Preventing Leprosy: Epidemiological and immunological aspects of chemo- and immunoprophylaxis in leprosy patients' contacts

Richardus, R.A.

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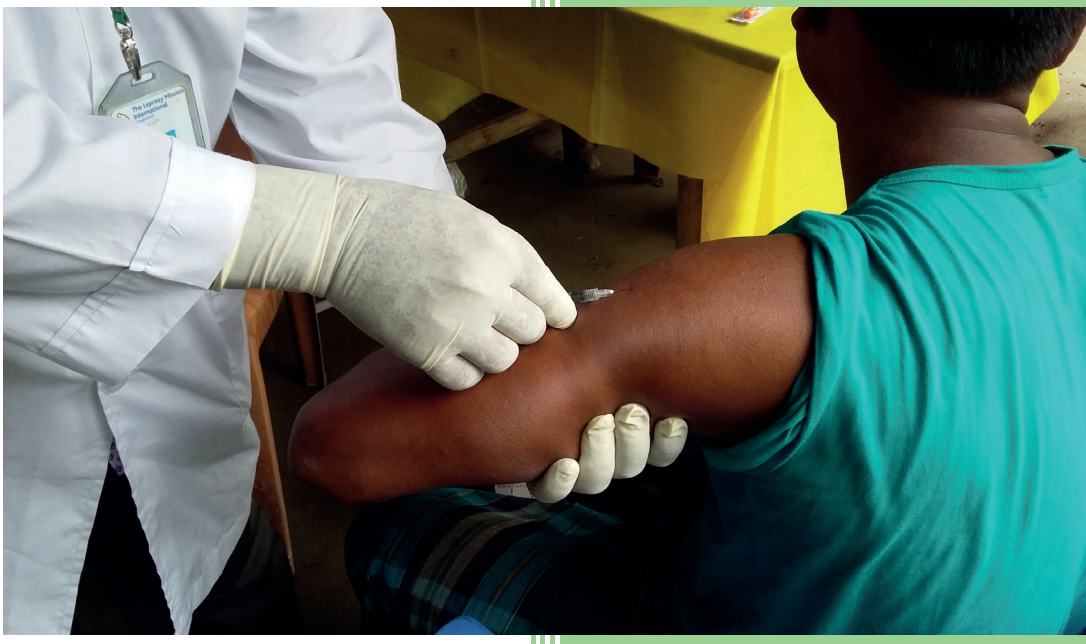

Renate Alicia Verbiest-Richardus

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COLOFON

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Voor Jelle en onze kinderen:

Misha, Josia, Matthea en Josephine

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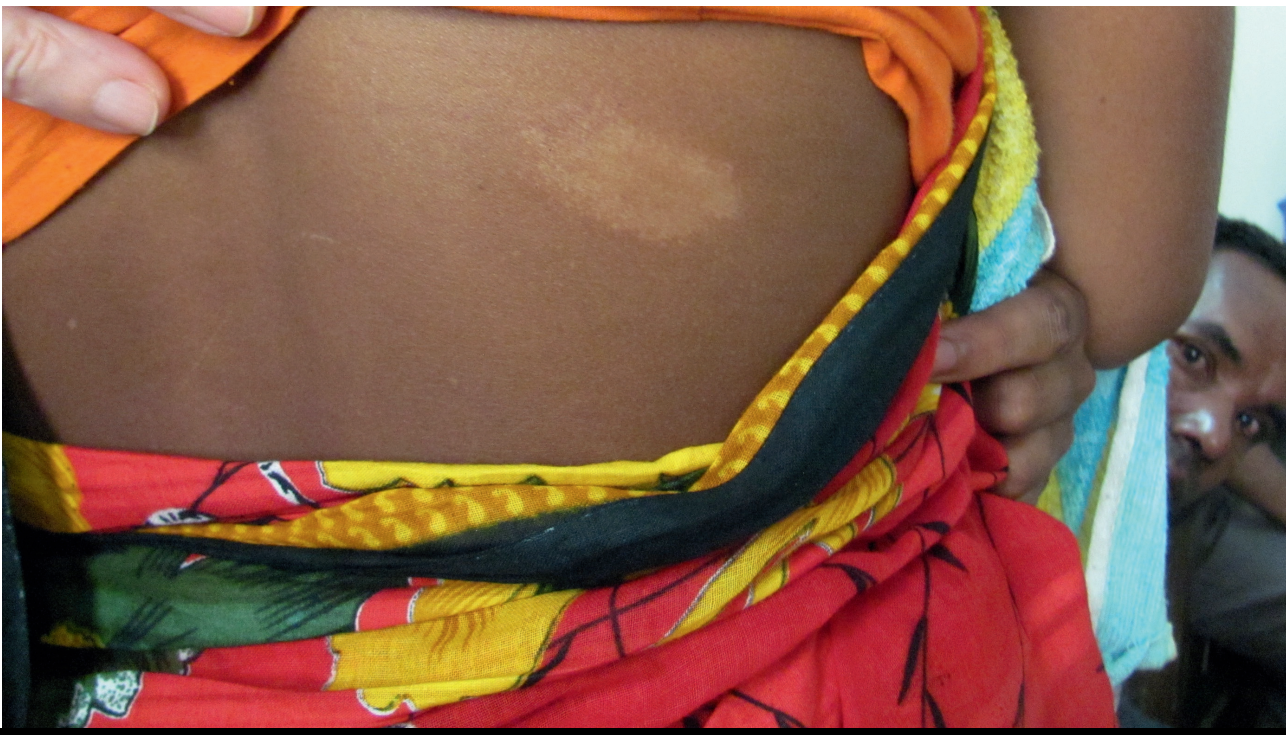
CHAPTER 1

General Introduction



CHAPTER 1: GENERAL INTRODUCTION

Leprosy

Leprosy is caused by *Mycobacterium leprae*, and primarily affects the skin and nerves. Leprosy is feared because of the deformities it can cause, consequently inducing social stigma and discrimination[1]. Grade 2 disability is defined as visible deformities in hands/feet and/or visual impairment as a result of leprosy. In order to prevent nerve damage, early diagnosis and subsequent treatment with multi-drug treatment (MDT), is crucial. Leprosy still presents a significant public health problem. Around 200,000 new cases of leprosy are detected each year, with highest numbers in India, Brazil and Indonesia[2]. *Mycobacterium leprae* closely resembles *Mycobacterium tuberculosis*, the bacillus causing tuberculosis (TB). *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccination is known as a vaccine against tuberculosis[3], but is also known to cross-protect against leprosy[4]. New TB vaccines often contain antigens with homologs in *M. leprae*, implying there is room to integrate new TB and leprosy vaccine research[5].

Classification of leprosy

Leprosy is diagnosed when at least one of the following cardinal signs is present: one or more pale or reddish skin lesions with definite sensory loss; thickened peripheral nerves; and a positive skin smear result for acid-fast bacilli. Patients with negative smear results at all sites and who have no more than five skin lesions are defined as having paucibacillary (PB) leprosy, and those showing positive smear results at any site or who have more than five skin lesions as multibacillary (MB) leprosy[6]. The proportion of PB versus MB leprosy cases varies per geographic region. In Bangladesh approximately two thirds of the leprosy patients present with PB leprosy, whereas one third develops MB leprosy[2]. Worldwide, however, these proportions are more evenly divided, with a little over 50% of the patients categorized as MB leprosy[2].

Besides the pragmatic division into PB and MB leprosy as provided by the World Health Organisation (WHO), the Ridley-Jopling classification[7] classifies leprosy based on histopathological features, bacillary load and immunological response into tuberculoid leprosy (TT), borderline tuberculoid leprosy (BT), borderline borderline leprosy (BB), borderline lepromatous leprosy (BL) and lepromatous leprosy (LL). In smears stained by the Ziehl-Neelsen method, living leprosy bacilli appear as solid staining, bright pink rods. These slit-skin smears are usually taken from 6 places, including both earlobes and active sites of infection, through a small incision in the skin, from which dermal tissue is taken. The Bacterial Index (BI) indicates the number of leprosy bacilli in smears. According to

Ridley's logarithmic scale, it ranges from zero to 6+ and is based on the number of bacilli seen in an average microscopic field of the smear. In practice, however, often only clinical criteria are used for classifying individual patients, since skin-smear services are not always available and dependable, but this may depend on the possibilities in a country or region. Since PB leprosy is characterized by a low bacterial load and thus bacilli-negative smears, diagnosis of leprosy in Bangladesh, as a country with a majority of PB cases, is extra complicated. Other skin diseases, such as fungal infections, nutritional deficits, vitiligo, pityriasis alba and versicolor, psoriasis, post-kala-azar dermal leishmaniasis, etcetera, may also complicate diagnosis.

Immunopathology of leprosy

Most individuals who have been in contact with the leprosy bacterium, clear the bacteria and never develop an infection. In the remaining small percentage, one or a few ill-defined hypo-pigmented or faintly erythematous patch(es) of indeterminate leprosy (I) may develop. Leprosy often starts with the indeterminate form and is therefore often not recognized. Indeterminate leprosy may heal without treatment, persist as indeterminate leprosy or become one of the definite (determinate) types of the disease. It is also possible for individuals to develop definite types of leprosy directly; and also for PB leprosy to spontaneously heal again[8].

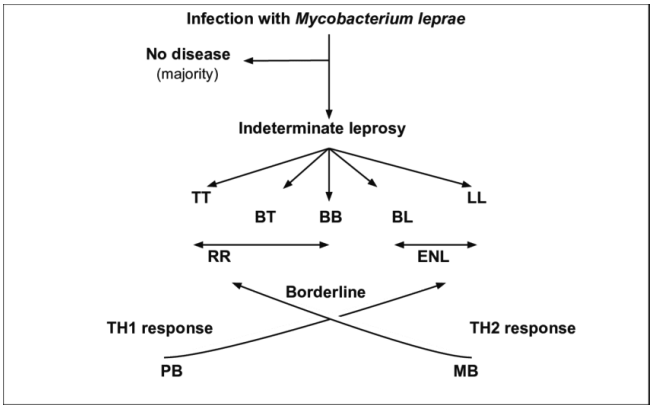


Figure 1. The Ridley-Jopling classification of leprosy.

Neder L, Rondon D, Cury S, da Silva C. Musculoskeletal manifestations and autoantibodies in children and adolescents with leprosy. Jornal de pediatria 90(5)·April 2014. DOI: 10.1016/j.jped.2014.01.007..

Leprosy depends on the infected individual's resistance to the disease. Macrophages have a classical activation phenotype (M1) in tuberculoid leprosy, while in lepromatous disease there is a pathway of alternative activation (M2). The M1 pathway stimulates $CD4^+$ T helper 1 (Th1) cells to produce pro-inflammatory cytokines such as interferon- γ (IFN-γ). These cytokines activate macrophages to

eliminate bacilli[9], leading to bacterial control, but also to secondary tissue damage due to the inflammation. Together, these macrophages form well characterized granuloma with few bacilli, leading to few and well-defined skin lesions[7].

The pronounced and local immune reaction in PB leprosy causes damage to melanocytes, sensory nerves, sebaceous glands and sweat glands. The skin lesions of TT leprosy consist of a single or few hypo-pigmented, oval or round, well-defined patches. Less melanocytes leads to hypopigmentation. Sensation is impaired, and the lesions are either hairless or with sparse hairs. Sweating is impaired in the affected area, causing the leprosy patch to be drier than the skin surrounding it. Sometimes an enlarged cutaneous nerve enters the lesion visibly. The related peripheral nerve trunk is usually enlarged. Damage to nerves leads to loss of sensation, pain, tingling and muscle weakness or paralysis.

In primary neuritic leprosy one or more peripheral nerve trunks are involved, without evidence of skin lesions. First sensory loss occurs, then motor loss; paralysis may lead to disabilities like claw hand or toes, wrist- or foot-drop, lagophthalmos, etc.

T cells of lepromatous leprosy patients are anergic to *M. leprae* and their tissues are ideal for the multiplication of leprosy bacilli. However, patients with lepromatous leprosy are not immune deficient in general. Macrophages in lepromatous disease prefer alternative activation (M2), which is not favorable for induction of Th1 responses[10 11]. Additionally, suppressor type CD8 + T cells also play a role in T cell anergy towards M. leprae in LL, which downregulate macrophage (M1) activity[12-15]. Furthermore, the production of anti-inflammatory cytokines such as interleukin-10 (IL-10) results in disseminating, progressive infection[16 17]. Patients with lepromatous leprosy also have higher levels of regulatory CD25+ CD4 + T-cells (Tregs), which play a role in *M. leprae*-Th1 unresponsiveness in lepromatous leprosy[18-20]. In lepromatous leprosy, there is also a high production of antibodies, which leads to accumulation of immune complexes, activating the complement system[21].

In lepromatous leprosy, granulomas are disorganized and filled with foamy macrophages and numerous bacilli. Skin lesions are more numerous, shiny, symmetrical and nodulous, furthermore they are less well defined and less anaesthetic. Loss of eyebrows and –lashes may occur. Patients with lepromatous leprosy have more elaborate and serious effects of nerve damage.

Leprosy Reactions

During the usually chronic course of leprosy, acute episodes (reactions) may occur[22 23]. It is mainly the borderline forms of leprosy that are immunologically unstable and therefore most likely to develop leprosy reactions[23]. Reactions may occur spontaneously, but are also associated with co-infections with helminths or HIV [23-27] and genetics[28]. Reversal reactions usually occur in the first 6 months of starting treatment. This is probably due to the bactericidal effect of rifampicin, which kills high amounts of bacilli. *M. leprae* antigens are released, which in turn trigger inflammatory reactions. The precipitating factors may not be obvious in some cases. There are two kinds of hypersensitivity: Type 1 (reversal reaction), which occurs mostly in patients with borderline tuberculoid leprosy, as a result of inflammation in skin and nerves caused by Th1 helper cells[29]; and Type 2 reaction (erythema nodosum leprosum), which occurs mostly in patients with borderline lepromatous leprosy, in which antigen and antibody complexes of the humoral immunity cause damage in tissues with systemic complications[30]. During reactions, inflamed skin lesions and nerves can be very painful and tender; irreversible nerve damage may occur if treatment with prednisone is not started soon enough. Reversal reactions must be differentiated from relapses, so that proper treatment can be given. Individuals who have received inadequate chemotherapy or those that have drug-insensitive organisms are more at risk of relapse.

Leprosy epidemiology

The global number of new leprosy cases has remained relatively stable over the past years (figure 1)[31], suggesting that transmission is ongoing; treatment of new cases alone seems insufficient. In order to be able to interrupt the transmission of leprosy, it is necessary to know the transmission routes of *M. leprae* and the risk factors of developing leprosy. A combination of factors (see below) play a role; predicting which *M. leprae* exposed individuals will progress to disease is therefore complicated.

The highest numbers of new leprosy cases are detected in India (135,485 in 2016), Brazil (25,218 in 2016) and Indonesia (16,826 in 2016)[31]. In Bangladesh, the number of new cases was 3,000 in 2016, compared to 3,141 new cases in 2013[31]. The incubation period of leprosy is generally between 3 and 5 years, although a great variability is known (between a few weeks to 45 years have been described)[32]. The exact mechanism of transmission of *M. leprae* is not known. Possibly, the bacterium is transmitted by skin-to-skin contact between cases of leprosy and healthy persons or by the respiratory route[33]. The respiratory route is considered the most important.

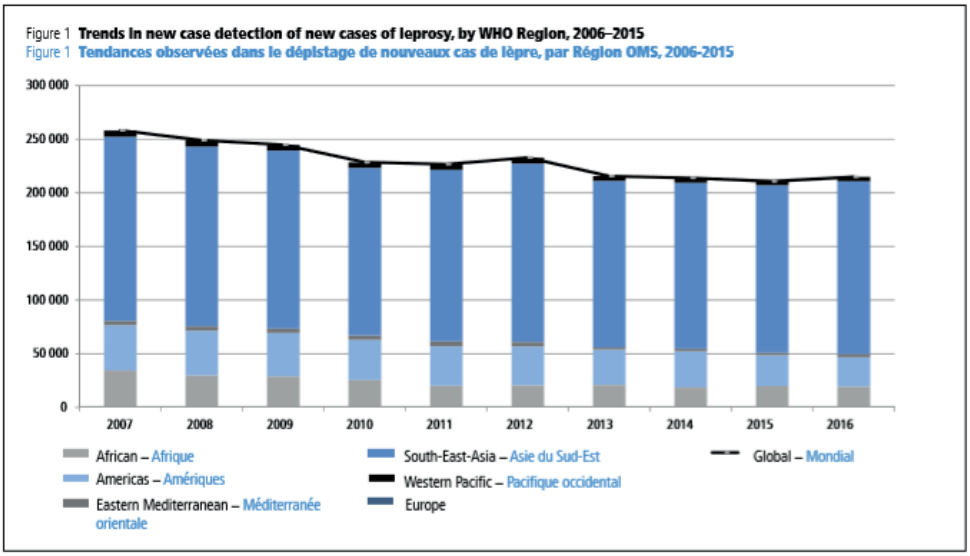


Figure 2. Trends of new case detection of leprosy between 2006 and 2015 by WHO region[31].

Nowadays the main reservoir for *M. leprae* is most likely human. Undetected (MB) cases in the community probably cause continued transmission[34]. Household contacts of MB patients have a higher risk of developing leprosy than PB cases, probably due to the high bacterial load[35]. Armadillo's are another possible reservoir of leprosy in the Southern States of the USA[36] and Brazil[37], although a recent article showed that *M. leprae* infected armadillo's may not always represent a source of infection in a specific area in the Brazilian Amazon region[38]. Possible, viable *M. leprae* resides in the soil and water mainly in areas with high prevalence of leprosy[39 40]. In Bangladesh, *M. leprae* DNA was found in 16.0% of soil from houses of leprosy patients[41]; possibly environmental sources can be (temporary) reservoirs for *M. leprae*, although further research is needed.

A low socioeconomic status and specifically nutritional deficits are known risk factors for leprosy in general[42 43]. A recent period of food shortage probably reduces the cell mediated immunity of individuals which harbour *M. leprae*, leading to clinical leprosy disease[43]. In tuberculosis, malnutrition is associated with low levels of leptin, a hormone secreted by adipose tissue[44]. Low leptin concentrations in turn, suppress macrophage functions due to elevated glucocorticoid levels, leading to decrease of bacterial killing and increased risk of disease[44]. Possibly, similar processes play a role in leprosy. Genetics[45] and co-infections[46] are also risk factors for developing leprosy. Helminth infections, specifically, have immunomodulatory properties, and can skew the host immune response

towards Th2[47 48]. The Th1 response is crucial in combatting mycobacterial infection, therefore helminth co-infection may stimulate *M. leprae* growth by upregulating Th2 cytokines or CD4 + CD25+ regulatory T cells (Tregs).

The main risk of exposure to *M. leprae* is in close contacts of new, untreated cases. Higher age of the contact, a higher bacterial load of the index patient, and close physical and genetic distance are independently associated with the risk of a contact acquiring leprosy[35]. The chance of finding a household contact with previously undiagnosed leprosy is ten-fold higher compared to the general population, for different categories of neighbours and social contacts this is between three and five-fold[49]. Contact management therefore seems an important pillar in stopping the spread of leprosy. *M. leprae* infected individuals may carrying large numbers of bacteria, without showing clinical symptoms, leading to a continued transmission. The WHO declared leprosy eliminated in 2000, after this there was a dramatic decline in leprosy control activities. This has led to a decrease in contact management and lower new case detection rates. Possibly worldwide large numbers of unreported cases remain undetected[34], also causing continued transmission.

Tools for early detection of leprosy

Enzyme-Linked Immunosorbent Assay (ELISA) techniques are used in Interferon-Gamma Release Assays (IGRAs) and are used more frequently than before. However, laboratory facilities are needed that are not found at all health centers in leprosy-endemic areas. Lateral flow assays (LFAs) are easily usable immunochromatographic assays, which find target analytes in samples without needing expensive equipment. Since diagnosing leprosy as early as possible is critical, LFAs provide new possibilities for rapid detection of leprosy patients in early stages of the disease or of *M. leprae* infected individuals without clinical symptoms[50-52 53 54]. Identification of predictive biomarkers is complicated, due to the long incubation time and low incidence of leprosy. By comparing immune profiles of leprosy contacts and following them over longer periods of time, it is becoming possible to identify which biomarkers correlate with progression to disease[50]. However, this requires large numbers of new cases indicating long-term follow-up studies in multiple endemic areas of which this study is one of the very few examples in leprosy research.

The role of phenolic glycolipid I

M. leprae phenolic glycolipid I (PGL-I) is an antigen found on the outer surface of the mycobacterium[55]. The finding of high levels of IgM antibodies (Ab) to PGL-I in leprosy patients, lead to the the development of several tests that were investigated extensively for diagnostic purposes[51]

[55 56]. In contrast to MB leprosy patients, anti-PGL-I Ab titers are not useful in detecting PB leprosy patients, since they develop cellular (not humoral) immunity and for this reason often lack Abs to PGL-I[51 55 56]. Schuring *et al.*[57] confirmed that anti-PGL-I seropositivity was associated with BI, which explains why the vast majority of PB patients have negative anti-PGL-I Ab titers.

The role of lateral flow assays using leprosy-specific biomarker profiles

Because of the broad disease spectrum of leprosy, biomarkers for both cellular and humoral mediated immunity are necessary in diagnostic tests in order to detect of *M. leprae* infection . This was demonstrated in a study[50] that used lateral flow assays (LFAs) for four immune markers (anti-PGL-I antibodies, IL-10, CCL4 and IP-10) in a field-trial in Bangladesh. Different biomarker profiles, not single markers, distinguished groups that were infected with *M. leprae* from those that were not, patients from household contacts and endemic controls, or MB from PB patients. This study is an example of how field-friendly LFAs are helpful tools in efficiently monitoring the different stages of infection and disease in leprosy contacts, facilitating early treatment of infected contacts and preventing the development of actual disease.

The role of BCG in leprosy protection

There are many studies into the use of immunoprophylaxis (vaccination) and chemoprophylaxis to prevent leprosy in contacts of leprosy patients. *Mycobacterium bovis* BCG vaccination is known as a vaccine against tuberculosis[3] and is part of the neonatal immunization scheme in a lot of areas in the world. In Bangladesh, the coverage of BCG vaccination at birth is estimated to be 98% (http://www.who.int/immunization/monitoring_surveillance/data/bgd.pdf). BCG is also recognized as protecting against leprosy[58-60]. The reason BCG can be used as a vaccine against leprosy is because of the many homologous antigens present in *M. bovis* (found in BCG vaccines) and the *M. leprae* and the *M. tuberculosis* genomes[4]. This gives a cross-reactive, protective immune response to *M. leprae* after BCG vaccination. Furthermore, live-attenuated vaccines such as BCG can give non-specific effects (NSE), besides protection against the specific micro-organisms for which it was meant[61]. The first possible immunological mechanism to explain NSE is heterologous immunity, in which T-cell memory responses to a specific antigen also cross-protect against other pathogens[62]. The second hypothesis is ‘trained immunity’, in which immunological memory is developed by the innate immune system[63].

One meta-analysis[58] showed that BCG vaccination offers an average protection of 26% against leprosy in experimental studies and 61% in observational studies; the observational studies thus

overestimating the protective effect of BCG vaccine in leprosy. The protection was better for MB leprosy compared with PB leprosy, since BCG could lead to an increase in the milder tuberculoid and indeterminate forms of leprosy, since host immunity may have improved after BCG vaccination[58]. Another meta-analysis[59] found a protective effect of BCG of 41% for trials and 60% for observational studies. There was a greater variability of the BCG vaccine effect against PB forms; for MB leprosy the estimates were more homogeneous, but a statistically significant different effect was not found. The protective effect of the BCG vaccination was significant higher if studies were conducted among household contacts instead of the general population. This is shown in a cohort study of 3536 contacts of 1161 leprosy patients in Brazil[60], whereby the protection conferred by a booster BCG vaccination was 56% and not clearly affected by previous BCG vaccination. This effect was 83-85% for the indeterminate and MB forms, but a non-significant effect of 26% was found for the PB forms. The risk of tuberculoid leprosy in the first months was high among BCG vaccinated contacts: among the 58 new cases detected during 18 years of contact follow-up, leprosy was diagnosed in 21 of these contacts (36%) within 2-10 months after vaccination; 18 out of these 21 contacts had PB leprosy.

Merle *et al*[59] performed a meta-analysis which showed no statistical difference in BCG protection in studies where patients are vaccinated once versus twice or more. The two large trials compared in this meta-analysis had very different results. The first was a cluster randomized trial[64] in Brazil among 99,770 school children aged 7–14 years, which were followed for 6 years. In the vaccinated group, an incidence rate ratio of leprosy of 0.99 was found compared to the control group, showing that revaccination did not give extra protection. By contrast, a randomized controlled trial[65] in northern Malawi showed that a second BCG vaccination gives a 49% protection against leprosy. The main difference between these two studies are the characteristics of the revaccinated population: in Brazil only school children were studied, whereas in Malawi infants to adults took part. Revaccination might give extra protection to adults for whom the first vaccination has become less effective over time, but revaccination might be less useful in school children[59].

Concluding, BCG vaccination provides a variable protective effect against leprosy in different studies[58-60] and seems better for protection against MB than PB leprosy, since improved host immunity after BCG vaccination could lead to an increased occurrence of milder forms of PB. The benefit of BCG seems dependent on the population receiving vaccination, with more benefit in adults than in children[59].

The role of SDR and previous BCG vaccination in leprosy prevention

Chemoprophylaxis entails the use of a drug to prevent the development of a disease. Dapsone and rifampicin (together with clofazimine) are drugs that are used as part of the MDT cocktail to treat newly diagnosed leprosy patients. However, these antibiotics have also been studied singularly as chemoprophylactic drugs in contacts of new leprosy patients since the 1960s. A meta-analysis of three studies using dapsone[66-68] as chemoprophylaxis, showed a reduction of 40% of leprosy incidence amongst contacts using dapsone versus placebo. A chemoprophylaxis trial in five Indonesian islands[69] was started in 2000, in which a blanket group (rifampicin prophylaxis given to all eligible persons), was compared to a contact group (rifampicin prophylaxis given to all eligible contacts of former/treated and newly diagnosed leprosy patients) and a control group (no chemoprophylaxis given). After three years, the cumulative incidence of leprosy was significantly lower in the blanket group, but no difference was found between the contact and control groups. Thus, rifampin prophylaxis seems most effective in communities where everybody was given the prophylaxis in contrast to only household contacts and direct neighbors[69]. A possible explanation is that the bacillary load in close contacts is already too high to be eliminated by a single dose of rifampicin.

In the COLEP study[70], a cluster randomized controlled trial conducted in a leprosy endemic area in the Northwest of Bangladesh between 2002 and 2009, the effect of single dose rifampicin versus placebo in preventing leprosy in close contacts of newly diagnosed leprosy patients was studied. The COLEP study showed that a single dose of rifampicin (SDR) in contacts of newl leprosy patients reduced the incidence of leprosy in the first two years with 57%[70]. In the subgroup analysis it was discovered that those contacts with a low *a priori* chance of developing leprosy, benefited most of the chemoprophylaxis (i.e. if the contact was not blood-related to the index patient, if the index patient had PB leprosy, and if the contact was a social contact rather than a household contact or neighbor). Furthermore, the COLEP study showed that the effect of SDR depended on the BCG status of the contact. If the contact had received BCG vaccination as neonate (presence of a BCG-scar), the protective effect of SDR was 80%[71]. Childhood BCG vaccination and SDR both had a protective effect for leprosy in contacts of about 60%, but if a contact who had previously received BCG vaccination also received SDR, the protective effect appeared to be additive. Based on these experiences, a trial was started in Bangladesh to assess the effectiveness of a combined strategy (the MALTALEP study). In this cluster randomized controlled trial, contacts of newly diagnosed leprosy patients received either BCG alone, or BCG plus SDR. In particular, the main aim was to determine whether the excess cases in the first year after immunoprophylaxis[60] can be prevented by chemoprophylaxis with SDR.

Recently, the World Health Organisation (WHO) has included SDR as guideline in their leprosy elimination strategy[72]. Implementation studies on SDR as leprosy post-exposure prophylaxis (LPEP)[73] are currently ongoing, which are designed to study the effectiveness, impact and feasibility of contact tracing and PEP for leprosy. However, the direct immunological effect of SDR on infection has not yet been investigated, nor its effect on *M. leprae* infection in the community.

Aims and outline of the Thesis

Chapter 1 gives an introduction on leprosy. Furthermore, tools are described for early detection of leprosy. The role of BCG and SDR in leprosy prevention is discussed. Finally, the rationale behind a combined strategy is introduced.

Chapter 2 describes the design and purpose of the MALTALEP trial, a cluster randomized controlled trial in the Northwest of Bangladesh among around 15,000 close contacts of new leprosy patients, to evaluate the effect of BCG only versus BCG and SDR as prophylactic measure to prevent the development of leprosy.

Chapter 3 provides an analysis of the clinical and demographic parameters of the unexpectedly high proportion of healthy contacts of leprosy patients presenting with paucibacillary leprosy within 12 weeks after receiving BCG vaccination in the first 1,5 years of the MALTALEP trial (0,40% of vaccinated contacts). It also describes the various immunological mechanisms that could underlie this phenomenon.

Chapter 4 describes the immune- and genetic profiles associated with adverse events after BCG vaccination in a leprosy endemic area in Bangladesh. Cytokine profiles induced by BCG vaccination in whole blood assays of contacts with and without vaccine-associated complications are compared.

In Chapter 5, the anti-PGL-I antibody levels of leprosy contacts are followed to determine whether these can be utilized as a prognostic biomarker for leprosy by predicting which individuals will progress to disease.

In Chapter 6, the primary and secondary outcomes of the MALTALEP trial are described. The difference between the number of new leprosy patients among leprosy contacts that emerge in either of the two intervention arms (BCG only versus BCG and SDR) within two years of intake is compared. Secondary data analysis is carried out in order to define special groups at risk for developing leprosy.

Finally, in Chapter 7, the following three research questions are discussed.

Research Questions:

1. What are the potential causative mechanisms underlying the development of leprosy following BCG vaccination?
2. Do the results of our trial justify the introduction of a combination of BCG and SDR in leprosy health care programs in Bangladesh to prevent the development of leprosy amongst household contacts of new leprosy patients?
3. Can immune markers be identified in contacts of leprosy patients that predict the development of clinical leprosy?

CHAPTER 2

2

Longitudinal assessment of anti-PGL-I IgM serology in contacts of leprosy patients in Bangladesh

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Abstract

Background: Despite elimination efforts, the number of *Mycobacterium leprae* (*M. leprae*) infected individuals who develop leprosy, is still substantial. Solid evidence exists that individuals living in close proximity to patients are at increased risk to develop leprosy. Early diagnosis of leprosy in endemic areas requires field-friendly tests that identify individuals at risk of developing the disease before clinical manifestation. Such assays will simultaneously contribute to reduction of current diagnostic delay as well as transmission. Antibody (Ab) levels directed against the *M.leprae*-specific phenolic glycolipid I (PGL-I) represents a surrogate marker for bacterial load. However, it is insufficiently defined whether anti-PGL-I antibodies can be utilized as prognostic biomarkers for disease in contacts. Particularly, in Bangladesh, where paucibacillary (PB) patients form the majority of leprosy cases, anti-PGL-I serology seems an inadequate method for leprosy screening in contacts as a directive for prophylactic treatment.

Methods: Between 2002 and 2009, fingerstick blood from leprosy patients' contacts without clinical signs of disease from a field-trial in Bangladesh was collected on filter paper at three time points covering six years of follow-up per person. Analysis of anti-PGL-I Ab levels for 25 contacts who developed leprosy during follow-up and 199 contacts who were not diagnosed with leprosy, was performed by ELISA after elution of bloodspots from filter paper.

Results: Anti-PGL-I Ab levels at intake did not significantly differ between contacts who developed leprosy during the study and those who remained free of disease. Moreover, anti-PGL-I serology was not prognostic in this population as no significant correlation was identified between anti-PGL-I Ab levels at intake and the onset of leprosy.

Conclusion: In this highly endemic population in Bangladesh, no association was observed between anti-PGL-I Ab levels and onset of disease, urging the need for an extended, more specific biomarker signature for early detection of leprosy in this area.

Trial registration Current Controlled Trials: ISRCTN61223447.

Registration date: 19/12/2005. Retrospectively registered.

Keywords: biomarkers, COLEP, contact study, diagnosis, fingerstick blood, leprosy, longitudinal analysis, *M. leprae,* phenolic glycolipid-I (PGL-I)

Authors Summary

Leprosy is an infectious disease caused by the bacterium *Mycobacterium leprae*, which causes skin and nerve damage. Despite worldwide efforts to eliminate leprosy, the number of infected individuals who develop leprosy, is still substantial. Household contacts of new leprosy patients are especially at risk. Early diagnosis of leprosy is key in preventing lifelong handicaps as well as transmission. This requires field-friendly tests that identify individuals at risk of developing the disease before they develop clinical symptoms so that they can receive (prophylactic) treatment. Measuring antibody levels directed against the *M.leprae*-specific phenolic glycolipid I (PGL-I) provides an indication of the bacterial load. To identify whether anti-PGL-I Ab levels correlate with the development of leprosy in contacts of newly diagnosed leprosy cases, we analyzed these levels in 25 contacts who developed leprosy during 6 years of follow-up and 199 contacts who were not diagnosed with leprosy at 3 time points in 6 years. This study showed that anti-PGL-I Ab levels at intake did not significantly differ between contacts who developed leprosy during the study and those who remained free of disease. Therefore, anti-PGL-I Ab levels alone do not represent a practical tool for prediction of which household contacts will develop leprosy in an endemic area such as Bangladesh, with high levels of patients with paucibacillary leprosy. This stresses the need for a diagnostic test composed of a biomarker signature consisting of multiple biomarkers.

Introduction

Leprosy is an infectious disease caused by *Mycobacterium leprae* (*M. leprae*), which causes damage to the skin and peripheral nerves[1]. The highest numbers of new leprosy cases are detected in India (127,326 in 2015), Brazil (26,395 in 2015) and Indonesia (17,202 in 2015)[2]. Bangladesh also has highly endemic areas, with a number of new cases of above 3,000 per year[2]. Although leprosy prevalence has decreased tremendously along with the widespread availability of multidrug therapy (MDT) in endemic areas, detection of new cases worldwide has shown only a modest decline in the last five years, and has stabilized in some countries[3]. In Bangladesh, the number of new cases was 3,976 in 2015, compared to 3,848 new cases in 2010[2]. Indirect evidence indicates that worldwide millions of unreported cases linger undetected as a gradual result of a decline in leprosy control activities after the disease was declared eliminated[1]. The continued transmission is probably largely due to *M. leprae* infected individuals, carrying substantial numbers of bacteria but (yet) lacking clinical symptoms. Thus, early detection and subsequent (prophylactic) treatment of asymptomatically infected individuals as well as subclinical disease is essential to reduce transmission.

Diagnosis of leprosy is still largely dependent on clinical signs and symptoms and detection of acid fast bacteria. However, user friendly lateral flow assays provide new possibilities for rapid diagnosis of leprosy patients in early stages of the disease or of *M. leprae* infected individuals without any symptoms[4 5]. Such assays are likely to contribute to reduction of current diagnostic delay in endemic areas and also aid classification of leprosy disease, allowing appropriate treatment. Currently, there is no specific and sensitive test available that can detect asymptomatic *M. leprae* infection or predict progression to clinical disease[6]. In view of the long incubation time of leprosy (typically 3-5 years) as well as its low incidence, identification of predictive biomarkers requires longitudinal monitoring of *M. leprae*-specific immunity in those at risk of developing disease. Therefore, investments in large-scale longitudinal follow-up studies, allowing intra-individual comparison of immune profiles in leprosy patients' contacts, is essential to evaluate which markers correlate with progression to disease and may be used as predictive biomarkers.

M. leprae phenolic glycolipid I (PGL-I) is an extensively studied antigen on the outer surface of the mycobacterium[7]. The existence of high levels of IgM antibodies to PGL-I[5-7], has allowed the development of several tests that were investigated broadly for diagnostic purposes[7-10]. Although useful for identifying multibacillary (MB) leprosy patients, anti-PGL-I antibody (Ab) titers have little value in detecting PB leprosy patients, since the latter develop cellular rather than humoral immunity and therefore often lack antibodies to PGL-I[5].

In a previously conducted cluster randomized controlled trial, designated the COLEP study, the effect of single dose rifampicin versus placebo in preventing leprosy in close contacts of newly diagnosed leprosy patients was studied between 2002 and 2009 in a leprosy endemic area in the Northwest of Bangladesh[11 12]. To investigate whether anti-PGL-I Ab seropositivity can be used as a predictive biomarker for progression to leprosy in contacts, the current study compared anti-PGL-I Ab levels of the prospective cohort at intake and at three time points covering six years of follow-up per contact.

Methods

Study participants. Contacts of leprosy patients were voluntarily recruited as part of the COLEP study (a cluster randomized controlled trial) in 2002 and 2003 in the districts Rangpur and Nilphamari in the northwest of Bangladesh, which is a leprosy endemic area[11 12]. Eligible participants (patients and contacts) were informed verbally about the study and invited to participate. Written consent was obtained from all participants at recruitment or from the parent or guardian of under 18s. Contacts were followed prospectively from 2002/2003 to 2008/2009 for the development of leprosy. Blood samples were collected by spotting on Whatman filter paper (Sigma) and subsequently stored at -80 °C. Blood samples were collected at 4 time points: recruitment into the study, follow-up 1 (FU1; two years after intake), follow-up 2 (FU2; four years after intake) and follow-up 3 (FU3; six years after intake)[12]. Leprosy was diagnosed when at least one of the following signs was present: one or more skin lesions with sensory loss, thickened peripheral nerves, or a positive skin smear result for acid-fast bacilli. Patients with negative smear results and no more than five skin lesions were classified as PB leprosy, and those with a positive smear or more than five skin lesions as MB leprosy[12]. Clinical and demographic data was collected in the COLEP study database[11].

Test group selection. A random sample was taken from 28,092 contacts of leprosy patients recruited within the COLEP study[11]. A total of 239 contacts developed leprosy within the six years of follow-up. 25 contacts were included into this sub-study who were diagnosed with leprosy at either FU1, FU2 or FU3 and for whom filter papers of at least three different time points were available. Out of the contacts who did not develop leprosy, 199 were randomly included using the RAND formula (Excel 2010), aiming for an equal ratio of three age groups (0-14, 15-29, and 30+ years).

The COLEP study represents a unprecedented field trial for leprosy, because it includes valuable longitudinal analysis of contacts and thus is uniquely suited to identify the predictive value of biomarkers. However, the COLEP study did not collect blood samples from contacts as the only samples collected was blood on filter paper. Therefore, this limited biomarker analysis to anti-PGL-I Ab only.

Leprosy prevalence. In this part of the country, the new case detection rate of leprosy was 3.21 per 10,000 in 2002 (DBLM Annual Report 2002). In these cases leprosy was diagnosed by active and passive case detection. In 2002 and 2003 random samples from the general population were taken to calculate the prevalence of previously undiagnosed leprosy (PPUL). In the contact group of the COLEP

study, the PPUL rate was 73/10,000, compared to 15.1/10,000 in the samples taken from the general population. These cases were found by active door-to-door screening[13].

Synthetic PGL-I. Disaccharide epitope (3,6-di-O-methyl-β-D-glucopyranosyl(1→4)2,3-di-O-methylrhamnopyranoside) of *M. leprae* specific native PGL-I glycolipid was synthesized and coupled to human serum albumin (synthetic PGL-I; designated ND-O-HSA). This was generated with support from the NIH/NIAID Leprosy Contract N01-AI-25469 and obtained through the Biodefense and Emerging Infections Research Resources Repository (http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx).

PGL-I ELISA. Antibodies (IgM, IgG, IgA) against *M. leprae* PGL-I were detected as described previously[5 14 15]. ND-O-HSA was coated onto high-affinity polystyrene Immulon 4HBX 96-well Nunc ELISA plates (Thermo Scientific, Rochester, NY) using 500 ng per well in 50 μl of 0.1M sodium carbonate/bicarbonate pH 9.6 (i.e. coating buffer) at 4°C overnight. Unbound antigen was removed by washing six times with PBS (phosphate buffered saline) with 0,05% Tween 20 (wash buffer). The wells were blocked with PBS containing 1% BSA (bovine serum albumin) (Roche Diagnostics, Germany) for 1 hour at room temperature (RT). Bloodspots were punched from filter papers. Three punches (2 mm each) per individual were added to 100 μl PBST (PBS/0,1% Tween20) and incubated at 4°C in 24 wells plates. After overnight incubation, 50 μl PBST/NRS (PBST + 10% normal rabbit serum) was added to each well and the plates were shaken gently for 1 hour at RT. The eluate was added to the ELISA plates (50 μl/ well) and incubated for 2 hours at RT. After incubating with the primary antibody, the wells were washed six times with PBS with 0.05% Tween 20 (wash buffer), followed by the addition of 50 μl of a 1:8,000 dilution of the secondary antibody anti-human (Dako P0212) for two hours. Following washing the wells with the wash buffer six times, 50 μl of *p*-nitrophenylphosphate substrate (Kirkegaard and Perry Labs, Gaithersburg, MD) was added. Antibodies (IgM, IgG, IgA) against *M. leprae* PGL-I were detected as previously described[14]. Absorbance was determined at a wavelength of 450 nm. Samples with an optical density at 450 nm (OD_{450}), after correction for background OD above 0.150, were considered positive. This threshold was determined by a threefold multiplication of an average EC value.

As quality control, anti-PGL-I IgM levels were determined for 10 Dutch leprosy patients by ELISA using serum as well as blood spots on filter paper: Although IgM levels were higher for 9 individuals

in sera, all seropositive individuals were also positive using blood spots and OD_{450} values correlated well (R^2=0,80).

Statistical analyses. Multivariable logistic regression was used to calculate adjusted odds ratios for the level of ant-PGL-I Ab levels at intake, and corrected for age and sex. A p-value ≤ 0.05 was used as a cut-off for statistical significance. To investigate the association of changes in anti-PLG-I Ab from baseline to the time of development of leprosy, generalized linear mixed models were used. The dependent variable was the development of leprosy at a time point and the differences in anti-PLG-I Ab levels from baseline were included as independent variables. To adjust for the correlation between intra-individual measurements we included a random intercept for each subject. The difference between the anti-PGL-I Ab levels between contacts of MB or PB index patients was calculated using a t-test comparing averages. All analyses were performed in R version 3.2.0 (R, Vienna, Austria; https://www.R-project.org).

Results

From the 28,092 contacts of leprosy patients recruited within the COLEP study[11], 239 contacts developed leprosy within the six years of follow-up. For 25 contacts who were diagnosed with leprosy during follow-up and 199 contacts who remained free of leprosy, good quality filter paper was available for at least three different time points during follow-up.
Characteristics of the study populations are shown in Table 1 and Table 2. Of the 25 contacts who developed leprosy, 10 contacts developed leprosy at 2 years after intake (FU1), 7 contacts at 4 years after intake (FU2) and 8 contacts at 6 years after intake (FU3). Four contacts (16%) developed MB leprosy and 21 (84%) developed PB leprosy. This is the same proportion of MB versus PB as in the total group of new leprosy cases diagnosed within the COLEP study[12] 4 years after intake (24 MB contacts versus 126 PB contacts; 16% versus 84%). The group was evenly distributed for sex (M/F = 1.17:1) and age categories. For 10 contacts the index patient had MB leprosy, whereas for 15 contacts this was PB leprosy.

Table 1. Contact characteristics.

	Contacts who developed leprosy (n=25)	Contacts who did not develop leprosy (n=199)
Male	14	91
Female	11	108
Age (1-15 years)	8	65
Age (16-30 years)	9	54
Age (31+ years)	8	80
Leprosy at FU1	11	0
Leprosy at FU2	7	0
Leprosy at FU3	7	0
MB	4	0
PB	21	0

Legend to Table 1. Characteristics of the contacts of new leprosy patients that either developed (n=25) or did not (n=199) develop leprosy. Sex, age group, time point of leprosy development (FU1: two years after intake; FU2: four years after intake; FU3: six years after intake) and type of leprosy developed (MB: multibacillary leprosy; PB: paucibacillary leprosy) were specified.

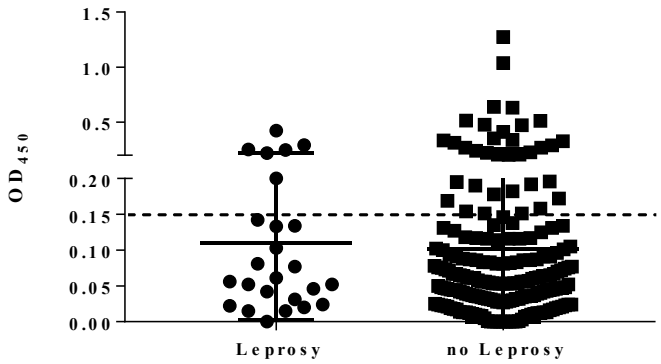
Table 2. Ridley Jopling classification of contacts with leprosy.

Ridley-Jopling Classification	Contacts with PB leprosy (n=21)	Contacts with MB leprosy (n=4)
I	1	0
TT	3	0
BT	17	4

Legend to Table 2. Ridley-Jopling classification of contacts (n=25) of new leprosy patients who developed leprosy at follow-up. Contacts developed either paucibacillary leprosy (PB): indeterminate (I), tuberculoid (TT) or borderline tuberculoid (BT) leprosy; or multibacillary leprosy (MB): borderline tuberculoid (BT) leprosy.

The anti-PGL-I Ab levels at intake were compared between the two groups of contacts (Fig 1). In the group of contacts who developed leprosy, the average anti-PGL-I Ab titer at intake was 0.11, and varied between zero and 0.424. 6 of these 25 (24%) contacts who developed leprosy had a positive anti-PGL-I Ab level of >0.150 at intake. In the group who did not develop leprosy, the average anti-PGL-I Ab titer was 0.10 and varied between zero and 1.275. 35 out of 199 (17.6%) contacts who did not develop leprosy had a positive anti-PGL-I Ab level of >0.15 at intake. No significant association was observed for the anti-PGL-I Ab levels at baseline (OR: 1.01 (0.78, 1.31), 95% CI p=0.94) between the two groups.

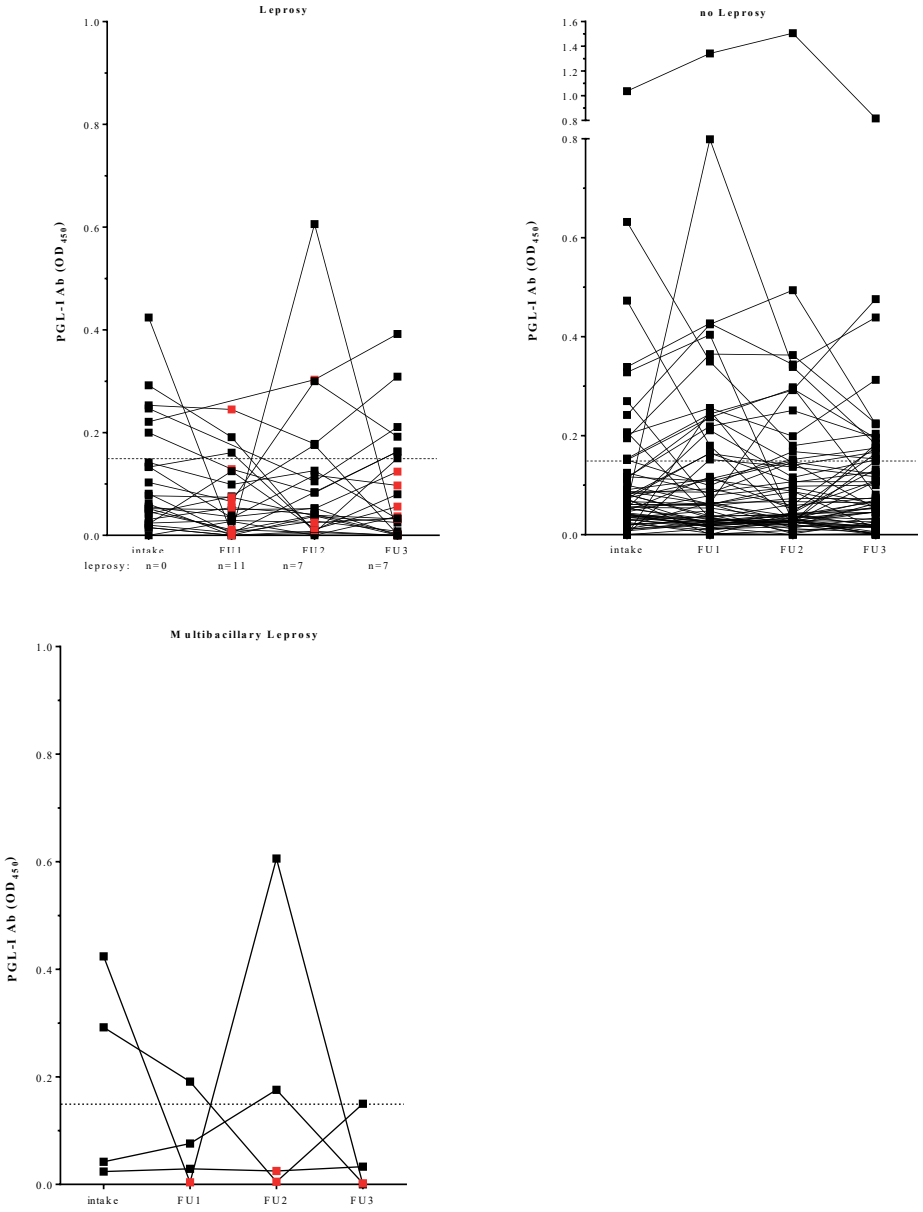
Figure 1. Cross-sectional analysis of anti-PGL-I Ig antibody levels at intake.



Legend to Figure 1. Anti-PGL-I antibodies at intake for contacts of leprosy patients who developed leprosy during the study (●; n= 25) and contacts who remained free of leprosy disease (▪; n=198) were detected by ELISA using natural disaccharide of PGL-I linked to HSA (ND-O-HSA). Optical density readings were performed at 450nm (OD_{450}) and corrected for background levels. Median values per group are indicated by horizontal lines. The cut-off for positivity is indicated by the dashed horizontal line.

To further analyze the longitudinal pattern of PGL-I serology in contacts, the anti-PGL-I Ab levels are depicted at different follow-up times, comparing the titers of contacts developing leprosy (Fig 2A) to the titers of contacts without leprosy (Fig 2B). The difference between anti-PGL-I Ab level at diagnosis was compared to the anti-PGL-I Ab level at intake. This difference was minus 0.047, indicating that the level of anti-PGL-I Ab titer was lower at time of diagnosis compared to time of intake. Next we calculated the difference between anti-PGL-I Ab titer at various time points of follow-up to the anti-PGL-I Ab level at intake of the contacts who did not develop leprosy using a generalized linear mixed model analysis. Thus, for all contacts who did not develop leprosy, we compared the anti-PGL-I Ab level at FU1 to intake, the level of FU2 to intake and the level at FU3 to intake. If a contact developed leprosy at FU2 (or FU3), we also included the difference between anti-PGL-I Ab titer at FU1 (and FU2) and intake into the group of contacts who did not develop leprosy. Differences in anti-PGL-I Ab levels had no significant association with the development of leprosy at either of the three follow-up time points (OR: 0.62 (0.15, 2.62), p=0.52). Thus changes in anti-PLG-I Ab levels are not predictive of disease progression in contacts of new leprosy patients in Bangladesh. Since MB patients harbor a higher quantity of bacteria than PB, we separately considered the longitudinal pattern of the anti-PGL-I Ab levels in the four contacts who developed MB leprosy (Fig 2C). The mean OD_{450} at the time of diagnosis for both MB/BT and PB patients was below threshold for positive (< 0.15). Also, no increase in anti-PGL-I Ab levels was observed at the moment of leprosy diagnosis; actually, anti-PGL-I Ab levels were often even lower at diagnosis time compared to intake. The findings indicate that not only for newly diagnosed PB, but also for MB patients, anti-PGL-I Ab levels do not represent a practical tool for prediction of leprosy.

Figure 2. Longitudinal analysis of anti-PGL-I Ig antibody levels.



Legend to Figure 2. Anti-PGL-I antibodies for all contacts of leprosy patients who developed leprosy during the study (**A**; n= 25) and contacts who remained free of leprosy disease (**B**; n=199) and 4 contacts who developed MB leprosy (**C**; n=4) were determined by ELISA using natural disaccharide of PGL-I linked to HSA (ND-O-HSA).

Sera were tested at three follow-up time points; FU1: 2 years after intake, FU2: 4 years after intake, FU3: 6 years after intake. Optical density readings were performed at 450nm (OD_{450}) and corrected for background levels. Anti-PGL-I Ab levels at the time point of leprosy diagnosis are indicated in red (**A** and **C**). The cut-off for positivity is indicated by the dashed horizontal lines.

Discussion

Although several studies described that positive anti-PGL-I Ab titers in household contacts of leprosy patients were related to a higher risk of developing leprosy[16-19], reports also indicated that more than half of the individuals with antibodies against PGL-I will never develop leprosy[16 17]. Besides, diagnosis based only on seropositivity for anti-PGL-I Abs would leave more than half of the new leprosy cases undetected[16 18 19]. To study the value of anti PGL-I Ab as a predictor of leprosy in those at risk of developing leprosy in a highly endemic area, we here analyzed the anti PGL-I Ab levels in the blood of 224 contacts of leprosy patients in the Northwest part of Bangladesh. However, no association was found between anti-PGL-I Ab levels and onset of disease in this population.

As part of a variety of studies investigating the use of serology for prediction of leprosy in those at risk of developing disease, a study in the state of Minas Gerais, Brazil[18] suggested that anti-PGL-I serology in household contacts of leprosy patients can be used to identify leprosy at a preclinical stage. This study identified more contacts with suspected leprosy in the group with positive anti-PGL-I levels (9.62%) than in the test-negative group (1.76%). However, out of the 52 contacts with positive anti-PGL-I serology, only 5 had leprosy. The anti-PGL-I seropositivity was higher in those contacts exposed to patients with MB leprosy than PB leprosy, which is probably due to the higher bacterial load in MB patients and therefore higher exposure rates of their contacts.

In another Brazilian study[17], performed in Rio de Janeiro, leprosy diagnosis had a strong association with anti-PGL-I seropositivity at intake. A significantly higher proportion of healthy contacts with anti-PGL-I Abs (5.6%) developed leprosy during the follow-up period compared with those without (2.3%). Anti-PGL-I seropositive contacts had a 3.2-fold higher risk of developing leprosy compared with seronegative contacts.

A third study performed in Cebu (the Philippines)[16] showed that household contacts of MB leprosy patients with anti-PGL-I Abs have a 7.65-fold-higher risk of developing leprosy in the six years of active surveillance than seronegative contacts. It is noteworthy that out of the 27 contacts developing leprosy, 13 remained seronegative, indicating that half of the new leprosy cases would not be detected when solely anti-PGL-I serology would be used as a predictive diagnostic tool. This particularly applies to PB cases, as all of the 10 newly diagnosed MB patients were or became seropositive. On the other hand, 85 out of the 99 anti-PGL-I Ab positive contacts never developed leprosy, implying a false positivity rate of 86% when using anti-PGL-I serology as a predictive marker for leprosy.

Barretto *et al.*[20] showed that the odds of seropositive versus seronegative school children developing leprosy within two years is 2.7 times higher in an hyperendemic region in the Amazones of Brazil (State of Pará). Thus, this would indicate a > 90 % probability of detecting at least one new case among 10 seropositive individuals in 2 years. On the other hand, 5 of 11 new cases found amongst school children in these high-risk areas in Brazil tested negative for anti-PGL-I Abs. Furthermore, no significant difference between the median anti-PGL-I Ab titer of new cases and of healthy school children was observed. Of note is that a significant increase in the anti-PGL-I IgM titers was found at the time of diagnosis compared to intake. The group that did not develop leprosy also demonstrated an increase in their average antibody titers, although the most significant increase was observed in the group that developed disease. These findings in Brazil stand in contrast to our current study in Bangladesh, in which hardly any difference or even a slight decrease in the anti-PGL-I Ab levels was observed in the contacts who developed leprosy.

A recent meta-analysis among household contacts of new leprosy patients in French Polynesia, Zaire, Papua New Guinean, Venezuela, Brazil, India and Philippines[19] shows that the risk of developing leprosy is about three times higher in those who are positive for anti-PGL-I Abs compared to the seronegative group, with the odds ratio varying from 2.72 to 3.53. However, the sensitivity of anti-PGL-I Ab tests as predictor of the development of clinical leprosy was found to be lower than 50% in all studies. Thus, selecting contacts with anti-PGL-I antibodies for prophylaxis, although possibly beneficial for reduction of transmission, would only prevent less than half of the leprosy cases among contacts. Our findings in contacts in Bangladesh are in line with those of the meta-analysis by Penna *et al.*[19] as well as the other studies discussed above, since development of leprosy was not associated with the level of anti-PGL-I seropositivity at intake, clearly indicating that also in Bangladesh anti-PGL-I Ab tests lack the ability to early diagnose leprosy amongst leprosy contacts[21-23], if used as a stand alone tool.

Most of the leprosy patients' contacts in our study developed PB leprosy (21 out of 25), which offers an explanation for the lack of increase of anti-PGL-I titers at leprosy diagnosis. Importantly, in Bangladesh, the percentage of PB cases amongst new leprosy cases is generally higher than in other countries in Asia, especially southeast Asia where predominantly MB patients are found[2]. This phenomenon is probably due to a combination of genetic factors as well as early case detection. Bangladesh is a high endemic area with a high rate of active case-finding, which leads to a lot of PB cases being found. In contrast, low endemic areas with little active case-finding have higher numbers of MB cases, since PB is often self-healing. In our study, only four household contacts developed MB leprosy (out of the 25 total number of new leprosy patients). PB leprosy in general is characterized by low levels or absence of antibodies against *M. leprae* antigens[16], which is in line with our finding that

there was no significant difference in anti-PGL-I Ab level at intake compared to leprosy diagnosis. Schuring *et al*[24] found that anti-PGL-I seropositivity was associated with bacterial index (BI). However, most contacts in our study had PB and therefore an undetectable BI. Separate evaluation of the four MB patients did not show any differential increase in anti-PGL-I Ab level in this group either. This is in line with the findings of van Hooij et al[4 25], showing low levels of anti-PGL-I Ab in all patients, including MB. Moreover, anti-PGL-I IgM levels could not be used to discriminate PB patients or household contacts from endemic controls. In leprosy endemic countries other than Bangladesh, where MB leprosy is more prevalent, the longitudinal pattern of anti-PGL-I Ab levels could hold more diagnostic value. Besides this, anti-PGL-I antibodies can represent a useful tool for monitoring effectiveness of treatment of leprosy (reactions), since effective treatment is associated with decrease in antibody levels[26].

As a part of the COLEP trial, half of the new leprosy contacts received placebo and the other half single dose rifampicin. It can be expected that single dose rifampicin could lower the anti-PGL-I antibody level in subjects with a relatively high bacterial load. However, it is unknown how soon and to which extent the antibody titre is suppressed. Furthermore, there is also the possibility that subjects become re-infected with *M. leprae* due to continued exposure to an unknown source. Also, in the absence of complete 'sterilisation' of *M. leprae* in these subjects, the bacterium may start to multiply again after the effect of rifampicin has waned. So although the antibody titre may certainly have decreased due to single dose rifampicin, it is unknown whether this effect would be apparent after 2 years, at the moment of first blood sampling.

Furthermore, it is worthy to note that leprosy is a complicated disease with different immunological processes playing a role in disease progression, which in turn are affected by factors such as genetics[27], co-infections[28] as well as food-shortage[29]. The combination of these factors with the long incubation time that elapses before leprosy becomes clinically manifest, makes predicting which *M. leprae* exposed individuals will progress to disease complicated. For example, certain helminth-derived proteins can bias the host immune response towards an anti-inflammatory Th2 response, which may facilitate *M. leprae* growth or progression to MB leprosy[28]. Furthermore, a period of food shortage can reduce cell mediated immunity of individuals incubating *M. leprae*, causing the development of clinical disease[29].

Recent advancements in leprosy biomarker research[15] have shown that IFN-γ responses measured after stimulation with leprosy-unique antigens can be used as a measure for *M. leprae* exposure. In particular, the combination of humoral and cellular biomarkers increased efficiency to distinguish *M. leprae* infected from non-infected individuals, patients from contacts, or lepromatous from

tuberculoid patients compared to serology alone[4 15]. In view of the findings in this study as well as our previous studies on cellular biomarkers[4 15 26 30], field-friendly tests using a biomarker signature would improve identification of contacts who are at risk of developing leprosy as well as asymptomatic, infected individuals who can transmit bacteria. In current longitudinal studies on biomarker identification, a new lateral flow test format is used (UCP-LFA)[4 25], that not only allows field-use but also provides a permanent record as the luminescent signal on the LF strips does not fade. Such tests would represent a useful contribution to current pilot studies on the effectiveness of SDR as leprosy post-exposure prophylaxis (LPEP)[31], allowing more selective targeting for prophylaxis as well as preventing overtreatment.

Conclusion

In view of the dichotomy of the leprosy spectrum in terms of immunity against *M. leprae*, current research is focused on identification of predictive biomarker profiles associated with early stage leprosy, consisting of multiple cellular and humoral (disease-specific) biomarkers. Early diagnosis of leprosy and subsequent appropriate multidrug therapy (MDT) will not only decrease severe nerve damage and subsequent lifelong handicaps, but also significantly contribute to further decrease of *M. leprae* transmission. This study shows that measurement of anti-PGL-I Abs alone is not sufficient to predict the development of clinical leprosy amongst household contacts of newly diagnosed leprosy cases in (highly) endemic area such as Bangladesh. Because of the high number of PB patients in Bangladesh, using anti-PGL-I titers as a screening test to discriminate which contacts to treat, may lead us to miss a lot of potential new cases.

Ethics Approval and Consent to Participate. Ethical clearance was obtained from the Ethical Review Committee of the Bangladesh Medical Research Council in Dhaka (ref. no. BMRC/ERC/2001-2004/799). All subjects were informed verbally in their own language (Bengali) about the study when they were invited to participate. Written consent was received from each adult, while a parent or guardian had to sign the consent form for children who participated in the study.

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CHAPTER 3

3

The combined effect of chemoprophylaxis with single dose rifampicin and immunoprophylaxis with BCG to prevent leprosy in contacts of newly diagnosed leprosy cases: a cluster randomized controlled trial (MALTALEP study)

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Abstract

Background: Despite almost 30 years of effective chemotherapy with MDT, the global new case detection rate of leprosy has remained quite constant over the past years. New tools and methodologies are necessary to interrupt the transmission of *M. leprae*. Single-dose rifampicin (SDR) has been shown to prevent 56% of incident cases of leprosy in the first two years, when given to contacts of newly diagnosed cases. Immunization of contacts with BCG has been less well documented, but appears to have a preventive effect lasting up to 9 years. However, one major disadvantage is the precipitation of excess cases within the first year after immunization. The objective of this study is to examine the effect of chemoprophylaxis with SDR and immunoprophylaxis with BCG on the clinical outcome as well as on host immune and gene profiles in contacts of new cases of leprosy. We hypothesize that the effects of both interventions may be complementary, causing the combined preventive effect to be significant and long-lasting.

Methods/design: Through a cluster randomized controlled trial we compare immunization with BCG alone with BCG plus SDR in contacts of new leprosy cases. Contact groups of around 15 persons will be formed for each of the 1300 leprosy patients included in the trial, resulting in a total of around 20,000 contacts. The intervention group will be given BCG immunization followed by SDR, 2 months later. The control group will receive BCG only. In total 10,000 contacts will be included in each intervention arm over a 2-year period. Follow-up will take place one year and two years after intake. The primary outcome is the occurrence of clinical leprosy within two years. Simultaneously with vaccination and SDR, blood samples for laboratory tests will be taken from 300 contacts participating in the trial to determine the effect of chemo- and immunoprophylactic interventions on immunological and genetic markers of infection.

Discussion: Combined chemoprophylaxis and immunoprophylaxis is potentially a very powerful and innovative tool aimed at contacts of leprosy patients that could reduce the transmission of *M. leprae* substantially. The trial intends to substantiate this potential preventive effect. Evaluation of immunological- and genetic biomarker profiles will allow identification of pathogenic vs. (BCG-induced) protective biomarkers and could lead to effective prophylactic interventions for leprosy by optimizing tools for identification of individuals who should best be targeted for prophylactic treatment.

Trial registration: Netherlands Trial Register: NTR3087

Keywords: leprosy, *M. leprae*, BCG vaccine, Rifampicin, prevention, RCT, Study protocol

Background

The global number of new leprosy cases has remained constant over the past years[1], indicating that the transmission of *Mycobacterium leprae,* the causative agent of leprosy, is ongoing in many endemic countries. The basic intervention is multidrug therapy (MDT) given to newly found leprosy cases, but this seems to be insufficient to decrease the number of new cases.

The main risk of exposure to *M. leprae* is in close contacts of new, untreated cases. Epidemiological studies have shown that the chance of finding a household contact with previously undiagnosed leprosy is ten-fold compared to the general population, and the chance for finding leprosy among different categories of neighbors and social contacts is between three and five-fold [2 3]. It has therefore been suggested that contacts should be the main focus of a future leprosy control strategy. Such strategy should have three basic pillars: 1) case detection; 2) case management; and 3) contact management.

In the past years, many studies have been done into the use of immunoprophylaxis (vaccination) and chemoprophylaxis to prevent leprosy. These interventions have focused primarily on contacts of leprosy patients. Bacillus Calmette-Guérin (BCG) vaccination is known as a vaccine against tuberculosis and is routinely given to infants as part of the neonatal immunization scheme in many parts of the world. BCG is also recognized as protecting against leprosy [4 5]. Over the years several vaccine trials using BCG have been performed to establish its protective effect against leprosy, often in combination with *M. leprae* or related mycobacterium vaccines. BCG was as good as, or superior to the other mycobacterium vaccines [6].

BCG efficacy appeared to be significantly higher among contacts of leprosy patients than among the general population: 68% *vs.* 53% [4]. In Brazil, the government officially recommends BCG to protect household contacts of leprosy cases. This policy was assessed in a cohort study showing that the protection conferred by BCG was 56% and was not substantially affected by previous BCG vaccination [7]. The risk of tuberculoid leprosy during the initial months was high among those vaccinated with no previous BCG vaccination; 21 of 58 new leprosy cases (36%) occurred in the first year. This risk however, had substantially declined by the first year and in the following years the protection rate in this group reached 80% [7]. The results of this study are not conclusive due to some methodological inconsistencies. In particular, the issue of increased risk of tuberculoid leprosy in the first months after BCG vaccination needs further evaluation.

With regard to chemoprophylaxis, the COLEP study showed that the use of a single dose of rifampicin (SDR) in contacts of newly diagnosed leprosy patients reduced the overall incidence of leprosy in the first two years with 57% [8]. Furthermore, this study showed that the effect of SDR depended on the BCG status of the contact [9]. If the contact had received BCG vaccination as part of a childhood vaccination program (as established by the presence of a BCG-scar), the protective effect of SDR was 80%. Childhood BCG vaccination and SDR both have a protective effect for leprosy in contacts of approximately 60%, but if a contact who had previously received BCG vaccination also received SDR, the protective effect appears to be additive.

Based on the experiences with BCG vaccination and SDR chemoprophylaxis in preventing leprosy among contacts of leprosy patients, a trial was initiated in Bangladesh to assess the efficacy of a combined strategy (acronym: MALTALEP study). The objective of this paper is to describe the design of a cluster randomized controlled trial, in which contacts of newly diagnosed leprosy patients will either receive BCG alone, or BCG plus SDR. In particular, it is important to determine whether the excess cases in the first year after immunoprophylaxis can be prevented by chemoprophylaxis.

Methods/design

Objectives and hypothesis. The objective of this study is to examine the combined effect of chemoprophylaxis with single dose rifampicin and immunoprophylaxis with BCG, in contacts of new cases of leprosy. Both interventions are known to have a preventive effect and we hypothesize that these effects may be complementary, so that the combined effect may be significant and long-lasting.

Study design. The intervention consists of a cluster randomized controlled trial, with two treatment arms, to study the effectiveness of the Bacillus Calmette-Guérin (BCG) vaccine versus BCG in combination with single dose rifampicin (SDR) in the prevention of leprosy under contacts of newly diagnosed leprosy patients.

Setting. The study takes place in the districts of Nilphamari, Rangpur, Thakurgaon and Panchagarh in northwest Bangladesh. Patients will enter into the trial through the Rural Health Program (RHP) of The Leprosy Mission International Bangladesh (TLMIB), located at the Nilphamari Hospital, a referral hospital specialized in the detection and treatment of leprosy. The population of the four districts is around 7,000,000 (Bangladesh Bureau of Statistics: Bangladesh Population & Housing Census 2011; http://www.bbs.gov.bd; accessed 9 Sept 2013) and 800-900 new leprosy patients are detected per year. The population in the four districts is mainly rural, but also includes six main towns.

Participants. Newly diagnosed leprosy patients will be included in the trial that have the diagnosis leprosy according to the Rural Health Program guidelines, which follow those of the National Leprosy Control Program [10 11]. All new leprosy patients are confirmed by a medical officer, and this confirmation is written on the patient card. Around 1,300 consecutive leprosy patients will be enrolled into the study. After a patient is diagnosed, patient details will be recorded (Table 1). Multidrug therapy (MDT) will be started according to the national guidelines. Intake of single-lesion PB (SLPB) patients will be stopped when 500 such patients have been included; the same will apply to the group of other PB patients (PB2-5, with two to five skin lesions on physical examination). This will ensure an intake of at least 300 multibacillary (MB) patients. Within two weeks after the new leprosy patient has received the second dose of MDT (four weeks after the first dose), a survey will

be performed under all household contacts. During this survey, contact groups will be formed consisting of around 15 persons for each patient. Thus, the total number of contacts included will be around 20,000.

Table 1. Patient and contact data recorded.

1	Personal data of patient and all selected contacts: name, year of birth, sex and relation of contact to the selected patient
2	Brief information regarding medical history of all contacts (liver disease, malignancies, HIV, TB, leprosy, pregnancy, vaccination status and medication use) to ensure that the participants have no contraindications for BCG vaccination or use of the medicine rifampicin
3	Results of physical examination on signs and symptoms of leprosy (including leprosy classification and WHO disability grade) and actions taken accordingly
4	Interventions: BCG vaccination, medication provided, blood sample taken
5	Record of any adverse reactions and actions taken accordingly
6	Report of follow up visits

Exclusion criteria for patients are as follows: any patient who refuses examination of contacts, any patient who suffers from the pure neural form of leprosy, any patient who resides only temporarily in the study area, any new patient found during contact examination of the index case, any new patient living less than 100 m away from a patient already included in the study or first and second degree relatives of a patient already included in the study.

The following categories of contacts of new leprosy patients have been distinguished for inclusion: those living in the same house (household members), those living in a house on the same compound, sharing the same kitchen, and direct neighbors (first neighbors). Exclusion criteria for contacts are as follows: any person who refuses informed consent, any woman indicating that she is pregnant, any person currently on TB or leprosy treatment, any person below 5 years of age, any person known to suffer from liver disease or jaundice, any person residing temporarily in the area, any person suffering from leprosy at the initial survey (these patients will be referred to the clinic for leprosy treatment) and any person who is a contact of another patient and is already enrolled in the contact group of the other patient.

Randomization. Each contact group will be randomly allocated to one of the two study arms (Arm 1: BCG only, or Arm 2: BCG plus SDR) by means of computer generation with a 1:1 ratio for each arm. The allocation to receive SDR is stamped on the data collection forms of each contact group. Immunoprophylaxis with BCG will be given at the moment of the contact survey to all included contacts in both arms of the trial, followed by chemoprophylaxis with SDR eight weeks later in contacts of Arm 2.

A schematic representation of the trial is given in Figure 1 (left side), together with a non-intervention group (right side) and the sampling framework for analysis of host immune and gene profiles, which is part of the IDEAL study (see below).

Outcome measures. The primary outcome measure is the number of new leprosy patients emerging from the contact groups. The proportions between the two arms of the trial will be compared after one and two years.

Secondary data analysis will be carried out in order to define special groups at risk for developing leprosy and blood sample analysis of host immune and gene profiles.

Intervention implementation and data collection. The medication provided in the trial is rifampicin. Rifampicin comes in capsules of 150 mg and the dosage is the same as recommended in the guidelines of the national leprosy control program of Bangladesh and RHP (Table 2). According to body weight and age, 2 to 4 capsules are taken by the contact under direct supervision of a RHP staff member.

Table 2. Dosage of rifampicin chemoprophylaxis according to age and body weight.

Age/weight	Dose of rifampicin
Adult >35 kg	600 mg
Adult <35 kg	450 mg
Child 10–14 years	450 mg
Child 5–9 years	300 mg

The vaccine provided in the trial is BCG. The BCG vaccine is applied by trained research assistants to all included contacts. 0.1 ml of BCG vaccine is given by intradermal injection. The BCG vaccine used in the trial (and in routine neonatal vaccination in Bangladesh) is produced at the Japan BCG Laboratory and is a freeze-dried glutamate BCG vaccine (Japan), composed of 0,5 mg/ampule live bacteria of Calmette-Guérin (as approximately 70% moist bacteria) and 2,0 mg/ampule sodium glutamate (as a stabilizer). Vaccines are stored at the State Immunisation Programme facilities.

All eligible patients and their contacts will be informed verbally about the study through the reading of the consent form, and then invited to participate. Before inclusion, the patient and their contacts are asked to sign a form if they agree to participate in the study. For illiterate people a thumb print will be taken, and for minors under 16 years of age, the guardian's additional consent will be taken. Contacts explicitly give consent for BCG vaccination and SDR, and for blood drawing. Furthermore, the researcher has to sign that he/she has accurately read or witnessed the accurate reading of the consent form to the participants, that the individuals have had the opportunity to ask questions and they have given consent freely. Participants will also be informed that they will be offered free consultation and treatment in the case of adverse events following BCG vaccination. They are provided with a vaccination card with details on how to reach the researcher if they have any concerns. Also, participants are informed that their participation is completely voluntary and that they may choose not to participate or stop at any point of time. Their decision not to volunteer, or to refuse to answer particular questions, will not affect their relationship with the researchers or other staff members of RHP.

At the initial contact survey, BCG will be given to all included contacts, followed by chemoprophylaxis with SDR two months later in those groups randomized to receive it (FU1). Follow-up examinations will be carried out one year (FU2) and two years (FU3) after receiving BCG. The three follow-up moments will be used to investigate whether the contact has developed leprosy or may be a suspected leprosy case (primary outcome measure). These patients will be sent to Nilphamari hospital or a local clinic for further investigation and treatment of leprosy. At these moments both groups will also be examined for adverse events following the BCG vaccination. Blood samples will be taken from 300 randomly chosen contacts for further molecular and immunological testing. Subjects not available for follow-up during the house visits will be contacted in order to plan another house visit. The trial started in July 2012 and will have duration of intake of 24 months. With a total observation period of 2 years after intake, the study will thus be completed after 48 months.

A separate database has been designed for the trial, which is linked to the database already in use at the RHP. Data are entered in the field onto purpose designed data sheets during clinic visits and

contact group surveys. These data are sent to the RHP center in Nilphamari, where they are entered into the database. All paper forms are scanned and filed on hard disk and CD. The paper copies of the data will be retained for at least 15 years after completion of the study. An electronic copy of the database is sent to the department of Public Health of Erasmus MC in the Netherlands on a monthly basis. Modern back-up facilities are available at Nilphamari as well. Protection of privacy of patients in the database will be according to Erasmus MC standards.

Blinding. Ideally, we would like to have set up a (double) blinded trial. However, this is not possible, since there are no placebo tablets of rifampicin available and we have not been able to locate any company that could produce these especially for this trial.

Adverse effects. Rifampicin can give adverse events, such as gastro-intestinal complaints, skin rash, elevated liver enzymes, headache, dizziness, influenza-like syndrome, acute loss of kidney function, thrombocytopenia, asthma-like symptoms and shock [12]. Also, rifampicin can cause urine, saliva, tears and faeces to turn an orange or red colour. However, the chance of developing these symptoms is low, especially when giving a single dose of rifampicin only. In a previous trial, in which over 20,000 contacts of leprosy were given SDR, no adverse events were reported, apart from innocent red discoloration of the urine (for which the contacts were forewarned) [8 13].

Serious complications of BCG vaccination are uncommon. Although localized skin reactions occur frequently; less than one in 1000 people vaccinated develop significant local reactions, such as abscesses or regional lymphadenitis [13 14]. More serious adverse effects include osteitis, osteomyelitis and disseminated infection, but these are rare [15-17]. As many as 95% of BCG recipients have an insignificant, local reaction at the site of inoculation, however, lesions typically heal by three months with permanent residual scarring at the puncture site.

Both interventions (BCG and SDR), have separately been used widely in contacts of leprosy patients, with minimal adverse effects [8 18]. There is no reason to expect any serious difficulties from the combined interventions, as they will be given two months apart. However, strict monitoring of adverse events will take place in the trial. Leaflets containing information about the aims and the methodology of the trial, and describing potential adverse reactions will be given to all contacts included in the trial. These leaflets request that contacts report any suspected adverse reactions to the responsible researcher. The responsible researcher will then examine all contacts with reported adverse reactions. All contacts will also be examined two months, one year and two years after

administration of the BCG vaccine. Data on adverse events is collected on the Contact Registration Forms of the trial. In the event of minor side effects, contacts will be referred to a State Tuberculosis Medical Officer for treatment, but the trial will not be stopped. In case of serious adverse effects the PI will stop the trial and initiate an individualized treatment scheme. All costs for treatment will be refunded.

Data analyses. Statistical analyses will be done using SAS software. We use techniques for the analysis of survey samples to account for the clustering at the level of the index patient in the sample. Bivariate associations are investigated using "proc surveyfreq" and the Rao Scott $\chi 2$ instead of the Pearson $\chi 2$. We also use "proc surveylogistic" instead of the ordinary logistic regression procedure. We report odds ratios, but because of the low prevalence of the outcome these are comparable with relative risks. The number needed to treat (NNT) is calculated per subgroup of contacts. A significance level of 5% is used in all tests.

Sample size calculation. In our power calculation, heterogeneity in the chance of contacts to develop clinical symptoms of leprosy was taken into account, but no major effect on the numbers needed was found. In the earlier COLEP trial [8] we found an incidence rate (IR) of leprosy among household contacts and direct neighbors of 4 per 1000 per year in the untreated group over the first two years. We hypothesize that in contacts receiving BCG only, this number will be the same in the first year or possibly increase slightly. Also based on the previous trial, we expect a 50% reduction through the SDR intervention (IR of 2 per 1000). On the basis of these figures (with $\alpha = 0.05$ two-sided, power = 0.80), a total of about 10,000 contacts will be necessary in each group in order to detect reliably the expected protective effect of the BCG plus SDR combination of 50%, even taking into account an expected 10% loss to follow-up of contacts.

Blood samples for analysis of host immune and gene profiles. Early detection of *M. leprae* infection (before clinical manifestations occur) is vital to reduction of transmission. However, current diagnosis relies on detection of clinical signs, since there are no tests available to detect asymptomatic *M. leprae* infection or predict progression to leprosy. Furthermore, although BCG vaccination and rifampicin chemoprophylaxis are both proven strategies for leprosy prevention, it is not known how the immunological and genetic biomarker profiles of infection are influenced by these (combined)

interventions. Identification of such profiles will enable distinguishing pathogenic from protective biomarkers and lead to effective prophylactic interventions for leprosy.

In this study we intend to evaluate and optimize diagnostic tools for identification of individuals who should best be targeted for prophylactic treatment. In order to develop improved diagnostic tests based on reliable biomarkers that are detectable in blood samples, this study will analyze immune and genetic host markers in order to identify biomarkers that distinguish individuals controlling bacterial replication from those developing disease using the following assays:

1. Whole blood assays (WBA):Upon recruitment 4 ml venous blood will be drawn and used directly in three WBA, using tubes pre-coated with *M. leprae* WCS, ML2478/ ML0840 recombinant proteins or without stimulus. Each tube will be marked with a colored cap specific for one of these stimuli. After 24 hour incubation at 37 °C, tubes will be frozen and stored for analysis of cellular markers [19] and/or analysis in the recently developed field-friendly lateral flow assays for detection of Th1/Th2 cytokines as well as anti-PGL-I Ab [20].

2. Dual color Reverse Transcription Multiplex Ligation dependent Probe Amplification (dcRT-MLPA). From each individual venous blood (app. 2.5 ml) will be added to a PAXgene® tube and stored at -80 °C. Total RNA will be extracted, purified and used to identify differential gene expression by dcRT-MLPA [21] using 179 selected target genes (Geluk A, Van Meijgaarden KE, Wilson L, Van der Ploeg- van Schip JJ, Bobosha K, Quinten E, Dijkman K, Franken KLMC, Haisma I, Haks MC *et al*: longitudinal Immune Responses and Gene expression Profiles during Development of Type I Leprosy Reaction. In preparation).

Blood samples will be taken from 150 randomly selected contacts in both arms of the trial (total 300) 6 weeks after BCG vaccination (Figure 1). In addition, blood will be taken from any contact developing leprosy during the observation period of 24 months at the time of diagnosis before treatment. The aim of this part of the study is to identify:

1. Host immune and gene expression profiles specific for pathogenic and protective immune responses to *M. leprae* by comparison of profiles of patients *vs.* contacts.

2. Effect of chemo- and immunoprophylactic interventions on markers of infection and clinical disease by comparison of profiles of BCG-vaccinated *vs.* non-vaccinated contacts.

As part of our study on host immune and gene profiles in a non-intervention group, conducted by the IDEAL (Initiative for Diagnostic and Epidemiological Assays for Leprosy) consortium, similar blood samples will also be taken from a cohort of 500 new leprosy patients, 5000 of their contacts, and from new cases of leprosy arising from this contact group during a 24-month observation period. As a referent group (endemic controls), 250 healthy individuals from the general population will be sampled as well.

Preparations and process evaluation. The trial is conducted according to detailed research protocols that were developed in close consultation with the senior staff of RHP. In addition, an online Good Clinical Practice (GCP) course was completed by all PI's. All research assistants received training in research protocol procedures and giving BCG. They were also assisted in the field by the staff of the national EPI program when giving the BCG, until they were well enough trained to do this independently. Training (both theoretical and practical) was also given in the venapunction of blood for the additional molecular and immunological tests to be performed later. All researchers have a professional background in the diagnosis and treatment of leprosy and received refresher courses on this.

Quality checks on all aspects of the data collection and entry are performed regularly, and feedback on the results is given to the field staff and the data entry manager. For this purpose Erasmus MC has employed a medical doctor as independent Trial Monitor in Bangladesh to perform supervision tasks on a monthly basis to ensure optimal compliance to the study protocol.

Discussion

Combined chemoprophylaxis and immunoprophylaxis is potentially a very powerful and innovative tool aimed at contacts of leprosy patients, which could reduce the transmission of *M. leprae* substantially. The trial intends to substantiate this potential preventive effect.

Childhood BCG vaccination and SDR both have a protective effect for leprosy in contacts of approximately 60%[7,8]. But if a contact who had previously received BCG vaccination also received SDR, the protective effect appears to be up to 80%[9]. However, the Brazilian trial[7] showed that there was an increased risk of tuberculoid leprosy in the first months after BCG vaccination, even though this was fully compensated later on. Because this trial was not conclusive, it is important to determine whether the excess cases in the first year after immunoprophylaxis can be prevented by chemoprophylaxis.

Evaluation of immunological and genetic biomarker profiles will allow identification of pathogenic versus (BCG-induced) protective biomarkers and could lead to effective prophylactic interventions for leprosy using optimized tools for identification of individuals who are most at risk of developing disease.

The global number of new leprosy cases has remained constant over the past years, indicating that the transmission of leprosy in close contacts of new, untreated cases is still ongoing. The combined use of BCG and rifampicin could be a powerful to tool in routine leprosy control to interrupt the transmission of leprosy.

Ethical approval

The national Research Ethics Committee (Bangladesh Medical Research Council) has approved the study protocol (Ref no. BMRC/NREC/2010-2013/1534).

Competing interests

The authors declare that they have no competing interests. The BCG vaccine will be provided free of charge by the Government of Bangladesh.

Authors' contributions

All authors contributed to the design of the study and manuscript preparation. All authors have read and approved the final manuscript.

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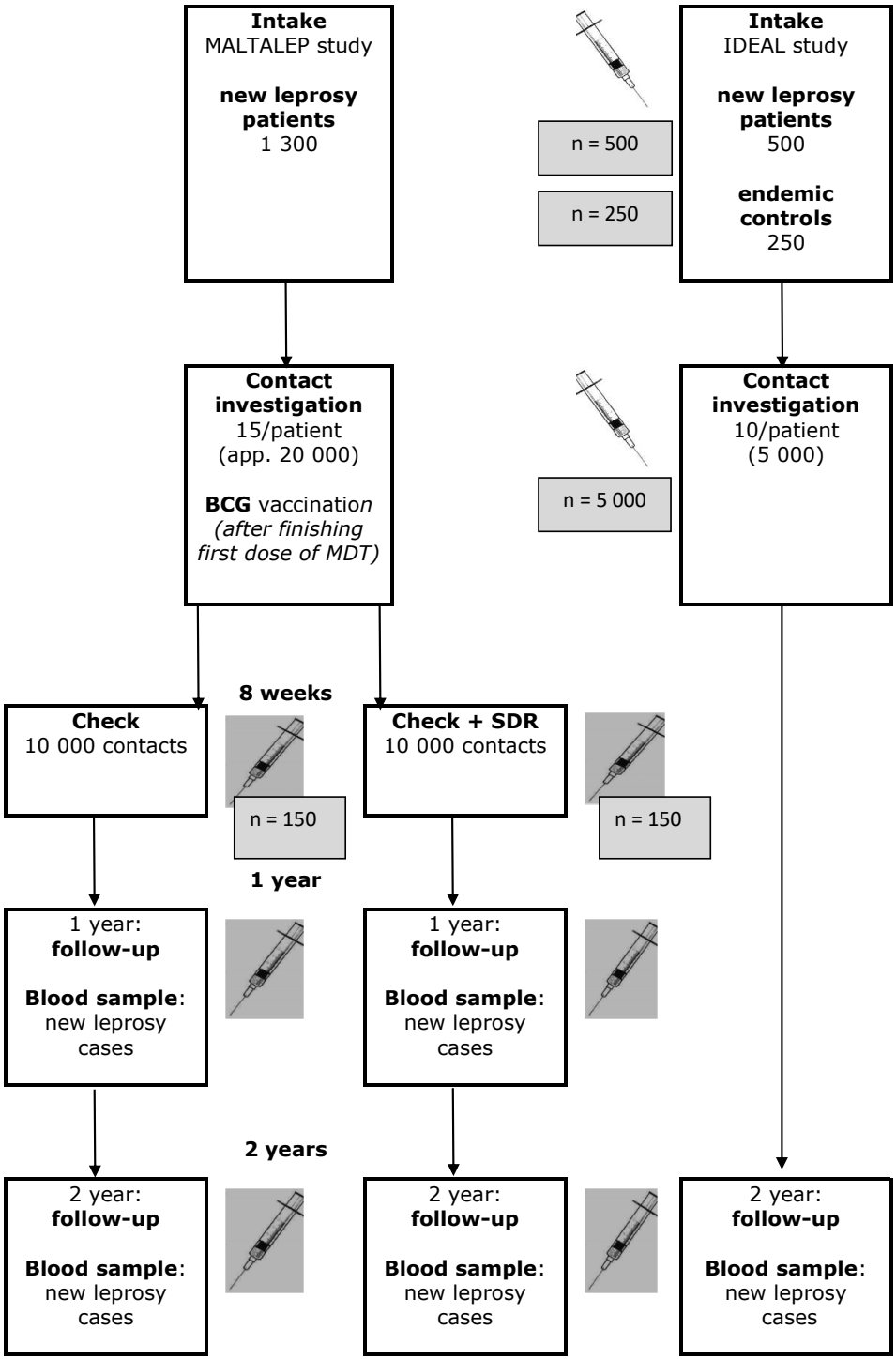
Figure 1. Schematic representation of the trial (MALTALEP study), together with the blood samples taken for analysis of host immune and gene profiles from subjects in the trial and in a non-intervention group (IDEAL study)


Intake
MALTALEP study
new leprosy patients
1 300
n = 500
n = 250
Intake
IDEAL study
new leprosy patients
500
endemic controls
250
Contact investigation
15/patient
(app. 20 000)
BCG vaccination
(after finishing first dose of MDT)
n = 5 000
Contact investigation
10/patient
(5 000)
8 weeks
Check
10 000 contacts
n = 150
Check + SDR
10 000 contacts
n = 150
1 year
1 year:
follow-up
Blood sample:
new leprosy cases
1 year:
follow-up
Blood sample:
new leprosy cases
2 years
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follow-up
Blood sample:
new leprosy cases
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CHAPTER 4

Clinical manifestations of leprosy after BCG vaccination: an observational study in Bangladesh

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Abstract

Background: Although BCG is used as a vaccine against tuberculosis, it also protects against leprosy. Previous evaluation over 18 years of an intervention of two doses BCG for 3536 household contacts of leprosy patients showed that 28 (23%) out of 122 contacts diagnosed with leprosy, developed symptoms 2-10 months after vaccination. This study describes contacts of leprosy patients in Bangladesh who developed leprosy within 12 weeks after receiving a single BCG dose.

Methods: A cluster RCT in Bangladesh aims to study the effectiveness of the BCG vaccine versus BCG in combination with single dose rifampicin (SDR) given 2 to 3 months after BCG, in the prevention of leprosy among contacts of newly diagnosed leprosy patients. During the first 1,5 years of this ongoing trial we identified contacts who developed leprosy within the first 12 weeks after receiving BCG vaccination, the timeframe before SDR is given.

Results: We identified 21 contacts who developed leprosy within 12 weeks after BCG vaccination among 5,196 vaccinated contacts (0.40%). All 21 cases presented with paucibacillary (PB) leprosy, including children and adults. About half of these cases had previously received BCG vaccination as indicated by the presence of a BCG scar; 43% presented with signs of nerve function impairment and/or Type 1 (reversal) reaction, and 56% of the index patients had multibacillary (MB) leprosy.

Conclusion: An unexpectedly high proportion of healthy contacts of leprosy patients presented with PB leprosy within 12 weeks after receiving BCG vaccination, possibly as a result of boosted cell-mediated immunity by homologues of *M. leprae* antigens in BCG. Various immunological mechanisms could underlie this phenomenon, including an immune reconstitution inflammatory syndrome (IRIS). Further studies are required to determine whether BCG vaccination merely altered the incubation period or actually changed the course of the infection from self-limiting, subclinical infection to manifest disease.

Key words: leprosy, BCG, contacts, *M. leprae,* prevention, prophylaxis

Introduction

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) remains the only available vaccine against tuberculosis (TB) today. It is routinely administered to infants in many countries worldwide and confers significant protection against severe forms of TB, mostly miliary and meningeal in young infants. BCG-induced immunity has been shown to decline with time and is generally thought to last no more than 10-15 years, differs between ages and endemic areas, and offers poor protection against contagious pulmonary TB in adulthood [1-4].

Despite being known primarily as a vaccine against TB, BCG also protects against leprosy (caused by *Mycobacterium leprae*), especially when given to household contacts of leprosy patients [5 6]. In fact, to date, BCG has been shown to be the best available vaccine for prevention of leprosy, superior to other mycobacterium containing vaccines, including combination vaccines with BCG and *M. leprae* specific vaccines [7 8]. The rationale for the use of BCG as a vaccine against leprosy relies on the occurrence of many highly homologous antigens present in the *M. bovis* genome (the progenitor for the BCG vaccine) and the *M. leprae* and the *M. tuberculosis* genomes [9 10], which induce cross-reactive, protective immune responses to *M. leprae* following BCG vaccination.

Because of BCG's protective effects against leprosy, Brazil has officially recommended BCG since the early 1970s for household contacts of leprosy cases, as a boost to routine BCG vaccination in new-borns as a TB prophylactic vaccine. Since 1991, the Brazilian Ministry of Health has advised two doses of BCG to be administered to both current household contacts and contacts of index cases who were diagnosed within the previous five years. This policy was assessed in a cohort study of 3536 contacts of 1,161 leprosy patients in Brazil [11], showing that the protection conferred by a booster BCG vaccination was 56% and was not substantially affected by previous BCG vaccination. Among the 122 new cases detected during 18 years of contact follow-up, leprosy was diagnosed in 28 of these contacts (23%) relatively soon after vaccination (2-10 months). Due to incomplete follow-up, the study needs to be interpreted with caution, and in particular the increased risk of tuberculoid leprosy in the first months after BCG vaccination needs further substantiation.

The COLEP study in Bangladesh showed that the use of a single dose of rifampicin (SDR) in contacts of newly diagnosed leprosy patients reduced the overall incidence of leprosy in the first two years by 57% [12]. Furthermore, this study showed that the effect of SDR depended on the BCG status of the contact: If the contact had received BCG vaccination as part of a childhood vaccination program (as established by the presence of a BCG-scar), the protective effect of SDR was 80% [13]. And if not, the protective effect of BCG alone was 57%.

In view of the above findings regarding BCG vaccine and SDR in contacts of leprosy patients, a cluster randomized controlled trial was initiated in Bangladesh in 2012 with the aim to study the effectiveness of the BCG vaccine versus BCG in combination with SDR given 2 to 3 months after BCG, in the prevention of leprosy among contacts of newly diagnosed leprosy patients [14]. In this trial special attention is given to the occurrence of clinical manifestations of leprosy in the first 12 weeks after the contacts received BCG vaccination, the timeframe before SDR is given. Here we report the occurrence of 21 cases of leprosy (among 5,196 vaccinated contacts) during this first period after BCG vaccination and describe the characteristics of these patients and their disease symptoms. Furthermore, the possible underlying immunological mechanisms and implications for public health practice are discussed.

Methods

The study is part of the MALTALEP trial [14] that is currently conducted in the districts of Nilphamari, Rangpur, Thakurgaon and Panchagarh in northwest Bangladesh. Leprosy patients are recruited into the trial through the Rural Health Program (RHP) of The Leprosy Mission International Bangladesh (TLMIB), located in Nilphamari; a referral centre specialized in the detection and treatment of leprosy. The population of the four districts is around 7,000,000 (2011 census [15]) and approximately 600 new leprosy patients were detected per year between 2011 and 2013. The population in the four districts is mainly rural, but also includes six main towns.

The MALTALEP trial is a cluster randomized controlled trial. The aim is to study the effectiveness of the BCG vaccine alone versus BCG in combination with single-dose rifampicin (SDR) in the prevention of leprosy among contacts of newly diagnosed leprosy patients. Full details of the trial protocol were described previously (9). In summary, contact groups of approximately 15 persons are established for each of the 1,300 newly diagnosed leprosy patients (index cases) included in the trial, which will result in roughly 20,000 contacts in total. The contact groups are divided randomly over the two arms of the trial with approximately 10,000 contacts each. Contacts who have been diagnosed with leprosy in the past, are diagnosed at the intake examination (i.e. co-prevalent cases) or are clinically considered to be leprosy suspects at intake examination, are excluded from the trial. All contacts are screened by trained and experienced health workers at intake, to ensure they had no apparent signs of leprosy at the time of intake. After written informed consent was obtained, BCG was administered to all subjects (i.e. healthy contacts) followed by SDR 8-12 weeks later in the intervention group. Subsequent follow-up takes place one year and two years after intake. The primary outcome is the occurrence of clinical leprosy within two years of intake. Individuals who are suspected to have leprosy at any of the follow-up time points or who present to a health clinic between follow-ups are sent to the specialised leprosy hospital in Nilphamari or a local clinic for confirmation of their disease by a specialist clinician and for treatment. Intake for the trial was started in August 2012 and is expected to be completed in 2015.

In this paper we report on incidental observations during the ongoing trial of all new leprosy cases among healthy contacts who were diagnosed within 12 weeks after receiving BCG (and before receiving SDR) between December 2012 and May 2014. We present demographic and clinical data of the patients as recorded in our database as a routine procedure for the purpose of the trial.

Results

A total of 21 contacts (0.40%) were diagnosed with leprosy within 12 weeks after receiving BCG vaccination, out of 5,196 contacts who had received BCG and were screened after 8-12 weeks.

Table 1 shows the characteristics of the healthy contacts who developed leprosy within 12 weeks after BCG vaccination. Of these contacts, 10 (48%) were male and 11 (52%) female. Table 2 shows the characteristics of the contacts who received BCG vaccination but who did not develop leprosy. The differences between the groups do not show statistical significance (P>0.05) due to the low number of contacts with leprosy, but some of the observed group characteristics are worth noting. The male-female distribution is also nearly equal in this group (47% and 53%, respectively). The average age at registration was 29 years (range: 10 – 70 years) among the contacts who developed leprosy, and 28 years (range: 5 – 90 years) in the group of contacts who did not develop leprosy. There were 8 children (≥ 5 to < 16 years of age) who developed leprosy within 12 weeks after BCG vaccination, representing 38% of the new cases. Among the contacts who did not develop leprosy, 34% were children. Nine (43%) of the new patients were household contacts to the index patient, sharing either the same kitchen or roof, or both. The remaining 12 (57%) were direct neighbours of the index patient. In the group of contacts who did not develop leprosy, 31% were household contacts of the index patient, a lower proportion. Nine contacts who developed leprosy (43%) were known to be blood relatives to the index patient, 3 were other relatives (unclear if blood relative or not), or in-laws. In the group of contacts who did not develop leprosy, 25% were blood relatives to the index patient. Twelve (57%) contacts developing leprosy had probably received BCG for the first time or no sufficient response was induced upon initial vaccination, since no BCG scar was observed. The other 9 (43%) had a BCG scar and were thus revaccinated. In the group of contacts that did not develop leprosy, the proportion with a BCG scar was higher (56%). These differences are also apparent in the proportion of leprosy among household contacts (0.55%) and neighbours (0.34%), blood related (0.69%) and not blood related relatives (0.30%), and those with (0.31%) and without (0.53%) a BCG scar (Table 2).

The average time from BCG to first suspicion of leprosy by the field staff was 9 weeks (range: 3-11 weeks) (Table 1). Two of these contacts came to a clinic on their own initiative before the planned follow-up time, because they detected leprosy patches themselves (3 and 9 weeks after BCG). When asking the contacts how long after having received BCG the patch had appeared, 7 contacts could not provide a clear answer as to when they first discovered a patch or they had only noticed it at follow-up time point when the staff pointed it out. The remaining 14 recalled having first seen the patch between 2 and 11 weeks after receiving BCG, although few could recollect an accurate date.

Table 1. Characteristics of new cases of leprosy among contacts within 12 weeks of BCG vaccination.

Contact No.	Age	Sex	Blood relation to index*	Contact level**	BCG scar (Y/N)	Time from BCG to patient first noticing patch (in weeks)	Time from BCG to first suspicion of leprosy by clinician (in weeks)	Smear result (BI)	Classification (Ridley Jopling)	Nerve involvement (Y/N)	Reversal reaction (Y/N)
1	24	F	N	H	Y	Unknown	10	negative	TT	N	N
2	10	F	N+	N	Y	10	10	negative	TT	N	N
3	11	F	Y	H	N	Unknown	9	refused	BT	N	N
4	40	M	N	N	Y	Unknown	10	negative	BT	N	N
5	60	F	Y	N	N	Unknown	10	negative	BT	Y	N
6	70	M	N	N	N	2	10	negative	TT	N	N
7	12	F	Y	H	Y	7	9	refused	TT	N	N
8	40	F	N	N	N	10	10	negative	BT	N	N
9	55	M	Y	N	N	Unknown	9	negative	BT	Y	Y
10	34	M	Y	N	N	4	9	negative	BT	N	Y
11	12	F	N+	H	Y	Unknown	10	negative	BT	Y	N
12	35	F	Y	H	N	11	11	negative	TT	N	N
13	38	M	N	N	N	4	9	negative	BT	N	N
14	27	F	N	N	Y	4	9	negative	BT	N	Y
15	15	F	N	N	Y	2	9	negative	BT	Y	N
16	16	M	Y	H	Y	Unknown	9	negative	I	N	N
17	12	M	Y	H	Y	10	10	negative	I	N	N
18	12	M	N+	H	N	4	10	negative	I	N	N
19	12	M	N	N	N	4	10	negative	BT	Y	N
20	22	M	Y	H	N	3	3	negative	BT	Y	N
21	60	F	N	N	N	4	9	negative	TT	N	Y

*Blood related contact: child (son/daughter), parent (father/mother), brother or sister; +Other relative: unclear if blood related or not**H: household contact; sharing either the same roof or kitchen, or both; N: neighbour living next door to patient`s house.

Table 2. Characteristics of contacts with leprosy within 12 weeks after BCG vaccination, compared to those contacts who received BCG vaccination but who did not develop leprosy.

Contact characteristics	Contacts with leprosy		Contacts without leprosy		All contacts	Contacts with leprosy****
	N	% ***	N	%***	N	%***
Number	21	-	5175	-	5196	0.40%
Male	10	48%	2426	46.9%	2436	0.41%
Female	11	52%	2749	53.1%	2760	0.40%
< 16 years	8	38%	1742	33.7%	1750	0.46%
≥ 16 years	13	62%	3433	66.3%	3446	0.38%
Household contact*	9	43%	1620	31.3%	1629	0.55%
Neighbour**	12	57%	3555	68.7%	3567	0.34%
Blood related	9	43%	1301	25.1%	1310	0.69%
Not blood related or unknown	12	57%	3874	74.9%	3886	0.30%
BCG scar	9	43%	2906	56.2%	2915	0.31%
No BCG scar	12	57%	2269	43.8%	2281	0.53%
Average age at registration	29 years		28 years			

*Household contact: sharing either the same roof or kitchen, or both
**Neighbour next to patient
*** χ^2 test: none of the differences in percentages between the two groups are statistically significant (P>0.05)
****Contacts that developed leprosy in each subgroup as a percentage of the total number of contacts in the same subgroup

All contacts with leprosy were classified as paucibacillary (PB). According to the Ridley-Jopling classification [16], 6 (29%) contacts were classified as tuberculoid (TT), 12 (57%) as borderline tuberculoid (BT), and 3 (14%) as indeterminate (I). Six contacts (29%) presented with nerve involvement, but only one had disability (partial foot drop). This contact (#9 in Table 1) asserted that the foot drop was present before BCG vaccination, but it was not noted by the staff at contact registration time. Possibly he was a co-prevalent case incorrectly registered at intake. The fact that he did not recover on steroids indicates that it was possibly a late-stage nerve function impairment. All known skin smears were negative, two contacts refused skin smears (because of young age).

Of the 21 contacts who developed leprosy after BCG, 4 (19%) had Type 1 (or reversal) reaction requiring steroids on initial presentation, including the patient described above with neuritis and partial foot drop. Three other patients (14%) who had no nerve involvement presented with a red, hot, swollen, anaesthetic patch indicating a mild Type 1 reaction. One of these had a second episode of reaction during the study requiring steroids and responded well. In July 2014, 6 of the contacts completed multidrug therapy without having any signs of reaction. Others were still on treatment.

Table 3 shows the characteristics of the 18 index patients of the contacts diagnosed with leprosy in the first 12 weeks after BCG vaccination. In the case of two index patients, multiple contacts were found with leprosy within 12 weeks (2 and 3 contacts, respectively). Of the remaining 16 index patients each had one contact that developed leprosy. The average age at registration of the index patient was 33 years (of which 3 index cases were younger than 16 years). This resembles closely the average age (35 years) of all new patients that were registered by the Rural Health Program in 2013 (data not shown). Among the index patients 8 (44%) were male and 10 (56%) female. In the group of all patients registered in 2013, the percentage of males and females was nearly equal. Of 18 index patients, 8 (44%) were classified as PB and 10 (56%) as MB leprosy. In the group of all patients registered in 2013, these percentages were the other way around, 66% and 34% for PB and MB, respectively. According to the Ridley-Jopling classification, all index patients were BT, except for one borderline lepromatous (BL) and one lepromatous (LL) patient. The bacterial index (BI) for most index patients was negative except for the one BL patient with a BI of 4 and the LL patient with a BI of 6. One patient refused to have a smear taken. In the 16 index patients symptoms were detected at an average of 38 months before diagnosis (range 5 to 120 months). The duration of delay was 18 months (range 1 to 264 months) in the group of patients registered in 2013. At intake six contacts (other than the contacts who were found to have leprosy at 8-12 weeks after BCG) of four index cases gave a history of leprosy in the past, but no details were available. One family represented an exception to this finding: the father was a smear positive MB case who was released from treatment in 1985 and restarted MB-MDT in 2013, and thus probably was the primary source of infection. One of his sons was the index case at intake of the trial and one of the other sons developed leprosy within 12 weeks after BCG vaccination. In this family there were two more family members with a history of leprosy. The father is included in Table 3 as one of the 3 contacts ever found with leprosy.

Table 3. Characteristics of the index cases according to new cases found among healthy contacts (see Table 1 for serial number of the new cases).

Index patient No.	Contact patient No.	Sex	Age	Classification (PB/MB)	Classification (Ridley-Jopling)	Smear result (BI)	Duration of symptoms before diagnosis (in months)	No. of contacts found at intake who ever had leprosy	No. of co-prevalent cases (contacts found with leprosy at intake)
1	1	M	23	MB	BL	4	36	1	0
2	2	F	55	PB	BT	0	12	0	0
3	3	F	30	MB	BT	0	72	0	0
4	4	M	26	PB	BT	0	not available	0	0
5	5	F	13	PB	BT	0	12	0	0
6	6	M	29	PB	BT	0	12	0	0
7	7	F	16	PB	BT	not taken	24	1	0
8	8	M	19	PB	BT	0	24	1	0
9	9	M	61	MB	BT	0	36	0	0
10	10	M	27	MB	LL	6	12	0	0
11	11	F	50	MB	BT	0	84	0	0
12	12	M	9	MB	BT	0	5	0	0
13	13	F	45	MB	BT	0	84	0	0
	14								
	15								
14	16	M	27	MB	BT	0	12	0	0
15	17	F	65	PB	BT	0	12	0	0
	18								
16	19	F	51	MB	BT	0	120	0	0
17	20	F	11	MB	BT	0	not available	3	1
18	21	F	40	PB	BT	0	48	0	0

Discussion

We found that 21 out of 5,196 (0.4%) healthy contacts of newly diagnosed leprosy patients in the ongoing BCG intervention trial in Bangladesh developed clinical evidence of leprosy within 12 weeks after receiving BCG. All these 21 contacts presented with PB forms of leprosy (I, TT and BT), with a nearly equal number of males and females, and including both children and adults. Nearly half (43%) presented with signs of nerve function impairment and/or Type 1 reaction. Among the contacts with leprosy there was a high number with MB index cases (56%) and with a long average duration of symptoms before diagnosis, possibly indicating that these contacts experienced a high level of exposure over a long time.

The reported prevalence of leprosy in the four districts of northwest Bangladesh in 2013 was 0.74 per 10,000 population and the new case detection rate 0.84 per 10,000 (source: Rural Health Program). Considering the high prevalence of leprosy in this area, it is not surprising that there are many people with subclinical leprosy, some of whom may present clinical signs and symptoms for the first time after receiving BCG. Since all of these 21 cases were tuberculoid forms of leprosy, the increase of *M. leprae*-reactive cellular immunity may result from boosting of cell-mediated immunity by homologues *M. leprae* antigens present in BCG. Alternatively, BCG vaccination has been shown to induce epigenetic reprogramming of innate cells leading to increased cytokine production in response to related and nonrelated pathogens for up to 3 months after vaccination, a phenomenon called trained immunity [17].

Past studies have shown sporadically that BCG may induce clinical expression of leprosy skin lesions in the short term [18 19]. It fact, this phenomenon was discussed as early as 1960, when an editorial in the International Journal of Leprosy addressed 'BCG-induced activations' and referred to two case reports in the French literature in 1958 [18]. Data from the Karonga Prevention Trial between 1986 and 1989 in Malawi indicated that protection against leprosy is afforded by a repeated BCG vaccination, even during the first year after revaccination, but that the case series is too small to confirm early 'induction' of leprosy after BCG [20]. The main reason for paucity of information in literature about this issue is that most trials only include long-term follow-up, often starting 1 year after vaccination. Taking into account in particular the data described for BCG vaccination of contacts in Brazil [11], we anticipated a probable increase in new leprosy patients in the first year after BCG, although we had not expected this to occur as early (within 12 weeks) after BCG vaccination, as was observed in the current study. Düppre et al. [11] hypothesized that the accelerated manifestations of tuberculoid leprosy after BCG vaccination found in their study in Brazil, reflected the influence of BCG in catalyzing the existing anti-mycobacterial immunity in subjects infected with *M. leprae* before or

immediately after BCG vaccination. In line with the Brazilian study, we also found predominantly tuberculoid forms of leprosy. The incidence rate in the Brazilian study in the first year was higher among the contacts without a BCG scar than among those with a scar. We found a similar tendency in our study, although the difference was not very large. Finally, among the contacts who developed leprosy soon after BCG, there was a relative high number of contacts with manifestations of Type 1 reaction, which was not described in the Brazilian study.

Live vaccines, in particular BCG, have a nonspecific beneficial effect on overall mortality when administered early in life, more than can be explained by the targeted infection [21]. In fact children with a scar or a positive skin test resulting from BCG vaccination, exhibit an overall reduction in child mortality of around 50% [22]. In adults, immunization with BCG causes increased levels of pro-inflammatory cytokines TNF and IL-1β in response to BCG-related stimuli that is maintained for up to three months after vaccination [23]. The adaptive immune response after BCG vaccination is clearly Th1-skewed and results in *Mtb*- and *M. leprae*-specific, IFN-γ producing $CD4^+$ T cells that provide an early response to these mycobacteria and are associated with some degree of protection [24]. However, as is evident from several studies, the IFN-γ response induced by BCG vaccination does not correlate with protection [25-27]. In addition, Th17 helper cells producing IL-17 and IL-22 are produced as well which are beneficial for protection against pathogens at mucosal sites [28].

In 1989, Bagshawe et al. [29] also already hypothesized that prevailing immunity to mycobacterial antigens is largely responsible for clinical manifestations of PB leprosy and that the non-specific immune stimulation induced by BCG vaccination can precipitate clinical signs and symptoms of leprosy in people incubating the disease and cause upgrading of established lesions, especially in indeterminate or borderline leprosy. In the Karimui trial in Papua New Guinea [29], a 47% protection against clinical leprosy by BCG was demonstrated. However, they provided evidence for accelerated manifestation of tuberculoid leprosy in children vaccinated when under 5 years of age. In our study, children less than 5 years old were excluded, but we observed this phenomenon among all other ages.

Among the index cases in our study more than half had MB leprosy, with an average duration of symptoms before diagnosis of over three years, compared with 18 months in all newly registered leprosy patients in the Rural Health Program in 2013. We also found that in the group of 21 contacts that developed leprosy, a higher proportion were blood relative and/or a household contact of the index patient than in the group of contacts that did not develop leprosy. These factors represents a high level of exposure over a long duration and possibly increased susceptibility for leprosy, but definite conclusions on the relationship between level of exposure and chance of contacts to develop

leprosy soon after BCG vaccination cannot be drawn until the trial is completed and immunological and gene expression data are available.

Presentation of leprosy as part of an immune reconstitution inflammatory syndrome (IRIS) in HIV infected individuals or AIDS patients starting their highly antiretroviral active (HAART) therapy has been described [30 31]. Previously, Deps et al. [30] proposed the case definition for IRIS in leprosy as leprosy and/or Type 1 reaction and erythema nodosum leprosum (ENL or Type 2 reaction) developing within 6 months after initiation of HAART. They found that 89.5% of the leprosy/IRIS cases presented a histopathological diagnosis of TT or BT leprosy. The mean time until onset of IRIS after initiating HAART was 8.7 weeks. Fifty-seven percent of the leprosy patients presented within 8-12 weeks after initiating HAART [31]. Two main forms of leprosy as IRIS occurring in the first few months of HAART were identified [30]. The first type is an inflammatory 'unmasking' of a previously untreated *M. leprae* infection, the second (less commonly occurring) is a paradoxical clinical deterioration in pre-existing leprosy during which the patient developed HAART-associated Type 1 reaction. We propose that a comparable process leads to presentation of clinically apparent leprosy after BCG vaccination of contacts of leprosy patients.

In our trial we found an unexpectedly high proportion of new leprosy patients among apparently healthy household contacts of leprosy patients in the first 12 weeks after receiving BCG vaccination. When all follow-up data of the trial are available, we will compare PB/MB proportions in new cases arising among contacts at different time points after BCG vaccination and in a group without BCG vaccination. If a higher proportion of contacts present with PB leprosy in the first 12 weeks after BCG and later (in the following 1-2 years) a higher proportion of contacts present with MB leprosy, this would support the theory that BCG accelerates the immune response and reveals highly immunologically active forms of subclinical leprosy first. In fact BCG vaccination given to household contacts of leprosy patients could actually identify this important group, who will then receive proper treatment at an early stage. However this does not imply that BCG should be seen as a legitimate diagnostic test for pre-clinical leprosy. Further investigation including analysis of the cytokine/chemokine range induced after BCG vaccination [32], is necessary to understand this phenomenon. Differentiation of the patients through epidemiological and immunological studies will be undertaken, in order to carefully consider the implications of giving BCG vaccination to contacts of newly diagnosed leprosy patients as immunoprophylaxis as part of a leprosy control programme.

Ethical approval

The national Research Ethics Committee (Bangladesh Medical Research Council) has approved the study protocol (Ref no. BMRC/NREC/2010-2013/1534).

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Conflicts of interest

The authors declare that they have no conflict of interest.

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CHAPTER 5

5

BCG and Adverse Events in the context of Leprosy

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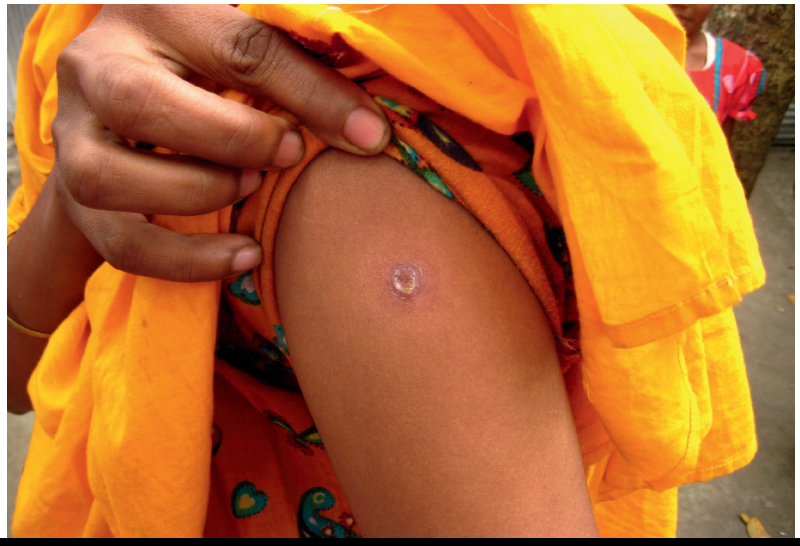

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Abstract

Background: Notwithstanding its beneficial immunoprophylactic outcomes regarding leprosy and childhood TB, BCG vaccination may cause adverse events, particularly of the skin. However, this local hyper-immune reactivity cannot be predicted before vaccination, nor is its association with protection against leprosy known. In this study we investigated the occurrence of adverse events after BCG (re)vaccination in contacts of leprosy patients and analyzed whether the concomitant systemic anti-mycobacterial immunity was associated with these skin manifestations.

Methods: Within a randomized controlled BCG vaccination trial in Bangladesh, 14,828 contacts of newly diagnosed leprosy patients received BCG vaccination between 2012 and 2017 and were examined for adverse events 8 to 12 weeks post-vaccination. From a selection of vaccinated contacts, venous blood was obtained at follow-up examination and stimulated with *M. leprae* antigens in overnight whole blood assays (WBA). *M. leprae* PGL-I specific antibodies and 32 cytokines were determined in WBAs of 13 individuals with and 13 individuals without adverse events after vaccination.

Results: Out of the 14,828 contacts who received BCG vaccination, 50 (0.34%) presented with adverse events, mainly (80%) consisting of skin ulcers. Based on the presence of BCG scars, 30 of these contacts (60%) had received BCG in this study as a booster vaccination.

Similar to the pathological T-cell immunity observed for tuberculoid leprosy patients, contacts with adverse events at the site of BCG vaccination showed elevated IFN-γ levels in response to *M. leprae* specific proteins in WBA. However, decreased levels of sCD40L in serum and GRO (CXCL1) in response to *M. leprae* simultaneously indicated less T-cell regulation in these individuals, potentially causing uncontrolled T-cell immunity damaging the skin.

Conclusion: Skin complications after BCG vaccination present surrogate markers for protective immunity against leprosy, but also indicate a higher risk of developing tuberculoid leprosy.

Trial registration: Netherlands Trial Register: NTR3087.

Keywords: adverse events, BCG (re)vaccination, biomarker profiles, household contacts, protective immunity, leprosy, *M. leprae*

Introduction

Despite effective treatment of leprosy patients with multi-drug therapy (MDT), the global number of new cases has not declined during the past decennium[1]. A plausible explanation for this status quo could be that contacts of leprosy patients are prolonged and repetitively exposed to *Mycobacterium leprae* (*M. leprae*) before treatment of index cases is initiated, leading to continued bacterial transmission. Therefore, new tools and methodologies, such as immuno- and chemoprophylaxis regimens, are needed to interrupt transmission.

BCG vaccination offers variable protection against tuberculosis[2] and other mycobacterial diseases such as leprosy[3] and Buruli ulcer[4]. Moreover, recently it has become clear that BCG can modulate the innate immune system also leading to protection through a mechanism referred to as trained immunity [5-7]. The protective effect against TB thus induced in children by neonatal BCG vaccination, influences cytokine responses to heterologous pathogens, an effect that is reported to be characterized by decreased anti-inflammatory cytokine responses, but increased IL-6[8].

In a previous study, immunoprophylaxis by BCG vaccination of contacts of newly diagnosed leprosy patients in Bangladesh conferred 56% protection, but was not affected by previous childhood BCG vaccination[9].

Although chemoprophylaxis does not protect a given individual from subsequent exposure to bacilli, the use of a single-dose rifampicin (SDR) in contacts in that study, showed prevention of 56% in the first two years after chemoprophylaxis and treatment of the index case[10]. Strikingly, if contacts had received BCG vaccination as part of a childhood vaccination program (as determined by the presence of a BCG-scar), the protective effect of SDR even reached 80%.

To investigate whether the effects of SDR and BCG can be complimentary, a cluster randomized controlled BCG vaccination trial is currently conducted in Bangladesh, analyzing the potential synergetic effect of these chemo- and immunoprophylactics by comparing the effect of BCG vaccination alone versus BCG followed by SDR after 8 to 12 weeks to prevent leprosy in contacts of new leprosy cases (designated the MALTALEP trial)[11].
In Bangladesh, BCG is routinely given to infants as part of the neonatal vaccination scheme as a prophylactic vaccine against tuberculosis. The coverage of BCG vaccination is estimated to be 98% (http://www.who.int/immunization/monitoring_surveillance/data/bgd.pdf). Based on the visibility of BCG vaccination scars, 8,430 out of 14,779 contacts (57%) within this trial had received BCG

vaccination at birth. However, since not all individuals receiving BCG develop a visible scar[12], this number is probably higher.

BCG vaccination has been reported to cause adverse effects within BCG childhood vaccination programs in endemic areas[13-16] as well as in BCG naïve individuals in leprosy and TB non-endemic areas[17-20]. In the current study, we investigated the number and nature of adverse events occurring after BCG vaccination in the MALTALEP trial.

In addition, to investigate whether these adverse events can provide further insight into the protective effect of BCG, we analyzed cytokine production in *M. leprae*-antigen stimulated whole blood assays (WBA) of 13 contacts developing adverse events and 13 contacts matched for age and gender, lacking such complications.

Material and Methods

Study population. Newly diagnosed leprosy patients and their household contacts (HC) were recruited on a voluntary basis between 2012 and 2017 (Table 1). Leprosy was diagnosed based on clinical and bacteriological analysis and classified according to Ridley and Jopling[21]. Leprosy patients were treated according to WHO standards. Contacts of consecutively diagnosed new leprosy patients were included in the districts of Nilphamari, Rangpur, Thakurgaon and Panchagarh, in the northwest of Bangladesh[11]. Each contact group consisted of around 15 contacts, and were randomly assigned to receive BCG or BCG plus rifampicin. Immunization with BCG was given to all included contacts, when the index case received the second dose of MDT. At intake, before BCG vaccination, all contacts were examined for a BCG scar on the left upper arm. After 8 to 12 weeks, vaccinated contacts were reviewed for adverse events during follow-up examination. Contacts were categorized as household members (sharing either roof, kitchen or both) or direct neighbors. Contacts were excluded from the study according to criteria described previously.

Leprosy prevalence. During this study the prevalence in the four districts (Nilphamari, Rangpur, Panchagarh and Thakurgaon) in the northwest of Bangladesh was 0.82 per 10,000 with a new case detection rate of 0.98 per 10,000 (monthly report of the Rural Health Program of these 4 districts).

Ethics. The MALTALEP trial is performed according to standard Good Clinical Practice (GCP) guidelines (www.ich.org). Participants were informed about the study objectives, the samples, and their right to refuse to take part in or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment from all participants. For illiterate people a thumb print was taken, and for minors under 16 years of age, the guardian's additional consent was obtained. All patients received treatment according to national guidelines. Participants were informed about the potential adverse events of the trial, that free consultation and treatment would be offered in case of adverse events and requested to report any suspected adverse events to the responsible field worker. Ethical approval of the study-protocol was obtained through the National Research Ethics Committee (Bangladesh Medical Research Council; protocol ref no. BMRC/NREC/2010-2013/1534).

BCG vaccination. Vaccination was performed between September 2012 and February 2017. BCG was administered intradermally. The BCG vaccine used in this trial (Japan BCG Laboratory, Tokyo, Japan) is also used in the routine neonatal vaccination program of Bangladesh. Vaccines were stored at the State Immunization Program facilities in the four different districts of the study area and kept at 0 - 4 °Celsius throughout the fieldwork.

Adverse events. All contacts receiving vaccination were provided with a vaccination card with details on how to reach the researcher in case of questions or adverse events. Contacts with self-reported adverse events were examined by field staff. Additionally, all contacts were examined 8 to 12 weeks after administration of the BCG. Data on adverse events were collected on the MALTALEP Contact Registration forms and on a separate BCG complication form[11]. In the case of an adverse event following BCG complication, contacts were referred to the state tuberculosis medical officers for treatment. Ulcers were considered abnormal if they were larger than 10 mm diameter in size, or if they presented in combination with fever and malaise. Contacts were also checked for the presence of lymphadenopathy, abnormal scarring and keloids and if the course of the complication was different than normal. To document the size of the ulcers, pictures were taken of each BCG complication case and stored in a database.

Samples for immunological analysis. Blood was drawn from 15 contacts who developed an adverse event after receiving BCG vaccination. Two contacts were excluded from the analysis, because they later developed leprosy. Cytokine levels in whole blood assays of 13 contacts with adverse events were analyzed and compared to those in contacts without (a scar or ulcer of less than 10 mm). Whole blood assays were performed for both groups and anti-PGL-I serology cytokines and chemokines concentrations in supernatants were assessed.

Whole blood assays (WBA). Venous blood was drawn from contacts at the time BCG complications occurred, which was on average 7.9 weeks after receiving BCG. As a control group, contacts without complications were tested. Controls were matched for age and gender as well as time point at which blood was drawn (on average 7.7 weeks; Table 2). Heparinized blood (4 ml) was directly added to microtubes pre-coated with *M. leprae* whole cell sonicate (designated WCS), *M. leprae*-unique recombinant proteins ML2478 and ML0840 (designated Mlep)[22] or without antigen stimulus (designated NIL)[11 23]. After 24 hours incubation at 37°C materials were frozen at -20°C, shipped on dry ice to the LUMC and stored at -80°C until analysis.

Cytokine-chemokine analysis. sCD40L, EGF, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-17A, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, PDGF-AB/BB, PDGF-AA, RANTES, TGF-α, TNF-α, TNF-β, VEGF and Eotaxin (CCL11) in whole blood assay supernatants were measured with the Milliplex magnetic bead kit (Merck, USA) on 96 well multiscreen filter plates (Millipore, USA) using the Bio-Plex-100-suspension-array-system (BioRad, Veenendaal) and analyzed using the Bio-Plex Manager software 6.1 (Bio-Rad Laboratories, Veenendaal, The Netherlands)[22]. After pre-wetting the filter with assay solution, supernatant samples (25 µl) were added to the plates, together with 25 µl assay buffer and 25 µl beads, and the plates were incubated overnight at 4°C. After two washing steps with 200 µl wash buffer using a vacuum pump (Millipore, USA), 25 µl detection Ab mixture was added per well, and plates were incubated at room temperature in the dark for 1 hour on a plate shaker at 300 rpm. Streptavidin-PE solution (25 µl per well) was added and incubated for 30 min at room temperature in the dark. After two washes, 150 µl Sheath Fluid was added to each well, and the plates were placed in the Bio-Plex System. From each well, a minimum of 50 analyte-specific beads was analyzed for fluorescence. A curve fit was applied to each standard curve according to the manufacturer's manual. Sample concentrations were interpolated from these standard curves. Analyte concentrations outside the upper or lower limits of quantification were assigned the values of the limits of quantification of the cytokine or chemokine.

PGL-I and M. leprae whole cell sonicate (WCS). Synthesized disaccharide epitope (3,6-di-O-methyl-β-D-glucopyranosyl(1→4)2,3-di-O-methylrhamnopyranoside), similar to *M. leprae* specific PGL-I glycolipid, coupled to human serum albumin (synthetic PGL-I; designated ND-O-HSA) and *M. leprae* whole cell sonicate (WCS), generated with support from the NIH/NIAID Leprosy Contract N01-AI-25469, were obtained through the Biodefense and Emerging Infections Research Resources Repository (24).

PGL-I ELISA. IgM and IgG antibodies against synthetic PGL-I were detected as previously described adapted for the use of specific IgM and IgG antibody detection[22 25]. A synthetic analog of the *M. leprae*-specific phenolic glycolipid-I (PGL-I; ND-*O*-HSA), was coated onto high-affinity polysorp Immulon 4HBX 96-well Nunc ELISA plates (Thermo Scientific, Rochester, NY) using 200 ng/well in 50 µl 0.1 M sodium carbonate/bicarbonate pH 9.6 (i.e. coating buffer) at 4°C overnight. Unbound Ag was removed by washing with PBS containing 0.05% Tween 20 (washing buffer) six times and wells were blocked with PBS containing 1% BSA (Roche Diagnostics, Germany) and 0.05% Tween 80 for 1

hour at room temperature. 50 µl of 1:400 diluted serum/plasma (PBS/0,01% BSA as dilution buffer) was added to the wells and incubated for 2 h at room temperature. After incubation, wells were washed six times with washing buffer, followed by the addition of 50 µl of 1:8000 anti-human IgM-HRP (Sigma A6907) or 1:4000 anti-human IgG-HRP (DAKO P0214) and incubated for 2 hours at room temperature. Following washing the wells with the wash buffer, 50 µl 3.3',5.5'-Tetramethylbenzidine (TMB) was added and the color reaction was stopped using H2SO4 after 10–15 minutes. The absorbance was determined at wavelength of 450 nm. Samples with an optical density (OD_{450}), after correction for background, above 0.20 were considered positive. The cut-off for positivity was determined by a threefold multiplication of the average value for nonendemic control individuals.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, San Diego,CA, USA; http://www.graphpad.com), SPSS Statistics 24 (http://www.spss.com.hk) and R Version 3.3.0 (R, Vienna, Austria; http://www.R-project.org). A Chi-square test was performed for contacts who developed BCG complications to identify potential differences compared to the control contacts' characteristics (Table 1). A significance level of $p \leq 0.05$ was used.

For identification of an immune biomarker signature associated with skin complications after BCG vaccination, a global test was used[26], which provided hierarchical clustering of the cytokines/chemokines based on absolute correlation difference and average linkage. Moreover, the Mann-Whitney U test was performed to identify differences in group mean levels of host markers. The statistical significance level used was $p \leq 0.05$. For significantly different markers in both the global test and Mann-Whitney U test the diagnostic potential was assessed by receiver operating characteristic curve (ROC) analysis to determine the area under the curve (AUC). The cut-off values for optimal sensitivity and specificity were determined by calculating the Youden's Index[27]. To construct a biomarker profile, a linear discriminant analysis (LDA) was performed in SPSS. Analytes were ranked based on the pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. The six most contributing analytes to the discriminant function were selected to construct a biomarker profile. The profile was constructed stepwise, determining the optimal sensitivity and specificity for each step. The optimal cut-off was determined per analyte after which each individual was designated positive or negative for all analytes separately.

Results

Occurrence of adverse events after BCG vaccination. Out of the 14,828 contacts who received BCG vaccination within the trial, 50 (0.34%) presented with vaccination-related adverse events (Table 1). The most common adverse events were skin ulcers (Table S1, Figure 1A). A total of 40 contacts (80%) developed large skin ulcers varying between 10 and 35 mm; 4 of these also had axillary lymphadenopathy and one had enlarged lymph nodes. One ulcer was 8 mm, but was included as adverse event because the contact also reported malaise and mild fever. Keloids (Figure 1B) were present in 8 contacts, of whom 3 were small (<1 cm) and 3 were >1 cm. One contact developed a persistent keloid, which was first signaled one year after receiving BCG vaccination. When excluding the contact with persistent keloid, the average time between BCG vaccination and initiation of complication in the 50 contacts was 5.5 weeks.

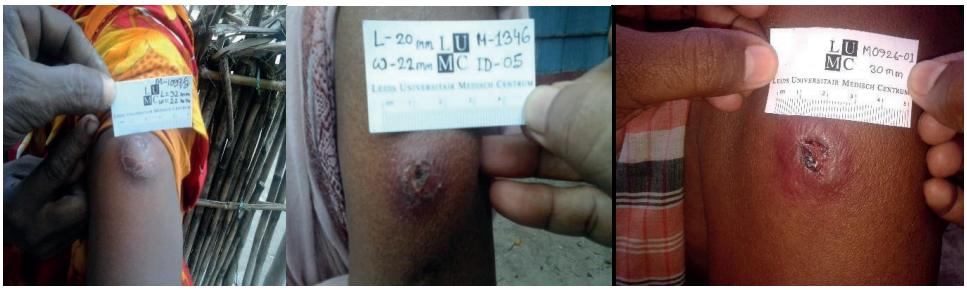
Table 1. Characteristics of contacts with or without complication after BCG vaccination.

	Contacts with complication after BCG (% of total)	**Contacts without complication after BCG**	**Total contacts who received BCG**	**p-value**
Contacts	50	14,778	14,828	n.a.
Male	23 (0.34%)	6677	6700	0.91
Female	27 (0.33%)	8101	8128	
Child (5-16 yrs)	21 (0.43%)	4829	4850	0.16
Adult	29 (0.29%)	9949	9978	
No BCG scar visible	20 (0.32%)	6336	6356	0.68
BCG scar present	30 (0.35%)	8430	8460	
Vaccination status unknown	0	12	12	n.a.
Index with MB	19* (4.08%)	447	466	0.08
Index with PB	26** (2.42%)	1047	1073	

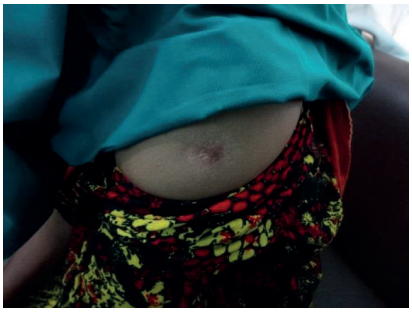
*One household with a multibacillary (MB) index had two contacts with a BCG complication

**One household with a paucibacillary (PB) index case had two contacts with a BCG complication, another household even had four contacts with a BCG complication

Figure 1. Representative examples of skin complications after BCG vaccination.



A.



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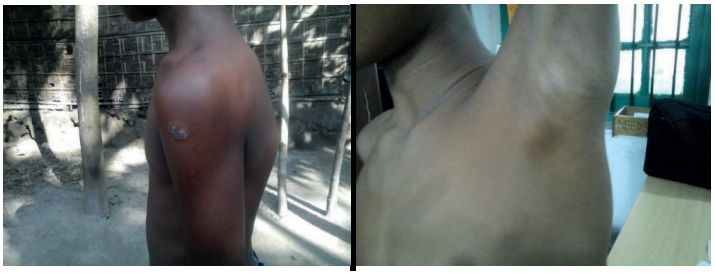


C.

Figure A: three contacts with big ulcers (>10mm)

Figure B: a contact with keloid (picture taken before operation).

Figure C: a contact with an ulcer and lymphadenitis who developed leprosy at follow-up.

Variations in BCG-vaccination-related adverse events. In four contacts adverse events manifested differently: one woman developed an abscess, which was incised and drained at home 3 months after vaccination, then developed intermittent fever and was treated unsuccessfully with various antibiotics of unknown kind provided by different doctors. After one year the contact was admitted for investigation, because of an erythematous nodule (2x2 cm) surrounded by scarring. She was re-incised by a plastic surgeon upon suspicion of a deep-seated abscess. The histological report of the biopsy showed a keloid scar (Figure 1B).

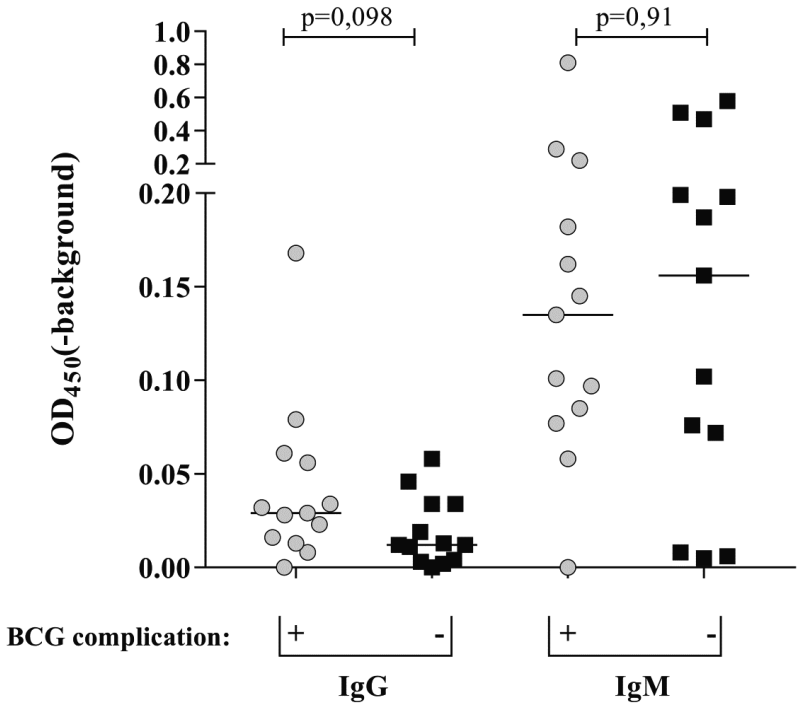
A second contact had a persistent pustule of 5 mm 5 months after receiving BCG, felt weak and had coughed for the past two months. She only had a two-day history of fever and was tested sputum-negative for acid-fast bacilli (AFB). The pustule was not opened, but kept clean and dry and healed after a course of flucloxacillin. A third contact had developed a large scar (12x10 mm) and many small ulcers on both arms and legs after receiving BCG. She received unknown medication from an outside doctor and the lesions healed. Finally, the fourth contact presented with an ulcer at the BCG injection site of 10x15 mm and mild left axillary lymphadenopathy. Already before BCG vaccination, the contact had a history of occasional fever and pain palpable on the ribs, which was treated with pain killers. He had no known contact to TB patients, and was sputum– and X-ray negative for TB. Besides adverse events, two contacts also developed leprosy following BCG vaccination (Figure 1C). One had a small keloid, the other an ulcer of 15x20 mm with lymphadenitis (Figure 1B).

The average age at the time of the adverse event was 30 years, with a range of 6 to 80 years. Similar numbers of males and females were identified with adverse events (Table 1). More than half (60%) received a revaccination, based on the presence of a BCG scar. A higher number of children between 5 and 16 years old (as under-fives were excluded) developed BCG adverse events compared to adults (0.43% versus 0.29%), however, this number was not statistically significant (p=0.16; Table 1). A slightly higher but statistically not significant number of contacts who received BCG for the second time, developed adverse events compared to those who lacked a BCG scar (0.35% versus 0.32%; p=0.68). Despite that an almost double amount of contacts developed adverse events when the index patient had multibacillary (MB) leprosy, compared to paucibacillary (PB) leprosy, this increase was not statistically significant either (p=0.08).

Among the 13 contacts with an adverse event after BCG from whom blood was analyzed, 9 had large ulcers >10mm, one patient had an ulcer of 8 mm, but with general malaise, one had a keloid, one a big scar and one an enlarged lymph node.

Anti-PGL-I IgM levels. To estimate whether the extent of seropositivity in contacts of leprosy patients could already indicate whether complications could occur after BCG vaccination, the levels of anti-*M. leprae* PGL-I IgM antibodies, as estimated by the optical density at 450 nm (OD_{450}), were measured in sera of 26 individuals; 13 with and 13 without BCG complications (Table 2, Figure 2). Three contacts from both groups were seropositive for IgM against PGL-I ($OD_{450} > 0.2$), but no significant differences were observed between both groups.

Figure 2. M. leprae phenolic glycolipid-I (PGL-I) specific antibodies in contacts of leprosy patients with or without BCG-induced skin complications.



IgG and IgM antibodies directed against synthetic PGL-I (ND-O-HSA) were determined by ELISA. Samples with OD_{450} (corrected for background OD) > 0.2 were considered seropositive. No statistically different levels of IgG and IgM antibodies were observed between the contacts with (+; grey dots) or without (- ; black squares) complications.

Table 2. Characteristics of contacts with BCG-related complications and matched controls.

	Complications	No complications
Number of contacts	13	13
Average age (years)	33.8	36.2
Number of females	8	8
Number of males	5	5
Average no. of weeks between BCG and WBA	7.9 (1.0 - 13.5)	7.7 (4.0 - 10.0)
Presence of BCG scar before study	8	6
Average size of BCG scar/ulcer (in mm)	14.8 (4.5 - 27)	3.4 (2.5 – 4.5)
Received SDR before blood drawing	1**	3*
Received no SDR	12	10

*All controls received SDR 2 weeks before blood was drawn.

**The contacts with a complication after BCG vaccination received SDR 4 weeks before experiencing the adverse event at 13 weeks post vaccination.

Immune profiles coinciding with adverse events after BCG vaccination. To assess what type of immune profile (i.e. combinations of cytokines in *M. leprae* stimulated WBA) is associated with BCG-related complications, a global test[26] was performed on all 32 cytokines stratified by stimulus used in the WBA (Figure 3). This analysis showed that three analytes were significantly different between the two contact groups: decreased levels of $sCD40L_{NIL}$ (soluble cluster of differentiation ligand 40, without stimulation) and GRO_{WCS} (growth-regulated oncogene, in response to *M. leprae* WCS) were significantly associated with occurrence of BCG complications (p=0.03 and 0.013 respectively; Figure 3 and 4). In contrast, increased levels of IFN-γ in response to *M. leprae* specific proteins (IFN-γ_{Mlep}; p=0.012) were observed in individuals developing BCG complications (Figure 3 and 4). Individually these three markers enable a good distinction between contacts with BCG-related complications and those without, showing an AUC of 0.75 for sCD40L and 0.78 for both GRO_{WCS} and IFN-γ_{Mlep} (Figure 3). Using a linear discriminant analysis (LDA) three additional markers $CCL4_{NIL}$, IL-6_{Mlep} and $GCSF_{NIL}$ that

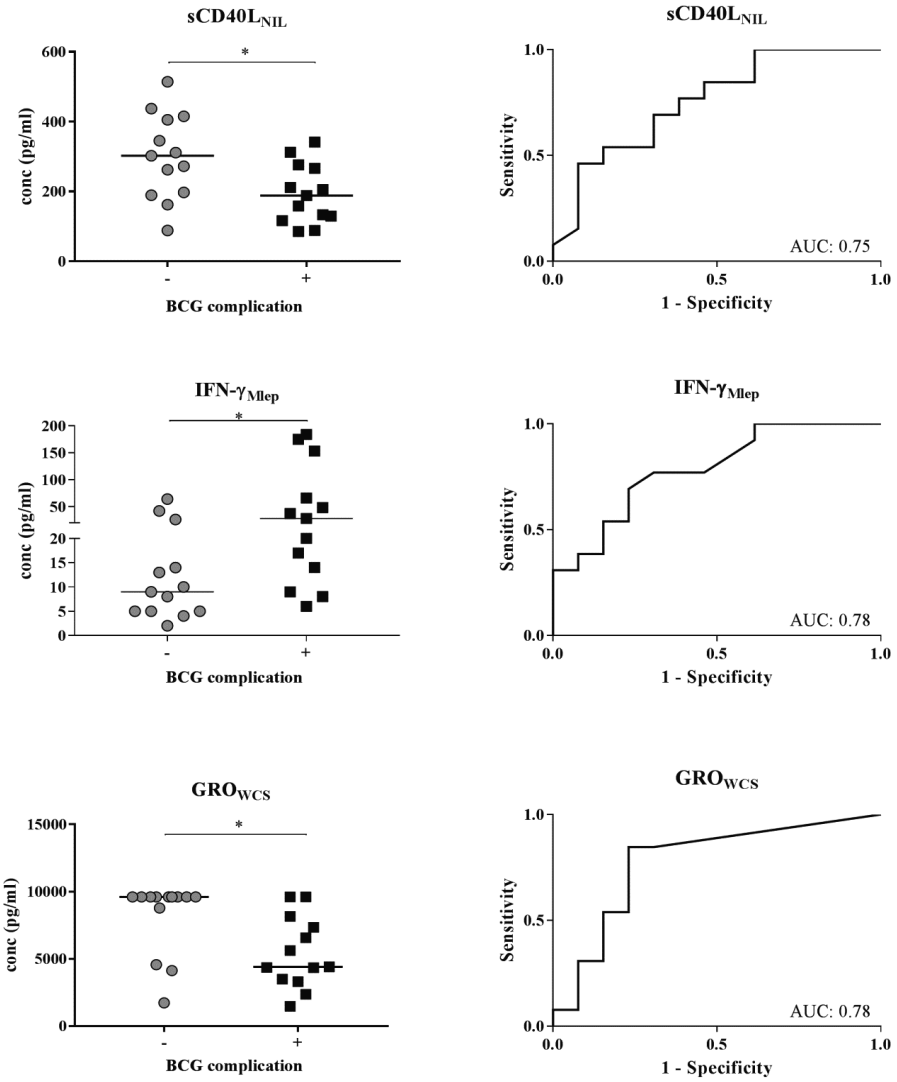
were decreased in individuals with adverse events, were identified, that improved the signature for adverse events. Next, the six analytes were ranked based on their contribution to the discriminant function and sequentially added to the biomarker profile (Table 3) and scored for each individual as positive or negative based on the optimal cut-off. This showed that optimal sensitivity (100%) was observed for the combination of $sCD40L_{NIL}$, IFN-γ_{Mlep} and GRO_{WCS} showing 76% specificity and an AUC of 0,94 ($p<0.0001$). On the other hand, optimal specificity (100%) was achieved by a five marker profile ($sCD40L_{NIL}$, IFN-γ_{Mlep}, GRO_{WCS}, $CCL4_{NIL}$ and IL-6_{Mlep}), with a sensitivity of 84% and an AUC of 0.96. The cut-off of >3.5 indicates that none of the contacts without complications scores positive for more than 4 out of 5 markers, thereby showing addition of markers improves the specificity. The five marker profile was optimal, as addition of a sixth marker slightly decreased the AUC from 0.96 to 0.93 (Table 3).

Table 3. Ability of analytes to distinguish contacts with adverse events in WBA.

	Single markers				**Signature**			
step	**analyte**	**correlation**	**stimulus**	**p-value**	**AUC**	**sens.**	**spec.**	**cut-off**
1	sCD40L	0.086	NIL	0.0262	0.75	85%	54%	<289
2	IFN-γ	0.076	Mlep	0.0124	0.83	62%	92%	>1.5
3	GRO	0.070	WCS	0.0126	0.94	100%	76%	>1.5
4	CCL4	0.066	NIL	0.1254	0.94	92%	85%	>2.5
5	IL-6	-0.055	Mlep	0.2234	0.96	84%	100%	>3.5
6	GCSF	0.043	NIL	0.2428	0.93	85%	92%	>3.5

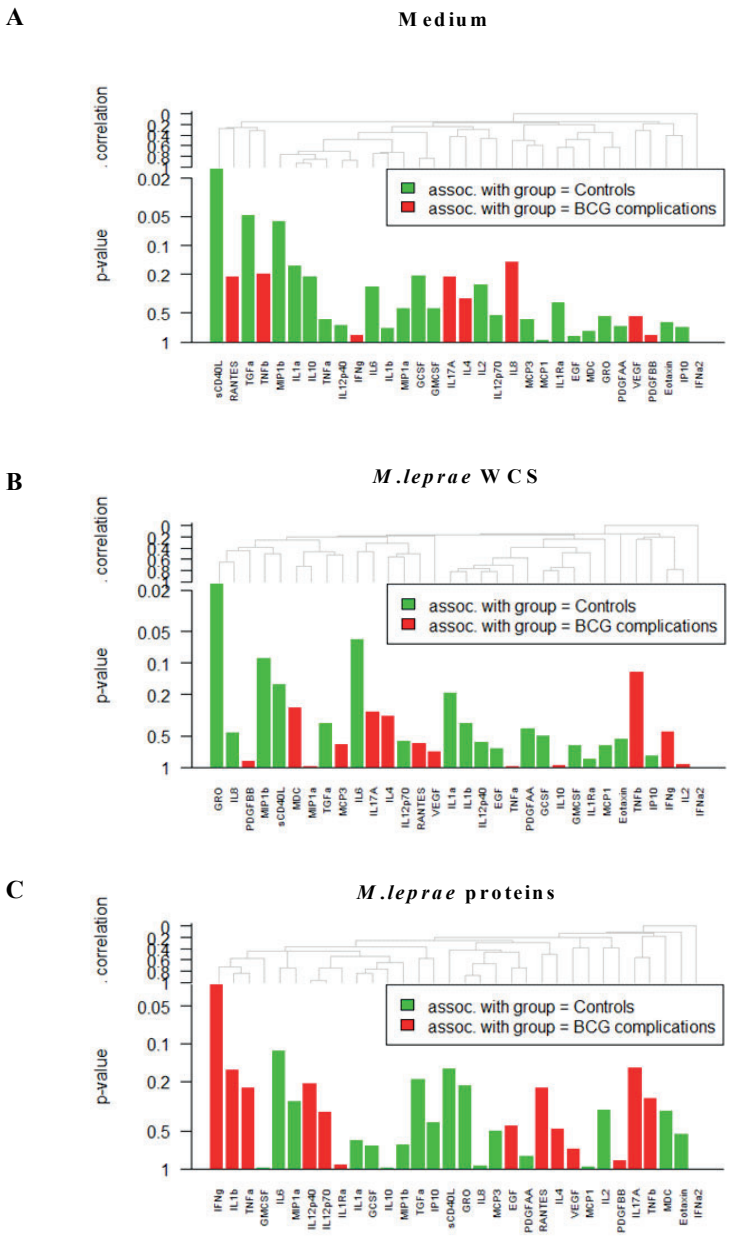
Step by step addition of analytes ranked by absolute size of correlation within discriminant function. For each step the analyte that was added to the signature specific for occurrence of BCG vaccination-related adverse events, the absolute size of correlation generated from the linear discriminant analysis, the stimulus, p-value (Mann-Whitney U test), area under the curve (AUC) and the sensitivity (sens.) and specificity (spec.) based on the optimal cut-off are shown. The three different stimuli used were: M. leprae whole cell sonicate (WCS), ML2478/ ML0840 recombinant proteins (Mlep) or without antigen stimulus (NIL).

Figure 3



Cytokine concentrations in 24h whole-blood assays (WBA) with or without stimulation with M. leprae unique proteins (Mlep) or M. leprae whole cell sonicate (WCS) in contacts with and without BCG complications (left panels). The global test[26]indicated that sCD40Lmed, GROwcs and IFN-γ_{Mlep} were significantly different between BCG vaccinated contacts of leprosy patients with BCG-related complications and those without. This was confirmed by a Mann-Whitney U test (*p-value <0.05-0.01). Receiver operating characteristic curves (ROC) were computed and the area under the curve (AUC) is indicated for each analyte (right panels). The limits of detections for sCD40Lmed were 1.5-10,000, for GROwcs were 12.5-9,600 and IFN-γ_{Mlep} were 2-10,000.

Figure 4



Results of whole-blood assays (WBAs) in contacts with and without BCG complications in **A**: medium (designated NIL); **B**:M. leprae whole cell sonicate (designated WCS); **C**: ML2478/ ML0840 recombinant proteins (designated Mlep) (C).

Discussion

Within a cluster randomized controlled BCG vaccination trial in contacts of leprosy patients in Bangladesh, adverse events were observed in 0.34% of the recipients. These complications consisted primarily (80%) of skin ulcerations and were associated with increased Th1 immunity, inflammation and reduced T-cell regulation in WBA.

Although serious adverse events after BCG vaccination are rare, as many as 95% of BCG recipients have an uncomplicated, local reaction at the site of inoculation, characterized by the appearance of a pustule in combination with pain, swelling and erythema within two to three weeks after vaccination. In approximately 70% of the cases, ulceration with drainage occurs at the vaccine site after about 6 weeks, resulting in a lesion of about 5 mm in diameter. Lesions usually heal within three months with permanent residual scarring at the vaccination site. Rare local abscesses and ulcers usually occur between one and five months post-vaccination, but adverse events have also been reported after longer periods of time[28]. Lymphadenopathy occurs in the drainage area of the vaccinated site, so is most common in the axilla and sometimes in the cervical lymph nodes[28]. Even more uncommon are serious adverse events such as osteitis, osteomyelitis and disseminated infection[19]. Disseminated disease following BCG vaccination occurs usually with immunosuppression, such as HIV-infection[16] or genetic immune deficiency[29], which develops in less than one in a million[20]. The incidence of adverse events of 0.34% in this study is comparable with the 0.02% to 5% described in previous studies[13-15 18 28]. A trial evaluating the incidence of adverse events to primary and booster BCG vaccination in schoolchildren in Salvador, Bahia (Brazil)[14], observed a rate of 0.35 per 1,000 vaccinations, without lethal cases or disseminated infections. Although not statistically significant, adverse events after booster vaccinations were approximately twice the rate compared to primary vaccination with BCG. The median time to onset of complications was 26 days, 12 days shorter than observed in Bangladesh. Similarly, 0.38 out of 1,000 vaccinated individuals developed complications in a study in the Brazilian Amazon[15]. In contrast, the risk in the group receiving a revaccination was only 1.05 higher than in the group receiving a first dose, similar to what we found in our Bangladesh study (0.35% versus 0.32%; p=0.68).

The presence of a BCG scar is considered a highly sensitive indicator of the vaccination status as 92% of individuals aged 1-4 months at vaccination, develops a visible scar at 7-12 months of age, which declines to 84% at 4 years[12]. When BCG is given to an infant before they are one month old, 90% has a scar at 7-12 months of age and 76% at 4 years. In this study, we used the absence of a BCG scar to

designate the lack of previous (childhood) vaccination. However, since 16-24% of BCG vaccinated individuals do not develop a scar, it could be that a larger number of individuals actually received a BCG booster in the MALTALEP trial than is estimated solely based on the presence of a scar.

The development of leprosy after BCG vaccination can be considered an ultimate adverse event. In a previous study[30], we observed an unexpectedly high proportion of new leprosy patients (mainly PB and leprosy type 1 reactions) among apparently healthy household contacts of leprosy patients within the first three months after BCG vaccination (0.4% of vaccinated contacts). Of these, 43% had a BCG scar before vaccination in the trial. However, it remains unclear whether BCG vaccination merely catalyzes the formation of clinical symptoms in individuals who are bound to develop leprosy, or whether patients would not have developed the disease without this vaccination.

Several recent studies show that BCG alters the innate immune system by trained immunity[5-7]. The protective effect against TB induced by neonatal BCG vaccination coincides with protection against heterologous pathogens. This effect is characterized by decreased anti-inflammatory cytokine responses, but increased IL-6 in unstimulated samples[8]. In another study, an BCG vaccination-induced increase in IL-6, EGF and PDGF-AB/BB and decrease in IP-10, IL-2, IL-13, IL-17, GM-CSF and GRO was observed in response to various non-specific innate immunity stimuli (PAM3Cys, C. albicans and S. aureus). Along with this cytokine biomarker signature, increased CD69 expression on NK cells was observed as well{Dockrell, 2017 #483}.

T helper 1 (Th1) host-cellular immunity is generally considered to be key in controlling mycobacterial infections[31]. However, clinical presentation of tuberculoid leprosy as well as type 1 (reversal) reactions also coincides with strong *M. leprae*-specific Th1 immunity and high levels of pro-inflammatory cytokines[32].

Despite the apparent homology between the mycobacteria, BCG but not *M. leprae* can stimulate monocytes to initiate a protective type 1 cascade[33]. Moreover, *in vitro* exposure of monocytes from healthy donors to *M. leprae* (or *M. leprae* PGL-I) reduced levels of Th1-type cytokines and expression of macrophage type 1 (Mϕ1) cell surface markers[33]. In contrast, *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) with BCG or purified protein derivative of tuberculin (PPD) from 10-weeks old infants in South Africa, who had received neonatal BCG vaccination, showed upregulation of mϕ1-associated genes whereas mϕ2 associated genes were down-regulated[34], indicating BCG-induced protective immunity. Also, in response to *M. leprae*, monocytes from these infants released higher levels of inflammatory cytokines TNF-α and IL-1β compared to monocytes

from unvaccinated infants[33]. Similarly, cytokine profiles of infants from the United Kingdom receiving BCG vaccination[35] showed that a higher number of IFN-γ^+ TNF-α^+ IL-2^+ multifunctional $CD4^+$ T-cells was associated with growth inhibition of mycobacteria. Although T-cell activation ($HLA\text{-}DR^+CD4^+$ T-cells) was a risk factor for TB disease, increased numbers of BCG-specific T-cells secreting IFN-γ were detected in BCG vaccinated infants without TB[36]. These studies indicate that pro-inflammatory Th1 immunity, although not the only factor, is associated with BCG-induced protection against tuberculosis. Similarily, the Mitsuda reaction measures whether an adequate immune response to an intradermal injection of the heat-killed leprosy bacilli (lepromin) is initiated, as it has a good prognostic value for susceptibility (when negative) or resistance (when positive) to the lepromatous form of leprosy[37]. In line with that it was also observed that individuals that showed large local reactogenicity after intradermal BCG administration or lepromin injection are reported to have less risk for leprosy onset[38].

In a BCG vaccination study in 12 tuberculin skin test (TST) and Quantiferon negative, BCG-naive adults in The Netherlands, local skin reactions varied strongly between individuals[17]. It was observed that BCG vaccination induced significant Th1-type immunity ($CD4^+$ IFN-γ^+, IL-2^+ TNF-α^+ and $CD8^+$ IFN-γ^+ T-cells) in those that presented with high local inflammation responses, with a peak 8 weeks post-vaccination. Of note is that BCG vaccination significantly increased regulatory $CD8^+$ T-cells such as $CD25^+$ $Foxp3^+$ $CD39^+$ $CD8^+$ T cells as well as $CD25^+$ $Foxp3^+$ $CD39^+$ LAG-3^+ $CCL4^+$ $CD8^+$ T cells in low inflammation responders.

Similarly, individuals who developed (skin) complications in Bangladesh also produced higher levels of IFN-γ in response to *M. leprae* antigens around 8 weeks (average 7.9) post-vaccination, although at least 8 out of 13 contacts with BCG complications were not BCG-naïve and the a priori chance of exposure to mycobacteria was considerably larger. In contrast to the Dutch cohort, CRP levels were high in both groups and did not differ significantly (Supplementary Figure 2).

Of note in the current study are the lower levels of $sCD40L_{NIL}$ and GRO_{WCS} that were significantly associated with BCG complications, concomitantly with elevated IFN-γ levels in response to *M. leprae* unique proteins (IFN-γ_{Mlep}). GRO (CXCL1) is expressed by macrophages, neutrophils and epithelial cells and has neutrophil chemoattractant activity. Although the role of GRO in leprosy pathology has not been investigated, increase in GRO levels can reduce severity of multiple sclerosis[39]. This neuroprotective role for CXCL1 could well be consistent with the onset of complications upon its reduction after *M. leprae* WCS stimulation as observed in our study. Moreover, in UK-born, BCG-

vaccinated infants the levels of GRO in response to non-specific innate immunity stimuli were suppressed as well, in line with our finding in Bangladesh[5].

Recently, it was shown that higher levels of sCD40L present in serum of patients with Behçet's disease caused a strong stimulus on the production of reactive oxygen species[40]. Thus, the reduction in sCD40L observed in contacts with complications could indicate a weaker ability to combat BCG bacilli locally leading to tissue destruction at the vaccination site.

Besides induction of activated T-cells, BCG vaccination can also induce Tregs, in particular $CD8^+$ T cells which dampen the inflammatory response to mycobacteria[41 42] and lead to inadequate killing of mycobacteria[43]. Likewise, Tregs have been isolated from lepromatous leprosy patients, who in contrast to tuberculoid patients display reduced Th1 immunity and capacity to kill *M. leprae* bacteria[44]. The breakdown of T-cell regulation, in favour of inflammation, underlies the aetiology of tissue damage in tuberculoid leprosy and leprosy reactions[45].

Regulatory T-cells can suppresses Th1 cells through the secretion of CC chemokine ligand 4 (CCL4)[42]. In this study, a reduction in CCL4 (although not significant) could indicate decreased T-cell regulation in individuals with complications, causing a shift in the equilibrium towards excessive Th1-type immunity with corresponding inflammation at the BCG vaccination site. However, further research will be required to identify in detail the cellular subtypes involved. Furthermore, the leprosy contacts with high inflammatory responses after BCG vaccination could therefore also be more likely to develop tuberculoid leprosy. In line with this hypothesis are the two cases out of the 50 contacts in this study with BCG complications, who developed border line tuberculoid leprosy (BT).

Conclusion

The rate of documented adverse events after BCG vaccination in the studied Bangladesh cohort of leprosy patients' contacts was low (0,34%), and comparable to studies in other countries.

Contacts with BCG complications showed increased *M. leprae*-specific Th1-type immunity but a tendency of reduced T-cell regulation in WBA with corresponding inflammation at the BCG vaccination site indicating improved protection against *M. leprae*. In addition, these individuals may also be at a higher risk of developing tuberculoid leprosy after *M. leprae* infection.

Ethics Statement:

The MALTALEP trial is performed according to standard Good Clinical Practice (GCP) guidelines. Participants were informed about the study objectives, the samples, and their right to refuse to take part in or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. For illiterate people, a thumb print was taken, and for minors under 16 years of age, the guardian's additional consent was taken. All patients receied treatment according to national guidelines. Participants were informed about the potential adverse events of the trial, that free consultation and treatment would be offered in case of adverse events and requested to report any suspected adverse events to the responsible field worker. Ethical approval of the study-protocol was obtained through the National Research Ethics Committee (Bangladesh Medical Research Counci; Protocol no. BMRC/NREC/2010-2013/1534).

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Author Contributions

This research project was designed by the authors JR and AG. Patients were enrolled and a clinical diagnosis was performed and registered by the field staff under supervision of KA and RA. The laboratory testing was done by AH, SE and LW. The data were analyzed by RR, AH, JR and AG. The paper was written by RR and AG. All authors agreed with manuscript results and conclusions.

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Supplementary Material

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CHAPTER 6

6

Effectiveness of BCG vaccine with or without single dose rifampicin in preventing leprosy in close contacts of patients with newly diagnosed leprosy: a cluster randomized controlled trial (MALTALEP study)

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ABSTRACT

Objective: To assess the effectiveness of SDR after BCG-vaccination in preventing leprosy in contacts.

Design: Single-centre, cluster-randomized controlled trial.

Setting: Leprosy control programme in northwest Bangladesh.

Participants: 14,988 contacts of 1,552 new leprosy patients; randomized in the SDR- arm (7,379) and SDR+ arm (7,609).

Interventions: Intervention group: BCG-vaccination followed by SDR 8-12 weeks later. Control group: BCG only. Follow-up: at one and two years after intake.

Main outcome measure: The occurrence of leprosy.

Results: The incidence rate per 10,000 person-years-at-risk was 44 in the SDR- arm and 31 in the SDR+ at 1 year, and 34 in the SDR- arm and 41 in the SDR+ arm at 2 years. There was a statistically non-significant (p=0.148; 42%) reduction for PB leprosy in the SDR+ arm at 1 year. Of all new cases, 33.6% appeared within 8-12 weeks after BCG-vaccination.

Conclusion: In the first year, SDR after BCG-vaccination reduced PB leprosy incidence among contacts by 42%. This was a statistically non-significant reduction due to the limited number of cases after SDR was administered. To which extent SDR suppresses excess leprosy cases after BCG-vaccination is difficult to establish because many appeared before the SDR intervention.

Trial registration: Netherlands Trial Register: NTR3087

INTRODUCTION

The global number of new leprosy cases has remained stable over the last decade[1], indicating that transmission of *Mycobacterium leprae* (*M. leprae*), the causative agent of leprosy, is ongoing in many endemic countries. The basic intervention in leprosy control is multidrug therapy (MDT), but this appears insufficient to decrease new cases numbers and achieve the WHO target of reducing the burden of leprosy[2].

Close contacts of untreated leprosy cases are exposed considerably to *M. leprae*. Age of the contact, bacterial load of the index patient, and close physical and genetic distance are independent risk factors for development of leprosy[3]. Household contacts of newly diagnosed patients have a ten-fold higher risk to develop leprosy compared with the general population[4]; for different categories of neighbours and social contacts this is three to five-fold higher[3 4].

Many studies regarding immunoprophylaxis (vaccination) and chemoprophylaxis aiming to prevent leprosy focused primarily on contacts of leprosy patients. Bacillus Calmette-Guérin (BCG) vaccination is known as a vaccine against tuberculosis and is routinely given to infants as part of the neonatal immunization scheme in many parts of the world. Moreover, BCG is also recognized as protecting against leprosy[5 6]. Several vaccine trials using BCG have established its protective effect against leprosy, often in combination with *M. leprae* or related mycobacterium vaccines[5 7 8 9 10 11]. Brazil has officially recommended BCG since the early 1970s for household contacts of leprosy cases, as a booster to routine neonatal BCG-vaccination against TB. Since 1991, the Brazilian Ministry of Health has advised two doses of BCG to be administered to household contacts. This policy was assessed in a cohort study in Brazil[12], and showed 56% protection by a booster BCG-vaccination. The risk of tuberculoid leprosy during the initial months was high among BCG-vaccinated contacts. Due to incomplete follow-up, the increased risk of paucibacillary (PB) leprosy in the first months after BCG requires further substantiation.

Regarding chemoprophylaxis, a study in Bangladesh (acronym: COLEP) showed that a single dose of rifampicin (SDR) in contacts of newly diagnosed leprosy patients reduced the overall incidence of leprosy in the first two years with 57%[13]. Furthermore, this study showed that the effect of SDR depended on the BCG-status of the contact[14]: if the contact had received BCG-vaccination as part of a

childhood vaccination program, the protective effect of SDR was 80%. Contacts that received SDR without prior BCG vaccination had a protective effect of 58%. Recently, the WHO has included SDR as recommendation in their guidelines [15].

Based on earlier studies with BCG-vaccination and SDR chemoprophylaxis in preventing leprosy among contacts, a trial was initiated to assess the efficacy of a combined strategy (acronym: MALTALEP). The main objective of this trial was to assess the effectiveness in preventing leprosy in close contacts of patients with newly diagnosed leprosy of SDR given after BCG-vaccination, and specifically to determine whether possible excess cases in the first year after immunoprophylaxis, as observed previously in Brazil[12], can be prevented by chemoprophylaxis.

MATERIALS AND METHODS

Trial design. The intervention was a cluster randomized controlled trial with two treatment arms, to study the effectiveness of single dose rifampicin (SDR+ arm) given after BCG-vaccination in the prevention of leprosy among contacts of newly diagnosed leprosy patients, *versus* BCG-vaccine alone (SDR-arm) (Figure 1). At the initial contact survey, BCG was given to all eligible contacts, followed by chemoprophylaxis with SDR 8-12 weeks later in those contact groups randomized to receive this (FU1). Follow-up examinations were at one year (FU2) and two years (FU3) after receiving BCG. The three follow-up moments were used to investigate whether contacts had developed leprosy (primary outcome measure). Also, contacts were examined for adverse events at the different follow-up points. Due to operational difficulties caused by political instability in the country, it was not always possible to provide SDR exactly 8 weeks after BCG, so we broadened the range to 8 to 12 weeks after BCG.

Eligibility criteria for participants. Newly diagnosed leprosy patients were included who had been diagnosed with leprosy according to the Rural Health Program (RHP) guidelines, which follow those of the National Leprosy Control Program[16 17]. Diagnosis of leprosy was made when at least one of the cardinal signs was present: one or more skin lesions consistent with leprosy and with definite sensory loss; thickened peripheral nerve(s); and a positive skin smear result for acid-fast bacilli. We grouped patients with negative smear results and five or less skin lesions as PB leprosy, and those with positive smear results or more than five skin lesions as multibacillary (MB) leprosy according to the WHO treatment criteria. MDT was started according to the national guidelines. Within two weeks after newly diagnosed leprosy received the second dose of MDT (four weeks after the first dose), a household survey was performed. Contact groups were formed of around 10-15 persons for each patient.

Exclusion criteria for patients and contacts are summarized in our methodology article[18]. Only close contacts were included, i.e. household contacts and next-door neighbours. Contacts were categorized according to their physical and genetic distance to the index patient. For physical distance we defined four categories based on the local housing situation: shares a house and kitchen; shares a kitchen only; shares a house but not kitchen (together called household contacts); and next-door neighbours. For genetic distance we defined two groups: blood-related (parent, child, or sibling); and not blood-related or unclear (all others). Written informed consent was obtained from

all patients and their contacts. For illiterate people a thumb print and for minors under 16 years of age, the guardian's additional consent was obtained.

Study setting. The study was in the districts of Nilphamari, Rangpur, Thakurgaon and Panchagarh in northwest Bangladesh. Patients entered the trial through the RHP of The Leprosy Mission International, Bangladesh (TLMI,B), based at the DBLM Hospital in Nilphamari, a referral hospital specialized in the detection and treatment of leprosy. The population of the four districts at the start of intake was around 7,000,000 and 800-900 new leprosy patients were detected per year[19]. The prevalence rate of HIV in adults aged 15 to 49 in Bangladesh in 2018 was <0,1[20].

Interventions. The BCG-vaccine was applied by trained research assistants to all included contacts; 0.1 ml of BCG-vaccine by intradermal injection. Two different BCG-strains were used in the trial (and in routine neonatal vaccination in Bangladesh). The Indian vaccine was used between 2011 and 2015 (Moscow strain 361) and the Japanese vaccine in 2016 and 2017 (Tokyo strain 172). These are freeze-dried glutamate BCG-vaccines composed of 0,5 mg/ampule live bacteria of Calmette-Guérin (as approximately 70% moist bacteria) and 2,0 mg/ampule sodium glutamate (as a stabilizer). The BCG-vaccine was stored at the Government Immunisation Programme facilities.

Rifampicin comes in capsules of 150 mg and the dosage is the same as recommended in the guidelines of the national leprosy control program of Bangladesh and RHP (Table 1).

Table 1. Dosage of rifampicin chemoprophylaxis according to age and body weight.

Age/weight	**Dose of rifampicin**
Adult >35 kg	600 mg
Adult <35 kg	450 mg
Child 10–14 years	450 mg
Child 5–9 years	300 mg

Outcomes. The primary outcome measure was the number of new leprosy patients emerging from the contact groups. The proportions between the two arms of the trial is compared after one and two years.

Sample size. In the earlier COLEP trial[13] we found an incidence rate (IR) of leprosy among household contacts and direct neighbours of 40 per 10,000 per year in the untreated group over the first two years. We hypothesized that in contacts receiving BCG only, this number would be similar in the first year or possibly slightly increased. Also based on the previous trial, we expected a 50% reduction through the SDR intervention (IR of 2 per 1000). Based on these figures (with $\alpha = 0.05$ two-sided, power = 0.80), a total of about 10,000 contacts would be necessary in each group to detect reliably the expected protective effect of the BCG plus SDR combination of 50%, considering an expected 10% loss to follow-up of contacts.

Intake took place between July 2012 and January 2017. The intake took longer than originally planned, since the required number of contacts according to the power calculation had not yet been reached. Nevertheless, it was necessary to end recruitment in 2017 for budgetary reasons. Follow-up after two years was completed in January 2019.

Interim analyses and stopping guidelines. Because the trial was not blinded, it was possible to assess the outcomes during the study. This was done annually. The main stopping criterion was the occurrence of more serious adverse reactions to BCG-vaccination among contacts than described in literature.

In the first year of the trial, we found an unexpectedly high proportion of healthy contacts of patients (0.4%) presenting with PB leprosy within 12 weeks after receiving BCG-vaccination (the timeframe before SDR was given)[21]. Since it was too early in the trial to draw definite conclusions about this finding, the study was continued according to protocol.

Randomisation. Each contact group was randomly allocated to one of the two study arms (Arm 1: BCG only, or Arm 2: BCG plus SDR) by means of computer generation with a 1:1 ratio for each arm. A block size of 10 was used. A randomization table was created with 2000 sequential study numbers (one for each contact group). Each study number received a random number generated in MS Excel and this was fixed. The table was then sorted by block number and random number. Within each block of 10 study numbers, the highest 5 random numbers were assigned SDR, the lowest 5 were assigned no SDR. The allocation was generated by the database manager (RF), participants were enrolled by field staff. On inclusion of a new index patient, the local database manager (KK) entered the index into the database. A randomization into an arm of the trial was achieved by automatically

assigning each next study number to the contact group, thus assigning the pre-allocated randomization group of the study number.

Blinding. Blinding was not possible because there were no placebo capsules of rifampicin available and we were not able to locate any company that could produce these especially for this trial.

Statistical methods. For the calculation of the primary outcome measure, we started at FU1, the time when SDR was provided in the treatment (SDR+) arm of the trial. Contacts who developed leprosy after BCG-vaccination, but before FU1, were not included in the calculation of the primary outcome measure. Incidence rates per 10 000 person-years-at-risk were calculated for year 1 (FU2) and year 2 (FU3) of follow-up. The numbers at risk were calculated by adding the number of new cases of leprosy to the number of contacts without leprosy at the same follow-up moment. The probability of developing leprosy at 2 years was converted to incidence rates assuming a constant hazard during the period (rate = -log (1-leprosy/total)/2). To obtain confidence intervals we applied the standard errors of the probability of developing leprosy (sqrt(1/leprosy + 1/no leprosy)) around the log(rate). Additionally, the number needed to treat for BCG + SDR was estimated. A significance level of 5% was used in all tests. Statistical analyses were done with SAS 9.4. We used techniques for the analysis of survey samples to account for clustering at the level of the index patient in the sample. Bivariate associations are investigated using proc surveyfreq and the Rao Scott $\chi 2$ instead of the Pearson $\chi 2$.

Additional analyses. The effectiveness of BCG alone and BCG with SDR were investigated in different subgroups and odds ratios were reported, which are comparable to relative risks due to the low prevalence of leprosy. Additionally, we reported the Number Needed to Treat (NNT) per subgroup of contacts. Clustering is accounted for through using proc survey logistic instead of ordinary logistic regression.

RESULTS

Participants flow

We included a total of 1,552 index patients, of whom 1,077 (70%) were PB patients and 475 (30%) MB patients. Intake of PB index patients was intentionally ended when around 1,000 had been included, to insure an intake of at least 300 MB patients. The number of participants in each arm of the trial is shown in Figure 1. A total of 20,947 eligible household contacts were identified. Reasons for exclusion were: steroid use (n=9), pregnancy (n=241), liver disease or jaundice (n=70), malignancies (n=7), history of or under treatment for tuberculosis (n=122), history of leprosy (n=462), leprosy patient or suspect at intake (n=228), refusal of informed consent (n=1,136), under 5 years old (n=1,900), residing temporarily in the area (n=1,314), or suffering from another serious illness (n=673). Some contacts were excluded because they had more than one exclusion criteria. HIV was not tested within the trial, but when reported was used as exclusion criterion. After exclusion, 14,988 contacts entered the trial.

The contacts in both arms of the trial were well-balanced (Table 2). Of the 14,988 contacts included, 7,245 contacts in the SDR- arm were checked at FU1, 7,033 at FU2, and 6,898 at FU3 (Figure 1). A total of 7,322 contacts in the SDR+ arm received SDR at FU1, 7,042 were checked at FU2 and 6,906 at FU3. Of 7,322 contacts randomized to receive SDR, 283 did not receive it for various reasons. These contacts have not been included in the effect calculations.

Among the included contacts, 27 new leprosy patients were found in the first year (at FU2) in the SDR- arm, and 19 in the SDR+ arm. Subsequently, 24 new patients were found in the second year (at FU3) in the SDR- arm, and 29 in the SDR+ arm (Table 3). The incidence rate of leprosy per 10,000 person-years-at-risk (PYAR) was 44 PYAR in the SDR- arm, and 31 PYAR in the SDR+ arm at 1 year, and 34 PYAR in the SDR- arm and 41 PYAR in the SDR+ arm at 2 years. The reduction in incidence of leprosy in the SDR+ group compared to the SDR- group was 42% (95% confidence interval -13% to 70%); Rao Scott $\chi 2=2.1$ (df=1), P=0.148; overall number needed to treat was 714 (95% confidence interval -2000 to 313)) for PB leprosy in the first year. The reduction of new PB cases in the BCG and SDR group occurred in the first year after treatment; in year 2 no statistically significant difference was found between the number of new PB cases in the groups.

Supplementary Table S1 and S2 (appendix) show the effect of BCG only and BCG with SDR prophylaxis by variable category one and two years after BCG-vaccination. No significant differences of interest were found. A negative NNT indicates a statistically non-significant difference.

Table 4 shows the number of new cases at the different follow-up points including FU1 at 8-12 weeks after BCG. This table shows that 50 out of a total of 149 new cases (33.6%) occur within 3 months after receiving BCG. These are all (except one) PB cases; later in the trial more MB cases arise (8 MB cases after 1 year, and 6 after 2 years).

The rate of documented adverse events after BCG in the trial was low (0.34%) and comparable to studies in other countries[22-25]. These complications consisted primarily (80%) of skin ulcerations, which are known, common and benign adverse event after BCG-vaccination, which we have described previously[26]. Except for the orange urine discolouration caused by rifampicin, no adverse events were reported after SDR.

Figure 1. Flow of participants through the trial (MALTALEP study).

Figure 1

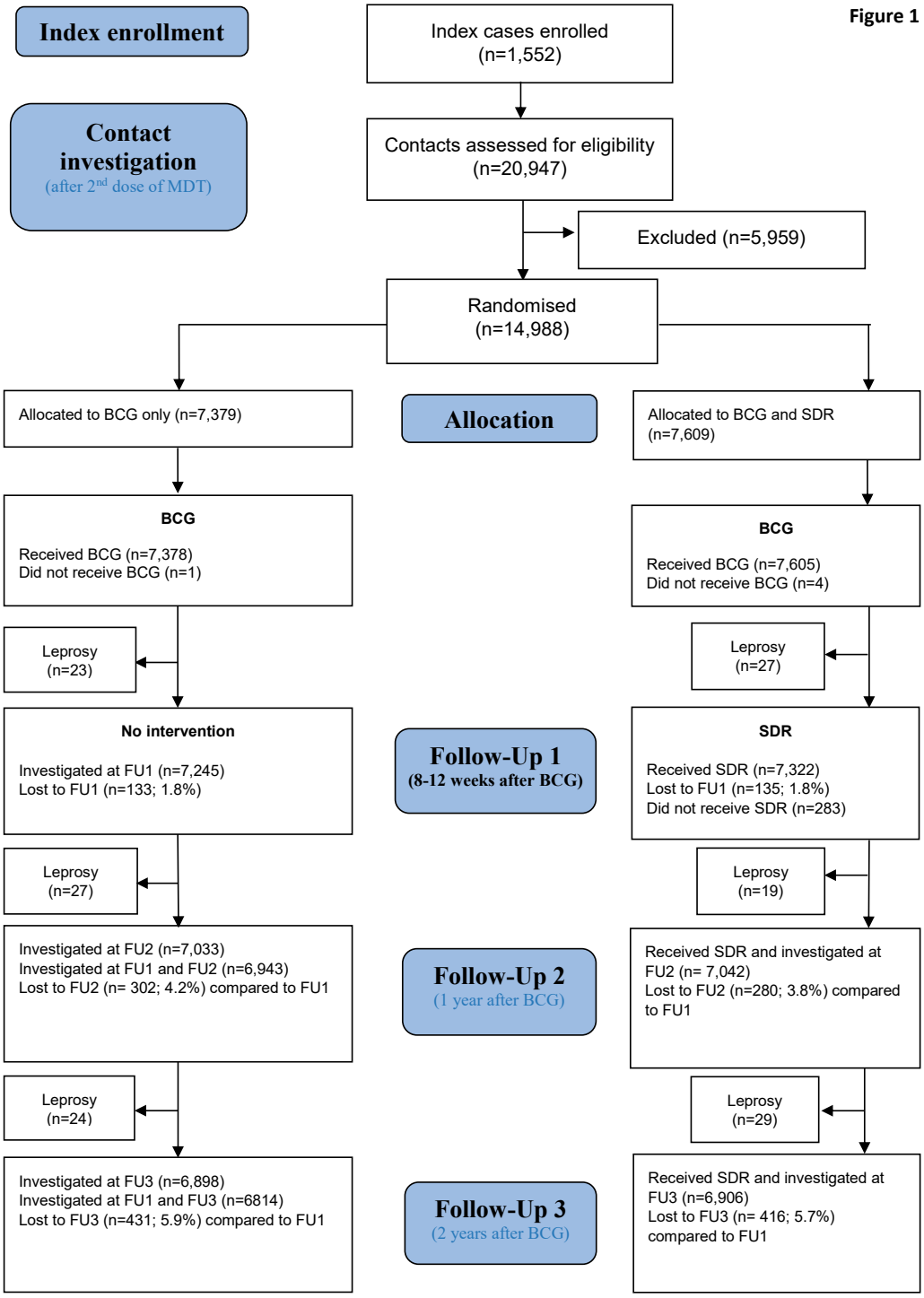
Index enrollment
Index cases enrolled (n=1,552)
Contact investigation (after 2nd dose of MDT)
Contacts assessed for eligibility (n=20,947)
Excluded (n=5,959)
Randomised (n=14,988)
Allocation
Allocated to BCG only (n=7,379)
Allocated to BCG and SDR (n=7,609)
BCG
Received BCG (n=7,378)
Did not receive BCG (n=1)
BCG
Received BCG (n=7,605)
Did not receive BCG (n=4)
Leprosy (n=23)
Leprosy (n=27)
No intervention
Investigated at FU1 (n=7,245)
Lost to FU1 (n=133; 1.8%)
Follow-Up 1 (8-12 weeks after BCG)
SDR
Received SDR (n=7,322)
Lost to FU1 (n=135; 1.8%)
Did not receive SDR (n=283)
Leprosy (n=27)
Leprosy (n=19)
Investigated at FU2 (n=7,033)
Investigated at FU1 and FU2 (n=6,943)
Lost to FU2 (n= 302; 4.2%) compared to FU1
Follow-Up 2 (1 year after BCG)
Received SDR and investigated at FU2 (n= 7,042)
Lost to FU2 (n=280; 3.8%) compared to FU1
Leprosy (n=24)
Leprosy (n=29)
Investigated at FU3 (n=6,898)
Investigated at FU1 and FU3 (n=6814)
Lost to FU3 (n=431; 5.9%) compared to FU1
Follow-Up 3 (2 years after BCG)
Received SDR and investigated at FU3 (n=6,906)
Lost to FU3 (n= 416; 5.7%) compared to FU1

Table 2. Baseline characteristics at intake of contacts of newly diagnosed leprosy patients (n=14,988) by treatment allocation.

Variable	BCG (n=7,379)*	%*	BCG and SDR (n=7,609)	%	p-value
Age at intake (in years)					
5-14	2,203	29.85	2,302	30.25	
15-29	2,051	27.80	2,113	27.77	
30-44	1,586	21.49	1,610	21.16	
≥45	1,539	20.86	1,584	20.82	0.68
Gender					
Male	3,358	45.51	3,407	44.78	
Female	4,021	54.49	4,202	55.22	0.27
Genetic distance to index patient					
Blood-related	1,662	22.52	1,647	21.65	
Not blood-related (or unclear)	5,717	77.48	5,962	78.35	0.34
Type of leprosy in index patient					
Paucibacillary	5,009	67.88	5,367	70.53	
Multibacillary	2,370	32.12	2,242	29.47	0.31
BCG scar					
Present	4,201	56.93	4,369	57.42	
Absent	3,172	42.99	3,236	42.53	
Unknown	6	0.08	4	0.05	0.67
Physical distance to index patient					
Household contact	2,192	29.71	2,117	27.82	
Neighbour	5,187	70.29	5,492	72.18	0.09

*Values are numbers and percentages of total numbers of contacts

Table 3A. Analysis of all cases of leprosy in contacts of patients with newly diagnosed leprosy*

Treatment	Leprosy	No leprosy	Total no. at risk	Incidence Rates per 10,000 PYAR	95% CI
BCG					
1 year FU	27	7,250	7,277	44	30-64
2 year FU	24	7,118	7,142	34	23-50
1-2 years FU	51				
BCG and SDR					
1 year FU	19	7,228	7,247	31	20-48
2 year FU	29	7,087	7,116	41	28-59
1-2 years FU	48				

Table 3B. Analysis of PB leprosy in contacts of patients with newly diagnosed leprosy*

Treatment	PB	No leprosy	Total no. at risk	Incidence Rates per 10,000 PYAR	95% CI
BCG					
1 year FU	24	7,253	7,277	39**	26-58
2 year FU	24	7,118	7,142	34	23-50
1-2 years FU	48				
BCG and SDR					
1 year FU	14	7,233	7,247	23**	13-38
2 year FU	23	7,093	7,116	32	22-49
1-2 years FU	37				

Table 3C. Analysis of MB leprosy in contacts of patients with newly diagnosed leprosy*

Treatment	MB	No leprosy	Total no. at risk	Incidence Rates per 10,000 PYAR	95% CI
BCG					
1 year FU	3	7,274	7,277	4.9	1.6-15
2 year FU	0	7,142	7,142	0.00	-
1-2 years FU	3				
BCG and SDR					
1 year FU	5	7,242	7,247	8.1	3.4-20
2 year FU	6	7,110	7,116	8.4	3.8-19
1-2 years FU	11				

*Numbers are provided by treatment arm at one and two years' follow-up, with incidence rates per 10,000 person-years at risk (95% confidence interval); **Overall reduction in incidence of PB leprosy in SDR+ group in year 1: 42% (95% confidence interval -13% to 70%); Rao Scott $\chi 2=2.1$ (df=1), P=0.148; overall number needed to treat 714 (95% confidence interval -2000 to 313).

Table 4. New leprosy cases among contacts of newly diagnosed leprosy cases identified according to the time points of diagnosis.

	8-12 weeks	1 year	2 years	Total
BCG				
PB	23	24	24	71
MB	0	3*	0	3
BCG and SDR				
PB	26	14	23	63
MB	1	5	6	12
Total	50	46	53	149

*Only 1 new MB leprosy case had a BI of 2+ (BL), the rest of the MB cases were smear negative (MB BT).

Supplementary Table S1. Protective efficacy of BCG versus BCG and SDR prophylaxis in contacts of newly diagnosed leprosy patients by variable category at one year follow-up.

	BCG		BCG and SDR					
Age group (years)	**No leprosy**	**Leprosy (n)**	**No leprosy (n)**	**Leprosy (n)**	**OR (95% CI)***	**p-value**	**p-value interaction**	**NNT****
5-14	2,171	5	2,212	5	0.98 (0.28, 3.40)	0.98	0.85	23,425.62
15-29	2,005	10	1,958	5	0.51 (0.18, 1.50)	0.22		410.86
30-44	1,561	7	1,527	6	0.88 (0.29, 2.61)	0.81		1,801.70
≥45	1,513	5	1,531	3	0.59 (0.14, 2.48)	0.47		743.39
Gender								
Female	3,952	15	4,013	6	0.39 (0.15, 1.03)	0.06	0.10	434.71
Male	3,928	12	3,215	13	1.11 (0.48, 2.58)	0.81		-1,011.58
Blood relative								
Yes	1,623	13	1,562	7	0.56 (0.22, 1.40)	0.22	0.47	283.41
No	5,627	14	5,666	12	0.85 (0.38, 1.93)	0.70		2,701.91
Type of leprosy index patient								
PB	4,927	12	5,099	9	0.73 (0.28, 1.87)	0.51	1.00	1,491.41
MB	2,323	15	2,129	10	0.73 (0.30, 1.75)	0.48		568.14
Physical distance								
Household	2,130	19	2,005	10	0.56 (0.24, 1.31)	0.18	0.28	254.28
Neighbour	5,120	8	5,223	9	1.10 (0.43, 2.86)	0.84		-6,224.80
BCG scar								
Present	4,314	15	4,154	8	0.53 (0.22, 1.28)	0.16	0.34	644.66
Absent	3,110	12	3,072	11	0.93 (0.40, 2.18)	0.86		3,599.82

*Odds Ratio (with 95% confidence interval), ** Numbers Needed to Treat

Supplementary Table S2. Protective efficacy of BCG versus BCG and SDR prophylaxis in contacts of newly diagnosed leprosy patients by variable category at two years' follow-up.

	BCG		BCG and SDR					
Age group	**No leprosy**	**Leprosy**	**No leprosy**	**Leprosy**	**OR (95% CI)***	**p-value**	**p-value interaction**	**NNT****
5-14	2,135	10	2,182	6	0.59 (0.21, 1.61)	0.30	0.12	517.04
15-29	1,960	5	1,900	8	1.65 (0.54, 5.03)	0.38		-602.59
30-44	1,541	2	1,506	9	4.61 (0.99, 21.4)	0.05		-213.76
≥45	1,482	7	1,499	6	0.85 (0.26, 2.70)	0.78		1,387.58
Gender								
Female	3,889	12	3,940	15	1.23 (0.53, 2.90)	0.63	0.96	-1,386.04
Male	3,229	12	3,147	14	1.20 (0.54, 2.65)	0.66		-1,365.45
Blood relative								
Yes	1,591	8	1,529	10	1.30 (0.51, 3.30)	0.58	0.87	-661.40
No	5,527	16	5,558	19	1.18 (0.56, 2.48)	0.66		-1,909.80
Type of leprosy index patient								
PB	4,832	11	4,995	25	2.20 (0.86, 5.64)	0.10	0.01	-366.50
MB	2,286	13	2,092	4	0.34 (0.11, 1.02)	0.06		264.92
Physical distance								
Household	2,084	14	1,967	13	0.98 (0.46, 2.09)	0.97	0.42	9,191.09
Neighbour	5,034	10	5,120	16	1.57 (0.62, 4.02)	0.34		-878.34
BCG scar								
Present	4,057	15	4,066	17	1.13 (0.54, 2.36)	0.74	0.75	-2,067.40
Absent	3,056	9	3,019	12	1.35 (0.54, 3.38)	0.52		-971.06

*Odds Ratio (with 95% confidence interval), ** Numbers Needed to Treat

DISCUSSION

In the first year after provision of SDR to contacts who had first received BCG-vaccination, the number of PB patients was reduced by 42% compared to the group that did not receive SDR. No additional effect of SDR was seen in the second year. A large proportion (33.6%) appeared within 8-12 weeks after vaccination, the window period between vaccination and provision of SDR.

By providing rifampicin (a bactericidal drug) 8-12 weeks after BCG-vaccination, we envisaged preventing new leprosy cases among contacts in the first year after the BCG. This was described in Brazil by Duppre *et al.*[12], who showed that the risk of PB leprosy was high during the initial months among those contacts vaccinated with BCG: among the 58 new cases detected during 18 years of contact follow-up, leprosy was diagnosed in 21 of these contacts (36%) relatively soon after vaccination (2-10 months); 18 out of these 21 contacts had PB leprosy. We also found an unexpectedly high proportion of new PB cases following BCG-vaccination; however, this phenomenon already occurred in the period between BCG-vaccination and SDR provision. We had designed this time interval to ensure that rifampicin would not affect the efficacy of BCG, which is a live vaccine. At the time of the conceptualisation of the trial, we had no indication to expect this would occur this early after BCG. Most trials only include long-term follow-up, often starting 1 year after vaccination. The Brazilian trial[12] diagnosed the new leprosy cases 2-10 months after BCG-vaccination, which was also later than what we found in our trial. In previous studies the number of cases were either too low to confirm early 'induction' of leprosy after BCG[27 28] or did not specify when exactly leprosy occurred after vaccination[29 30]. So, at the time SDR was provided in the current study, most excess cases had probably already become manifest.

What would have been the result of the trial if SDR was given before BCG-vaccination? There was no published evidence to support our decision on the order of BCG and SDR. We simply followed the logic of the primary research question whether SDR would suppress the excess cases after BCG-vaccination and designed the study in that order. Also, the intervention strategy considered the bactericidal effect of SDR on live bacteria such as BCG. In hindsight it could have been preferable to first provide SDR, and this should be explored in a future study.

The level of protection offered by SDR in our study is 42%, which is less that the COLEP study (57%) conducted 10 years previously in the same population[13]. However, our contact population only included household and first neighbour contacts, while the COLEP study also included second neighbours and social contacts. The further contacts are physically removed from the index case, the more pronounced the effect of SDR is in protecting against leprosy. This is probably due to a lower exposure rate and hence a lower bacterial load of these further distanced contacts, rendering a single dose of rifampicin more effective[13 31]. Immunological screening of the effect of SDR on *M. leprae* infection in contacts can provide insight to what extent, how fast and how durable *M. leprae* infection is reduced by this single dose of antibiotics.

The observations from this trial give rise to interesting hypotheses regarding the immunological mechanisms underlying the effect of BCG-vaccination given to contacts of newly diagnosed leprosy cases. Possibly BCG accelerates pro-inflammatory T-helper 1 (Th1) immunity to *M. leprae* antigens, thereby revealing incipient forms of PB leprosy. Alternatively, BCG-vaccination is also known to induce trained immunity and thereby nonspecifically activates protective innate responses[32 33]. In a previous study[26] we showed that BCG-vaccination induced significant Th1-type immunity (higher levels of IFN-γ) in those who presented with high local inflammation responses, implicating that efficient protection against *M. leprae* is dependent on an adequate Th1 response[34], although the concomitant inflammation may result in collateral tissue damage[35].

This study investigated the effect of BCG with or without SDR in one highly endemic area in the Indian sub-continent with a specific PB:MB ratio (2:1 instead of the usual 1:1 reported world-wide)[36-38], a low socioeconomic status, and specific demographic, genetic and cultural characteristics. Whether BCG would give similar protection in other areas of the world is questionable. Furthermore, in Bangladesh the Moscow strain 361 and Tokyo strain 172 are used, elsewhere the use of other BCG-strains for vaccination could lead to different results[39 40].

Our trial was not designed to establish the protective effect of BCG against leprosy. We assumed this is a given based on literature[5 27 41] and had therefore not included an arm in the trial without BCG. However, we doubt that the protective effect of BCG alone was large in our study. The incidence rate of leprosy at 2 years among the household contacts and next-door neighbours in the non-intervention arm in the COLEP study was 39.35 per 10,000 PYAR[13]. The incidence rate is 33.72 per

10,000 PYAR in the BCG only arm at 2 years of the MALTALEP trial. This implies a 14.3% reduction of leprosy incidence by BCG vaccination compared to no intervention. A Brazilian trial[12] showed that the protection conferred by a booster BCG-vaccination was 56% and was not substantially affected by previous BCG-vaccination. More specifically, this effect was 83-85% for the indeterminate and MB forms of leprosy, but a non-significant effect of 26% was found for the PB forms. This might explain the lack of effect of BCG in our trial when compared to no intervention; in Bangladesh most patients have the PB form of leprosy[1].

In a subgroup analysis (supplementary data), we found no significant difference between the development of leprosy in revaccinated (BCG-scar positive) versus primarily vaccinated (BCG-scar naïve) contacts. In their meta-analysis, Merle et al.[5] also found no statistical difference in BCG-protection against leprosy between studies where individuals are vaccinated once and studies where individuals receive a booster vaccination on top of the neonatal vaccination.

There may be better alternatives to BCG-vaccination as immunoprophylaxis in leprosy, with new candidate leprosy vaccines in the pipeline, such as MIP[10] and LepVax[42 9 10]. The MIP vaccine has only been evaluated in Uttar Pradesh, India, when both patients and contacts were vaccinated. The protective efficacy was 68%, 60%, and 28% after three, six, and nine years, respectively[10]. For LepVax, post-exposure prophylaxis tested in nine-banded armadillos appears safe and, unlike BCG, diminishes the neurologic disruptions caused by *M. leprae* infection[42]. Further trials are needed to investigate these vaccines before they can be introduced in the field.

Strengths of our trial is that it is randomized-controlled and field-based. An extensive number of leprosy contacts (14,988) were included. Also, because it is based in a leprosy-endemic area, implementation lies close to clinical field practice. Our loss to follow-up was less than 6%, which was less than expected. A limitation is that it was not possible to make it double-blind (placebo was not available), which may bias the results. Even when using a harmless dose of a dissimilar vitamin pill to prevent participants from knowing whether or not they had been given an intervention, this would not have prevented bias by the field staff since they would know the difference. For instance, the field staff may expect and look more closely for signs and symptoms of leprosy in those that have not received SDR. Furthermore, a limitation was that intake took longer than expected and therefore we

could not reach the 10,000 contacts per arm we set out to include, leading to less power and therefore less statistically significant results.

CONCLUSION

It is difficult to establish the extent to which SDR suppresses excess leprosy cases among contacts in the year after BCG-vaccination. Based on this study we cannot recommend BCG-vaccination followed by SDR as routine intervention in leprosy control. However, we do advise contact surveys followed by SDR to eligible contacts of new leprosy cases. Recently, the WHO included SDR as guideline in their leprosy elimination strategy[15]. Implementation studies on the effectiveness of SDR as leprosy post-exposure prophylaxis (LPEP) are currently ongoing [43 44].

Ethical approval

The national Research Ethics Committee (Bangladesh Medical Research Council) has approved the study protocol (Ref no. BMRC/NREC/2010-2013/1534).

Competing interests

The authors declare that they have no competing interests. The BCG-vaccine will be provided free of charge by the Government of Bangladesh.

Authors' contributions

All authors contributed to the design of the study and manuscript preparation. All authors have read and approved the final manuscript.

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CHAPTER 7

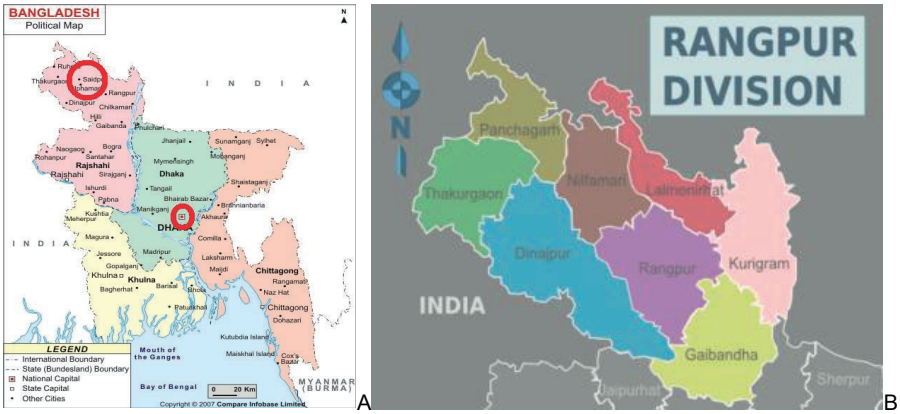
7

General Discussion

CHAPTER 7: GENERAL DISCUSSION

This thesis is centered around the MALTALEP trial, a cluster randomized controlled trial conducted from 2012 to 2019 in northwest Bangladesh among 15,000 close contacts of new leprosy patients, to evaluate the effect of BCG only versus BCG and SDR as prophylactic measure to prevent the development of leprosy.

Figure 1A: Map of Bangladesh, with the research area indicated with the larger circle (the smaller circle indicates the capital city Dhaka).
Figure 1B: the MALTALEP study was conducted in the districts of Nilphamari, Rangpur, Thakurgaon and Panchagarh in northwest Bangladesh.



A B

Figure 2: Population and number of new cases in the four districts Nilphamari, Rangpur, Thakurgaon and Panchagarh in the northwest of Bangladesh.

	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
Population	7214063	7320833	7429183	7539136	7650715	7763948	7878854	7973399	8069079	8190035
New cases	1043	867	572	651	641	765	801	666	862	967

This thesis is centered around the following three research questions:

1. What are the potential causative mechanisms underlying the development of leprosy following BCG vaccination?
2. Do the results of our trial justify the introduction of a combination of BCG and SDR in leprosy health care programs in Bangladesh to prevent the development of leprosy amongst household contacts of new leprosy patients?
3. Can immune markers be identified in contacts of leprosy patients that predict the development of clinical leprosy?

Research Question 1: What are the potential causative mechanisms underlying development of leprosy following BCG vaccination?

In 1989, Bagshawe[74] already said that BCG vaccination may precipitate clinical signs and symptoms of tuberculoid leprosy in people carrying *M. leprae* and cause upgrading of existing lesions. Duppre *et al*[60] also hypothesized that the high number of cases with tuberculoid leprosy found 2-10 months after BCG vaccination in Brazil, is caused by BCG stimulating the already present anti-mycobacterial immunity in individuals infected with *M. leprae* before or immediately after BCG vaccination. In line with these studies, we also found an unexpectedly high proportion of healthy contacts of leprosy patients presenting with PB leprosy within 12 weeks after receiving BCG vaccination (33,6% of all cases diagnosed during the 2-year observation period)[75 83].

It is well known that in tuberculoid leprosy, macrophages have a classical activation phenotype (M1), while macrophages in lepromatous disease show alternative activation (M2)[76-79]. BCG has shown to direct macrophages preferentially towards pro-inflammatory M1 activation[76]. In the case of subsequent *M. leprae* infection, higher levels of inflammatory cytokines will be released; the immune system can then clear the bacilli more effectively or tuberculoid leprosy may develop[76]. Thus, BCG vaccination causing increased pro-inflammatory immune responses, may also render already infected contacts more prone to developing PB leprosy by shifting the immune response to destructive Th1 responses. Rhodes *et al*[80] showed that BCG revaccination provides a higher and longer lasting IFN- γ+ CD4+ T-cell response than primary vaccination in humans, with a peak around 30 days.

When addressing the research question what immunological processes play a role in leprosy activation following BCG vaccination, it is instructive to observe immunological responses during BCG-related complications: large local reactogenicity after BCG vaccination possibly correlates with an adequate immune response, and thus less chance of developing the more severe lepromatous forms of leprosy, which are an expression of a suppressed immune response.

In our trial, adverse events were observed in 0.34% of the contacts of leprosy patients that received BCG vaccination[75]. These complications consisted primarily (80%) of skin ulcerations. We investigated what type of immune profile is associated with BCG-related complications[75]. Similar to the increased pro-inflammatory Th1 immunity and high levels of pro-inflammatory cytokine observed for tuberculoid leprosy patients, contacts with adverse events at the site of BCG vaccination showed elevated IFN-γ levels in response to *M. leprae* specific proteins in whole-blood assays (WBA). In addition to the elevated IFN-γ levels, we found that lower levels of $sCD40L_{NIL}$ and GRO_{WCS} were significantly associated with BCG complications. sCD40L and GRO (CXCL1) both play a role in T-cell regulation; a decrease in their levels may potentially cause uncontrolled T-cell immunity damaging

the skin. Finally, regulatory T-cells secrete CC chemokine ligand 4 (CCL4), which in turn suppresses Th1 cells[12]. In our study[75], a reduction in CCL4 (although not significant) was found in those contacts that developed complications after BCG. This may indicate decreased T-cell regulation and a shift towards excessive Th1-type immunity with inflammation at the BCG vaccination site as a result. Skin complications after BCG vaccination therefore may be surrogate markers for protective immunity against leprosy, although there may be a higher risk of developing tuberculoid leprosy.

The Mitsuda reaction shows if an appropriate immune response to an intradermal injection of lepromin (which is a heat-killed leprosy bacilli) is formed. In case of a negative skin reaction, it has a good prognostic value for susceptibility to the lepromatous form of leprosy; a positive skin reaction indicates resistance to lepromatous leprosy[77]. In the same way, individuals with high local reactogenicity after intradermal BCG administration have less risk for lepromatous leprosy onset[78]. In 12 tuberculin skin test and Quantiferon negative, BCG-naive adults in the Netherlands, BCG vaccination induced significant Th1-type immunity in those with large local inflammation responses. However, in low inflammation responses, significantly increased regulatory $CD8^+$ T-cells were found[79].

The sudden increase in leprosy patients after BCG vaccination is caused by a boosted cell-mediated immunity by homologues of *M. leprae* antigens in BCG. The mechanism may be similar to the immune reconstitution inflammatory syndrome (IRIS) seen in human immune deficiency virus (HIV) patients on highly active antiretroviral treatment (HAART), who develop leprosy. Deps *et al.*[81] defined IRIS in leprosy as leprosy and/or Type 1 reaction and erythema nodosum leprosum (ENL or Type 2 reaction) within 6 months after start of HAART. 89.5% of the leprosy/IRIS cases presented with TT or BT leprosy. After starting HAART, IRIS was initiated after a mean of 8.7 weeks. Immune restoration in leprosy as IRIS after starting HAART is based on an increase in circulating $CD4^+$ T cells[81].

We suggest that a comparable process takes place, namely stimulation of the Th1 cascade, leads to presentation of clinically apparent tuberculoid leprosy and augmentation of type 1 reactions after BCG vaccination in contacts of leprosy patients. Our trial is unique with respect to several aspects, one of which is that it has its first follow-up moment relatively soon after BCG vaccination (within 8-12 weeks). By finding the new cases at an early stage, early treatment was also possible, possibly preventing complications. In fact, BCG vaccination given to household contacts of leprosy patients could actually identify this important group. However, it should not be used as a legitimate diagnostic test for pre-clinical leprosy, since it is unclear whether BCG vaccination only alters the incubation period or changed the course of the infection from self-limiting, subclinical infection to manifest disease.

Research Question 2: Do the results of our trial justify the introduction of a combination of BCG and SDR in leprosy health care programs in Bangladesh to prevent the development of leprosy amongst household contacts of new leprosy patients?

The MALTALEP trial[82] was designed to evaluate whether combining SDR with BCG (re)vaccination provides additional value in preventing leprosy. Strengths of our trial[83] is that it is a randomized-controlled trial that includes an extensive number of leprosy contacts (14,988). Also, because it is based in a leprosy-endemic area, implementation lies close to clinical field practice. However, on the basis of the trial results, we cannot justify the introduction of a combination of BCG and SDR in the field to prevent the development of leprosy amongst household contacts of new leprosy patients. This is due to several reasons:

Firstly, in the first year after BCG (re)vaccination, the reduction in incidence of leprosy in the SDR+ arm was 42% compared to the SDR- arm, which shows a clear effect of this chemoprophylactic intervention[83]. However, the low number of cases prompt us to designate this effect as a trend, as it was not statistically significant ($p>0.05$), due to lack of power. No additional effect of SDR was seen in the second year, which is within the line of expectation because SDR is not a vaccine and no long-lasting immunological response is induced[84]. The COLEP trial describes an overall effect of SDR of 57% in the first two years[70]. However, chemoprophylaxis with SDR was most effective in contact groups with relatively low perceived *a priori* risks, such as contact groups of PB index patients, in contacts not living in the same household, or without close blood relationship to the index patient. It is assumed that infected contacts in these groups have had less exposure to *M. leprae* prior to SDR provision and therefore lower bacterial loads than those who are closer to an index patient. Thus, one single dose of rifampicin should be enough to clear the bacterial load in these low-risk groups, whereas for more heavily infected individuals (due to either genetic susceptibility and/or long-term exposure to an untreated MB patient), treatment with SDR is less effective. In the COLEP trial in the group of blood-related household contacts the effect of SDR was around 25% only, while it was around 50% in non-blood-related and neighbouring contacts, and up to 75% in social contacts[70 85]. For the high-risk group that is possibly incubating MB leprosy, a diagnostic test indicating the extent of infection would justify an extended treatment regimen, possibly a full course of MDT. Contact screening including a field-friendly diagnostic test could represent an efficient strategy to reduce transmission of *M. leprae* in the community. The goal of early contact tracing and subsequent provision of SDR is three-fold: firstly, with contact tracing early cases of leprosy can be detected; secondly, provision of SDR prevents leprosy in infected contacts without clinical signs of leprosy; and thirdly it contributes to the interruption of transmission of *M. leprae* to others[84]. The finding that SDR

is efficient for contacts who are not living in the same household is in line with the few other studies available on this subject. These show that blanket chemoprophylaxis of a whole community, mostly including individuals with less exposure and as such with no or low bacterial load, is more effective than chemoprophylaxis to household contacts only in reducing new case detection rates in the community[69 86]. Our study[83] had a larger proportion of household contacts, probably explaining why SDR was less effective in our study (42%), when compared to COLEP (57%), where a wider group of contacts were included[70].

Secondly, the effect of BCG in protecting against leprosy among leprosy contacts in the MALTALEP trial appeared to be smaller than anticipated[83], compared to previous studies on BCG immunoprophylaxis, for example from Brazil[60]. The incidence rate in our study in Bangladesh is 33.72 per 10,000 PYAR in the BCG only arm at 2 years of the MALTALEP trial[83]. The incidence rate of leprosy at 2 years among the household contacts and next-door neighbours in the non-intervention arm in the COLEP study was 39.35 per 10,000 PYAR[70]. This implies a 14.3% reduction of leprosy incidence by BCG vaccination compared to no intervention. In the Brazilian study amongst leprosy contacts[60], the protection conferred by a booster BCG vaccination was 56% and was not substantially affected by previous BCG vaccination. This effect was 83-85% for the indeterminate and MB forms, but a non-significant effect of 26% was found for the PB forms. This might also explain the lack of effect of BCG in the MALTALEP trial when compared to no intervention: in Bangladesh a larger proportion of leprosy patients develop the PB form (66% of total leprosy patients) when compared to other parts of the world (50% of total leprosy patients)[2]. Furthermore, the BCG strain used may also have effect on the efficiency of BCG vaccination[87]. In Bangladesh, the Moscow strain 361, Tokyo strain 172 and the Aventis-Pasteur strain are used. Elsewhere the use of other more virulent BCG strains for vaccination could lead to different results. The Tokyo strain, for example, is known to be a non-virulent strain; restoration of its lost T-cell epitopes in the future may lead to new, more powerful BCG vaccine strains[88]. Furthermore, genetic or nutritional differences between populations, environmental influences such as sunlight exposure (vitamin D), poor cold-chain maintenance, or exposure to environmental mycobacterial infections may lead to variation in the efficacy of BCG[89].

Thirdly, we found no statistically significant difference between the development of leprosy in contacts who were revaccinated as part of the trial (with visible BCG scar) *versus* contacts who were vaccinated for the first time in their life as part of the trial (BCG scar naïve)[83]. This is in line with the conclusions of Merle *et al.*[59] that revaccination might give extra protection to adults for whom the effectiveness of the first vaccination decreased over time, but there may be no use of revaccination when it is performed in school children. Our study[83] has a relatively high proportion of school

children between 5 and 14 years of age (15%), which may explain why revaccination had little benefit.

Finally, we had expected to prevent the excess cases in the first year after BCG as described previously[60 74], by giving chemoprophylaxis in the form of SDR 8-12 weeks after BCG vaccination. We had not anticipated however, to find such a large proportion of new leprosy cases (33.6%) in the three-month observation period before providing SDR (Table 2[83]). This renders the bactericidal capacity of SDR redundant and therefore less prominent.

Table 2: New leprosy cases among contacts of newly diagnosed leprosy cases identified according to the time points of diagnosis

	8-12 weeks	1 year	2 years	Total
BCG				
PB	23	24	24	71
MB	0	3*	0	3
BCG and SDR				
PB	26	14	23	63
MB	1	5	6	12
Total	50	46	53	149

*Only 1 new MB leprosy case had a BI of 2+ (BL), the rest of the MB cases were smear negative (MB BT).

The Brazilian trial[60] only described an augmentation of new leprosy cases 2-10 months after BCG vaccination. It is possible that this trial did not describe earlier cases, because follow-up did not occur in the first two months, although this is not mentioned specifically in the trial description. Future trials could consider providing SDR before BCG. Giving BCG and SDR at the same time within the MALTALEP trial was not possible, due to the bactericidal effect of SDR on BCG, which is a live vaccine. When designing the trial, we chose not to administer SDR before BCG, because of the logistic implications as an extra follow-up time point would have been necessary, which is difficult in a country like Bangladesh with a limited infrastructure and political instability.

Although we do not recommend a combined strategy with BCG and SDR based on the results of our trial, there is sufficient evidence to continue advising administration of SDR to household contacts of new leprosy cases. However, the direct immunological effect of SDR on infection has not yet been investigated, nor its effect on *M. leprae* infection in the community. Future studies will assess this in more detail in Bangladesh in the next three years. The advantage of targeting household contacts in general is that they are a clearly defined group, who are easily reachable. Because of the social stigma associated with leprosy, new leprosy patients may be less willing to give disclosure when

asking people outside the direct contacts to participate in prophylactic campaigns. Furthermore, since new leprosy cases are becoming rarer in most endemic countries, it is not cost-effective to apply interventions such as chemoprophylaxis and immunoprophylaxis to total populations within a blanket approach due to the enormous numbers needed to treat to prevent a case of leprosy. Targeted interventions towards well-defined high-risk groups is preferable.

Research Question 3: Can immune markers be identified in contacts of leprosy patients that predict the development of clinical leprosy?

It has been established that antibodies directed aganst the *M. leprae*-specific phenolic glycolipid I (PGL-I) cannot be applied in predicting the development of clinical leprosy in the Bangladeshi context. Combined biomarker tests, however, are increasingly proving to be useful.

Our findings in contacts in Bangladesh[90] are in line with previous literature[91-96], where it has been shown that the development of leprosy amongst leprosy contacts was not associated with the level of anti-PGL-I seropositivity among these contacts at intake. Although positivity to anti-PGL-I and development of leprosy in healthy contacts was associated, choosing contacts for prophylaxis based on anti-PGL-I response would miss more than half of future leprosy cases, particularly PB[91-94]. To understand the value of anti-PGL-I Ab as a predictor of leprosy in those at risk of developing leprosy, we analyzed the anti PGL-I Ab levels in the blood of 224 contacts of leprosy patients in a highly endemic area in the northwest part of Bangladesh and followed them over a period of 6 years[90]. Six of these 25 (24%) contacts who developed leprosy had a positive anti-PGL-I Ab level of >0.150 at intake. Thirty five out of 199 (17.6%) contacts who did not develop leprosy had a positive anti-PGL-I Ab level of >0.15 at intake. No significant association was found for the anti-PGL-I Ab levels at baseline (OR: 1.01 (0.78, 1.31), 95% CI p=0.94) between the two groups. Furthermore, changes in anti-PLG-I Ab levels did not predict disease progression in contacts of new leprosy patients in Bangladesh. These results clearly indicate that also in Bangladesh anti-PGL-I Ab tests alone are not able to diagnose leprosy amongst leprosy contacts at an early time point.

Most of the leprosy patients' contacts in our study, however, developed PB leprosy (21 out of 25), which offers an explanation for the lack of increase of anti-PGL-I titers at leprosy diagnosis. In this respect it is important to realize that in Bangladesh the percentage of PB cases amongst new leprosy cases is generally higher (67%) than in other countries in Asia or the rest of the world (on average around 50%)[2]. In southeast Asian countries, such as Indonesia, predominantly MB patients are found, which is probably due to a combination of genetic factors as well as lack of early case detection, since more PB cases are found when active case finding strategy is applied[84]. Therefore, in other leprosy endemic countries, where more MB leprosy occurs, the longitudinal pattern of anti-PGL-I Ab levels could have more prognostic value. Furthermore, anti-PGL-I antibodies could be a useful tool for monitoring how effective the treatment of leprosy (reactions) is, since effective treatment leads to a decrease in antibody levels[97].

Importantly, combining humoral and cellular biomarkers (instead of serology alone) gives more possibilities in distinguishing *M. leprae* infected from non-infected individuals, patients from

contacts, or lepromatous from tuberculoid patients[50 98]. Field-friendly tests based on a recently developed lateral flow test format (UCP-LFA) using biomarker signatures instead of single markers, were useful in identifying which contacts are at risk of developing leprosy, as well as individuals infected with M. leprae without clinical symptoms[50 98]. Other studies by our group have focused on this immunodiagnostic research line as part of the IDEAL project[50 99] and projects situated in leprosy endemic areas outside Bangladesh[54].

SUMMARY

Leprosy is an infectious disease caused by *Mycobacterium leprae (M. leprae)*, which can cause damage to the skin and peripheral nerves. The global number of new leprosy patients has remained constant over the past decennium, with a total number of around 200,000 of which 10% are children. This indicates that transmission of *Mycobacterium leprae* is ongoing in many endemic countries. People living in the same household as untreated leprosy cases have the highest risk of infection with *M. leprae* and developing disease. Therefore, it is essential that leprosy control strategy is focused on this risk group. This strategy has three basic pillars: 1) identifying new leprosy patients; 2) treating new leprosy patients; and 3) treating contacts of new leprosy patients.

In the past years, several studies have investigated the use of immunoprophylaxis (vaccination) and chemoprophylaxis (medication) to prevent the spread of leprosy among contacts of leprosy patients. Bacillus Calmette-Guérin (BCG) is the most frequently given vaccine in the world. It is known as a vaccine against tuberculosis and is routinely given to infants in countries endemic for tuberculosis as part of the neonatal immunization. BCG is also recognized as protecting against leprosy. In Brazil, the government officially recommends BCG (re)vaccination as prophylaxis to protect household contacts of newly diagnosed leprosy cases. This policy showed a 56% protection rate in a Brazilian cohort study. However, a high number of new leprosy patients were found amongst the contacts in the first 2-10 months after BCG vaccination.

The COLEP trial, performed in the northwest of Bangladesh between 2002 and 2009, showed that the use of a single dose of the antibiotic rifampicin (SDR) as chemoprophylaxis in contacts of new leprosy patients reduced the incidence of leprosy in the first two years after intake with 57% compared to placebo; after four and six years no additional effect was seen. BCG vaccination and SDR each had a protective effect in contacts of around 60%, but the COLEP study also showed an additional additive protective effect of SDR (80%) in contacts that had received BCG vaccination in the past.

Based on the experience with BCG vaccination and SDR chemoprophylaxis in preventing leprosy among contacts of leprosy patients, a trial was started in Bangladesh to assess the efficacy of a combined strategy (the MALTALEP study). The MALTALEP study is a cluster randomized controlled trial in the northwest of Bangladesh between 2012 and 2018, in which around 15,000 contacts of newly diagnosed leprosy patients received either BCG alone, or BCG plus SDR. The primary outcome was the development of leprosy within two years after receiving BCG with or without SDR.

Chapter 1 gives a general introduction to the thesis. Chapter 2 describes how the blood of symptom-free contacts of new leprosy patients of the COLEP study was collected and analyzed at three time points over a period of six years. This showed that the anti-PGL-I antibody rates at intake did not significantly differ between contacts that developed leprosy during the study and those that remained symptom-free. Also, the presence of anti-PGL-I antibodies could not predict leprosy in this population, since no significant correlation was found between anti-PGL-I antibody rates at intake and when leprosy was developed. Chapter 3 of this thesis describes the methods section of the MALTALEP trial.

In Chapter 4 we described 21 contacts of new leprosy patients who developed PB leprosy within 12 weeks after BCG vaccination (0,4% of vaccinated contacts). This relatively high percentage is possibly caused by stimulation of the cell-mediated immunity by homologues of *M. leprae* proteins (antigens) in BCG. When BCG is given to contacts who have previously been exposed to *M. leprae*, this stimulates an immune reaction that may give rise to clinical leprosy.

In Chapter 5 we described the adverse events that occurred amongst contacts that had received BCG (in 0,34% of vaccinated contacts), which consisted mainly of skin ulcers. Comparable to the pathological T-cell immunity in PB leprosy patients, contacts with adverse events had elevated Th1 rates in reaction to *M. leprae* specific proteins in whole blood assays. However, for serum proteins associated with T cell regulation, lower levels were found in reaction to *M. leprae* antigens, possibly pointing to uncontrolled T-cell immunity that destroys the skin.

Chapter 6 describes the results of the MALTALEP trial. SDR reduced the number of new PB leprosy cases amongst contacts that had been vaccinated with BCG with 42%. Unfortunately, this effect is not significant, because the number of new leprosy patients was too low. Also, a large proportion of the new leprosy patients (33.6%) arose between 8-12 weeks after BCG vaccination, the time frame between vaccination and SDR. Therefore, it is difficult to say if SDR can suppress the augmentation of new leprosy patients amongst contacts in the first year after BCG. Based on this study, we cannot advise the introduction of BCG followed by SDR as a routine intervention to prevent the spread of leprosy. SDR as chemoprophylaxis, however, has been become part of the guidelines of the WHO, because monotherapy gives a 57% reduction in leprosy amongst contacts of new leprosy patients.

Finally, in the discussion, the three research questions as described in the introduction are addressed in the light of the data obtained within this thesis.

NEDERLANDSE SAMENVATTING

Lepra is een besmettelijke infectieziekte die wordt veroorzaakt door *Mycobacterium leprae (M. leprae)*, die schade toe kan brengen aan de huid en de perifere zenuwen. Het wereldwijde aantal nieuwe leprapatiënten per jaar is het afgelopen decennium vrij constant gebleven, met een totaal aantal van ongeveer 200,000, waarvan 10% kinderen zijn. Dit geeft aan dat *M. leprae* nog steeds verspreid wordt in landen waar lepra endemisch is. Personen die in hetzelfde huishouden leven als onbehandelde leprapatiënten hebben de hoogste kans om geïnfecteerd te worden met *M. leprae* en om lepra te ontwikkelen. Daarom is het essentieel dat lepra preventiestrategieën gericht zijn op deze risicogroep. Deze strategie heeft drie belangrijke pilaren: 1) het identificeren van nieuwe leprapatiënten; 2) de behandeling van nieuwe patiënten; 3) de behandeling van contacten.

De afgelopen jaren hebben een aantal studies het gebruik van immunoprofylaxe (vaccinatie) en chemoprofylaxe (medicatie) onderzocht om de verspreiding van lepra te voorkomen onder de contacten van leprapatiënten. Bacillus Calmette-Guérin (BCG) is het meest frequent gebruikte vaccin ter wereld. Het is bekend als een vaccin tegen tuberculose, en wordt als onderdeel van het neonatale vaccinatieschema routinematig gegeven aan pasgeborenen in landen waar tuberculose endemisch is. BCG beschermt ook tegen lepra. In Brazilië adviseert de overheid BCG (her)vaccinatie als profylaxe om contacten uit het huishouden van nieuwe leprapatiënten te beschermen. Een Braziliaanse cohortstudie die dit beleid evalueerde, toonde een bescherming van BCG van 56% aan. Wel werden er een verhoogd aantal nieuwe leprapatiënten gevonden onder de contacten binnen 2-10 maanden na BCG vaccinatie.

De COLEP studie, uitgevoerd in het noordwesten van Bangladesh van 2002 tot 2009, liet zien dat het gebruik van een enkele dosering van het antibioticum rifampicine (SDR) als chemoprofylaxe in contacten van nieuwe leprapatiënten de incidentie van lepra in de eerste twee jaar na toediening met 57% vermindert in vergelijking met placebo; na vier en zes jaar werd geen additioneel effect gezien. BCG vaccinatie en SDR hebben elk een beschermend effect van ongeveer 60%. Echter uit de COLEP studie bleek ook dat het beschermend effect van SDR zelfs cumulatief was bij contacten die in het verleden BCG vaccinatie hadden gehad (80%).

Gebaseerd op deze ervaringen met BCG vaccinatie en SDR chemoprofylaxe in de preventie van lepra onder de contacten van leprapatiënten, werd er een trial geïnitieerd in Bangladesh om de effectiviteit van een gecombineerde strategie te evalueren (de MALTALEP studie). De MALTALEP studie (2012 tot 2018) is een cluster gerandomiseerde trial in het noordwesten van Bangladesh,

waarbij ongeveer 15,000 contacten van nieuwe leprapatiënten of alleen BCG krijgen, of BCG met SDR. De primaire uitkomstmaat is het ontwikkelen van lepra binnen twee jaar na het ontvangen van BCG met of zonder SDR.

In hoofdstuk 1 van het proefschrift wordt een algemene introductie gegeven. In hoofdstuk 2 wordt beschreven hoe het bloed van contacten van nieuwe leprapatiënten zonder klinische symptomen van lepra uit de COLEP studie wordt verzameld en geanalyseerd op drie verschillende tijdspunten over een periode van zes jaar. Hieruit blijkt dat de anti-PGL-I Ab waarden tijdens intake niet significant verschilden tussen contacten die lepra ontwikkelden tijdens de studie en diegenen die symptoomvrij bleven. Bovendien kon de aanwezigheid van anti-PGL-I antilichamen lepra niet voorspellen in deze populatie, aangezien er geen significante correlatie werd gevonden tussen anti-PGL-I Ab waarden bij intake en bij het ontwikkelen van lepra. Hoofdstuk 3 beschrijft de methodologie van de MALTALEP trial.

In hoofdstuk 4 worden 21 contacten van nieuwe lepra patiënten beschreven die PB lepra ontwikkelden binnen 12 weken na BCG vaccinatie (0,40% van gevaccineerde contacten). Dit relatief hoge percentage wordt mogelijk veroorzaakt door een stimulatie van de celgemedieerde immuniteit door homologen van *M. leprae* eiwitten (antigenen) die voorkomen in BCG. Als BCG gegeven wordt aan contacten die eerder aan *M. leprae* zijn blootgesteld, kan dit een immuunreactie geven die leidt tot klinische symptomen van lepra.

In hoofdstuk 5 worden de bijwerkingen beschreven die ontstonden onder de contacten die BCG hadden ontvangen (bij 0,34% van gevaccineerde contacten), die voornamelijk bestonden uit huidulceraties. Vergelijkbaar met de pathologische T-cel immuniteit in PB leprapatiënten, hadden contacten met bijwerkingen verhoogde Th1 waarden als reactie op *M. leprae* specifieke eiwitten in volbloed testen. Echter, voor serumeiwitten geassocieerd met T-cel regulatie werden verlaagde waarden gevonden in reactie op *M. leprae* antigenen, hetgeen mogelijk ongecontroleerde T-cel immuniteit veroorzaakt die de huid beschadigt.

In hoofdstuk 6 worden de uitkomsten van de MALTALEP trial beschreven. SDR verminderde het aantal nieuwe PB lepra gevallen onder de contacten die eerst gevaccineerd waren met BCG met 42%. Helaas was dit effect niet statistisch significant, doordat het aantal nieuwe leprapatiënten te laag was. Bovendien ontstond een groot deel van de nieuwe leprapatiënten (33.6%) binnen 8-12 weken na de BCG vaccinatie, de periode tussen vaccinatie en inname van SDR. Het is daarom moeilijk om te zeggen in hoeverre SDR de toename van nieuwe leprapatiënten onder contacten in het eerste jaar na BCG kan onderdrukken. Gebaseerd op deze studie kan geen aanbeveling worden gedaan over het

geven van de combinatie van BCG vaccinatie gevolgd door SDR als routine interventie om de verspreiding van de leprabacterie tegen te gaan en daarmee lepra te voorkomen. Wel is SDR als chemoprofylaxe sinds kort opgenomen in de richtlijnen van de WHO, omdat het als monotherapie een 57% reductie geeft in het voorkomen van lepra onder contacten van nieuwe leprapatiënten.

Tenslotte worden in de discussie de drie onderzoeksvragen (zoals geformuleerd in de inleiding) besproken aan de hand van de data die in het proefschrift aan het licht is gekomen.

LIST OF ABBREVIATIONS

Ab	antibody
BB	borderline borderline leprosy
BCG	Bacillus Calmette-Guérin
BI	Bacterial Index
BL	borderline lepromatous leprosy
BT	borderline tuberculoid leprosy
CCL4	chemokine (C-C motif) ligand 4
CMI	cell-mediated immunity
ELISA	Enzyme-Linked Immunosorbent Assay
ENL	erythema nodosum leprosum
HAART	highly active antiretroviral treatment
HIV	human immune deficiency virus
I	indeterminate leprosy
IDEAL	Initiative for Diagnostic & Epidemiological Assays for Leprosy
IFN-γ	interferon-gamma
IGRAs	Interferon-Gamma Release Assays (IGRAs)
IL-10	interleukin-10
IP-10	IFN-γ -inducible protein 10
IRIS	immune reconstitution inflammatory syndrome
LL	lepromatous leprosy
(L)PEP	(leprosy) post-exposure prophylaxis
M1	type 1 macrophages
M2	type 2 macrophages
MALTALEP	the Order of Malta-Grants-for-Leprosy-Research
MB	multibacillary
MDT	multidrug therapy
M. leprae	*Mycobacterium leprae*
NSE	non-specific effects
PGL-I	phenolic glycolipid I
PB	paucibacillary

SDR	single dose rifampicin
Th1	T helper 1
Th2	T helper 2
Treg	regulatory T-cells
TT	tuberculoid leprosy
UCP-LFA	up-converting phosphor technology lateral flow assay
WBA	whole-blood assay
WHO	World Health Organisation

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CURRICULUM VITAE

Renate Alicia Verbiest-Richardus was born in Rotterdam, the Netherlands, on the 13th of September 1983. She graduated cum laude from her secondary education (tweetalig-VW0) in 2001, which included an International Baccalaureate in English A1. She started medical school at the University of Leiden, and received her Master (Artsexamen Geneeskunde) in 2007. After an internship in internal medicine, she decided to specialize in Tropical Medicine and focused on mother-child health in the tropics, completing internships in both gynaecology and paediatrics (IJsselland Ziekenhuis, Capelle aan den IJssel; Amphia Ziekenhuis, Breda). This included a three months intensive training course in Tropical Medicine and Hygiene at the Koninklijk Instituut voor de Tropen in Amsterdam, The Netherlands. From 2012 to 2016 she moved to the northwest of Bangladesh with her family, and worked as a medical doctor on the gynaecology and paediatric department of LAMB Hospital. Alongside this, she supervised the MALTALEP/IDEAL trial from 2012 onwards, which had just started in Nilphamari Hospital, as part of the Rural Health Program (RHP) of The Leprosy Mission International Bangladesh (TLMIB). This is a collaboration of TLMIB, the department of Public Health at the Erasmus MC, University Medical Center Rotterdam and the Department of Infectious Diseases at the Leiden University Medical School (LUMC). In 2016 she started as an official PhD candidate at the Department of Infectious Diseases at the LUMC under supervision of Professor dr. Annemieke Geluk. Since 2017, Renate is trained at the LUMC to be a general practitioner.

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When I moved to Bangladesh together with my family in 2012 to work at LAMB Hospital, I never thought to be involved in leprosy research, let alone complete a doctoral thesis. However, when my predecessor Sabiena Feenstra left Bangladesh, someone was needed to take over from her and supervise the MALTALEP trial, which had just started in Nilphamari Hospital. Since the hospital was only an hour drive from our home, and the combination of research activities with my clinical activities at LAMB was appealing and feasible, I agreed to the project. And what a trip it has been! The four years in Bangladesh became extra special with the monthly visits to Nilphamari, sometimes together with Jelle and our children. After moving to the Netherlands, I visited Nilphamari five times in order to continue supervising the ongoing field work. And now, in my second year of GP training, the end of this 7 year trip is drawing to an end!

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