow up for helminth evaluation which was equally distributed in the albendazole (49/72) compared to the placebo group (46/68). In a meta-analysis, albendazole showed increased efficacy against helminth infections caused by hookworm, Ascaris lumbricoides compared to mebendazole, and both drugs showed lower efficacy against Trichuris trichiura [232]. In accordance to the reported efficacy of albendazole, we could observe a tendency for persistence for trichuris relative to the other helminthes in our trial after week 12. This shows that anti-helminthic drugs do not have similar potency against helminthic parasites. A similar result was shown in a deworming trial conducted in HIV patients in Kenya [129], where a helminth prevalence of 21.6% vs. 40% was observed in the albendazole treated and placebo groups, respectively, after 12 weeks of deworming. Thus, the efficacy of deworming in our study was higher at week 12 compared to previous studies although the helminth load and the composition of the infecting helminth species could have an impact on this difference.

Albendazole treatment did not show any significant difference in the TB-score change comparing week 8 to the baseline in the albendazole treated group compared with placebo (5.6 ±2.87 points vs. 5.9 ±2.54, p=0.59). We further performed a subgroup analysis in a subset of patients given albendazole treatment (n=30; HIV=21%) and placebo (n=33; HIV=17%) who had Ascaris infection. No significant difference in the TB-score change from baseline was observed between the two subgroups (4.89± 2.08 vs. 5.58 ± 4.7, p= 0.45). The failure to detect a clinical difference with regards to the TB-score might be due to the fact that it is not sensitive enough to measure subtle clinical changes from helminth infection during active TB disease in our limited sample size. The effect of anti-TB treatment itself could mask any clinical changes from helminth infection as a marked decline in TB-score was observed after 8 weeks of anti-TB treatment in both treatment groups. In general, the TB-score is an appropriate tool for clinical TB monitoring and could be applicable in clinical trials as has been indicated in Paper II. However, its usefulness to detect subtle clinical changes due to effects other than anti-TB treatment might need further refinement.
Regarding changes in the secondary outcomes, a significant decline in peripheral blood eosinophil counts was noted in the albendazole-treated group compared to placebo (week 12: 237 cells/mm$^3 \pm 226$, n=52 vs. 384 cells/mm$^3 \pm 401$, n= 52, p= 0.02). We previously showed a significant correlation of eosinophilia with helminth infection (Paper III). Thus, even in asymptomatic helminth infection during TB, albendazole treatment may induce a significant reversal of immunological changes as measured by a reduction in eosinophil counts. The decline in the peripheral eosinophil counts could be associated with the reduction in the recruitment and infiltration of eosinophil granulocytes because of the significantly reduced rate of helminth co-infection in the albendazole treated TB patients. The impact of such findings for the outcome and immune response to TB is not known but the response as such is indicative of a role for deworming in optimizing the immunity against TB. This finding is in agreement with a previous study conducted in infants with helminth infection in which only a decline in eosinophilia was noted after anti-helminthic treatment, but not in the levels for IFN-γ, IL-5, IL-10 and IL-13 cytokines [233].

A non-significant increase in the absolute CD4+ T-cell counts change from baseline was observed in patients given albendazole at evaluation after 3 months, (absolute mean CD4+ change of +107 CD4+ T-cells/mm$^3$ vs. +42 CD4+ T-cells/mm$^3$; p=0.16). This effect was present primarily in HIV negative albendazole treated patients compared with the placebo group (+122 CD4+ T-cells/mm$^3$ vs. +42 CD4+ T-cells/mm$^3$; p=0.17). The impact of increased CD4+ T-cell counts in patients with TB following albendazole treatment is not clear but may indicate a restoration of T-cell subsets following clearance of helminth infection. A similar effect of albendazole treatment on CD4+ T-cells was shown previously in a randomized trial conducted in Kenya [129] in helminth co-infected HIV patients. A significant increase in CD4+T-cell counts following albendazole treatment were detected in individuals infected with *Ascaris lumbricoides*. In that study, the mean absolute change in the CD4+ T-cell counts in the albendazole treated group was +109 CD4+ T-cells/mm$^3$ higher than in the placebo arm at 12 weeks of albendazole treatment. The effect of increased CD4+ T-cells count after albendazole treatment may have important immunological implications although the composition of the increased CD4+ T-cells compared to placebo was not investigated. Moreover, the response in the peripheral blood compared to the site of the disease in terms of host immunity and immune cell expansion may show discrepancies [234]. The CD4+ T-cells are specialized in providing supporting signals to
other cells including macrophages, dendritic cells, B-lymphocytes and CD8+ T-cells through either secretion of cytokines or direct cell-to-cell interactions. The trend for increased CD4+ T-cells merit further studies to assess whether albendazole could lead to such increment through deworming or if the observed effect is related with the pharmacokinetics of albendazole.

No significant effects as a result of albendazole treatment were detected for other secondary outcomes than eosinophil counts, including changes in chest x-ray findings, smear conversion and IgE level. As part of secondary outcome measurement, immunological analysis for Tregs as well as ELISPOT analysis of IFN-γ, IL-5 and IL-10 producing PBMCs was performed in a subset of 35 helminth positive TB patients. The HIV co-infection rate for this subset in albendazole and placebo groups was 50% (6/12) vs. 15% (3/20), respectively (p=0.04). Thus, an unfortunate balance in the rate of HIV infection was present. Nonetheless, in the baseline measurements no significant impact of HIV infection was present on the Treg levels. Reports have shown that the ability of HIV to directly infect Tregs is controversial [235,236]. On the other hand, it has been reported that exposure of Tregs to HIV selectively promotes their survival, which is supported by the observation that Tregs are accumulated in the gut or in the tonsils of HAART-naive HIV positive patients where active viral replication is taking place [236]. Similarly the kinetics of Tregs lacks a consensus in HIV-positive patients. Increased Tregs numbers was reported independently of HIV treatment [237], whilst others showed an increase and a subsequent return to levels comparable to controls [238]. In this study, no significant difference in Tregs, or IFN-γ, IL-5 or IL-10 response was observed between the two groups.

Sub-group analysis for Tregs, IFN-γ, IL-5 and IL-10 was done for TB patients with Ascaris infection (n=12 in total), but did not show any significant differences between the albendazole and the placebo group. This is in agreement with previous studies conducted in HIV patients in which albendazole treatment of HIV patients with Ascaris infection showed no difference in the levels of Th1/Th2 cytokines (such as IFN-γ, IL-4, IL-5, IL-13), but where a decline in the IL-10 levels was observed in albendazole treated patients compared to the placebo group [233,239]. In accordance to these findings, the results from the present study, excluding patients with persistent helminth infection at week 12 in a multivariate analysis adjusted for baseline level of IL-10, age, sex, and HIV, showed an increased trend towards significantly lower levels of IL-10 in the albendazole group compared to placebo (p=0.08). In animal models, an elevated IL-10
level was associated with susceptibility to TB, and mice lacking IL-10 were shown to clear *M. bovis* BCG infection more efficiently [240]. Additionally, mice over expressing IL-10 were more susceptible to *M. tuberculosis* infection, exhibiting an impaired Th1 response as characterized by decreased numbers of activated T-cells in the peripheral blood and lung tissue [111]. Overall, the finding of a decrease in IL-10 following deworming compared to placebo support the previous experimental data.

**Regulatory T-cells, IFN-γ, IL-5 and IL-10 are increased in helminth co-infected TB patients compared to helminth negative TB patients and healthy subjects**

There is emerging evidence regarding the influence of Tregs and associated cytokines during infectious diseases in facilitating pathogen persistence and modulating the host immune response to infection [241]. The significant increase in Tregs and associated cytokines in TB patients co-infected with helminths in paper V strengthens this notion. We observed a significant increase in the frequency of Tregs in helminth co-infected TB patients compared to helminth-negative TB patients, (2.2±1.5, n=25 vs. 1.2±1.1, n=16; p=0.03) and to helminth-positive community controls, (1.1±0.99, n=14; p=0.018, (Table 3a and 3b).

The increased expansion of the Treg population in the present study was accompanied by an increased level of IL-5 and IL-10, among helminth-positive TB patients compared to helminth-negative TB patients. In animal models, it has been described that T-cells are highly associated with helminth infection and are in great number in areas prevalent in helminths. Tregs are common in the gut and most are Foxp3+ Tregs that express CD4. In the colon mucosa, more than 50% of the Foxp3+ T-cells also produce IL-10. Our finding supports the strong association of helminths with Tregs and the Th2 associated cytokines IL-5 and IL-10 which have the capacity to modulate the host immune response. The observed increase in baseline IFN-γ response in helminth infected TB patients might be associated with induction of a Th1-type response during the early stages of helminth infection, as previously indicated [242,243]. After analyzing the distribution IFN-γ responses, it was observed that the increase in the helminth-positive TB patients is due to the marked increased (more than two standard deviations higher than the mean) levels of this cytokine in a few patients which were outliers (6/36).
Table 3a. Baseline characteristics and levels of Tregs, IFN-γ, IL-5 and IL-10 producing peripheral blood mononuclear cells (PBMCs) in patients with TB

<table>
<thead>
<tr>
<th></th>
<th>He+/TB</th>
<th>SD</th>
<th>n</th>
<th>He-/TB</th>
<th>SD</th>
<th>n</th>
<th>p*</th>
<th>HIV-negative</th>
<th>He+/TB</th>
<th>SD</th>
<th>n</th>
<th>He-/TB</th>
<th>SD</th>
<th>n</th>
<th>p**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29</td>
<td>10</td>
<td>40</td>
<td>34</td>
<td>14</td>
<td>23</td>
<td>NS</td>
<td></td>
<td>28</td>
<td>11</td>
<td>30</td>
<td>32</td>
<td>16</td>
<td>18</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (% males)</td>
<td>55</td>
<td>40</td>
<td>57</td>
<td>23</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td>63</td>
<td>30</td>
<td>56</td>
<td>18</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ T-cells (cells/mm³)</td>
<td>446</td>
<td>278</td>
<td>40</td>
<td>402</td>
<td>201</td>
<td>19</td>
<td>NS</td>
<td></td>
<td>552</td>
<td>236</td>
<td>30</td>
<td>463</td>
<td>179</td>
<td>18</td>
<td>0.22</td>
</tr>
<tr>
<td>TB-score (points)</td>
<td>8.1</td>
<td>2.4</td>
<td>40</td>
<td>7.6</td>
<td>2.0</td>
<td>19</td>
<td></td>
<td></td>
<td>8.3</td>
<td>2</td>
<td>30</td>
<td>7.6</td>
<td>2</td>
<td>18</td>
<td>NS</td>
</tr>
<tr>
<td>HIV (%)</td>
<td>25</td>
<td>40</td>
<td>18</td>
<td>23</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>18</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. lumbricoides (%)</td>
<td>43</td>
<td>0</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>43</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hook work (%)</td>
<td>22</td>
<td>0</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. stercoralis (%)</td>
<td>20</td>
<td>0</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs (%/CD4+ T-cells)</td>
<td>2.2</td>
<td>1.5</td>
<td>25</td>
<td>1.2</td>
<td>1.1</td>
<td>16</td>
<td>0.021</td>
<td>2.0</td>
<td>1.5</td>
<td>18</td>
<td>1.3</td>
<td>12</td>
<td>14</td>
<td>0.150</td>
<td></td>
</tr>
<tr>
<td>IFN-γ, Unst. (SFU)</td>
<td>87</td>
<td>76</td>
<td>37</td>
<td>77</td>
<td>70</td>
<td>15</td>
<td>NS</td>
<td></td>
<td>83</td>
<td>70</td>
<td>28</td>
<td>69</td>
<td>78</td>
<td>11</td>
<td>0.596</td>
</tr>
<tr>
<td>IFN-γ, PPD (SFU)</td>
<td>212</td>
<td>112</td>
<td>37</td>
<td>125</td>
<td>108</td>
<td>15</td>
<td>0.020</td>
<td>210</td>
<td>114</td>
<td>28</td>
<td>113</td>
<td>124</td>
<td>11</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>IL-5, Unst. (SFU)</td>
<td>33</td>
<td>49</td>
<td>37</td>
<td>29</td>
<td>53</td>
<td>15</td>
<td>0.080</td>
<td>54</td>
<td>50</td>
<td>28</td>
<td>20</td>
<td>30</td>
<td>11</td>
<td>0.044</td>
<td></td>
</tr>
<tr>
<td>IL-5, PPD (SFU)</td>
<td>141</td>
<td>96</td>
<td>37</td>
<td>76</td>
<td>76</td>
<td>15</td>
<td>0.024</td>
<td>151</td>
<td>105</td>
<td>28</td>
<td>50</td>
<td>58</td>
<td>11</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>IL-10, Unst. (SFU)</td>
<td>139</td>
<td>166</td>
<td>37</td>
<td>21</td>
<td>27</td>
<td>15</td>
<td>&lt;0.001</td>
<td>143</td>
<td>180</td>
<td>28</td>
<td>19</td>
<td>30</td>
<td>11</td>
<td>0.030</td>
<td></td>
</tr>
<tr>
<td>IL-10, PPD (SFU)</td>
<td>189</td>
<td>182</td>
<td>37</td>
<td>102</td>
<td>136</td>
<td>15</td>
<td>0.240</td>
<td>197</td>
<td>187</td>
<td>28</td>
<td>61</td>
<td>84</td>
<td>11</td>
<td>0.027</td>
<td></td>
</tr>
</tbody>
</table>

He+/TB= Helminth co-infected TB patients; He-/TB= Helminth-negative TB patients; PPD=purified protein derivate, SFU=Spot forming units/250 000 peripheral blood mononuclear cells.

* Helminth-positive TB patients vs. Helminth-negative TB patients

** HIV-negative, helminth-positive TB patients vs. HIV-negative, helminth-negative TB patients

Unst. = Unstimulated
Table 3b. Baseline characteristics and levels of Tregs, IFN-γ, IL-5 and IL-10 producing peripheral blood mononuclear cells (PBMCs) in healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>HIV-negative</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>He+/CC</td>
<td>SD</td>
<td>n</td>
<td>He-/CC</td>
<td>SD</td>
<td>n</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (% males)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ T-cells (cells/mm³)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB-score (points)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. lumbricoides (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hookworm (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strongyloides (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs (%/CD4+ T-cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ, Unst. (SFU)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ, PPD (SFU)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-5, Unst. (SFU)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-5, PPD (SFU)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10, Unst. (SFU)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10, PPD (SFU)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

He+/CC= Helminth-positive controls; He-/CC= Helminth-negative controls.

PPD=purified protein derivate, SFU=Spot forming units/250 000 peripheral blood mononuclear cells.

*HIV-negative, helminth-positive TB patients vs. HIV-negative, helminth-positive control subjects.

Unst.= Unstimulated
We were not able to reach the target sample size in recruitment (n=210). The expansion and decentralization of anti-TB treatment centers allowed patients to complete the TB treatment in their vicinity, which made recruitment of the initial calculated sample size in the planned period unachievable at the study sites. Moreover, consideration of subgroups such as the impact of HIV infection in immunological and clinical evaluations is problematic and might need even larger sample sizes or even exclusion in preference of HIV-negative TB patients only. The lack of a previous study in relation to helminth infection, albendazole treatment and tuberculosis makes the power calculation challenging. However, we reasoned that the impact of deworming may be much less pronounced than from the anti-TB drugs. The marked decline in TB-score due to anti-TB treatment alone in patients allocated to albendazole and placebo groups could mask clinical effects due to deworming. It is difficult to exclude an undetected low grade helminth infection in the helminth-negative TB patients and controls, and a possible re-infection after albendazole treatment.

The major strengths of the study are the combinatory clinical and immunological evaluation. Compared to previous studies, the sample size for the analysis of Tregs as well as IL-5, IL-10 and IFN-γ was relatively large. Additionally, the approach to use a randomized design to investigate the effect of albendazole treatment on asymptomatic helminth infection is novel.

In conclusion, albendazole treatment did not affect the clinical outcome of helminth co-infected TB patients as analyzed by changes in TB-score after two months. Deworming of helminth co-infected TB patients induced a significant decline in peripheral eosinophil granulocytes, and a non-significant increase in peripheral CD4+ T-cells after 12 weeks. Tuberculosis patients who received effective albendazole treatment showed a borderline significant decrease in IL-10 levels at follow up. In patients with TB, we showed that asymptomatic helminth infection induces an increased Treg response and increased levels of IL-5 and IL-10 producing PBMCs. Taken together, a main conclusion of our study is that helminth infection significantly affects the immunological response to TB. Larger multi-center studies are warranted to further investigate the effect on deworming on clinical outcome of TB as well as the helminth-induced mechanism of immune modulation in active TB.
CONCLUSIONS

- During treatment of TB there is a significant decline in the proportion of QFN positive patients down to the baseline level similar to a healthy control group.
- The agreement between TST and QFN is poor in active TB but satisfactory in community controls.
- Early changes in the TB-score composed of clinical signs and symptoms of TB may be used to identify patients in need of closer follow up and hospitalization.
- More than one third of patients with active pulmonary TB were co-infected with helminths, which was significantly higher than in healthy controls.
- Tuberculosis patients with HIV infection had a significantly lower rate of helminth infection than HIV-positive TB patients.
- Eosinophilia and elevated IgE levels correlated with asymptomatic helminth infection in patients with active pulmonary TB.
- Tuberculosis patients with asymptomatic helminth infection had a significantly increased frequency of Tregs as well as increased levels of IL-5 and IL-10 PBMCs compared to helminth-negative TB patients and healthy controls.
- Albendazole treatment of helminth-positive TB patients resulted in a marked decline in the rate of helminth infection after three months compared to placebo whereas it did not significantly affect the primary outcome (change in TB-score between baseline and 2 months).
- Albendazole treatment resulted in a significant decline in eosinophil cells after 3 month compared to placebo as well as a trend towards a lower rate of IL-10 producing peripheral mononuclear blood cells after 3 months compared to placebo.
CONCLUDING REMARKS

The work presented in this thesis contributes to the understanding of the clinical and immunological impact of helminth co-infection in active TB. Previously, the clinical and immunological impact of helminth infection during TB was mainly described in experimental models or in cells from healthy individuals. The main conclusion of this work is that asymptomatic helminth infection has an immunological impact in active TB. This has important implications for the effect of the present BCG and future TB vaccines, but may also be of importance for the outcome of active TB infection. The randomized clinical trial of albendazole versus placebo in helminth positive TB patients is the first of its kind and included a thorough follow-up including immunological assessment. Larger multi-center studies with a sufficient representation of different helminth species in each treatment arm are important to make subgroup analysis including considerations of HIV co-infection.

Novel tools for treatment monitoring of TB were evaluated such as the TB-score and longitudinal QFN measurements. This has significant importance in the clinical care of patients. It fills a major gap in resource limited settings in particular where there is a deficiency in objective surrogate markers of clinical outcome in TB which could be monitored longitudinally. The available WHO recommended strategy of sputum AFB smears and radiology based assessment is only applicable to a limited subset of TB patients and suffers from limitations in terms of sensitivity, objectivity and logistics. Our findings evaluated the strength and limitations of the QFN assay and the TB-score tools for TB treatment monitoring and their relevance in clinical research. We showed that QFN results are influenced by HIV infection. Furthermore, after 2 to 6 months of TB treatment, TB patients showed a modest decline in the rate of QFN positive patients to a level comparable with that recorded for healthy control subjects from the same area. It was shown that TB-score is a potential marker for early prediction of patients at high risk of mortality and adverse outcomes. The findings of our helminth-TB co-infection studies add new clinical and immunological data, which were previously limited. The immunomodulatory effects of helminth infection have been demonstrated in several experimental studies. We showed an increased helminth burden in TB patients compared to healthy controls from the same area. It is not known whether asymptomatic helminth infection could increase susceptibility to TB disease following exposure. The observed higher burden of
helminth co-infection in active TB patients as well as the immunomodulation induced by helminths, merits further long-term follow-up studies of larger cohorts, including effects of deworming. Such studies also need to consider other confounders such as HIV co-infection and nutritional status including vitamin D deficiency. Our immunological findings strongly support the impact of asymptomatic helminth co-infection on host immunity to active TB. The increase in Tregs in helminth co-infected TB patients, suggests a significant immunological effect as the Tregs are known for their immunosuppressive role. Furthermore, the increased levels of the Th1 attenuating cytokine, IL-10, further underlines the Th1 attenuating immunological impact of helminths on the host immune response during active TB. The hypothesis generated is shown in Figure 11.
Figure 11: According to the general hypothesis of this thesis, chronic helminth infection induces Treg expansion and increased levels of the associated inhibitory cytokines, IL-10 and TGF-β. The strong Th2 response and suppressive effect from regulatory cells lead to an attenuation of the Th1 response. Thus, the increased frequency of Tregs, inhibitory cytokines and a skewed Th2 immune response in addition to other factors such as HIV, diabetes mellitus, malnutrition including low vitamin-D levels may attenuate a proper cell-mediated immune response against TB. This will in turn increase the risk of developing active disease from primary infection, and it may also increase the risk of developing active TB diseases through reactivation in patients with latent TB. After the occurrence of active TB disease, deworming of helminth co-infected TB patients may lead to better clinical outcome as helminth infection affects the immunological response during active TB.
REFERENCES


ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to everyone who has been involved in completing this work or taken part in my life in other ways during these years. I would first like to thank all the participants in the studies included in this thesis. I would especially like to mention the following people:

My supervisor, Thomas Schön for giving me the opportunity to be your student and believing in me. I greatly give much credit for your continuous effort in refining me. All your enthusiasm to research, encouragement and your impressive knowledge made it a pleasure working under your guidance. I am always inspired by your endless energy. Your knowledge is covering the basics and clinical sciences. I always bless our first meeting in Addis. It grossly changes my carrier and I am now happy to see the results of this choice in life! Thomas, I hope our collaboration will continue. It would be inappropriate not to mentioning your beloved family. They always surprise me from the first meeting to this time. They are loving and special. Thank you Maria, Freja, Algot and the lovely Tyra!

My co-supervisor Olle Stendahl, a man of a big pool of basic and clinical knowledge. I always admire your easy way of grasping complicated scientific concepts and analysis. You clearly showed me what a true professor is! I greatly appreciate your critical thinking and the untouchable scope you view things. I will not forget the encouragement and support you have given me especially in the process to complete the thesis write-up in such short time. Thank you Olle, for all your scientific and administrative support, without it I would not have managed!

Britt-Inger, I want to extend my appreciation also to you, for your kind personality and hospitality!

My co-supervisor Abraham Aseffa, my appreciation starts more than 15 years back. You are among a handful Ethiopians who can be perfectly described in every term. Your deep and sharp knowledge, critical thought and clearness are indescribable. Your kind personality and positive thinking always inspires me. I wish to give you more honor and working freedom to bring out all your potentials. Thank you Abraham!

Jonna Idh, my Swedish sister “Abelij”. You have a very big place in my PhD journey. We were swimming together for a couple of years, which enabled me to really get to know you. You are an intelligent, superb and loving sister. I want to think of many of our good memories and funny moments in a very calm mood after closing this chapter. Many thanks Jonna for all the unlimited untold support I got from you in all these time. My appreciation to your family, thank you Nina, for hosting me and the nice time we had in Stockholm! Thank you BG/Maria and (Rich)Richard- “kirstinaw lene new”.

Daniel Elias, for always believing in me and to our long term true friendship (from the time of “teacher” to “Dany boy!”). Thank you for bridging me to the excellent Swedish research team. I much appreciated your simple personality, focused thought and clarity. Share with Hiwot my appreciation!

Sven Britton, for your long way support and intellectual ideas and comments.
Mary Esping, for superb administrative service and help with all types of queries, whatever query you receive wherever from!

My lab and office companions, TB team and other friends in Sweden, Maria Lerm (I admire your strong spirit, enthusiasm and deep knowledge of TB basics. Thank you for the pulka!), Kalle Magnusson (I like to sit near you during fika for a relaxing atmosphere you always create), Tommy Sundqvist, I wish to have more time to listen to your impressive discussions about NO. Danne Eklund/Louise (say hi to the junior in October!), Robert Blomgran- I like your easy and relaxed personality which give comfort to anyone. Thank you for your help during optimization of flow assay. Congratulation for the job you did in Brussels), Johanna Raffetseder (you are extremely helpful and many thanks for the many translations, proof readings and inviting approach, ich danke Ihnen vielma(!)), Clara Braian, You were helpful all the time, thanks for the proof reading and the stroller, which makes life easy for my kid; Tack somnyket Clara!!), Thommie Karlsson, hope you restore your energy after the dissertation. I like the few minutes’ talk we usually had in the weekends. My office companions Tony Forslund, for a very pleasant personality and Maggan Lindroth, it makes me happy hearing your funny talks. You are so relaxing and create comfort to people surrounding you. Thank you both for the nice working atmosphere we have had in our office. Elisabet, Lena, Peter (A=A), Elena, Angelika, Henrik, Asia, Anders, Fredrik, Pontus, Magali, Yuhan, Sadaf, Ramala, Fanny (wish you a fruitful time here and in Peru!), Kajsa Holmgreen, Asa Keita, Olena Yakymenko, Katarina Tejle and Christina Samuelsson, for excellent technical assistance. The master students we have supervised over the years Märta, Petra, Sofia, Anna, Caroline and Jonas and all fellow Ph.D students at Medical Microbiology, old and new.

Peter Söderkvist and Asa from division of Cell Biology; Lars Brudin, Kalmar County Hospital and Mats Fredriksson, Linkoping for statistical assistance. To Daniel, a friend from Eritrea in Linköping.

To the Gondar research team for your unreserved effort in helping me with my work: Aschalew, Belay Anagaw, Martha, Tigist Feleke, Lamesgin, Meseret Senbeto, Meseret Atale, Tezera, Abye, Saba, Alemnesh, Kirkin ,Yeshy, Atanaw, Misanaw, Abebech, Bethelhem, Endale Zerfe, Dagne, all staffs in ART and main lab of Gondar hospital, and the Debark team! All staffs and colleagues at the school of Biomedical and Laboratory Sciences (the seniors-Prof. Moges, Dr. Feleke, Baye, Demekew, Aregawi, and Binyam, Abebe, Meseret,Wubet . Gizachew, and to all energetic young academician in the school!), and to GU/Gondar medical college as a whole, Drs. Sisay Yifr, Shitaye Alemu, Ermiyas Diro (Emry, I appreciate your enthusiasm to research and we should toast a glass for the nice and fruitful old friendship), Dr. Assefa Getachew for our successful scientific collaboration and for your unreserved effort in reading all the x-ray films. Oldies but goodies- Buddies: Afework Kassu/Berish, Gashaw Andarge (“Enkuan maryam marechish Atsedye”!), Dereje Nigusse, Mamo, Kalsay Hiruy, Jemal Ali, Andargachew Mullu, Belay Tessema, Yeshambel Belihun, Yifokir, for your friendship and all your support! All JHU staffs in particularl the Awassa team: Dr Girma Tesfaye,Mekete Reta, Jhony, Edris, Heny. My old true friend Alex (Cherkos)-wishing a better life over there.

All staffs and students at AHRI/ALERT, especially Martha Zewdie (thank you Marthy for all unreserved support during optimization of ELISSPOT and flow assays, you are really special!), Kidist, Liya, Hiwot, Elena, Markos,
Adane, Demis, Alemayehu, Almaz, Wude, Abebe, Menbere, Endalawaw, Haki (you are a superb secretary!), Jemal, Yonas, Yohannis, Binyam (thank you for handling the finance matters), Drs. Rawligh, Lawrence, Ruth Likassa and to all other AHRI/ALERT staffs!

A big thank to my amazing life time friends in Ethiopia Zeyede, Amare, Selamina, Yonas, Marthy², Endris, Guilat, Lule, Amha, Etsub and Geho (US). Kume, a new member but who laid the foundation.

To my entire family, to all your unconditional LOVE and support given to this time, Solome Ebba (my angel who is the battery of my motor and a source of happiness), my father, Abate Waktola (for all the untold support and assistance to this end), Helena/Nunish, Derese, Kuyu, Yared, Etaferwa, Kidus, Kidist, KB, Wengel, Barkot, Gelet, Hailu, Telile, Zewde, Mare, Belayneh, Ably (“enkuan aterefeh”), Mitaye, and the rest---

Last but certainly not least, thank you Mar/Mesy (what a wonderful wife you are!!!), many thanks for changing my life and showing the other parts of this world. Thank you Mar for all your patience and unreserved support and endless Love. I am the luckiest person in this world to have you as a wife!!! I will always Love you!