The Role of Nitric Oxide in Host Defence Against
*Mycobacterium tuberculosis*

Clinical and Experimental Studies

Jonna Idh
This thesis is dedicated to all
whom lost their health,
their love, or their life
due to TB.
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Financial enclosure

The work included in this thesis was supported by the Swedish Research Council, the Swedish Heart and Lung Foundation, King Oscar II Jubilee Foundation, SIDA/SAREC, Minor Field Studies (MFS/SIDA), European and Developing Countries Clinical Trials Partnership, (EDCTP, European Union), the Research Council of Southeast Sweden (FORSS), the Swedish Society of Medicine, the Lion Research Foundation, Knut and Alice Wallenberg Foundation and the Swedish Society of Tropical Medicine and International Health.
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Abstract

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), responsible for significant morbidity and mortality worldwide, especially in low-income countries. Considering aggravating factors, such as HIV co-infection and emerging drug resistance, new therapeutic interventions are urgently needed. Following exposure to M. tuberculosis, surprisingly few individuals will actually develop active disease, indicating effective defence mechanisms. One such candidate is nitric oxide (NO). The role of NO in human TB is not fully elucidated, but has been shown to have a vital role in controlling TB in animal models. The general aim of this thesis was to investigate the role of NO in the immune defence against M. tuberculosis, by combining clinical and experimental studies. In pulmonary TB patients, we found low levels of NO in exhaled air, and low levels of NO metabolites in urine. HIV co-infection decreased levels of exhaled NO even further, reflecting a locally impaired NO production in the lung. Low levels of exhaled NO were associated with a decreased cure rate in HIV-positive TB patients. Household contacts to sputum smear positive TB patient presented the highest levels of both urinary NO metabolites and exhaled NO. Malnutrition, a common condition in TB, may lead to deficiencies of important nutrients such as the amino acid L-arginine, essential for NO production. We therefore assessed the effect of an arginine-rich food supplement (peanuts) in a clinical trial including pulmonary TB patients, and found that peanut supplementation increased cure rate in HIV-positive TB patients.

We also investigated NO susceptibility of clinical strains of M. tuberculosis, and its association to clinical outcome and antibiotic resistance. Patients infected with strains of M. tuberculosis with reduced susceptibility to NO in vitro, showed a tendency towards lower rate of weight gain during treatment. Moreover, there was a clear variability between strains in the susceptibility to NO, and in intracellular survival within NO-producing macrophages. A novel finding, that can be of importance in understanding drug resistance and for drug development, was that reduced susceptibility to NO was associated with resistance to first-line TB drugs, in particular isoniazid and mutations in inhA.

Taken together, the data presented here show that NO plays a vital role in human immune defence against TB, and although larger multicentre studies are warranted, arginine-rich food supplementation can be recommended to malnourished HIV co-infected patients on TB treatment.
Sammanfattning på svenska

List of original papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

Paper I


Paper II


Paper III


Paper IV

INTRODUCTION

*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), remains a major worldwide health problem. Even though antibiotics can cure TB, the disease causes approximately 1.4 million deaths annually. The efforts to control TB are mainly hampered by: the prolonged time from initial symptom to diagnosis, logistic challenges in reaching TB-endemic areas, the extended time of treatment to eliminate the infection, the immunological impact of HIV infection, increasing drug resistance, and the fact that the vaccine against *M. tuberculosis* (BCG vaccine) has limited efficiency. To overcome these challenges, the development of new drugs and vaccines, more accurate and rapid diagnostic tools as well as research on therapeutic strategies that target the immune system, are urgently needed. This thesis focuses primarily on the role of nitric oxide (NO) in the immune defence against *M. tuberculosis*. Results presented here can be applied in the area of drug development, by investigating the ability of *M. tuberculosis* to withstand the toxic effects of NO, but also in the area of immunotherapy and vaccine development, by assessing aspects of NO-mediated immunity in the host defence against TB.
ABBREVIATIONS

AIDS  acquired immune deficiency syndrome
ART  anti-retroviral therapy
BCG  bacillus Calmette Guérin
BMI  body mass index
DC-SIGN  DC-specific intracellular-adhesion-molecule-3-grabbing non-integrin
DETA/NO  diethylenetriamine NONOate
DOTS  directly observed treatment, short-course
HIV  human immune deficiency virus
IGRA  interferon-gamma release assay
IFN  interferon
INH  isoniazid
iNOS  inducible nitric oxide synthase
MDR TB  multidrug-resistant tuberculosis
MOI  multiplicity of infection
NADPH  nicotinamide adenine dinucleotide phosphate
NO  nitric oxide
NO2⁻  nitrite
NO3⁻  nitrate
NOS  nitric oxide synthase
ONOO⁻  peroxynitrite
O2⁻  superoxide
PCR  polymerase chain reaction
PPD  purified protein derivative
RNS  reactive nitrogen species
ROS  reactive oxygen species
SIN-1  3-morpholino-sydnonimine
TB  tuberculosis
TLR  toll-like receptor
TNF  tumour necrosis factor
TST  tuberculin skin test
XDR TB  extensively drug-resistant tuberculosis
A voice of TB

“I had chest pain and dyspnoea but I didn’t know that it was TB. When the doctor told me I had TB I felt sad, because I then had to stay at home from school. I come from the countryside, and I had a family member with TB, I think I got it from that person. My parents are dead, so now I live with my sister in town. She is not afraid of getting sick, and we sleep in the same room. My school is in the countryside, but I can’t stay there since I need to be in town to take my drugs. I have four months left, and then I will return to school. I think I will be well after treatment.”

Abera, 18-year-old young man, student at elementary school
BACKGROUND

Tuberculosis

A historical view

Tuberculosis (TB) is an infectious disease caused by the microbe *M. tuberculosis* [1]. It has a history of thousands of years together with mankind, and was described by Hippocrates (400 B.C.) and has been found evident in antique Egyptian mummies (2000-3000 B.C.) [2]. Many names have been used to describe the disease over the years, such as “white plague”, “phthisis”, “wasting away”, “consumption”, “Pott’s disease”, “Gibbus deformity”, and “scrofula” [3]. In late 17th century a French pathologist, Franciscus Sylvius, found pulmonary nodules from TB patients and named these tubercula (small knots). He also observed how the tubercula developed into lung ulcers (cavities). After some years it was suggested that TB could be transmitted through the “breath” of a sick person [3] but it took nearly 200 years before Robert Koch managed to isolate the causative agent of TB. Shortly after, the sanatoria were introduced with a regimen of bed rest, preferably out in the open-air, and a nutrient-rich diet [4]. Many poets and writers have described the life at sanatoria and the struggle against TB. Examples from the Swedish literature are Edith Södergran [5], Sven Stolpe [6] and Olof Lagercrantz [7], who all fell victims to TB.

Epidemiology

The dynamics of TB in an epidemiological perspective depends greatly on socioeconomic factors [8]. From the 16th to the 18th century TB was responsible for one in four deaths in Europe, and reached a peak in the 19th century due to urbanization and crowded living conditions [2]. As housing, diet and sanitation improved during late 19th century, the TB incidence declined even before the discovery of antibiotics, stating the importance of social factors and nutrition in containing TB [9]. After the introduction of chemotherapy in the 1940s, there was a steady reduction in disease burden for several decades. However, as a consequence of the HIV/AIDS pandemic [10] and the development of drug-resistant strains, TB has returned as one of the deadliest infectious diseases in the world [3, 11, 12].
Despite treatment being available for more than 50 years, the World Health Organization (WHO) declared TB a global public health emergency in 1993, and prioritized TB in the Stop TB Partnership and Millennium Development Goals. Still, WHO estimated that TB caused the death of about 1.4 million people in 2010, and that 9 million cases of TB were diagnosed worldwide. Two-thirds of these TB cases occur in people between 15–59 years and are devastating for the financial systems in high incidence countries, as well as for the 10 million children that were estimated to be orphans as a consequence of parental death in TB (2009) [13].

In Sweden, the TB incidence is low with 7.3/100 000, and 683 actual cases prevalent in 2010 [14], as compared to a high endemic areas like Ethiopia with an incidence of 261/100 000 and a prevalence of 394/100 000 (figure 1). In a country the size of Ethiopia, this resulted in absolute numbers of 220 000 new TB cases, 330 000 people living with TB and 29 000 deaths due to TB (excluding HIV) in 2010.

Figure 1. Tuberculosis incidence rates per country in 2010 [13]. (Reprinted with permission from WHO Press.)
As a weak comfort to these significant numbers, WHO has presented a slow decrease in the absolute number of new TB cases since 2006 [13], but this is not in agreement with others [8] as it stands clear that to stop the TB epidemic there is a need for a more aggressive approach against HIV [10, 15]. In 2010, 43% of TB patients in Ethiopia were tested for HIV and of these 15% were HIV-positive [13]. Even though there have been improvements in screening for TB in HIV-positive individuals, as well as initiation of TB preventive therapy for HIV-positive patients without active TB, only 34% of TB patients were tested globally in 2010 [13].

Transmission

TB is almost entirely spread by air-borne droplets produced by a person with pulmonary TB, where cavitary disease and the presence of bacteria in sputum (sputum smear-positive), increase the risk of transmission [16]. Most people exposed to \textit{M. tuberculosis} will not develop active disease. As a result of a strong innate immune response, they will either clear the infection with no signs of \textit{M. tuberculosis} encounter, or they may contain the infection with no signs of disease (latent TB). Due to immune suppression later in life, the latent infection can reactivate to active disease [17] (figure 2). A latent TB infection can be detected by immune reactivity against antigens of \textit{M. tuberculosis}, with interferon-gamma release assays (IGRA) and/or tuberculin skin test (TST) [18]. It is not possible to detect the presence of the potentially well-contained bacteria themselves [1], but mycobacterial DNA has been found in lung tissue from asymptomatic individuals in high endemic areas, who died from causes other than TB [19].

There is a relatively high risk of transmission to close household contacts of TB patients [20, 21] and mathematical modelling of TB transmission and key factors in disease control, has led to the findings that members of larger families are responsible for more disease transmissions than those from smaller families [22]. However, in high endemic areas a significant part of the transmission occurs outside the household, such as on public transports [22], and the benefit of active case finding within the households in high-endemic areas has not proven as obvious as in low-endemic areas [23].
Figure 2. Transmission of *M. tuberculosis*. A strong innate immune response may eradicate *M. tuberculosis* instantly, and there will be no immunological or radiological sign of *M. tuberculosis* encounter. If the patient is infected, there are two possible scenarios. If the host manages to contain the infection through effective cellular immune responses, there will be no symptoms of disease, and this stage is called latent TB. Antigen recognition with tuberculin skin test (TST) and/or interferon-gamma release assays (IGRA), is a sign of latent TB, but may not always indicate that viable *M. tuberculosis* are present in the host. If the host is not capable of mounting an adequate immune response, bacteria will continue to replicate and cause active disease. Such a scenario may take place in small children and in late stage of HIV infection. If the immune response in latent TB is affected by factors such as old age or chronic diseases, the latent infection can reactivate and develop into active TB, and spread to a new host.
There are known risk factors for TB, but even when taking those factors into consideration, it is still not clear why the majority of individuals infected with *M. tuberculosis* will stay healthy while others will develop disease. It is of great importance to understand the underlying mechanisms behind the susceptibility or resistance to TB infection in order to prevent infection and disease [24].

There is a sex-related difference in TB incidence [25]. In Ethiopia there was a male to female ratio of 1.3 in 2010 [13]. The difference depends both on social structures and lower case findings among women, but also on biological differences [25, 26]. Chronic diseases known to be associated with increased risk of TB include diabetes [27-29], renal failure [30], haematological malignancies [31] and solid-organ malignancies due to immune suppression by the disease itself or by prescribed chemotherapy [32]. In recent years, increased use of biological immune modulators such as tumour necrosis factor (TNF) antagonists for the treatment of rheumatologic disorders is common, significantly increasing the risk of reactivating latent TB [33]. Other risk factors for TB are smoking [34], indoor combustion of biofuels [35], silicosis [36] and heavy alcohol consumption [1, 37].

The risk of developing TB after exposure to *M. tuberculosis* is greatest in the youngest children [38] and decreases from the age of 5 until adolescence, when the risk again increases with a peak in the age of 20-30 [25]. Elderly are at high risk due to dysfunction of the cellular immune functions, similar to that seen in young children [25]. Immune dysfunction due to HIV has lead to an epidemic of HIV-associated TB [10], where TB now is the leading cause of death among HIV-positive individuals [1]. The two diseases accelerate one another, by increased transcription of HIV in TB [39, 40] and decreased numbers of TB-protective T cells in HIV [41]. The TB incidence ratio between HIV-negative and HIV-positive individuals is around 20 to 37, depending on HIV prevalence [1]. Another co-infection, which could have an impact on the development of TB, is chronic worm infection. In TB endemic areas there is a high incidence of worm infection [42, 43] known to have immune modulatory effects, and a subsequent negative impact on host response to TB [43, 44], and on immunity induced by vaccination to protect against TB (BCG vaccination) [45].
TB was previously thought to be an inherited disease and that has now been substantiated in the last decades, since human genetics have shown to contribute to susceptibility to TB [46]. Variations in incidence of TB between regions may be explained by genetic differences [47]. On the other hand TB took the lives of millions of people during the pre-antibiotic era, resulting in a natural selection of individuals with immunity strong enough to withstand the infection or disease [24]. Excluding a few exceptions, one by one the genetic variants will not have a big impact on the risk of TB, but together on a population level, the impact may be profound [8]. Genetic identification of these risk factors could lead to individualized treatment and novel vaccination strategies [46]. Genes confirmed to be important in host defence in TB are the natural resistance-associated macrophage protein (NRAMP or SLC11A1) [48], the vitamin D receptor (VDR), the nuclear factor kappa B (NFκB) pathways, the human leucocyte antigen (HLA) region, pattern recognition receptors (PRR) such as DC-SIGN and toll-like receptors (TLRs), as well as the IFN-γ pathways [49]. There is also a genetic association between nitric oxide synthase (NOS2A) and tuberculosis confirming the role of NO in human disease [46].

**Nutrition and tuberculosis**

TB has been called “A poor mans disease” indicating risk factors for TB associated with poverty, like crowded living conditions and malnutrition [50]. Previously TB went under the name “Consumption” [51], describing the common symptom of weight loss seen in TB patients. The weight loss is due to loss of appetite, but also to malabsorption and altered metabolism [52]. Protein-energy malnutrition, micronutrients deficiencies and low body mass index (BMI) increase the risk of TB [53-55] and malnutrition is associated with early death during TB treatment [56]. Malnutrition affects the cell-mediated immunity (CMI), the principle host defence against TB [57] and deficiencies of micronutrient such as vitamin D, vitamin A and zinc reduce protective immune responses in murine TB models [58]. There is also an association between human TB and micronutrient malnutrition with low levels of substances such as vitamin A, vitamin D, iron, zinc, selenium, and plasma carotenoids [59-62]. In the pre-antibiotic era cod liver oil, now known to be rich in vitamin D, was included in the treatment of TB [63]. Phototherapy as sun exposure of the skin was also a common treatment of TB [64], and the effect is thought to be due to the conversion of vitamin D to its active form (1,25-dihydroxyvitamin D3) [65].
Several recent trials have investigated the issue of micronutrients in TB, and clinical effects of multi-nutritional supplements and specific minerals or vitamins such as zinc, copper, selenium, vitamin A, B complex, C, D, and E [66-72]. Epidemiological studies have found lower levels of vitamin D in TB patients compared to controls [73], but supplementation with vitamin D3 to patients treated for TB in Guinea Bissau showed no clinical effect [69]. In a similar study in London there was a significant effect on time to sputum conversion, but only in a subgroup of TB patients with a specific vitamin D receptor (VDR) polymorphism [74]. Despite that malnutrition may have a great impact on the TB epidemic, it has been difficult to convincingly show a beneficial effect of supplementation in clinical studies, as described in a recent Cochrane review [52]. This may be attributed to the relatively small sample sizes of clinical studies performed so far [75].

**Diagnosis**

Early diagnosis is a key strategy to control TB [76] but fast and accurate diagnostics are often not available in high-endemic areas [13]. The diagnosis of TB relies on direct microscopy of sputum smears, and where available, mycobacterial culture and polymerase chain reaction (PCR)-based detection. Even if culture is time-consuming (3-6 weeks), and for safety reasons a complicated procedure, it is the gold standard for diagnosis of TB [77]. For over a hundred years, the sputum smear microscopy has been the primary tool for diagnosing TB, and is still so in low income countries [78]. Typically a smear of a sputum sample is stained with Ziehl-Neelsen stain (figure 3), which is inexpensive, fast, and specific in TB endemic areas, but with low sensitivity (range 20 to 80%) especially in HIV co-infected TB patients [79].
In order to increase the sensitivity, sputum samples may be processed by chemical or physical methods [80] such as the concentration, sedimentation and the bleach method [81]. Staining with auramine-fluorescent dye requires a fluorescence microscope, but can increase the sensitivity further, even though still not satisfactory [82].

In 2010, 8 of the 22 high burden countries did not have even one laboratory with microscopy service per 100 000 inhabitants. Among the 36 countries of high burden and high multidrug-resistant (MDR) TB, less than 20 had one laboratory capable of performing culture and drug susceptibility testing per 5 million inhabitants. Instead the clinician’s evaluation of symptoms, sputum smear examination, and if available, chest X-ray is the most common way to diagnose TB in low-income countries. In contrast, high-income countries with a low incidence of TB have advanced techniques and sputum or tissue samples can be analysed with PCR within hours to determine, not only if the patient is infected with *M. tuberculosis*, but also if the strain is resistant to antibiotics [13].

In 1890, Robert Koch presented his findings of the liquid “tuberculin” that could be used as a diagnostic tool [3], and is so still today. The tuberculin (or purified protein derivate, PPD) is injected intradermally on the volar side of the lower arm (TST) [83]. An induration of more than 10 mm after 48-72 hours is considered as positive, but there may be false negative results due to anergy in a minority of patients with active TB, as well as false positive results due to exposure to environmental mycobacteria and BCG vaccination [84]. The IGRAs have been developed as an alternative to the TST for detection of latent TB. In contrast to TST, IGRAs measure immune response to antigens such as ESAT-6, CFP-10 and TB7.7 (p4) expressed, by members of the *M. tuberculosis* complex but only a very few other mycobacterial species, and notably not *M. bovis* BCG [85]. Two commercial methods are available, the T-SPOT.TB test (Oxford Immunotech, Abingdon, UK) and the QuantiFERON-TB Gold In-tube (Cellestis Ltd, Carnegie, Australia) [86]. Both IGRAs and TST have a modest positive predictive value around 3% [87]. Although IGRAs have no role in the diagnosis of active TB [88, 89], they might reduce the number of people considered for preventive treatment in low-incident countries [18, 86], due to their high negative predictive values [90]. In HIV-infected TB patients, IGRAs perform similarly or slightly better than the TST [77].
Another alternative marker for TB is the chemotactic protein IP-10 (IFN-γ-induced protein 10), secreted from antigen presenting cells upon activation by T cells. IP-10-based tests appear to perform comparably to the IGRAs, but may provide some additional information for young children and HIV-infected individuals [91].

Antibody as well as antigen detection has been tried in TB diagnostics. There are antibody-based TB tests available in the market, but of little or no diagnostic value [92], but detection of the TB antigen lipoarabinomannan (LAM) in urine of patients with TB has been a contribution in HIV co-infected patients, even if sensitivity still is low [93].

**Clinical presentation**

The lungs are the main site of disease for TB and are also the primary route of infection. Patients with active TB present a wide range of symptoms from severely ill to minimal complaints, which is also illustrated by the spectrum of host responses to the disease [94]. Common symptoms are cough, night sweats, weight loss and sometimes also haemoptysis, fatigue and fever [95]. In post-primary (reactivating) pulmonary TB, a chest X-ray will most commonly show an upper lobe infiltrate, or fibrosis with or without cavitation. It is also from here that the bacteria are shed to new hosts by the infected patient expectorating airborne droplets containing bacteria. Where resources allow, the chest X-ray may be complemented by computed tomography and magnetic resonance imaging increasing the sensitivity [77].

HIV and TB co-infection may lead to atypical symptoms and disseminated disease causing great difficulties in establishing a correct diagnosis [95]. For HIV-positive patients both chest radiography and sputum examination, the common diagnostic tools in high endemic areas, have an even poorer performance than in HIV-negative individuals. Other respiratory infections may also be misinterpreted as sputum smear negative TB [96]. Chest X-rays of HIV co-infected TB patients may show a pattern of primary TB with hilar lymphadenopathy but less consolidation and cavitation [97] and the infiltrates are more often located to the lower or middle lung fields than in HIV-negative TB patients [98] (figure 4).
Figure 4. Chest X-ray patterns of pulmonary TB. Minimal TB (A) with left upper lobe infiltrate in an HIV-negative patient. Far advanced TB (B) involving the whole right lung and left upper lobe with a cavity in the right upper lobe in an HIV-negative patient. Bilateral hilar enlargement (C) in an HIV-positive TB patient. (Patients from Gondar University Hospital, Ethiopia, examined by Dr Assefa Getachew.)

Extrapulmonary TB is defined as TB in any other organ than the lung and includes lymphatic, pleural, bone and/or joint, meningeal, visceral and genitourinary TB [99]. Extrapulmonary TB constitutes a major portion of TB among HIV-positive, elderly and children [25, 99]. Miliary TB is a severe form of extrapulmonary TB which may be associated with bacteraemia, fever, fatigue and weight loss which is primarily diagnosed in immunocompromised persons such as HIV-infected individuals or in young children [95].

From an era with no treatment available, time from symptoms to death or self-cure has been estimated to be approximately 3 years for both smear-positive and smear-negative TB [100]. If untreated, the 10-year case fatality is reported to be around 70% (ranging from 53% to 86%) in smear-positive TB without HIV and approximately 20% in culture-positive smear-negative TB.

**Treatment**

The drugs which are available today can lead to the cure of most TB patients harbouring susceptible strains, but with emerging drug resistance this may soon no longer be the case, and drug development is far behind the TB epidemic [13]. The discovery of drugs against TB and the use of combination therapy instead of monotherapy in the 1940s and 1950s dramatically reduced mortality in TB [13]. The first drugs in clinical use, around 1945, were streptomycin (SM) and para-aminosalicylate (PAS) [101].
In order to reduce disease burden and transmission, TB drugs need to rapidly kill dividing bacteria, prevent development of resistance and in the long run sterilize the tissue from slow growing bacteria. The mechanisms of the drugs used today are combined to cover all these tasks but unfortunately the time to achieve this goal is long and causes compliance problems [101].

The present standard treatment consists of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol for two months, followed by INH and RIF for another four months [102]. INH plays a critical role in the initial killing of replicating bacteria and has been in use since 1952. It is a prodrug that needs activation by the bacterial catalase-peroxidase enzyme (encoded by katG). The active form of INH targets mycolic acid synthesis, important for the bacterial cell wall, and mutations in the gene target for this process (inhA) is the other important factor in INH resistance [103]. RIF, introduced in 1966, acts by binding to bacterial RNA polymerase (encoded by rpoB), blocking RNA synthase, and appears to be a more effective sterilizing agent than INH. RIF discolours urine, faeces, sputum and tears in a red-orange colour [101]. PZA is the third most important drug in TB but its role is mainly to increase the sterilizing effect during the intensive phase (two first months). It has to be cleaved into its active compound pyrazinoic acid and needs an acidic environment to kill the bacteria by disruption of the cell wall. EMB was first reported in 1961 and is today used primarily to prevent development of drug resistance to the other drugs used [101]. EMB targets the synthesis of the bacterial cell wall components, arabinogalactan and lipoarabinomannan, by inhibition of arabinosyltransferases (EmbA, EmbB, and EmbC) [104].

Since symptoms resolve within a few weeks after treatment initiation, patients may be tempted to interrupt treatment leading to the survival of resistant subpopulations of the bacteria [105]. This acquired resistance is the reason why compliance is a key factor in reducing the burden of resistant TB [106]. To increase compliance and avoid development of resistance, WHO launched the concept of direct observed treatment short course (DOTS) that between 1995-2010 monitored 55 million TB patients around the world. Among new sputum-positive TB patients who were treated within the DOTS programme in Ethiopia in 2010, the success rate was 84%, similar to worldwide results. Among relapse cases (n=4 898) in Ethiopia 2010, 510 were tested for drug resistance and 121 were found to be MDR TB [13]. Besides improved case finding, DOTS is of major importance since treatment of patients with sensitive strains of TB can increase the MDR TB cases if there is a low compliance [107].
Drug susceptibility testing is also detrimental to stop the TB epidemic, since patients with resistant strains will otherwise be on ineffective treatment and transmission may continue [105]. The use of rapid molecular detection of resistance in smear-positive specimens or culture isolates has shown good accuracy. Mutations in \textit{rpoB}, \textit{katG} and \textit{inhA} are used for rapid PCR-based detection of resistance using the GenoType MTBDrplus assay [108-110]. Another widely spreading technique, because of its point of care strategy, is the Xpert MTB/RIF assay (a PCR-based assay for use in a GeneXpert device). It enables detection of TB and RIF resistance (\textit{rpoB}) on sputum samples without culturing or safety laboratories, facilitating rapid screening for TB and multidrug resistance. However false positive results for RIF resistance in low endemic areas of MDR TB, as well as issues of high running cost have been important concerns [111-113].

The last 20 years MDR TB, then extensively drug-resistant (XDR) TB [114], and recently strains resistant to all TB drugs have emerged [115]. The definition of MDR TB is \textit{M. tuberculosis} resistant to at least INH and RIF, and XDR TB is MDR TB additionally resistant to any fluoroquinolone (levofloxacin, ofloxacin or moxifloxacin) and one of three injectable aminoglycosides (capreomycin, kanamycin, and amikacin) [107]. As soon as INH and RIF cannot be used, the treatment duration will increase from 6 months to at least 18 months [101, 116]. Additionally, with the alternative drugs used in MDR TB and especially XDR TB, the patients are left with insufficient treatment, significant side effects and increased mortality rates, approaching 50-80% in some areas [117].

Even though no new molecule for treating TB have succeeded in reaching the market in the past 40 years there are a number of TB drugs (new or repurposed) in preclinical and clinical development. Bicyclic nitroimidazoles are drug candidates currently in phase II trials for the treatment of tuberculosis [118, 119]. They are prodrugs, and one of the active metabolites generates RNS thought to mediate the major part of the anaerobic effect that has been observed [120, 121].
Vaccination has long been considered the most cost-effective strategy against infectious diseases [122]. In early 1900s, Albert Calmette and Camille Guérin isolated the causative agent of bovine TB, *M. bovis*. At that time *M. bovis* was responsible for about 6% of all human TB deaths in Europe, due to the use of unpasteurised milk [1]. Calmette and Guérin observed that after growing the stain for some time they had selected an avirulent variant, which gave immunological protection also against *M. tuberculosis*. After a decade they had finally developed what we today call the BCG vaccine (bacille Calmette-Guérin) [3]. The loss of the region of difference 1 (RD1) in BCG resulted in its attenuation [123], and also made it possible to use the RD1-encoded 6-kDa early secretory antigenic target (ESAT-6) as an antigen for differentiating between immunity to BCG vaccination and *M. tuberculosis* exposure [124]. BCG is an intradermal vaccine, that it is cheap, safe and that protects children efficiently against the early manifestations of TB [125]. Unfortunately, efficacy of the vaccine against pulmonary TB in adults is highly variable and protection can decline fast. Large and well-controlled vaccine trials have estimated the protection to range from 0 to 80% [1].

When developing new vaccines against TB it must be taken into account that, especially in developing countries, the population can either be infected with *M. tuberculosis* but without symptoms, or be fully naïve. Today, four strategies can be identified in vaccine development: a replacement vaccine for BCG, a booster vaccine, a post-exposure vaccine or a therapeutic vaccine [122]. A newly developed booster vaccine delayed and reduced clinical disease in cynomolgus macaques exposed to *M. tuberculosis*, and also prevented reactivation of latent infection [126]. The vaccine consists of an adjuvant, containing two of the *M. tuberculosis* antigens secreted in the acute phase of infection (Ag85B and ESAT-6), and a nutrient stress-induced antigen (Rv2660c) [127]. Another promising approach is the recombinant live vaccine (VPM1002) based on the properties of listeriolysin O (LLO), a haemolysin that forms pores allowing the vaccine to translocate from the phagosome to the cytosol of the host cell, and thereby induce a strong T cell response [128]. However, no vaccine candidate, that today is in a Phase I or a Phase II trial, is estimated to be available earlier than 2020 [13].
**The genus Mycobacterium**

Mycobacteria show high tolerance to environmental exposures and inhabit various reservoirs such as water, soil, animals and humans and can be both commensals as well as highly successful pathogens, such as *M. tuberculosis*, *M. leprae* and *M. ulcerans* [3]. The *M. tuberculosis* complex includes strains of five species, *M. tuberculosis*, *M. canetti*, *M. africanum*, *M. microti*, and *M. bovis* and the two subspecies, *M. caprae* and *M. pinnipedii* [1, 129]. In contrast to *M. tuberculosis*, with an exclusive tropism for humans, *M. bovis* can cause both bovine and human TB and is the cause of 5%–10% of human TB cases [130]. *M. africanum* is geographically limited to West Africa where it may be responsible for up to 50% of the pulmonary TB cases [131]. There are many different strains of *M. tuberculosis*, but six main lineages associated with different geographical regions have been identified [132]. It is suggested that the Beijing family of strains from Asia, and a strain family called W and W-like are responsible for many drug-resistant cases and clonal clusters [133].

**Mycobacterium tuberculosis**

*M. tuberculosis* is an intracellular pathogen infecting macrophages (figure 5) and other cells, preferentially in tissues with high oxygen tension, such as the lungs [3]. The bacterium is non-motile, rod-shaped, 0.3 to 0.6 μm wide and 1 to 4 μm in length, with a complex cell wall. It is slow growing with a generation time of around 15-20 hours compared to *Escherichia coli* that divides every 20 minutes. The prolonged time to divide and the ability to stay in a latent state are some reasons for the extended treatment duration of both active and latent TB [101].

*M. tuberculosis* grows optimally under aerobe conditions but can switch to dormancy programs and survive under anaerobic conditions as well. Under aerobic growth, ATP is generated by oxidative phosphorylation from electron transport chains, while during anaerobic conditions, alternative electron transport chains are present, such as nitrate reductase [134].
Figure 5. *M. tuberculosis* within macrophages. Confocal microscopy analysis of the main effector cell against *M. tuberculosis*, the macrophage (stained red with rhodamine-phalloidin). Macrophages are designed to kill pathogens, but *M. tuberculosis* (green due to expression of the green fluorescent protein, GFP) can evade the killing mechanisms and instead allow replication within the cells. (*Provided by Johanna Raffetseder, Linköping University.*)

The sequencing of the whole genome of *M. tuberculosis* revealed 4,411,529 base pairs and around 4,000 genes. It differs from other bacteria in the very large coding capacity for the production of enzymes for lipogenesis and lipolysis. These enzymes are essential for lipolysis of lipids from the host cell, for metabolism and bacterial cell wall synthesis of *M. tuberculosis* [135]. The mycobacterial cell wall contains of long-chain fatty acids called mycolic acids, linked to the polysaccharide arabinogalactan that is attached to peptidoglycans [3]. Due to the lipid-rich cell wall, *M. tuberculosis* is nearly impermeable to basic dyes, and therefore described as acid-fast [1]. The low permeability of the cell wall also results in a high degree of intrinsic resistance to antibiotics [135].
Host immunity in tuberculosis

Innate immune responses

Epidemiological and experimental observations indicate effective innate immune mechanisms against *M. tuberculosis*, which can be strong enough to avoid infection or induce a total resolution of the infection in some individuals [136, 137].

Infection with *M. tuberculosis* is caused by inhalation of the bacteria in airborne droplets. Once across the barrier of the epithelium in the airways, alveolar macrophages take up the bacteria. Chemokines produced by infected epithelial cells and immune cells will recruit neutrophils and monocytes to the site of infection [138, 139]. The monocytes will differentiate into macrophages or dendritic cells within the tissue, and dendritic cells will migrate to the draining lymph nodes, where mycobacterial antigens are presented to T cells [1, 140, 141]. Activated T cells will proliferate and migrate into the infected tissue [142]. A strong cytokine response will be mounted, with locally high levels of IFN-γ that activate macrophages to produce antimicrobial substances [17, 137] (figure 6).

Neutrophils, recruited to the site of infection are initially beneficial for the host, but can also cause tissue destruction [144]. In the neutrophil the bacteria are exposed to reactive oxygen species (ROS) and antimicrobial molecules such as lactoferrin, defensins, lipocalin 2 and cathelicidin LL-37 [145, 146]. Neutrophils are present in sputum and BAL from TB patients and can be a niche for replication [147]. Even if neutrophils themselves cannot control the infection, apoptotic neutrophils containing *M. tuberculosis* can activate macrophages and dendritic cells [148, 149].

Subsets of NK cells and cytotoxic T cells (CD8+ T cells) are lymphocytes able to produce granulysin, a small granule-associated peptide that may kill *M. tuberculosis* and can lyse infected cells. Following delivery into the cell through the pore-forming perforin, granulysin binds to the bacterial cell surface, and disrupts the membrane causing osmotic lysis of the bacteria [146, 150].
Figure 6. Immune response in TB. Upon inhalation, *M. tuberculosis* is phagocytosed (1) by resident alveolar macrophages (MΦ) in close vicinity to the small airways. The macrophage may instantly kill the bacteria but in any case secrete pro-inflammatory cytokines (2) to recruit monocytes and neutrophils from the blood stream (3) to the site of infection. In the tissue, monocytes will differentiate into macrophages and dendritic cells (DC). Infected dendritic cells will migrate (4) to a regional lymph node to present mycobacterial antigens to T cells that will proliferate (5), and in turn migrate back into the infected tissue. A strong cytokine response of especially interferon-gamma (IFN-γ) (6) will activate macrophages to produce TNF-α and antimicrobial substances, and as more immune cells are recruited, a granuloma will form [17, 137, 143].
**Within the macrophage**

After phagocytosis, the macrophage phagosome will normally fuse with a lysosome to form a phagolysosome. However, *M. tuberculosis* is known to disturb this process [151]. The phagolysosome is the compartment in which the macrophage can use its effector function and still protect itself and surrounding cells from injury [152] (figure 7).

**Figure 7. Antimicrobial mechanisms within the phagolysosome.** The major defence mechanisms within the macrophage phagolysosome include low pH, reactive oxygen species (ROS) produced by NADPH oxidase, reactive nitrogen species (RNS) produced by inducible nitric oxide synthase (iNOS), iron (Fe³⁺) scavengers and exporters, proteases and antimicrobial peptides such as cathelicidin [152].
Within the phagolysosome, mycobacteria are deprived of essential nutrients such as iron. They are also exposed to anti-bacterial agents such as the vitamin D related peptide, cathelicidin LL-37 [153-155], and to an oxidative burst caused by production of reactive nitrogen species (RNS) and ROS [156]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex reduces molecular oxygen to ROS, toxic for many pathogens. It is found both at the plasma membrane, producing extracellular ROS and within the phagolysosome. The downstream superoxide products like hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl) or hydroxyl radical (OH•) are highly antimicrobial, even though the effect on *M. tuberculosis* is believed to be limited [140, 146, 157].

**T cell mediated response**

Macrophages and dendritic cells present *M. tuberculosis* antigen to T cells and B cells initiating an adaptive immunity with IFN-γ production from T cells and antibody production from B cells [17].

*M. tuberculosis*-specific lymphocytes appear approximately 2–3 weeks post-infection [158]. The fact that HIV-infected individuals, which exhibit decreased number and function of CD4+ T cells and defects in the IFN-γ signalling, are at high risk of TB, strongly suggests a vital role of adaptive immunity in TB [142, 158].

CD4+ T cells can differentiate into T helper 1 (Th1), Th2, Th17 and Tregs depending on stimuli. The Th1 response results in IFN-γ production and activation of macrophages, with subsequent killing of intracellular bacteria by phagolysosomal fusion and effector mechanisms of the macrophage. In contrast, the Th2 response produces B cell stimulating factors (IL-4, IL-5, IL-10 and IL-13) and suppresses the Th1 response [3, 137]. The role of B cells in TB is not clear, but has been recognized as potentially protective in TB [159, 160].

Depending on stimuli from T cells, macrophages polarize to M1 (classically activated) or M2 (alternatively activated) phenotype. M1 polarization is induced by Th1 cytokines and leads to iNOS expression, ROS production and anti-microbial activity. M2 polarization is induced by Th2 cytokines and has regulatory properties with up-regulation of arginase and limited antimicrobial activity [161] (figure 8). Macrophages are also able to switch from one activation state to another depending on stimuli [162].
A recently described subset of T cells, Th17 cells, recruits neutrophils and stimulates defensin production through the cytokines IL-17, IL-21 and IL-22. Both Th1 and Th17 cells can be downregulated by Treg cells, producing TGF-β and IL-10. The precise role of Treg cells in TB has not yet been elucidated, since inhibition of inflammation can both be beneficial and detrimental [137]. Upregulation of Treg cells is found in BAL from latently infected asymptomatic subjects, and may prevent further disease but also hinder complete sterilization [163].

Helminth infection gives a dominant Th2 type immune responses, chronic immune activation as well as up-regulated Treg activity [43]. By deworming before BCG vaccination, T cell proliferation and IFN-γ production in peripheral blood mononuclear cells (PBMC) stimulated with PPD could be increased [164]. Our group is now investigating the effect of deworming TB patients with asymptomatic worm infections, based on the hypothesis that worm infection impairs effective immunity against *M. tuberculosis* (ClinicalTrials.gov Identifier: NCT00857116).

Figure 8. Macrophage polarization. Stimulation of macrophages with IFN-γ, TNF-α or IL-1β will lead to the M1 phenotype, with up-regulation of iNOS, NO production and antimicrobial activity. If stimulated with IL-4, IL-10 or TGF-β the phenotype will be a M2, with increased arginase activity beneficial for tissue repair but not for bacterial killing [161, 165].
The granuloma

The granuloma is the classical hallmark of TB. Initially thought to be exclusively beneficial to the host, the role of the granuloma is now found to be more complex [166]. A granuloma is constantly remodelled, due to the balance between pro- and anti-inflammatory immune signals at the site of infection [158, 167, 168]. Varying proportions of macrophages, multinucleated giant cells, foamy macrophages, epithelioid macrophages, dendritic cells, T cells, B cells and neutrophils can be found within a granuloma, that can measure from just a millimetre to > 2 cm [167] (figure 9) [143, 151]. At the onset of adaptive immunity, more cells are recruited to the site of infection, extensively vascularised for this purpose. The granuloma then acquires a more organized, stratified structure with a macrophage-rich centre surrounded by lymphocytes that in turn may be covered with a fibrous cuff [158]. The classically described caseous granuloma indicates a central necrotic region rich in foamy macrophages [167] that may become hypoxic and induce a non-replicative state of the bacteria [169].

Figure 9. The granuloma. The recruitment of immune cells to the site of infection leads to the formation of a granuloma with varying cellular composition. To the left is a granuloma from a patient with skin TB, showing a central accumulation of macrophages, surrounded by a border of lymphocytes. To the right is a schematic illustration including macrophages, foamy macrophages, neutrophils and dendritic cells, both infected and uninfected, surrounded by lymphocytes and a fibrous cuff. [17, 143, 151, 167] (Left image provided by Thomas Schön, Linköping University and Kalmar County Hospital.)
At old age, malnutrition, or in advanced HIV co-infection, containment of the infection within the granuloma may fail and the capsule will rupture. Such extensive pathology may spill bacilli into the airways resulting in transmission to new hosts [17]. In early HIV infection, granulomas are still formed, but are found to be less organized as HIV proceeds [1]. The granulomas in a TB patient are a perfect environment for HIV to spread to uninfected immune cells [170]. *M. tuberculosis* itself also takes advantage of the high cell recruitment to the site of infection and infects new cells, and the mounted immune response can turn out to be favourable for both *M. tuberculosis* and HIV [170, 171].

It is difficult to study the phenomena of cavities during TB infection in humans, since different species present a variety of responses. Cattle and guinea pigs form caseous granulomas, but no cavities are seen in rabbits and non-human primates. Murine models (the most common *in vivo* model for TB) only present granulomatous necrosis and fibrosis, but in all species the granuloma formation is dependent on a cell-mediated response [172].
Nitric oxide

Biochemistry

In 1992, the gas nitric oxide (NO) was crowned as “molecule of the year” by Science magazine, due to the finding that NO was an endothelium-derived relaxing factor, a mediator of immune responses, a neurotransmitter, a cytotoxic free radical, and a signalling molecule [173]. Nitric oxide (NO) is a small (30 Da) free radical, with diverse and opposing biological activities. It is short-lived (lifetime of 1-1000 ms) and will quickly react with superoxide (O$_2^-$) to form peroxynitrite (ONOO$^-$). NO is soluble in both aqueous and hydrophobic environments, and passes freely across cell membranes at a speed of 5-10 cell lengths in one second [174].

NO is mainly generated by nitric oxide synthases (NOS). These large and complicated enzymes catalyse the oxidation of L-arginine to NO and L-citrulline in a NADPH- and O$_2$- dependent manner, in the presence of five cofactors (haem, tetrahydrobiopterin (BH$_4$), flavin mononucleotide (FAD), flavin adenine dinucleotide (FMN) and Ca$^{2+}$-calmodulin [175] (figure 10). In absence of L-arginine, NOS instead acts as a superoxide generator [176].

![Figure 10. Nitric oxide generation. Activated nitric oxide synthase (NOS) catalyses the formation of NO and L-citrulline from L-arginine in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) oxygen (O$_2$) and cofactors [175].](image-url)
The amount and profile of NO production varies widely depending on from which of the three NOS isoforms it is produced. Two of the isoforms are constitutive (NOS1 or neuronal, nNOS and NOS3 or endothelial, eNOS) and one is inducible (NOS2 or iNOS) [177], although eNOS is also able to change to an inducible form [178]. The main differences between the isoforms are duration and magnitude of the production. The constitutive enzymes can produce short bursts of NO while iNOS is permanently activated and can produce NO, for a prolonged period [174]. If cofactors are available, production of NO in the microenvironment depends on the presence of L-arginine and O2, localization of the NOS within the cell, arginase activity and other consumptive mechanisms, such as presence of ROS, interaction with red blood cells, and cellular metabolism [174].

Nitrite and nitrate were long thought to be end products of NO metabolism, but have been shown to be able to be recycled back to NO under certain circumstances. If both L-arginine and O2 are absent, NO can be produced by the acidification or reduction of nitrite, that in turn can be produced by reduction of nitrate [179].

**Nitric oxide in health and disease**

A lot of attention has been given NO in diverse aspects of health and disease. RNS such as NO, nitrite, nitrate, peroxynitrite, S-nitrosothiols, nitrated fatty acids and N-nitrosamines are continuously formed in the host. Effects range from important signalling events in the function of regulatory proteins, cell survival and cell proliferation to toxic effects [174, 175, 179, 180]. RNS target crucial enzymes by reacting with haem, iron-sulphur (Fe-S) clusters, cysteine and tyrosine residues, or with key metabolites such as the thiols in cysteine and glutathione [165, 181].

The in vivo concentration of NO can vary from basal levels produced by epithelial cells (< 2 nM) to that of an activated macrophage (> 1 μM) [174]. At lower concentrations, NO has anti-inflammatory properties and modulate T cell functions, whereas high concentrations of NO results in bacterial killing, T cell dysfunction as well as tissue injury [182]. In macrophages, iNOS mediated NO production is enhanced by IFN-γ, TNF-α, bacterial lipopolysaccharides (LPS), IL-1β, IL-6, and IL-17, whereas transforming growth factor beta (TGF-β), IL-4, IL-10, IL-11, and IL-13 suppress the induction of iNOS in macrophages [165, 183].
In vivo production of NO can be identified by the presence of tyrosine nitration and the relative stable metabolites of NO, nitrite and nitrate [180]. Endogenously produced NO will in most situations react with red blood cells, be reduced to nitrate, transported to the kidneys and excreted in the urine as such [184, 185]. Infected urine may contain considerable amounts of nitrite as a result of bacterial nitrate reductase activity, and detection of nitrite in urine is routinely used in the diagnosis of bacterial cystitis [186]. Acidification of urine results in NO production from nitrite, which might explain why urinary acidification is effective in the treatment of bacteriuria [187].

Nitric oxide can also be measured in exhaled air and is formed both in the upper and lower airways [188-190]. Asthma [191], viral respiratory tract infections [192] and exposure to dust [193] are associated with high levels of exhaled NO. Decreased levels of exhaled NO are seen in patients with HIV [194] and cystic fibrosis [195]. Cigarette smoking results in reduced levels of exhaled NO [196], but normalises after smoking cessation [197], and could be part of the explanation of the increased risk of respiratory tract infections and chronic airway disease seen in smokers [198, 199].

Low levels of the substrate for NO production, L-arginine, are found in patients with TB [200, 201], malaria [202] and sepsis [73]. Clinical studies on administration of intravenous or inhaled L-arginine have shown tumour reduction in breast cancer [203] improved endothelial function in malaria, reduced symptoms in intermittent claudicatio, and improved pulmonary function in cystic fibrosis [204, 205]. A diet with low L-arginine levels may impair NO synthesis [206] and L-arginine supplementation is popular in the same way as nitrate, for improving physical strength and to potentate sexual performance [179, 207, 208].

Nitric oxide in tuberculosis

In murine models it has been shown that NO is essential for host defence against *M. tuberculosis*, but the role of NO has not been fully established in man [209-211]. The antimycobacterial effects of RNS were first shown experimentally in murine macrophages infected with mycobacteria [212, 213]. Failure to express iNOS results in susceptibility to TB, rapid proliferation of *M. tuberculosis* and early death in iNOS -/- mutated mice [211, 214-216]. Murine latent TB infection can reactivate following treatment with an iNOS inhibitor [215], and upregulation of iNOS can on the other hand result in accelerated clearance of bacillary load [217].
At the site of TB infection in humans, the presence of iNOS and nitrotyrosine in macrophages has been shown as indicators for NO production [218, 219] (figure 11). Alveolar macrophages [220-222] as well as peripheral monocytes [223] from TB patients express iNOS to a higher extent than controls. Levels of nitrite, IL-1β and TNF-α were also elevated from alveolar macrophages of TB patients compared to normal subjects, and a high production of nitrite from alveolar macrophages was associated with increased resolution of disease [224]. Increased levels of NO in exhaled air, and NO metabolites in urine from TB patients indicate that the human immune defence is partly dependent on NO production in the control of *M. tuberculosis* [221, 222].

![Figure 11. Immunohistochemistry analysis of tissue samples from patients with TB. A caseous granuloma (A), with surrounding inducible nitric oxide synthase (iNOS)-positive macrophages (brown colour), from a patient with pleural TB. Alveolar macrophages (B) stain positive for nitrotyrosine in a lung biopsy from a Swedish patient with pulmonary TB. (Provided by Thomas Schön, Linköping University and Kalmar County Hospital.)](image)
Survival strategies of *M. tuberculosis*

*M. tuberculosis* has evolved to escape immune mechanisms and actually survives within macrophages [225]. Mycobacteria owe their success to their ability to persist, without symptoms, within the host for prolonged times, in an assumed viable but non-replicating (dormant) state. Upon immune suppression they are able to replicate and cause disease [226].

Mycobacteria have been considered non-sporulating but recent findings suggest that *M. marinum* and likely also *M. bovis* bacillus Calmette-Guérin can form spores as an adaptation to a stressful environment [227, 228]. Other virulence properties of *M. tuberculosis* include the ability to inhibit apoptosis, and induce necrotic cell death in neutrophils and macrophages [229, 230]. In this way the bacteria escape the cells, evade the host defences, and spread to uninfected cells [231] (figure 12).

![Figure 12. *M. tuberculosis* trafficking within the macrophage.](image)

During an effective immune response (left), phagocytosis of bacteria will lead to the formation of a phagosome followed by acidification and several maturation steps. Finally the phagosome will fuse with lysosomes to form a phagolysosome with a wide range of antimicrobial properties. However, *M. tuberculosis* can survive (right) by preventing phagosomal maturation, acidification and fusion with the lysosomes, or may escape into the cytosol [143, 151, 236].

By modulation of the inflammatory signal and by inhibition of phagosome-lysosome fusion, *M. tuberculosis* survives within macrophages [156, 232]. Attached to the cell wall of *M. tuberculosis* are lipoglycans including lipoarabinomannan (LAM) involved in this process [233]. ESAT-6 is a virulence factor, encoded by RD1 found in several mycobacterial species [234]. The virulence mechanisms for ESAT-6 include plasma and phagosomal membrane lysis that release the bacteria from the phagosome into the cytosol [151, 235, 236].
To survive within the phagosome *M. tuberculosis* also inhibits transportation of iNOS to the phagosome [237] as well as inhibits recruitment of H⁺-ATPase and acidification of the phagosome, retaining a suitable pH [152]. *M. tuberculosis* also carries scavenger functions against ROS and RNS in order not to face decreased growth rate, due to enzyme damage or nutrient depletion [181, 238-240]. Pathogenic mycobacteria are inherently more resistant than non-pathogenic to RNS and have several antioxidant systems [241-243] and clinical strains vary in susceptibility to NO produced by acidified nitrite [241, 244, 245]. The catalase peroxidase (*katG*), the alkyl hydroperoxide reductase subunit C (*ahpC*), superoxide dismutase (SOD), haem proteins, bacterial proteases, thioredoxin and lipoamide dehydrogenase (Lpd) can all scavenge ROS and RNS [246-251] (figure 13).

**Figure 13. Survival strategies of *M. tuberculosis*.** Bacterial defence mechanisms against the host immune response include inhibition of phagosomal acidification, bacterial proteases, modification of the bacterial cell wall to resist antimicrobial peptides, inhibition of the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as scavenger mechanisms (antioxidants) to protect DNA and proteins from RNS and ROS [152].
Aims

The overall aim of this thesis was to investigate the role of NO in host defence against *M. tuberculosis* by combining clinical and experimental studies.

In the clinical setting our first aim was to investigate local and systemic levels of NO during active pulmonary TB, and in household contacts to such infectious patients.

Our second aim was to assess the effect of an arginine-rich food supplement on NO-production and clinical outcome in pulmonary TB patients during standard treatment.

Our final aim was to investigate the potential variability of clinical strains of *M. tuberculosis* in susceptibility to NO, and if this variability correlated to antibiotic resistance and ability of macrophages to control the infection, or to clinical manifestations and treatment response in TB patients,
A voice of TB

“Before the diagnosis I suffered from chest pain, loss of appetite and I lost weight. When I heard I had TB I was afraid to transmit it to the rest of my family. I was very sad and wondered how I got this disease. I could not work properly because I was weak. When I came to the health office I saw more patients with TB. I felt comfort that I would recover, and now I’m not afraid of the disease.”

Demeku, 40-year-old, housewife
Materials and methods

Study site

Gondar College of Medicine, northern Ethiopia (figure 14), was the study site for the clinical part of this thesis. With a population of approximately 83 million (2011), Ethiopia has the second largest population in Africa. Apart from a few years occupation by Italy during the Second World War, Ethiopia has never been colonised and today owns a rich and well-preserved culture [252].

Figure 14. The Castle of Fasilades, Gondar town (left) and Simien Mountains National Park (right), northern Ethiopia. (Photo by Jonna Idh.)

Gondar College of Medicine and Health Sciences, University of Gondar Hospital is a referral hospital with 400 beds, serving approximately 6 million people and plays an important role in teaching students within the medical field since decades.

HIV treatment has been available in Gondar since March 2005, profoundly affecting the outcome of many patients including TB patients. In 2010, a TB isolation and treatment ward, with 28 beds capacity, was built to improve the hospital TB infection control. The need is much greater than the present beds available, but capacity building activities are noted, including a new hospital construction with 400 beds.

The work with \textit{M. tuberculosis} was taken place in BSL3 facilities at Armauer Hansen Research Institute (AHRI), Addis Ababa, Ethiopia (Paper III) and at Linköping University Hospital, Sweden (Paper IV).
Study subjects (Paper I-III)

Inclusion of TB patients and recruitment of household contacts took place at the DOTS clinic within the Gondar Hospital, Ethiopia. Only outpatients fulfilling the inclusion criteria of newly diagnosed sputum smear positive TB, age between 15 and 60, and willingness to take part in the study were included (ClinicalTrials.gov identifier: NCT00857402). In total 317 TB patients were eligible for inclusion (Paper I and II). Of the first 111 eligible patients, 95 were included in Paper I. In total 180 patients were included in Paper II. Smear-positive TB was defined as 2 of 3 morning sputum samples positive, or 1 of 3 positive with a chest X-ray and clinical symptoms, suggestive of active pulmonary TB. Exclusion criteria were admission to hospital, peanut allergy, pregnancy, previous treatment for TB, clinical signs or medical treatment indicating any concomitant disease other than TB and HIV. Household contacts (n=21) were defined as persons above 15 years of age, who continuously spend more than 12 hours per day together with a TB patient. The control group (n=63) was recruited from the blood bank, and as for household contacts, the inclusion criteria were no symptoms of TB and a chest X-ray without evidence of TB. Exclusion criteria were acute or chronic disease, smoking and antibiotic or corticosteroid treatment.

Chemotherapy and nutritional supplementation (Paper II)

The chemotherapy consisted of INH, PZA, RIF and ethambutol (EMB) during the intensive phase of 2 months, followed by INH and EMB for an additional 6 months. At initiation of TB treatment, patients were randomised (in blocks of six) to supplementation with 30 g of wheat crackers called “dabqolo” (0.1 g L-arginine, 623 kJ) or 30 g peanuts (1 g L-arginine, 750 kJ) daily for four weeks. Intake of all drugs and food supplementation was supervised daily. The final outcome following treatment at eight months was registered according to the WHO classification as cured, died, defaulted, treatment failure or transferred out [253].

Follow-up

AFB status by microscopy, erythrocyte sedimentation rate (SR or ESR), BMI, as well as clinical symptoms such as haemoptysis, cough, and fever were monitored from the DOTS centre throughout the treatment.
Chest X-ray

Chest X-ray grading was done according to the National Tuberculosis Association of the USA in normal, minimal, moderately advanced and far advanced TB [254] and during follow-up, the radiological response was classified as marked regression, regression, no change or progress. The same radiologist, blinded for HIV status and treatment assignment, read all chest X-rays.

Laboratory analyses (Paper II and III)

The HIV status of the TB patients was analysed with Enzygnost Anti-HIV 1/2 Plus (DADE BEHRING, Germany) and confirmed with Vironistika HIV Uni-Form II ag/ab (Biomérieux, France). Serum levels of TNF-α, IL-10 and IL-12 were analysed using commercial ELISA kits according to the instructions from the manufacturer. L-arginine was analysed by high-performance liquid chromatography (HPLC) using a modified version of the protocol described by Carlberg [255].

Measurement nitric oxide in exhaled air and nitric oxide metabolites in urine

NO in exhaled air was assessed using a chemiluminescence NO analyser (NIOX, Aerocrine AB, Sweden), approved by the FDA [256] at a flow rate between 45–55 ml/s during 10 s with dead space-time set to 0.5 s. The average of three measurements with the plateau concentration within 2.5 ppb (parts per billion) or 10% was recorded. Urinary nitrite and nitrate was measured with the Griess reaction after conversion of nitrate to nitrite by nitrate reductase in the presence of NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase, as described by Verdon et al [257].

Drug susceptibility testing and spoligotyping of M. tuberculosis (Paper III)

Sputum specimens (n=50) from patients with active TB were processed and inoculated on Lowenstein Jensen (LJ) slant media. M. tuberculosis was identified by a PCR-based approach built on genomic deletion analysis as described elsewhere [258]. Drug susceptibility testing for SM (2 mg/L), INH (1 mg/L), EMB (5 mg/L) and RIF (1 mg/L) was performed using the WHO-recommended indirect proportion method on LJ media [258-260]. Spoligotyping was performed with the oligonucleotides DRa and DRb to amplify the direct repeat (DR) regions [261].
**Susceptibility testing to nitric oxide (Paper III)**

Clinical isolates cultured on LJ medium were transferred to Middlebrook 7H9 broth and grown until early log phase. A final concentration of $10^7$ CFU/mL of bacteria was exposed in duplicates to 1 mM diethylenetriamine NONOate (DETA/NO) or to PBS (used as control). The antimicrobial effect of DETA/NO was determined by viable count (CFUs) in tenfold dilution on Middlebrook 7H10 plates.

**Luciferase-based viability assay (Paper IV)**

To assess viability, a luciferase-based method has proven useful for *M. tuberculosis* [262]. In principle, live bacteria carrying the pSMT1-plasmid encoding a hygromycin-containing gene for selection and the gene for *Vibrio harveyi* luciferase, will emit flash luminescence upon addition of the substrate n-decanal. The amount of light emitted can be measured by a luminometer (GloMax® Multi-Detection System, Promega). For this purpose 7 clinical strains of *M. tuberculosis* were transformed with the pSMT1-plasmid prepared from recombinant *E. coli* DH5a as described by Garbe [263]. In addition, H37Rv harbouring the pSMT1-plasmid was cultured on INH- and hygromycin-containing plates to select an INH resistant mutant. All strains carrying the pSMT1-plasmid were then analysed for the presence or absence of resistance mutations in *rpoB, katG* and *inhA* by GenoType MTBDRplus® (Hain Lifescience) according to the instructions from the manufacturer. At a final concentration of $10^7$ CFU/mL, strains were exposed to DETA/NO or peroxynitrite (3-morpholinosydnonimine or SIN-1 chloride) (0.1, 1 and 10 mM), with PBS used as the unexposed control.

**Infection of macrophages (Paper IV)**

Murine macrophages (RAW 264.7; American Type Collection) were prepared for infection by seeding in 96-well plates (25 000 cells/well) with or without stimulation (IFN-γ and LPS) 5 hours prior to infection. The macrophages were infected in triplicates in the 96-well plates at a multiplicity of infection (MOI) of 5. Bacterial uptake was assessed by luminometry after one hour of infection. Extracellular and intracellular growth was measured after two days, the latter after lysis of the cells with distilled H₂O. Nitrite and nitrate was measured in the supernatants (as previously described) to detect activation of the macrophages and subsequent NO production.
Statistics (Paper I-IV)

In Paper I data are presented as median and interquartile range (25–75%). To compare groups the Mann-Whitney U-test was used and correlations were tested with Pearson’s correlation test (r2). In Paper II effects of peanut supplementation, compared to daboqolo, were evaluated by Chi-square test or Fisher’s exact test for discrete variables and Students t-test for continuous variables. Continuous data in Paper II are expressed as means with 95% confidence intervals. Kinetics of exhaled NO was evaluated by repeated measures ANOVA. The experimental data in paper III are presented as median and range or first and third quartiles (Q1-Q3) if not stated otherwise. Numerical data were analysed with Mann-Whitney U-test, discrete data with Chi-square test and Fisher’s exact test. Multiple logistic regression analysis with a stepwise correction was applied on variables with a p-value less than 0.1 in the univariate analysis. In Paper IV, Students t-test was used for comparisons of two groups, a one-way ANOVA for comparison of multiple groups and a two-way ANOVA for comparison of multiple groups with different stimulations. A p-value of $\leq 0.05$ was regarded as statistically significant.

Ethical consideration

All patients gave oral and written consent prior to inclusion. For children below 18 years, informed and written consent was obtained from parents or guardians. The study including Paper I, II and III was approved by the Ethics Committees at Gondar College of Medical Sciences, Ethiopia (RPO/252/2005, GCMS), Ethiopian Science and Technology Commission (RDHE/5-57/04, ESTC) and Karolinska Hospital, Stockholm, Sweden (KIFEK: 03-331). For the sake of capacity building all laboratory analyses were initiated and performed at Gondar College of Medicine (Paper I and II).

All patients included were treated according to the DOTS program [253] with addition of a food-supplement rich in L-arginine (peanuts) or a wheat-based snack (daboqolo). L-arginine has no known side effects in humans, but peanuts can cause allergic reactions and all study subjects were asked about known allergy to peanuts before entering the study.
At the time of the study initiation, no ART (anti-retroviral therapy) was available in government health services in Ethiopia, and none of the study participants received ART until March 2005, when it became available at Gondar University Hospital. According to the hospital routines the TB nurse and responsible doctor followed all patients. TB patients and blood donors were offered pre- and post-test counselling, as recommended by WHO prior to HIV testing.

There was no additional cost or economic incentive for the patient participating in the study. Measurement of exhaled NO is a non-invasive and simple procedure with little discomfort. The inconveniences for the patients regarding blood and urine sample collection were regarded as small and in general patients found the close monitoring beneficial.
A voice of TB

I had cough and high fever and I thought that I had TB. I think that I got the disease as I was graining seeds at the mill. I am now unable to work and I have to stay at home. I get food from money that I have saved. I have one child and we stay in different rooms in the house, and the rooms are well ventilated. We don’t sleep together. My husband is not afraid to get TB, but I have my own cup and personal things. TB is a difficult disease, but if you get an early diagnose, you will get well after treatment.

Mitikie, 23-year-old, daily labourer
Results and discussion

Systemic levels of nitric oxide in pulmonary TB

To address the question of NO production during TB infection, we measured the NO metabolites nitrite and nitrate in urine. We analysed the data with respect to HIV status, since HIV per se is known to affect NO production. We found that HIV-negative TB patients had significantly lower levels of nitrite and nitrate in urine than controls, and that household contacts to TB patients had the highest levels (Paper I). Healthy blood donors also had higher levels of nitrite and nitrate in urine, indicating that effective immune responses with NO production might be protective against infection and active disease (figure 15).

Figure 15. Levels of urinary nitrite and nitrate. HIV-negative TB patients (HIV-/TB, n=58), HIV-positive TB patients (HIV+/TB, n=35), household contacts to smear-positive TB patients (HC, n=12) and blood donors (BD, n=45). Data are presented as median and interquartile range. * (p < 0.05).

Levels of nitrite and nitrate in urine from TB patients were previously found to be elevated [222]. This discordance could partly be explained by higher levels of urinary nitrite and nitrate in the control group in the present study. The significant increase in urinary nitrite and nitrate found in HIV-positive compared to HIV-negative TB patients is in line with the finding that HIV infection increases systemic production of NO [264, 265], and that macrophages infected with HIV express iNOS and produce NO [266].

There can be profound effects of diet on urinary levels of nitric oxide metabolites [267]. After a nitrate-rich meal, urinary levels return to baseline values within 24 hours with a maximum concentration at 4-6 hours after intake [268, 269], leading to between-day variations [267]. Approximately 65-70% of the dietary nitrate is excreted in the urine [268] with some loss in
faeces and sweat [185]. We have tried to limit the influence of diet on our results, by adapting an overnight fast and collecting the first morning urine. Although high outliers in nitrite and nitrate may be due to intake of a nitrate-rich meal, it is unlikely to introduce significant bias on comparisons at the group level.

**Local levels of nitric oxide in pulmonary TB**

In order to more accurately assess the NO production from the site of infection in pulmonary TB, we measured NO in exhaled air and found that HIV-positive TB patients had significantly lower levels of NO in exhaled air compared to healthy household contacts and controls. The same tendency was seen in HIV-negative TB patients (figure 16). This finding is well in line with our hypothesis that NO is essential for protection against *M. tuberculosis* infection.

![Figure 16. Levels of exhaled NO (FeNO). HIV-negative TB patients (HIV-/TB, n=59), HIV-positive TB patients (HIV+/TB, n=36), household contacts to smear positive TB patients (HC, n=17) and blood donors (BD, n=46). Data are presented as median and interquartile range. ppb (parts per billion). * (p < 0.05).](image)

In contrast to our finding, Wang et al [221] previously showed that pulmonary TB patients presented higher levels of exhaled NO compared to healthy controls. In the study by Wang et al, patients with poor nutritional status were excluded to avoid the influence of poor nutrition on immunity. In our study population, HIV-positive as well as HIV-negative TB patients had a median BMI of 16 kg/m² with an estimated weight loss of 5-6 kg. Hence, they were in a more deteriorated nutritional status, supporting the link between immune defence and nutritional status. Wang et al also found that alveolar macrophages from patients with more extensive disease had a lesser capacity to generate NO. This is in line with our finding that
patients with lower urinary nitrite and nitrate (< 1000 µM) had significantly higher levels of IL-10 (known to downregulate iNOS) [270, 271], compared to patients with higher urinary nitrite and nitrate (> 1000 µM) (Paper II).

Lower levels of nasal [272] and oral [194] exhaled NO have previously been shown in HIV patients without TB, supporting the effect of HIV infection on the immune system’s capacity to generate sufficient levels of NO in the lungs during TB infection. In line with this, there was a higher rate of HIV-negative TB patients with exhaled NO > 25 ppb even though there was no difference in median levels of exhaled NO between HIV-positive and HIV-negative TB patients. There was also a trend towards higher levels of the pro-inflammatory cytokine, IL-12 in HIV-negative patients (Paper I). Considering NO as protective against TB, this supports the fact that HIV-positive individuals are at higher risk of *M. tuberculosis* infection, or reactivation of infection, than HIV-negative individuals [1].

As described by Olivieri et al [273], it is difficult to determine reference values of exhaled NO for a healthy population, since there are several factors affecting the measurable levels, although a range from 2.6 to 28.8 ppb in men, and from 1.6 to 21.5 ppb in women has been suggested [274]. Levels of exhaled NO reported in Paper I and II are within this range, even though a well-defined reference range is more important in diagnostics or prognostics. In measurements of exhaled NO we used a flow rate of 45–55 ml/s, which is recommended for asthma [256]. As pulmonary TB infection is mainly localized to the lung parenchyma, a higher expiratory flow rate [190, 275] may be needed to measure NO production optimally in TB patients, although this might prove difficult in clinical practice since few patients may be able to comply with such measurements in the acute stage of the disease.

*Treating TB with peanuts – are you nuts?*

Malnutrition can lead to increased susceptibility to infections [54] and is known to be associated with TB [57]. To further investigate the cause of low NO levels during TB, we measured the precursor L-arginine in plasma from TB patients. We found no difference in levels of plasma L-arginine between HIV-positive and HIV-negative TB patients, but all TB patients had lower levels compared to a healthy cohort in the same area (60 µM vs. 119 µM) [200]. The same was observed by Zea et al [201], where TB patients had lower levels of plasma L-arginine than healthy controls.
Even though the contribution of dietary supplement must be seen in the perspective of the effective TB-drugs available [58, 75], supplementation of nutrients may shorten duration of treatment, as well as increase recovery in TB patients [54]. The hypothesis that supplementation of L-arginine may be beneficial for TB patients had previously been investigated by our group. Patients were then randomised to L-arginine or placebo and a significant improvement (shorter duration of cough, increased weight gain and higher sputum conversion rate) was seen in HIV-negative TB patients [276], thus we now wanted to investigate the effect of a food supplement rich in L-arginine in addition to the standard TB treatment.

Peanuts are known to be rich in L-arginine and are locally available at the study site in Gondar, and were therefore used as the arginine-rich supplement. As a control we chose another locally available snack of roasted wheat crackers called “daboqolo”. Our primary outcome was cure rate, but as previously described, the effect of TB drugs is strong, with a high cure rate by itself. Secondary outcomes were chosen to reflect a faster improvement of symptoms and subsequently, an improved immune status. No effect of the arginine-rich food was found either in primary or secondary outcome, but based on our previous study on L-arginine supplementation [276], and other studies on micronutrient supplementation [67, 71], HIV status is likely to have an impact. In the subgroup analysis of HIV status, HIV-positive TB patients receiving the food supplement rich in L-arginine showed a cure rate of 84% (31/37) compared to 53% (17/32) among the patients randomised to the low L-arginine supplement (table 1).

In the previous arginine study, improvement of L-arginine was seen in the HIV-negative TB patients instead of the HIV-positive TB patients. In the present study all patients received nutritional supplementation and to elucidate the impact of the increased food intake itself, we compared our findings to the follow-up of 60 smear positive TB patients, who did not receive any food supplementation, and observed that the food supplementation per se had an overall effect on sedimentation rate and the number of treatment failures.
Table 1. Effects of high (peanuts) versus low (daboqolo) L-arginine-containing food on clinical outcome in TB patients.

<table>
<thead>
<tr>
<th>Primary outcome (8 months)</th>
<th>Peanuts</th>
<th>Daboqolo</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cured</td>
<td>83.8 (31)</td>
<td>53.1 (17)</td>
<td>0.006</td>
</tr>
<tr>
<td>Not cured</td>
<td>16.2 (6)</td>
<td>46.9 (15)</td>
<td>0.005</td>
</tr>
<tr>
<td>Treatment failure</td>
<td>5.4 (2)</td>
<td>12.5 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>Defaulted</td>
<td>2.7 (1)</td>
<td>15.6 (5)</td>
<td>0.07</td>
</tr>
<tr>
<td>Died</td>
<td>8.1 (3)</td>
<td>15.6 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>Transfered out</td>
<td>0.0</td>
<td>3.1 (1)</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary outcomes</th>
<th>Peanuts</th>
<th>Daboqolo</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum smear conversion, 2m</td>
<td>88.9 (32)</td>
<td>86.7 (26)</td>
<td>NS</td>
</tr>
<tr>
<td>Weight &gt; 10 %, 2 m</td>
<td>44.4 (16)</td>
<td>20.7 (6)</td>
<td>0.05</td>
</tr>
<tr>
<td>Presence of cough, 2 m</td>
<td>50.1 (18)</td>
<td>25.2 (16)</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean DBO (mm/h), 2 m</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NR (mm/h), 2 m</td>
<td>57.9</td>
<td>60.0</td>
<td>NS</td>
</tr>
<tr>
<td>CXR improvement, 2 m</td>
<td>1.9</td>
<td>1.9</td>
<td>NS</td>
</tr>
<tr>
<td>eNO (ppb), 2 m</td>
<td>17.3</td>
<td>18.3</td>
<td>NS</td>
</tr>
<tr>
<td>uNOx (M), 2 m</td>
<td>21.5</td>
<td>21.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Wheat in “daboqolo” is rich in the amino acids glutamine and glutamate, which can be converted to L-citrulline in an intact intestinal mucosa, and in the kidney further to L-arginine [206]. It is possible that this is what happens in HIV-negative TB patients, thereby masking the effect of the arginine-rich food in this group. However in HIV-positive TB patients, enteropathy with reduced levels of serum citrulline is common [277], indicating a reduced conversion of glutamine in the intestine, thereby explaining the improvement seen after arginine-rich food supplement in this sub-group.

Nitric oxide – does it kill the bug?

Even though the host may be capable of fast and high production of NO, there is evidence that *M. tuberculosis* can resist the toxic effects of RNS and ROS [239]. The influence of strain variation on the outcome of infection with *M. tuberculosis* is an emerging area of research [278]. Identifying such resistance mechanisms, directed towards the immune responses, is of great interest when finding new drug targets and vaccines. To investigate this we cultured clinical sputum specimens from pulmonary TB patients, and exposed each strain to NO *in vitro* (Paper III).
All the isolates were identified as *M. tuberculosis* by PCR and by spoligotyping. Nine known clusters and 4 unique clusters were identified. In fifty clinical strains exposed to NO there was a time and dose-dependant susceptibility to NO. The median survival after 24 hours exposure to 1 mM DETA/NO, was 10% (range 0-60%) (figure 17). Even though dormancy regions are upregulated after NO exposure [240], suggesting that NO rather has a bacteriostatic than a bactericidal effect on *M. tuberculosis*, no viable bacteria following exposure to 10 mM DETA/NO were found even after 8 weeks of culture on solid media.

**Figure 17.** Reduced NO susceptibility in spoligotype-based clusters of *M. tuberculosis*. Survival of clinical isolates 24 hours after exposure to the NO donor DETA/NO. Presence of resistance to first-line TB drugs is indicated with circles; isoniazid (INH-R), streptomycin (SM-R) and rifampin (RIF-R). Each point represents a mean value of duplicates and the dashed line is the median survival of all 50 isolates.

**Nitric oxide resistance – from bench to bedside**

We hypothesised that with equal capacity to produce NO, a host infected with a NO tolerant strain of *M. tuberculosis* would present a more severe disease or slower recovery compared to a host infected with a NO susceptible strain.

We found no significant correlation between susceptibility of the strains to NO and treatment outcome or sputum conversion, but the four patients who did not increase in weight were all infected with NO tolerant strains. Three of these were HIV-positive, and HIV co-infection is known to be associated with weight loss in TB patients [95], although there was no significant
difference in rate of weight gain between HIV-negative and HIV-positive TB patients in the present study. In conclusion, we found no convincing impact of the level of NO tolerance of the infecting strain on treatment response, but to be able to adjust for factors such as HIV infection and antibiotic resistance larger sample sizes are needed.

**Antibiotic resistance associated to nitric oxide susceptibility – a novel finding**

Resistance to first-line TB drugs (INH resistance (4/50), SM resistance (3/50) and RIF resistance (1/50)) were distributed over 6 spoligotype-based clusters (figure 17). There was a clear association between NO tolerance and resistance to first-line TB drugs, INH in particular. INH-resistant strains showed a median survival of 53% (Q1-Q3, 32-57%) when exposed to NO compared to 10% (Q1-Q3, 10-50%, \( p=0.006 \)) in INH-susceptible strains.

Intrigued by the finding that reduced NO susceptibility could be linked to antibiotic resistance, and in particular to INH resistance, we used a newly developed model with luciferase-expressing *M. tuberculosis* to study bacterial viability upon NO exposure (Paper IV). This model avoids time-consuming culturing, which may take several weeks before reaching results, and enables direct measurement of viable *M. tuberculosis* [262]. Five INH-resistant and 2 fully susceptible clinical strains as well as H37Rv were transformed with the luciferase-expressing plasmid and exposed to NO. Again INH-resistant strains showed a trend towards increased tolerance to NO (figure 18). An important observation was also the variability in NO susceptibility among clinical isolates, and an increased tolerance to NO in some of the clinical strains compared to H37Rv. This indicates that this laboratory strain, widely used in TB research, may not reflect the phenotype of clinical isolates in terms of NO susceptibility.

Since NO readily reacts with superoxide to produce peroxynitrite, we also exposed the strains to this reactive compound (SIN-1). In our system NO was relatively more toxic than peroxynitrite, although the comparison is difficult to fully assess in our experimental system, considering the differences in chemical features and time of donation by the two compounds. The half-life of the NO donor used is approximately 20 hours [279], while the peroxynitrite generator will give a short burst of peroxynitrite for about 15-30 min [280]. *M. tuberculosis* will thereby be exposed to NO over a longer period, probably more effective than a short exposure time to SIN-1.
Among the 50 isolates from Ethiopian TB patients in paper III, there is no data on the exact mutations responsible for antibiotic resistance, but the most common gene involved in INH resistance in Ethiopia is katG [281]. Among the 7 luciferase-expressing strains, four strains with an inhA mutation showed significant increase in tolerance to NO, compared to inhA wild type (figure 18).

![Figure 18](image-url)  
**Figure 18.** Survival of INH-resistant and inhA-mutated strains of *M. tuberculosis* exposed to 0.1 mM DETA/NO. Experiments were repeated three times and each point on the graph represents the median of triplicates in one experiment. The horizontal lines represents the median value of all strains (n=9). Data are presented as % survival compared to unexposed control and a significant difference (p ≤ 0.05) is indicated with *.

In a further attempt to investigate the association of INH resistance to NO susceptibility, luciferase-expressing H37Rv was subcultured in INH to select a subset of the strain with resistance to INH. This was successfully achieved, but with no significant effect on the NO tolerance. No mutations were found in the katG or the inhA genes, suggesting that there are other mutations responsible for the INH resistance [103] without any association to NO tolerance.

INH is a prodrug that needs activation by the bacterial catalase-peroxidase enzyme (encoded by katG) [282]. As katG mutations lead to reduced activity, or total loss of bacterial catalase, they also increase the susceptibility of the bacteria to ROS and RNS [283]. This can be compensated for by ahpC upregulation and increased tolerance to NO [284]. Other mechanisms of INH resistance are mutations in ahpC, encoding for bacterial alkyl hydroperoxidase, ndh genes encoding NADH dehydrogenase [103], and inhA encoding enoyl-acyl reductase. The latter is a NADH-dependent enzyme essential in mycolic acid
biosynthesis for the bacterial cell wall [285] and is inhibited by catalase-mediated formation of an INH-NAD adduct [103, 286]. Since RNIs are potent lipid peroxidising and nitrating agents, affecting cell wall lipids [287], it could be speculated that the variation of NO susceptibility in inhA-mutated strain could be due to modifications of the cell wall, and compensatory upregulation of alternative defence mechanisms. This merits further investigation since the understanding of interactions between different drug resistance mutations and compensatory mutations are important to be able to make predictions about global epidemics of MDR TB and XDR TB [288], as well as for identification of new drug targets.

**Survival of M. tuberculosis within the macrophage**

To investigate if NO susceptibility had any impact on the ability of macrophages to control the infection, 7 clinical strains and H37Rv were investigated in activated macrophages. Even if human macrophages produce NO at the site of infection, it is not yet possible to explore this mechanism in vitro [289]. Thus we used the murine cell line (RAW 264.7) with the capacity to generate NO after stimulation with IFN-γ/LPS (figure 19).

![Figure 19. Effect of IFN-γ/LPS stimulation on growth control of clinical strains of M. tuberculosis in macrophages. The overall capacity to control M. tuberculosis infection in unstimulated and IFN-γ/LPS stimulated macrophages (RAW 264.7). Data are presented as fold change of total bacterial load (intra- and extracellular) day 2 divided by day 0 (ratio D2/D0) and bars represent median with error bars at min and max from three experiments run in triplicates.](image-url)
There was variability in the macrophage capacity to phagocytose the different strains, as well as differences in their efficiency to achieve growth control of the different strains. However, there was a general agreement with the cell-free system, that IFN-γ/LPS-stimulated macrophages (producing NO) controlled the infection more effectively. A limitation of our study is that we did not include an iNOS inhibitor to quantify at which level the reduction in growth within the macrophages was strictly NO-dependent. Preliminary experiments with H37Ra (an avirulent laboratory strain of *M. tuberculosis*) showed that macrophages treated with iNOS inhibitors were clearly less effective in controlling bacterial growth than NO-producing cells, which has been shown also with virulent strains of *M. tuberculosis* [213].

The inability to identify a significant correlation between survival to NO in a cell-free system with survival in activated macrophages, most probably reflects that macrophages do not only use NO, but several other bactericidal systems, such as ROS production and cathelicidin, to control infection [290, 291] and that the microenvironment where the NO exposure takes place affects the potency of NO [181].
Concluding remarks

This thesis provides insight into the role of nitric oxide in the immune defence against *M. tuberculosis* by applying preclinical findings in clinical studies.

We found that household contacts to TB patients had high levels of nitric oxide, and that active TB was associated with low nitric oxide levels, indicating a protective role of nitric oxide in TB. In HIV-positive TB patients, low levels of exhaled nitric oxide were associated with reduced cure rate, and an arginine-rich food supplement increased cure rate among these patients. We also found that nitric oxide is toxic for *M. tuberculosis* both *in vitro* and in activated macrophages, and that reduced nitric oxide susceptibility is associated with isoniazid resistance.

The novel finding of an association between reduced susceptibility to nitric oxide and isoniazid resistance in clinical strains of *M. tuberculosis*, could be of importance in identifying new therapeutic targets. Although new vaccines and antimicrobial drugs are urgently needed to combat TB, complementary ways to strengthen the host response is beneficial. Increasing nitric oxide production in macrophages at the site of infection could be one strategy to reach this goal, both in terms of protection against developing infection, as well as for increasing cure rates in those that already suffer from active disease (figure 20 and 21). Although larger multicentre studies are warranted, arginine-rich food supplementation can be recommended to malnourished HIV co-infected patients on TB treatment.

Take-home message

Nitric oxide kills *M. tuberculosis* and peanuts can contribute to the treatment of TB.
Figure 20. A model of the role of NO, generated by the work included in this thesis. Nitric oxide (NO) in the host is part of the immune defence in tuberculosis (TB). High levels of NO (↑↑↑) upon exposure to M. tuberculosis may clear the bacteria even before infection occurs. Intermediate levels of NO (↑↑) may lead to latent infection, where the cell-mediated immunity (CMI) and NO control the infection for a lifetime. In case of insufficient levels of NO production (↑↓) by a poor CMI, or because of NO resistance or other virulence factors in the bacterial strain, bacterial replication will continue and cause active disease. In latent disease, malnutrition may lead to deficiency of L-arginine, the substrate for NO production, and reduced levels of NO, with subsequent loss of control of the infection and development of active disease.
Future areas of interest

Figure 21. Future areas involving NO-based therapies. The outcome of the *M. tuberculosis*-human encounter depends both on host and bacterial characteristics, and can result in bacterial clearing or persistent infection. Four main areas can be identified as targets for new NO-based interventions. Immunization of the host (1) to prepare for an early and strong NO response upon *M. tuberculosis* encounter. Therapeutic strategies (2) to increase NO output post-exposure, either by L-arginine supplementation or iNOS up-regulation. The bacterial defence mechanisms (3) against NO and other anti-bacterial agents are potential targets for new drugs. Immune modulatory therapies (4), unleashing contained bacteria by inhibition of cell mediated immunity (CMI) of NO production, could be combined with new drugs to shorten treatment and to reach sterilization.
A voice of TB

“When I heard I had TB I knew that it was curable so I was not afraid. I don’t know if I had been in contact with any person with TB. In order to get the diagnosis I had to go to many health centres, but finally a private clinic and an X-ray confirmed my TB. The disease does not affect my life much since I work as usual with no difficulties and I am sure I will be well after the treatment.”

Himarim, 51-year-old, governmental employee
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Acknowledgements

I am sincerely grateful to everyone who has contributed to this thesis, and I would especially like to mention the following people:

My SUPER supervisor Thomas Schön for your never-ending energy, enthusiasm and knowledge (it has amazed me from day one, and still does), and for your support both when things go well and when life is tough.

Professor Olle Stendahl, my supervisor, for welcoming me to the department despite my more adventurous than scientific attitude (I hope the latter has improved a bit), for trust in independence and support when asked for (from wherever in the world you happened to be).

Professor Tommy Sundqvist, the ideas man, for an endless stream of hypothesis, for teaching me statistics and nitric oxide chemistry, and for finding something interesting in ever so boring data.

Ebba Abate, my co-pilot in this work and my new brother, you are the best!

Professor Sven Britton, the founder of the fruitful Swedish-Ethiopian collaboration, for the admirable attitude of not accepting things as they are but instead do all your best to change it. Daniel Elias, for introducing me to the Ethiopian culture and for continuing to take part in all the work despite your move to Denmark. Anna Westman, Karolinska Hospital, for your encouraging and warm attitude and for all the work you did during the start-up of the clinical studies. Mekidim Mekonnen, for your impressive and accurate works in the lab, I wish you all the best. Wassihun Wedajo, for contributing with your excellent lab skills and for a lot of good fun. Dr Abraham Aseffa, director of AHRI, for your dedication to science and to Ethiopia.

My friends and colleges at Gondar College of Medicine. Dr Feleke Moges, for taking excellent care of me my first time in Gondar. Dr Assefa Getachew, for examining hundreds of X-rays, but most for your hospitality, teaching attitude and positive spirit. Nurse Meseret Senbeto, for long and reliable work at the DOTS and for wonderful times with your family. Nurse Tezera Jemere, for excellent monitoring of patients at the DOTS. Nurse Meseret Atale, for being an extraordinary research nurse, for doing your very best during all circumstances and for being a true caring and responsible person. Assistant Saba Ekuby, for your extended service at the DOTS, helping out with all kinds of things, for your lovely way and for always opening your door for my family and me. Mrs Sabash, for inviting me to your home and for your way of communicating about serious things with no common language us between. Atanaw Kassahun, for not giving up your search for patients lost to follow-up. Yeshi Kassahun and Abebech Molla, for entering endless amounts of data. Belay Anagaw, for support in the lab and for always cheering me up. Aschalew Gelaw, my Ethiopian lab mate, for the all your work in the lab and for your wonderful sense of humour. Martha Alemayehu for excellent contributions in the lab and for being a very nice friend. Lamesgin Muniiken for your sharp way and accurate work in the lab. Tigist Feleke and Alemesh Kehal for support with samples and lab work and for being so friendly. Dr Ermias Diro, for taking medical responsibility at the study site and for interesting discussions in the field of research, medicine and life in general. Dr Shitaye Alemu, for your friendly attitude and your courage, inspiring many women around you. Addis Alemu, Endalkachew Melese, Yared Wondikun, Belete Ayele, Afework Kassu, Yohannes Asfawossen, Yohannes Mikena, Alemnigus Yigzaw, Yeneew Kebede, for all contributing to the clinical part of this thesis.
My friends and colleges at the Department of Medical Microbiology. Katarina Tejle, for helping me out with just about everything, lending me materials and sharing your skills in the lab, but most of all for becoming a close friend. Tony Forslund, for admirable patience in teaching me the basics in lab work and supporting me at the lab. Daniel Eklund, for sharing methods and skills and for your relaxed and friendly way. Robert Blomgran, for making long working hours in the office not so bad after all, and for correcting and encouraging me during the write-up of this thesis. Maria Lerm, with a capacity greater than most of us, for your open mind and valuable inputs. Johanna Raffetseder for invaluable support in the lab and for your positive, friendly and quick way. Elsje Pienaar, for your calm and sharp appearance, your genuine helpfulness, and for introducing me to Craig and his family. Clara Braian, for your friendly way and for proofreading this thesis, even though you were on maternity leave. Amanda Welin, for introducing me to macrophages and mycobacteria, for making complicated things seem easy and for you colourful and happy appearance. Alexander Persson, for fun times at work and at conferences, and for all the chitchatting when things were slow. Martin Winberg, for being such a nice roommate, we miss your singing. Hanna Abdalla for your friendliness and incredible ability to stay strong far from your family. Mary Esping, and previously Ingegerd Wranne, for your incredible capacity to keep papers and figures in order for us with absent minds. Kristina Orselius, for sharing your experience of laboratory work on nitric oxide. Lena Svensson, for the friendly and relaxed atmosphere that you bring, and for loving dogs. Elisabeth Hollén for supporting me during my first small steps into science and nitric oxide, for good talks and great music. Henrik Andersson, for sharing your extensive knowledge in immunology and for your friendly and helpful attitude. Christina Samuelsson, for excellent technical assistance and for making it more easy at work than at home. Medical students Märta Kroon, Petra Axenram, Anna Bornefall and Sofia Forsgren, for being so nice and contributing to the overall work on TB. TB lab personal: Britt-Marie, Sara, Ann-Marie, Eva, Slavica and Helena for coping with us around, and Michaela Nordvall, for efficient technical support. Marie Larsson, for your friendly way and for supporting me in the TB lab, I wish I had your lab skills. Jacob Paues, Lennart Persson and Sven-Göran Fransson, for providing a clinical connection to the TB research also in Sweden. Lars Brudin, for statistical support. Professor Pia Forsberg, my mentor, for support in both private and occupational matters, and for being an extraordinary person. Kristian Ångeby, Jim Werngren and Pontus Juréen, for providing strains, and for sharp reflections on results and methods. TB lab personal at Karolinska Hospital, for being so friendly during my stay time. Susanna Brighenti, for enthusiasm and fruitful discussions on TB.

My extended family of close and loved friends, especially Emma and Charlotte, Pia-Maria, Emma H, Joachim, Cecilia, Andreas, Anna-Kajsa, David, Ellinor, Jesper, Ulf, Åsa, Carl and Henrietta, Helena E, Helena R, the family Modin, Katarina, Marie and Lisa with families, Maj-Britt, the Kindgren family, the Lundberg family, the Lindstein family, the Hegethorn family, the Wareborn family, the Blomqvist family, and the extended Idh family, for your sweet support, excellent crisis management and for putting things in perspective.

Most of all, I want to thank my family. Pappa B-G, for love, understanding and for not giving up. Mamma Maria, for your love and encouragement. Lillasyster Yster (Nina), for unconditional love and for keeping me down on earth.

Last but not least, thank you Rickard, for taking my hand, and heading out on a joint journey through life.