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Determinants for the development and course of leprosy - findings from a prospective cohort study

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1.1 Mycobacterium family

Enviromental. Most species from the *Mycobacterium* family are free living or environmental, so typically present in soil and water. They can be ingested by phagocytic cells, which for environmental mycobacteria would be typically amoebae. Amoebae feed on micro organisms by engulfing them (phagocytosis) and digest them in stomach-like compartments (vacuoles). Some mycobacteria have the pathogenic ability to proliferate inside phagocytic cells. After phagocytosis, by either amoebae or other phagocytic cells, these pathogenic mycobacteria can interrupt the digestive processes of their host, and even start to use the intracellular nutrients, thereby feeding on the predator.

Human. Mycobacteria may enter the human body, for instance through aerosols. The human immune system includes phagocytes (white blood cells) which apply similar mechanisms as amoebae to kill invading micro organisms. [1-3] The ability to infect human phagocytes and cause disease has been shown for several mycobacterial species, including *Mycobacterium ulcerans,* cause of Buruli ulcer, *M.avium,* a significant cause of death in immunocompromised people, and the most famous ones, *M.leprae* and *M.tuberculosis,* causing leprosy and tuberculosis (TB), respectively. However, clinical disease is a rare outcome, especially if one takes into account the numerous interactions that occur daily between humans and (in particularly the environmental) mycobacteria. [4-6] Even when infected with TB one has a lifelong risk of only 10% to develop clinical disease. [7]

Unlike leprosy, TB is very infectious, potentially deadly, and highly prevalent throughout the world, effectively meaning it generates

more resources for drug development, disease control and research. Besides the higher incidence, TB can be grown *in vitro*, making it easier to study. Analogies between *M.leprae* and *M.tuberculosis* led to comparative studies, extrapolation of study results, like transmission routes and risk groups, [8-9] and even shared control strategies, like contact surveys and BCG vaccination. [10-11]

In the following paragraphs details on *M.leprae*, its effect on humans, disease characteristics, and progress in disease control are given, concluded with a summary of the current research needs and the outline of this thesis.

1.2 *Mycobacterium leprae* cell characteristics

General characteristics. M.leprae is an acid-fast, rod-shaped bacillus with the very impermeable cell wall that is typical for the mycobacterium family. Many favourable aspects are attributed to this cell wall, with respect that it allows a relative long survival in harsh conditions, like exposure to acids, alkalis, detergents, oxidative bursts, lysis by complement and (certain) antibiotics. [12]

Intracellular localization. M.leprae is an intracellular bacterium which evidently lost its capability to proliferate outside particular environments. [13] This has frustrated scientific progress, since *in vitro* culture has for a long time been a prerequisite to reveal metabolic pathways and virulence factors.
The bacterium targets human Schwann cells and phagocytes, like macrophages and dendritic cells. [14] While phagocytes live to ingest pathogens like *M.leprae*, the invasion of Schwann cells is an unusual trait. A possible mechanism for this may be the binding of specific *M.leprae* cell wall components like phenolic glycolipid-I (PGL-I) and 21kDa protein [15,16] to parts of the basal lamina layer of Schwann

cells. [17] Hereafter, uptake may be facilitated by alpha-dystroglycan. [18]

Slow growth. Within the mycobacterium family there is a distinction between rapid and slow growers. *M.leprae* has an exceptionally slow doubling time of 11-12 days (determined using a mouse model [19]). Since most of the pathogenic mycobacteria, like *M. tuberculosis*, *M.avium, M.bovis* and *M.leprae*, are slow growers, some have hypothesized that the (slow) growth rate itself or a growth rate determining virulent trait, may enhance pathogenicity. [12,20-22]

Temperature. While normal human body temperature does not affect the *M.leprae* membrane, the optimum metabolic activity is definitely not 37°C. Lahiri et al. (2005 [23]) concluded that the optimum growth temperature for *M.leprae* is 33°C or lower and a relatively short-term incubation at 37°C has clear deleterious consequences. [24] It is therefore not surprising that *M.leprae* is most prevalent in the cooler parts of the human body (such as fingers, toes, nose and earlobes), and that the nine-banded armadillo, which has a core body temperature of 33°C, supports massive systemic growth of *M.leprae*. [23]

1.3 Genome

Like most intracellular organisms, *M.leprae* has a relative small genome. The complete genome sequence revealed an extreme case of reductive evolution: 3.27 megabase for the *M.leprae* genome compared to 4.41 megabase *M.tuberculosis*. [13] Reductive evolution should be considered as a niche-specific refinement and not as decay to a certain limit. [25] The gene comparison with *M.tuberculosis* revealed that *M.leprae* has remarkably few protein-coding genes, suggesting that *M.leprae* encodes just enough to permit intracellular growth. [13] Although the *M.leprae* genome consists for about half of both

pseudogenes and non-coding regions, [13] this does not mean that this half is non-functional. Akama et al. (2009 [26]) pointed out that the relatively high amount of pseudogenes, and their altering expression level following macrophage infection, [27] does indicate some biological function. Moreover, the same authors stated that the high expression of unidentified non-coding regions may indicate a yet unrecognized function. [26]

All *M.leprae* strains known to date can be attributed to a single clone whose global spread from Eastern Africa or the near East happened after its reductive evolution. [28] The genome is now very stable and differences seem to have no known effect on disease outcome. [28,29] However, recently a new mycobacterium species, *M.lepromatosis sp novin*, was identified from two lepromatous leprosy patients, which led to the statement that some of the clinical and geographic variability of leprosy may be explained by different causative agents. [30]

The completion of the genome sequencing of a number of mycobacteria kick-started research into metabolic, biochemical and pathogenic parameters and pathways. [14] New leprosy biomarkers may be derived from essential metabolic pathways and likewise maybe reveal new targets for vaccines, drugs and diagnostics.

2 Reservoirs and transmission routes

Humans. M.leprae is present in patients, although this can be hard to prove: in about 70% of the patients the bacteria cannot be detected by microscopy on acid-fast stained smears or biopsies. [31] Polymerase chain reaction (PCR) and serological techniques may show the presence of *M.leprae* DNA or specific antibodies, but, again, not all

patients give PCR- or seropositive results. [32,33] This is illustrative for the low profile that the bacterium often maintains in the human host.
If bacterial presence can be established by microscopy, it is typically seen in the facial/nasal area, the peripheral nerves or skin lesions. Likewise, patients are generally thought to shed the bacteria via the nasal cavity and, potentially, the skin. [33]

It can be concluded from both leprosy and TB studies that the bacterial load of a patient is an important risk factor for transmission to contacts. However, it is certainly not the only risk factor, [8,34] suggesting that all patients should be considered as possible sources of infection, but the degree of infectiousness varies. As a consequence, the emphasis in leprosy control is very much on early detection and treatment of patients, since this stops their infection potential, which is thought to lead to a reduction in incidence. Screening of contacts of leprosy patients for early signs and symptoms is not systematically done in most control programs. Also, the absence of an early diagnostic test hampers the early identification of leprosy.

M.leprae is thought to be present in healthy individuals in endemic areas. Surveys with PCR and/or serology tests suggest that subclinical infection is far more common than overt disease. Studies showing presence of *M.leprae* DNA in the nasal cavity support this hypothesis. [35-40] Serological tests performed on endemic individuals showed that the antibody prevalence varies greatly from 1.7-30%. [41] It is currently still under debate whether transmission occurs through these sub-clinically infected individuals. [8]

Other (potential) hosts and sources: armadillos, soil and water. Besides humans, the only other naturally occurring reservoir host is the nine-banded armadillo, Dasypus novemcinctus, [42-44] which may host large numbers of *M.leprae*. [45] There is increased proof that humans can get infected by armadillos but at the moment it remains to be seen

if this transmission route needs to be—or even can be—controlled. [45-48] Others animals, like mice (mouse footpad) and mangabey monkeys, can be experimentally infected with *M.leprae* as well, but the disease prevalence in these animals under normal conditions is unknown.

Infection with *M.leprae* occurs from person to person, but indirect routes may be possible as well, since the bacterium is viable for some time outside the body. [49] Soil and water are considered as potential (intermediate) reservoirs, [50] in analogy to other environmental mycobacteria that occasionally cause disease in humans. [51] Lavania et al. (2008 [50]) showed, using DNA and RNA detection techniques, that viable *M.leprae* were present in 50% of soil samples collected in the direct vicinity of a leprosy patient. Furthermore, fewer, but still 15%, of samples taken from areas not directly associated with a leprosy patient, also showed presence of viable *M.leprae*. Although still preliminary, this may indicate a potential non-human intermediate environment. Whether *M.leprae* is capable of proliferation in an environmental phagocyte, like other mycobacteria, has yet to be determined. So far, the phagocyte *Acanthamoeba castellanii* was shown to ingest and support viability of *M.leprae*, [50] but whether proliferation of *M.leprae* in an environmental phagocyte is possible still remains to be determined. Moreover, considering its niche-specific reductive evolution one can wonder whether *M.leprae* remains sufficiently competitive with other organisms/mycobacteria to survive on a large scale outside known hosts.

No control policy is currently active to target non-human infection sources or transmission pathways.

3 Host – pathogen interaction

3.1 Entry of the pathogen into the host

More than 95% of the infected people do not develop overt disease [53,54] as the immune system will kill any invading *M.leprae* before disease symptoms occur. So, only in less than 5% of the infections *M.leprae* will have some degree of success in evading the human defence system. Although not completely understood, there are some insights in how *M.leprae* succeeds in this.

For instance, the surface molecules PGL-I and ManLAM are both involved in cleavage of C3, a protein from the complement system that promotes phagocytosis via complement receptors CR1 and CR3. However, phagocytosis via these receptors does not result in an effective oxygen burst, lacking reactive nitrogen which is needed to kill the bacteria. Mouse knockout models showed that *M.leprae* could not be killed by reactive oxygen alone, but that reactive nitrogen was required; [55,56] *M.leprae* PGL-I and superoxide dismutases SodC and SodA are responsible for neutralizing reactive oxygen. [14,57] Natural antibodies may even aid the bacteria in this favorable mechanism, since they were shown to facilitate cleavage of C3 to the *M.leprae* surface. [58]

When phagocytosis has taken place by effective receptors, like the mannose-, SIGN- or lagarin receptors, the initial phagosome-lysosome fusion may be hampered by live bacteria. [59] Additional stimulation by IFN-gamma, produced by other cells, is then required for secondary fusion. However, these cells have to be recruited first from the lymphoid tissue. And since the effect of IFN-gamma stimulation decreases in a time dependent manner with the intracellular presence of *M.leprae*, [59-61] it is clear that a quick response is essential for bacterial clearance. Thus, fast DC migration after encountering a

pathogen is thought to be essential for optimal immune response. Intracellular *M.leprae* may suppress antigen presentation needed for recruiting other immune cells by down-regulating the antigen presentation complex, MHC I/II in DCs. If ultimately the DC succeeds in expressing antigens, the expression of PGL-I on its cell surface has further immunosuppressing capabilities. This was demonstrated by Hashimoto et al., [62] who showed that after masking the expressed PGL-I, T-cell proliferation as well as IFN-gamma production were up-regulated.
Furthermore, innovative research by Van Helden [63,64] illustrate another possibility for a delayed immune response; bacteria lacking lipopolysaccharide (LPS) do not trigger DCs to lose their podosomes, which are surface molecules that prevent DCs to migrate quickly.

Initial interaction of *M.leprae* with dendritic cells (DC) can result directly in a favorable maturation process: Krutzik and coworkers (2003 and 2005 [65,66]) showed that interaction and maturation—mediated by the TLR1/2 pathway—led to the maturation of the DCs into the DC-SIGN and DC-CD1 subtypes. DC-SIGN will attempt to kill the bacteria through phagocytosis, but without help from the stimulus factors produced by DC-CD1 the bacteria may circumvent being killed. Successful killing requires enough DC-CD1 subtype cells that will recruit other immune cells and activate secondary phagosome-lysosome fusion. The DC-SIGN subtype has also been shown to cause IL-10 derived immunosuppressing by binding ManLAM of *M.leprae* or *M.tuberculosis.* [67-70]

Schwann cells. Besides for macrophages, *M.leprae* has a host tropism for the Schwann cells of peripheral nerves. Possibly, bacterial invasion occurs through direct binding to Schwann cells in the dermis or through accumulation in epineural lymph and blood vessels followed by entering of the endoneural area through the blood supply. [71] Intracellular proliferation proceeds slowly and at some point the

Schwann cell may express antigen on its surface with MHC II molecules. [72] Once recognized by cytotoxic T-cells, the Schwann cell is destructed and subsequent release of inflammation factors within the endoneural area can lead to further nerve damage. [73]

3.2 Host immunogenetics

Leprosy offers an opportunity to investigate the association between gene functioning and human immunology, since leprosy presents itself as an immunological spectrum from tuberculoid to lepromatous leprosy, which poles correlate with the two types of adaptive immune responses to *Mycobacterium leprae*. [74]

Genetic diversity of the host has great potential to modulate susceptibility to *M.leprae* infection, especially for those genes directly involved in the above described mechanisms, e.g. lysosome maturation, oxidative burst, and antigen presentation. Numerous genes have been reported to influence susceptibility or disease outcome, many in immunomodulating pathways of TLR/LIR-7, VDR, TNF-alpha and TGF-beta1. [75]

In the COLEP study the human PARK2/PACRG and Toll-like receptor (*TLR*) genes were studied, and as this thesis describes the results of the COLEP study, we will concentrate on these genes.

Sub-optimal protein degradation after oxidative stress and antigen presentation may be particularly important for *M.leprae* susceptibility. [76,77] *PARK2* and *PACRG* are genes linked to the ubiquitin tagging system, which tags unneeded proteins to be degraded. Whether mutations cause susceptibility for leprosy infection and/or modify clinical outcome is still under debate. [75] Unpublished results of the COLEP study indicate that the single nucleotide polymorphism (SNP)

PARK_e01(-2599) is related to susceptibility to leprosy per se (T allele OR 1.25; 95% CI 1.04-1.52) and the genotype TT is related to MB leprosy (OR 1.76; 95% CI 1.17-2.66). Interestingly, all eleven patients who had developed ENL reaction, had the TT genotype (unpublished results).

TLRs interact directly with the pathogen and initiate inflammatory responses. [78] Each TLR is able to recognize a specific class of pathogen ligands and subsequently two TLR-mechanisms can be activated. Firstly, stimulation of DC maturation enables adaptive immune responses, [79] and, secondly, recognition by TLR may lead to macrophage maturation and ensuing activation of antimicrobial activity and phagocytosis. [80,81] The TLR1/2 pathway was shown to lead to maturation of DC into the DC-SIGN and DC-CD1 subtypes, as described above. [65,66]

It is quite difficult to single out a genetic prognostic marker, because of the diversity of mechanisms and the possibility that they are interlinked, as well as possible other factors that may influence the course and outcome of infection and disease. [75] The availability of the whole human genome sequence, allowing gene comparison and genome wide-scans, may further increase our understanding of host immunology, potentially leading towards a multiple marker test.

4. Leprosy

4.1 Clinical leprosy

For most people the infection with and clearance of the bacilli and its antigens goes unnoticed (subclinical infection). However, depending on the immunological response of the host, a whole range of clinical and histopathological features may appear. Ridley and Jopling have

categorized clinical leprosy into five types: tuberculoid (TT) and lepromatous (LL) leprosy with three borderline groups, BT, BB and BL, and an indeterminate group (I) based on these patterns. [74] Tuberculoid leprosy patients have a fairly successful *M.leprae* specific cell-mediated immune response. Their lesions are characterized by epithelioid cell granulomas, participation of lymphocytes (mainly of Th1 type), and few if any detectable bacilli. In contrast, in the lepromatous form, the specific cell immunity against *M.leprae* is virtually absent [82], with diffuse dermal infiltrates characterized by poorly differentiated young macrophages with a heavy load of bacilli and a small number of T cells predominantly of the Th2 type. [83] In the spectrum of borderline leprosy there are varying degrees of cell-mediated immune response declining from the tuberculoid to the lepromatous pole. [74]

The balance between the immune response and *M.leprae* is not necessarily stable: spontaneous fluctuations known as leprosy reactions may occur. Reactions are acute events of inflammatory response, and since nerves may be involved they are considered as medical emergencies. There are two types of reactions: reversal reaction (RR) and erythema nodosum leprosum (ENL). Reversal reactions are a spontaneous increase of T-cell reactivity to *M.leprae* antigens that occur in 30% of the borderline patients. [73] Common clinical characteristics are neuritis and inflammation of skin lesions (swelling, redness, local heat, loss of sensation and tingling). [84] ENL is a systemic inflammatory response characterized by high circulating concentrations of TNF-alpha and systemic toxicity, which mainly occurs in BL and LL patients. [85,86] A common clinical characteristic are inflamed, painful and red nodules. Due to its systemic nature, ENL reactions affect the whole body and patients feel general malaise and fever. [87]

As described above the tropism of *M.leprae* for Schwann cells causes peripheral nerve damage. The risk for nerve function impairment (NFI) is highly increased during reactions. Preventing permanent

disabilities due to nerve function impairment remains a major concern in leprosy control, [88] in particular because NFI can take place before, during and/or after leprosy treatment. Early detection (within 6 months) and corticosteroid treatment may prevent further decline of or even revert nerve function. [84,89,90]

4.2 Diagnosis

Early diagnosis—of subclinical infection, disease, and reactions—is a major topic in leprosy control, since a patient may become a source of infection and develop nerve function impairment at an early stage. Bakker et al. reviewed several studies for the risk of PB and MB contacts and estimated that contacts of patients had an increased risk—PB contacts had a two times increased risk, MB contacts 5 to 8 times increased risk—compared to non-contacts. [34]

Diagnosis of subclinical infection. Diagnostic tools for the phase before clinical symptoms become apparent would be invaluable to prevent transmission. There are initiatives ongoing to develop such early diagnostic tools (e.g. the IDEAL collaboration [91]), but currently no test is routinely implemented, even though a number of targets are under evaluation.

For instance, Geluk et al. [92] report the development of a leprosy-specific T-cell assay with novel antigens selected from the genome sequence. Although some of these antigens showed to be highly sensitive for leprosy, many healthy controls also had a positive test result, decreasing the specificity. Duthie et al. [93,94] reported a good association with an *M.leprae* antigen-construct LID-1 (LID-1= fusion construct of ML0405 and ML2331) and future development of leprosy, but for PB patients the positive predictive value was rather low (6 out of 30).

The low incidence of clinical leprosy effectively means that a biomarker should be both very specific and sensitive, and have a good predictive value for the development of clinical disease. Moreover, there is always the possibility of spontaneous healing/bacterial clearance, decreasing the positive predictive value of any test. Also, immune responses may be different between subclinical infection and clinical disease, in the same respect as differences between PB and MB disease.

In general, antibodies are not effective against intracellular pathogens, which are shielded off by a host cell, but they may facilitate the non-lethal phagocytosis by complement receptors. [95] The most widely used serological test today is based on PGL-I. [41] Seropositivity indicates a non-protective, humoral immune response against *M.leprae*. Besides a sign of infection, antibodies may indicate successful proliferation of *M.leprae.* Potentially, initial seropositivity is an indicator for a continuing and later mainly humoral immune response of an individual with an increased susceptibility to develop clinical/MB disease. [35,96,97]

Diagnosis of clinical disease. Leprosy is diagnosed when finding any one of three cardinal signs, 1) one or more hypopigmented, anaesthetic skin lesions; 2) one or more thickened peripheral nerves; or 3) presence of acid-fast bacteria. [98]

Clinical features, in particular the skin lesions, are the most profound indicators for leprosy, but may take a long time to develop. [99]
Neuropathy assessment is normally done with a monofilament test and voluntary muscle test. [101] With the monofilament test, nylon monofilaments are used to monitor touch sensation on the hands and feet. [102] Instead of filaments, a regular ball-point pen may be used as well. [103]

The number of acid-fast bacteria is expressed in a bacterial index, a logarithmic scale ranking from zero to six. After acid-fast staining of skin smears or biopsies, the bacteria are counted under a microscopy. [100] As described above (section 4.1) histopathology can also be used for diagnosis and classification.

Anti-PGL-I IgM serology has a strong correlation with microscopy results and overall systemic bacterial load. [32] About 15-40% of the PB patients and 75-100% of the MB patients are seropositive. [41] The test is not suitable for diagnosis, but may contribute to correct classification [104-106] and the identification of high risk groups for NFI [107] and future development of leprosy. [108] And to detect a leprosy relapse in an early stage. [109]

Analogous to other micro-organisms, the detection of the pathogen by PCR could play an important role in diagnosis. A number of very sensitive PCR-techniques have been developed over the years, [110-112] but so far these are hardly used in routine leprosy control programs.

Diagnosis of reactions and nerve function impairment (NFI).

Management of nerve function impairment is an important aspect in leprosy control as timely treatment may prevent permanent damage. Neuropathy assessment can be performed as described above. A recent publication of the INFIR study group (2008 113), an initiative to compare diagnostic tests for neuropathy assessment, concluded that detection was best when using an electrophysiological test (sensory nerve conduction) or measuring thermal threshold (warm temperature perception test). Symptoms could be detected 12 weeks earlier than with the common monofilament test.

Testing all patients on a regular basis puts a high burden on health care workers. Therefore patients should be taught to inspect their body on a daily basis and attend to any injuries promptly. [114,134]

Self reporting and self care needs to be promoted continually to prevent permanent nerve damage.
NFI risk factors and an NFI prediction rule have been determined based on data from the Bangladesh Acute Nerve Damage Study (BANDS 115). The prediction rule categorizes patients into NFI risk groups based on their World Health Organization (WHO) classification (ie, PB or MB leprosy) and the presence of NFI at diagnosis.

4.3 Classification of disease

The classification according to Ridley and Jopling as described above is still in use, and is especially helpful for research. Nowadays, the most widely used classification system for treatment purposes is the one designed by the WHO, which is based on clinical features of skin lesions only. Microscopy results can be taken into account as well, but this is optional, since it requires laboratory facilities, which are often absent in the field. After a number of modifications, the WHO classification nowadays divides leprosy patients into multibacillary (MB) patients with 6 or more skin lesions and/or a positive bacterial index and paucibacillary (PB) patients who have up to 5 skin lesions and a negative bacterial index. [116]

4.4 Risk factors

Even though transmission patterns in leprosy are difficult to study, there are a number of well-recognized individual risk factors for leprosy such as sex, age, bacterial load of the index patient and genetic and physical distance to a patient. (reviewed by Bakker et al. and Moet et al. [8,34]) Males are at higher risk than females, especially for MB leprosy and children and elderly are frequently reported to have a higher leprosy incidence. [8,34] The exact causative mechanisms for these

increased risks are not always well-established, but are generally accepted to be related to immunological and/or exposure differences.

As described above, patients in which bacteria can be demonstrated are considered to be most infectious, thereby increasing the risk for their contacts. [8, 34] *M.leprae* specific antibodies were shown to be associated with leprosy in a few prospective studies. [32,35,93,94,96,117] Potentially, seropositivity is an indicator for a continuing humoral immune response in an individual, which is associated with (lepromatous or MB) leprosy.

Leprosy is often associated with poverty, but evidence is not easily obtained. [118] Ponnighaus et al. (1994 [119]) found an association with two poverty-related traits and leprosy development: lower house quality and less education independently increased the risk. Kerr-Pontes (2004 [120]) showed that inequality, population growth, and presence of a railroad were associated with higher leprosy prevalence. It was speculated that population growth and social-economic inequality may cause over-crowding, thus facilitating transmission of *M.leprae*. In addition, these inequalities may hamper social needs and so impair health. [121]

Lietman et al. (1997 [122]) reported that infection with *M.tuberculosis* protects against leprosy. Fine et al. (2001 [123]), showed evidence for cross-protection from natural exposure to certain environmental mycobacteria, which may explain the geographic distribution of mycobacterial diseases like leprosy and tuberculosis. Sterne et al. (1995 [124]) concluded that there is a marked geographic variation in the incidence of leprosy, not explained by socioeconomic or cultural factors. Besides, national/local policies and quality of leprosy control have an obvious impact on leprosy risk as early detection and treatment have an effect on ongoing transmission. So, policies on diagnosis, interventions (both prophylactic and curative), and contact

surveys influence infection risk and leprosy incidence. Finally, (the characteristics of) geographical location may be considered as a risk factor as well.

4.5 Global prevalence and incidence.

The current global leprosy situation is monitored by the WHO and shows a decrease in overall new case detection since 2001 (table 1). [125]

Before 2001, WHO have been reporting a decrease in the global disease burden from 5.2 million in 1985 via 805,000 in 1995 and 753,000 at the end of 1999. [126] Furthermore the WHO states that the global prevalence rate of the disease has dropped by the year 2000 to a level of less than 1 per 10,000 inhabitants. Subsequently, the WHO declared that leprosy has been eliminated as a public health problem at a global level (prevalence <1/10,000 cases). At a country level this was achieved in 113 out of 122 countries where leprosy was considered as a public health problem in 1985. [126]

However, the above statements undervalue that, despite the prevalence drop, the majority of leprosy endemic countries are still detecting new cases at a steady level, even though the numbers may be relatively low. India is the remarkable exception with incidence dropping from 473.658 in 2002 to 137.685 in 2007. [125] Since about two thirds of all new cases come from India, the statistics from this country have a large impact on global figures. The steep decline in South-East Asia—primarily India—is questioned since it is unlikely that the MDT policy and elimination activities changed transmission that dramatically. [127-129] Besides, overall numbers for regions can be misleading since control activities and political commitment may vary per country and over time.

Moreover, the number of reporting countries differs over time, making interpretation difficult. [130]

Table 1: Trends in the detection of new cases of leprosy, by WHO region, 2001-2007 (excluding European Region). [125]

	Number of new cases detected						
WHO region	2001	2002	2003	2004	2005	2006	2007
African	39 612	48 248	47 006	46 918	45 179	34 480	31 037
Americas	42 830	39 939	52 435	52 662	41 952	47 612	41 978
South-East Asia	668 658	520 632	405 147	298 603	201 635	174 118	171 552
Eastern Mediterranean	4 758	4 665	3 940	3 392	3 133	3 261	4 091
Western Pacific	7 404	7 154	6 190	6 216	7 137	6 190	5 867
Total	763 262	620 638	514 718	407 791	299 036	265 661	254 525

5. Leprosy control

For centuries leprosy was a mysterious, chronic and untreatable disease and exclusion from society was common practice. Modern disease control was unthinkable, until two turning points in leprosy history shed some light on the mystery and changed the perspective of patients: the discovery of the causative agent, *M.leprae* by Armauer Hansen (1873) established leprosy as an infectious disease, and the discovery of dapsone in the 1940's turned leprosy into a treatable disease.

Even without the benefits of this knowledge, leprosy was already largely gone from Europe by the end of the 17th century, [131] even though the reasons for this are poorly understood. Several hypotheses have been raised, yet proving them may be impossible. Were people less susceptible because of increased social-economic standards? [119,120] Or was the competition of other pathogen(s) interruptive enough to hamper *M.leprae* transmission? [122, 132] Was it the

isolation of patients [133] and/or improved hygienic behaviour? Although answers are lacking, the phenomenon does illustrate that elimination is possible.

5.1 Treatment

A major step forward was the introduction of multi drug treatment (MDT) in 1982, consisting of a regimen of rifampicin, dapsone and clofazimin for MB leprosy and rifampicin and dapsone for PB leprosy (see figure 1). [134]

Treatment with rifampicin leads to a large reduction of viable bacteria after the initial dose, [135,136] effectively stopping infection potential. The shortened treatment period compared to dapsone in combination with cleaning of registers resulted in a steep decline of registered patients in the 1980's. It was hoped that MDT would permit control of the disease and ultimately interruption of transmission. This led to the development of the concept of "leprosy elimination". [129]

"Leprosy elimination by the year 2000" was first proposed in 1986 and accepted at the 44th World Health Assembly in 1991, modified by the postscript "as a public health problem". Leprosy elimination was thus defined as a disease prevalence of less than one case per 10,000. The subsequent leprosy elimination activities had some notable successes, but also revealed the epidemiological, medical, and political problems of a time-bound concept. [137,138]

Several articles have been written stressing the weaknesses of the elimination strategy. [14,127,132,137] The elimination strategy includes several non-sustainable elements for the long term. [127] In addition, mathematical modelling by Meima et al. [133] calculated that the incidence decline with the elimination strategy would be gradual

The standard adult treatment regimen for MB leprosy is:

Rifampicin: 600 mg once a month
Clofazimine: 300 mg once a month, and 50 mg daily
Dapsone: 100 mg daily
Duration: 12 months (12 blister packs)

The standard adult treatment regimen for PB leprosy is:

Rifampicin: 600 mg once a month
Dapsone: 100 mg daily
Duration: six months (six blister packs)

Standard child (ages 10 – 14) treatment regimen for MB leprosy is:

Rifampicin: 450 mg once a month
Clofazimine: 150 mg once a month, and 50 mg every other day
Dapsone: 50 mg daily
Duration: 12 months (12 blister packs)

The standard child (ages 10 – 14) treatment regimen for PB leprosy is:

Rifampicin: 450 mg once a month
Dapsone: 50 mg daily
Duration: six months (six blister packs)

Figure 1: Treatment regimens for leprosy. [125]

(2-12%), even under favourable circumstances. Many questions [130,133,139] were thus raised for the steep prevalence declines reported by some countries in recent years. [130] Currently, the emphasis on "elimination" is abandoned in the WHO strategy guide. [134]

5.2 Prophylactic interventions

As mentioned in section 5.1, MDT did have an impact on prevalence of the disease, but its impact on incidence is debatable. Hence, the research community investigated novel approaches to reduce the incidence. One of the approaches studied was chemo- and/or immunoprophylactic interventions.

Chemoprophylactic regimens in leprosy are protective against leprosy. A meta-analysis by Smith and Smith (2000 [140]) showed protection by dapsone, and two recent studies showed that rifampicin chemoprophylaxis is protective for a limited period of time. [141,142] In a randomized controlled trial by Moet et al. (2008 [141]) a single dose of rifampicin gave a 57% reduction in leprosy incidence during the first two years. Bakker et al. (2005 [142]) showed 75% reduction after 33.5 months with two doses of rifampicin supplied to the complete population of three small islands; no reduction, however, was seen in a neighbouring island population where only spatially defined contacts of leprosy patients received rifampicin. [142] Both studies found that the protective effect was strongest in the contact groups furthest away from the index patients, suggesting that close contacts require a more extensive regimen, possibly due to higher initial bacterial load.

The World Health Organization's (WHO) Expanded Program of Immunization lead to the current widespread use of BCG vaccination and is thought to have had a major effect on leprosy incidence. [143] The protection of BCG vaccine is clearly demonstrated, Setia et al. (2006 [11]) and Zodpey (2007 [144]), who both published a meta-analysis. None of the analyzed studies reported a negative protective effect of BCG. The overall protective effect was lowest for experimental studies as reported by Setia et al. (26%; 95% CI 14-37%); the highest effect was seen for cohort studies by Zodpey (62%; 95% CI 53-69%). By

preventing disease, but also by altering PB/MB ratios, BCG is thought to give protection against the more infectious MB leprosy. [145]

6 Research themes for leprosy control

In the past years emphasis in leprosy control has been on the availability and accessibility of control activities, which include diagnosis, treatment with MDT, patient and family counseling, community education, prevention of disabilities/impairments, rehabilitation, and referral for complications. [134] While these control activities need to be continued, innovative approaches are necessary to further lower the incidence of leprosy.

A consultancy meeting [146] on "Innovative Approaches To Further Reduce Leprosy Burden In Countries", held in September 2008, postulated technical, operational and strategic needs for improving integration of leprosy services into the primary health care system, quality of services and monitoring. Among the given priorities were prevention and management of nerve function impairment and reaction (**theme 1**), improved chemotherapy (**theme 2**), operational research to improve sustainability and integration of leprosy services (**theme 3**), and diagnostics to identify individuals at high risk of developing leprosy (**theme 4**). The recommendations are likely to be included in the upcoming WHO strategy for 2011-2015.

Research needs were also postulated in the report of the 9th Technical Advisory Group meeting on Leprosy Control, which was held in March 2008. [147] The research needs were identified based on an analysis of the necessary criteria for "leprosy eradication". The research priorities are: a test for infection (**theme 5**), understanding transmission (**theme 6**), understanding the development of a protective immune response (**theme 7**), and development of effective, safe,

acceptable and inexpensive interventions (**theme 8**). Besides these research needs, feasibility of leprosy eradication mainly depends on the technical feasibility, economic resources and political commitment. The Technical Advisory Group considered leprosy to be not eradicable at this moment.

7 This thesis

Data from this thesis are derived from the COLEP trial in northwest Bangladesh, covering the data and samples collected during intake and the first two follow-ups. [148]

The COLEP study was designed to determine the effect of chemoprophylaxis with single-dose rifampicin. It was designed as a large double-blind and placebo-controlled trial. The study population consisted of newly diagnosed leprosy patients (1037) and contact groups, of roughly 20 contacts per patient (total contacts included: 21,708). In the second month after the start of treatment of the patient, all contacts were visited for inclusion. During this survey, an examination for signs and symptoms of leprosy was done, blood samples were collected and either prophylaxis or placebo was distributed to the whole contact group.

A sample from the general population was also included in the study to compare their characteristics with the patient and contact groups. In a random cluster survey, twenty clusters of 1000 people were examined during house-to-house visits.

Two follow-up surveys were performed after two and four years to enable comparison of new case detection rates between contact groups and the general population.

The study was conducted in northwest Bangladesh, in the districts Nilphamari and Rangpur. At a nation-wide level, Bangladesh has reached the elimination goal of the WHO, but some districts remain above the 1/10,000 prevalence level. By the end of 2002, the prevalence in Nilphamari was 3.0 and Rangpur 1.3 per 10,000. [149] The active surveys done for the COLEP study revealed that actual prevalence in the general population was six times higher than the registered prevalence. [150]

The field work was performed by the Rural Health Program (formerly DBLM), which started its leprosy control activities in 1977 in Nilphamari and in 1986 in Rangpur. This centre has experience with performing high-quality international research: it was involved in BANDS and the TRIPOD studies, both studies focusing on nerve function impairment.

This thesis addresses the following research themes:

- Chapter 2 addresses the influence of host genetics on susceptibility to leprosy (research themes 1, 4, 7).
- Chapter 3 describes the serological, demographic and clinical patients characteristics (research themes 2, 4).
- Chapter 4 proposes an improved prediction rule for nerve function impairment (NFI) and discusses its implications (research themes 1, 8).
- Chapter 5 shows the potential of two preventive strategies for leprosy: vaccination with BCG and chemoprophylaxis with rifampicin (research theme 8).
- Chapter 6 describes the contribution that serology can make to identify individuals at high risk to develop leprosy (research themes 4, 5, 6).

Compound of family houses with men made pool for bathing and fishing

Typical house, this one is used by two adults (one is a leprosy patient)

Data entry by Kallyan Kundu

Ziehl-Nielsen staining by Kanu Ram Chowdhury

Early start: pick up by driver from the guesthouse in Nilphamari

COLEP staff meeting during 3rd follow-up

COLEP staff meeting 3rd follow-up—staff making a group assignment

COLEP staff meeting 3rd follow-up—staff discussion on calculations

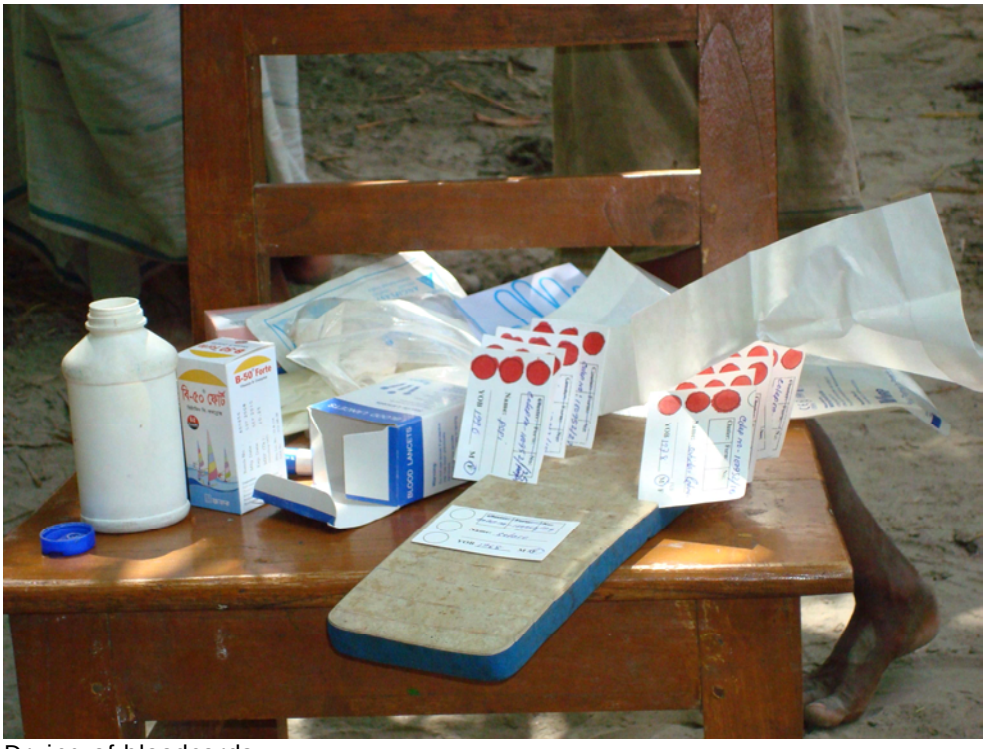
Field visit near Nilphamari—asking permission to enter family premises

Field visit near Nilphamari—gathering of the index patient's contacts

Pictures showing blood and data collection of the patient's contacts

Arriving in a village near Rangpur for another follow-up visit

Blood and data collection



Drying of bloodcards

Afterwards we were invited for tea and a photo was requested

Field visits in the hill-track area in Chittagong is done by boat
(Chittagong is not a part of the COLEP study area)

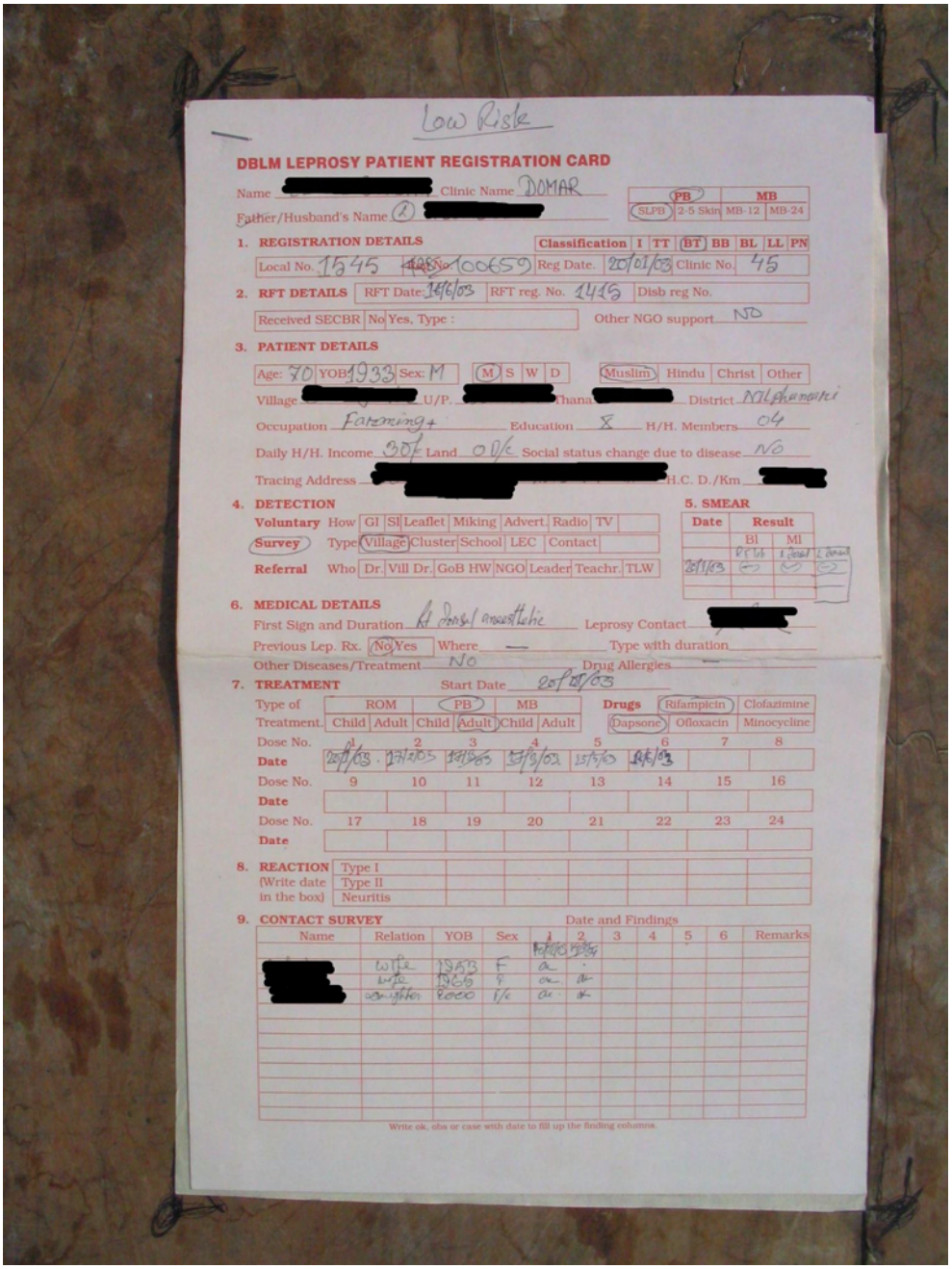
Arriving at a little island with a few houses

Arriving at a patient house where the local housing situation was shown

The kitchen

The bedroom

Staff helping with digitalization of patient information cards in 2005.



Low Risk

DBLM LEPROSY PATIENT REGISTRATION CARD

Name [redacted] Clinic Name DOMAR

Father/Husband's Name (2) [redacted]

PB: SLPB | 2-5 Skin — MB: MB-12 | MB-24

1. REGISTRATION DETAILS — Classification: I | TT | (BT) | BB | BL | LL | PN

Local No. 1545 — Reg Date. 20/01/03 — Clinic No. 45

2. RFT DETAILS — RFT Date: 14/6/03 — RFT reg. No. 1415 — Disb reg No.

Received SECBR: No | Yes, Type : — Other NGO support: NO

3. PATIENT DETAILS

Age: 70 — YOB 1933 — Sex: M — (M) | S | W | D — (Muslim) | Hindu | Christ | Other

Village [redacted] — U/P. [redacted] — Thana [redacted] — District Nilphamari

Occupation Farming+ — Education 8 — H/H. Members 04

Daily H/H. Income 30/= — Land 0 D/c — Social status change due to disease NO

Tracing Address [redacted] — H.C. D./Km [redacted]

4. DETECTION

Voluntary — How: GI | SI | Leaflet | Miking | Advert | Radio | TV

(Survey) — Type: (Village) | Cluster | School | LEC | Contact

Referral — Who: Dr. | Vill Dr. | GoB HW | NGO | Leader | Teachr. | TLW

5. SMEAR

Date	Result	
	Bl	Ml
20/1/03	(+)	(+)

6. MEDICAL DETAILS

First Sign and Duration Rt Dorsal anaesthetic — Leprosy Contact [redacted]

Previous Lep. Rx. (No) Yes — Where — — Type with duration —

Other Diseases/Treatment NO — Drug Allergies —

7. TREATMENT — Start Date 20/01/03

Type of Treatment: ROM (Child | Adult) — (PB) (Child | Adult) — MB (Child | Adult)

Drugs: (Rifampicin) | Clofazimine | (Dapsone) | Ofloxacin | Minocycline

Dose No.	1	2	3	4	5	6	7	8
Date	20/1/03	17/2/03	19/3/03	15/4/03	15/5/03	14/6/03		

Dose No.	9	10	11	12	13	14	15	16
Date								

Dose No.	17	18	19	20	21	22	23	24
Date								

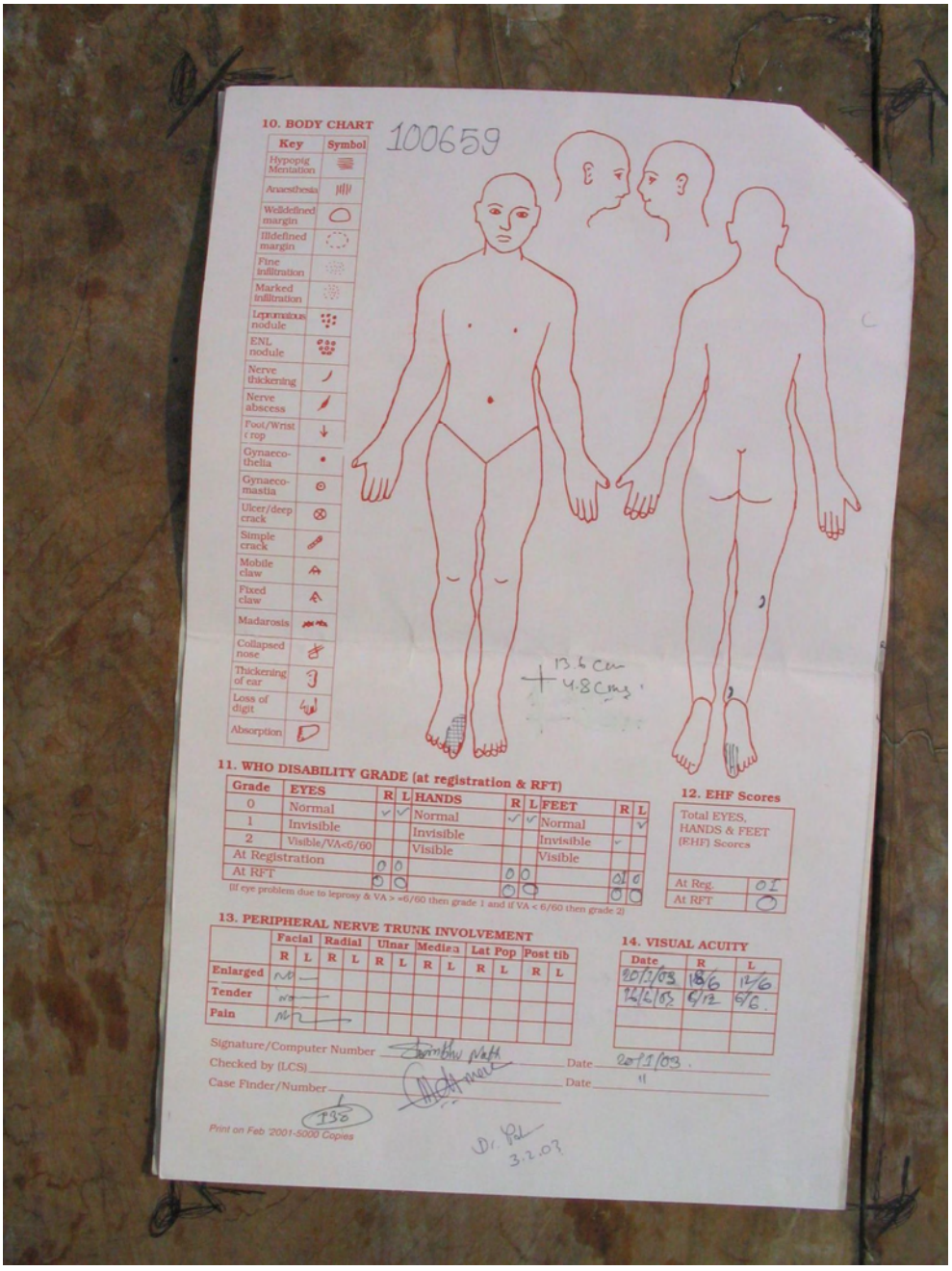
8. REACTION (Write date in the box) — Type I | Type II | Neuritis

9. CONTACT SURVEY — Date and Findings

Name	Relation	YOB	Sex	1	2	3	4	5	6	Remarks
				14/6/03	19/8/03					
[redacted]	wife	1953	F	ok	ok					
	wife	1963	F	ok	ok					
	daughter	2000	F/c	ok	ok					

Write ok, obs or case with date to fill up the finding columns.

Front page of patient information card, the so-called redcard



On the back page the clinical features of the patient are drawn. Here the lesion size is indicated: 13.6 by 4.5 cm

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Chapter 2

Polymorphism N248S in the human Toll-Like receptor 1 gene is related to leprosy and leprosy reactions

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Abstract

We investigated the association between a polymorphism of a key innate immunity receptor, Toll-like receptor 1 (*TLR1*) N248S, and susceptibility to leprosy and its clinical presentation. *TLR1* N248S has been shown elsewhere to diminish TLR1 signaling and subsequent leprosy disease. The homozygous genotype SS was more frequent (P = 0.012) and the heterozygous SN genotype was less frequent (P = 0.015) in patients with leprosy than in control subjects. Additional observed differences in allelic frequency in patients who experienced reversal reactions and/or erythema nodosum leprosum reactions indicates that altered TLR1 function, or at least a *TLR1* N248S–linked trait, may affect the progression from infection to disease as well as the disease course and the risk of debilitating reactional episodes in this population.

Introduction

The mechanisms underlying immune responses to pathogens are relevant to our understanding of human susceptibility to infectious diseases. The Toll-like receptors (TLRs) are of prime importance, because they recognize the pathogen-associated molecular patterns that initiate inflammatory responses. [1] Each TLR is able to recognize a specific class of pathogen ligands, after which it initiates cellular responses that contribute to host defense. First, stimulation of monocyte differentiation into macrophages leads to activation of antimicrobial activity and stimulation of phagocytosis. [2, 3] Second, stimulation of dendritic cell (DC) maturation enables adaptive immune responses. [4] Leprosy offers an opportunity to investigate the association between TLR functioning and these 2 mechanisms, because leprosy presents itself as an immunological spectrum that ranges from tuberculoid to lepromatous leprosy, which correlates with the 2 types of adaptive immune response to *Mycobacterium leprae*. [5] Patients with tuberculoid leprosy are relatively resistant, with a low bacterial load, localized infection, and relatively strong cell-mediated immunity involving T helper (Th) 1 cytokines, [6] whereas patients with lepromatous leprosy are characterized by a high bacterial load, systemic disseminated infection, and a propensity for nonprotective humoral response involving Th2 cytokines. The disease is not static, because reactions occur. Reversal reactions are the result of increased T cell reactivity, and patients may (temporarily) shift toward the tuberculoid pole of the spectrum; erythema nodosum leprosum (ENL) reaction is a systemic inflammatory reaction with immune complex and tumor necrosis factor–α involvement.

The course of leprosy is known to be influenced by host factors and genetic variation. [7] We hypothesize that the different clinical presentations of leprosy may be caused, completely or in part, by

differential TLR signaling. *M.leprae* is an intracellular mycobacterium, and macrophages have to be activated for its destruction by antigen-specific Th1 cells, which in turn are activated by DCs. Krutzik et al. showed, in vitro, that lepromatous patients fail to produce such $CD1b^+$ DCs after stimulation of the heterodimer formed by TLR1 and TLR2. [8] However, they could produce macrophages positive for DC-specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN). In vivo, in patients with tuberculoid leprosy and patients who were experiencing reversal reactions, both $CD1b^+$ DCs and $DC\text{-}SIGN^+$ macrophages were present in cutaneous lesions. In patients with lepromatous leprosy, $DC\text{-}SIGN^+$ macrophages, in which *M.leprae* was abundantly present, were found in the lesions.

M.leprae predominantly activates the TLR heterodimer TLR1/2. [9] Thus, alterations in either the *TLR1* or *TLR2* genes may alter susceptibility to *M.leprae*. Several studies report single-nucleotide polymorphisms (SNPs) in these 2 genes, which are involved in susceptibility and/or resistance to other infectious diseases. Omueti et al. described 3 SNPs in the *TLR1* gene, P315L, N248S, and H305L, which impaired the response to several bacterial agonists for this receptor. [10] All 3 SNPs are located in the extracellular domain, which is required for sensing bacterial lipopeptides, and do not influence cell surface expression. Johnson et al. reported that the I602S SNP in the *TLR1* gene is associated with abnormal trafficking of TLR1 to the cell surface, resulting in a reduced blood monocyte response to bacterial agonists. [11] Remarkably, the S602 allele was found to be associated with a decreased incidence of leprosy in a Turkish population. The authors suggested that "*M.leprae* subverts the TLR system as a mechanism of immune evasion". [11, p. 7520] Recently, Misch et al. added the finding that the S602 allele protects against reversal reactions. [12] However, no association was seen with the tuberculoid or lepromatous forms of leprosy. Bochud et al. analyzed polymorphisms in *TLR2* in Ethiopian patients with leprosy and control subjects. [13] A SNP 597T allele was

shown to protect against reversal reactions, and a homozygous 280-bp allelic-length microsatellite increased the risk of such reactions, indicating that TLR2-mediated pathways influence the occurrence of reversal reactions.

In this study, we focused on analyzing functionally relevant SNPs in the coding regions of *TLR1* and *TLR2*. Because the other SNPs were either absent or equally distributed, we studied the association of the *TLR1* N248S SNP and leprosy in a Bangladeshi population and performed an in-depth analysis of patient characteristics.

Materials and methods

The study population consisted of participants in a prospective (sero-) epidemiological study on contract transmission and chemoprophylaxis in leprosy (the COLEP study; International Standard Randomised Controlled Trials Number 61223447), which studied the effect of chemoprophylaxis in persons who have had regular contact with patients with leprosy. Written approval was granted by the Ethical Review Committee of the Bangladesh Medical Research Council (reference numbers BMRC/ERC/2001– 2004/799 and BMRC/ERC/2004–2007/120). All participants provided written informed consent.

For the study described here, 842 patients with leprosy who were registered with the Rural Health Program of the Leprosy Mission Bangladesh in 2002 and 2003, were followed up for 4 years and assessed for reactions. Patients were classified as having paucibacillary or multibacillary leprosy, in accordance with the 1998 World Health Organization classification. The control subjects were selected through a multistage cluster sampling procedure. Twenty clusters of approximately 1000 people each were randomly selected from 13 subdistricts in the study area. From 1 of 9 control subjects (n = 2203), a finger prick blood sample was collected on blotting paper (GB002 [0.37-mm thickness];

Schleicher & Schuell); the sample was air dried and stored in a plastic zip bag with silica gel at -20°C until use. Of these samples, 543 randomly selected samples were used for genetic analysis.

DNA isolation was performed using guanidium thiocyanate and silica particles, as described by Boom et al. [14] Genotyping of the SNPs *TLR1* N248S, *TLR1* I602S, and *TLR2* R753Q was achieved by melting curve analysis on genomic DNA using fluorescencelabeled hybridization fluorescent resonance energy transfer (FRET) probes and the LightCycler 480 system (Roche Diagnostics). The polymerase chain reaction for *TLR1* N248S contained primers for the sequences 5'-TTGGATGTGTCAGTCAAGACTGTAG-3' and 5'-GCTTCACGTTTGAAATT-GAG-3', along with FRET probes 5'-TTAAGGTAAGACTTGATAACTTTGG-3' (3'-labeled with fluorescein) and 5'-GTTTGAAGTTTCGCCAGAATACT-TAGG-3' (5'-labeled with LCRed60). The *TLR1* S248 (G allele) and *TLR1* N248 (A allele) gave rise to melting peaks at 58.2°C and 50.0°C, respectively.

The control population was evaluated for Hardy-Weinberg equilibrium by comparison of the expected and observed frequency of genotypes in a 2 x 3 X^2 table. The *TLR1* N248S SNP frequency of the control group was in Hardy-Weinberg equilibrium (X^2 = 0.74; P = 0.346). Furthermore, there was no indication that patients from certain geographical regions in the study area had a different allele distribution (data not shown). To determine the risk associated with the *TLR1* SNP, odds ratios (ORs) were calculated using the observed genotypes of the case patients, compared with those of the control subjects. Subgroup analysis of the patient population was performed using leprosy classification, reactional status, and serologic status as variables.

Results

The *TLR2* R753Q SNP, which occurs with an allelic frequency of 0.05 in Europe, was completely absent in the populations studied (not shown). The *TLR1* I602S was found with an allelic frequency of 0.064 in the patients with leprosy and 0.054 in the control subjects (P = 0.54). Further analysis also failed to show any correlation of this SNP with the clinical course of disease.

TLR1 N248S is a common SNP in our Bangladeshi study population (table 1). We found the S allele to be slightly more frequent among the patients with leprosy than among the control subjects (54% vs. 51%; OR, 1.12 [95% confidence interval {CI}, 0.97–1.31]). Analysis of genotype frequencies revealed that homozygous S248 was significantly associated with leprosy per se (OR, 1.34 [95% CI, 1.06–1.70]) (table 1). The heterozygous SN genotype was found to be protective against leprosy (OR, 0.78 [95% CI, 0.63–0.96]). In contrast, the homozygous N248 genotype was equally distributed among patients and control subjects (25%; OR, 1.01 [95% CI, 0.79–1.29]).

In-depth analysis of patient characteristics included leprosy classification, leprosy reactions, and serologic status (table 1). No difference in allele frequency or genotype was seen when patients with multibacillary or paucibacillary leprosy were compared or when seropositive and seronegative patients were compared. However, we observed an association between the *TLR1* N248S SNP and leprosy reactions; the S248 allele was more frequent among patients with reversal reaction (60% frequency) than among patients who had no reaction (54% frequency), but this difference failed to reach statistical significance (OR, 1.28 [95% CI, 0.91–1.81]). However, a significant association was found for patients who experienced ENL reactions, who were less likely to have the S248 allele (32% frequency) than were

patients who had no reaction (54% frequency)(OR, 0.40 [95% CI, 0.16–0.99]).

Table 1. Frequency of N and S alleles at aa 248 in Toll-like receptor 1 in study subjects, according to subgroup.

Group	Total subjects, no.	Allele frequency, no. (%)		Genotype, no. (%) of subjects		
		S	N	SS	SN	NN
Control subjects	543	553 (51)	533 (49)	146 (27)	261 (48)	136 (25)
Case patients	842	908 (54)	776 (46)	278 (33)	352 (42)	212 (25)
Comparison of patients and control subjects, OR (95% CI)		1.12 (0.97–1.31)		1.34 (1.06–1.70)	0.78 (0.63–0.96)	1.01 (0.79–1.29)
Case patient subgroups						
Leprosy classification						
Paucibacillary	702	760 (54)	644 (46)	232 (33)	296 (42)	174 (25)
Multibacillary	140	148 (53)	132 (47)	46 (33)	56 (40)	38 (27)
Comparison of MB and PB, OR (95% CI)		0.95 (0.73–1.23)		0.99 (0.67–1.46)	0.91 (0.63–1.32)	1.13 (0.75–1.70)
Leprosy reaction status[a]						
None	656	707 (54)	605 (46)	211 (32)	285 (43)	160 (24)
Reversal reaction	75	90 (60)	60 (40)	32 (43)	26 (35)	17 (23)
ENL reaction	11	7 (32)	15 (68)	1 (9)	5 (45)	5 (45)
Comparison of RR and no reaction, OR (95% CI)		1.28 (0.91–1.81)		1.57 (0.97–2.55)	0.69 (0.42–1.14)	0.91 (0.51–1.61)
Comparison of ENL and no reaction,OR (95% CI)		0.40 (0.16–0.99)		0.21 (0.03–1.66)	1.09 (0.33–3.59)	2.58 (0.78–8.58)
Serologic status[b]						
Negative	597	646 (54)	548 (46)	199 (33)	248 (42)	150 (25)
Positive	235	251 (53)	219 (47)	76 (32)	99 (42)	60 (26)
Comparison of positive and negative,OR(95% CI)		0.97 (0.78–1.20)		0.96 (0.69–1.32)	1.02 (0.75–1.39)	1.02 (0.72–1.45)

NOTE. CI, confidence interval; ENL, erythema nodosum leprosum; OR, odds ratio. [a] Reaction status was unknown for 100 patients. [b] Serologic status was unknown for 10 patients.

Discussion

Amino acid 248 of TLR1 is located in the external ligand binding site of the receptor. The S248 variant enables normal functioning of TLR1, whereas the N248 variant diminishes the response of TLR1 to bacterial agonists. [10] We found an association between the N248S SNP in the *TLR1* gene and leprosy in a Bangladeshi population. Genotype analysis of patients and control subjects revealed that the presence of the homozygous S248 genotype was positively associated with leprosy, whereas the heterozygous SN genotype was negatively associated. These findings are in line with the results of 2 recent reports concerning the I602S SNP in *TLR1*; [11, 12] a nonfunctional TLR1 (variant S602) was shown to lack cell surface expression and was found more frequently in control subjects. Surprisingly, the homozygous N248 genotype was equally distributed in patients and control subjects; this finding is unexpected, because the frequency of this genotype should be higher in control subjects. We currently have no explanation for this finding, but it indicates that the homozygous N248 genotype does not influence leprosy susceptibility.

Our analyses failed to reveal any differences in allele frequencies or genotypes associated with leprosy classification or serologic status. However, patients who experienced ENL reactions were more likely to have the N248 allele (68%) than were patients who had no reactions (46%); 10 of 11 patients who experienced ENL reactions had ≥1 N248 allele. Clearly, the function diminishing SNP *TLR1* N248 is strongly associated with ENL reactions.

In contrast, patients who experienced reversal reactions had a higher S248 allele frequency than did patients who had no reactions (60% vs. 54%). In line with these results, Misch et al. found that the normal-functioning *TLR1* I602 allele is more frequent among patients who experience reversal reactions. [12] Both findings indicate that the TLR1 signaling pathway accommodates reversal reactions.

The N allele and genotype SN, associated with decreased signaling function of TLR1, seems to give some protection against leprosy. The differences in allele frequency among patients who experienced different reaction types indicates that the distinct functions of TLR1 may influence the chance of these debilitating episodes. The genetic effect that has been detected may be related to another gene mutation in the vicinity of the N248S SNP. Various *TLR1* and *TLR2* SNPs have been repeatedly studied, and the association with leprosy per se is evident. Krutzik et al. showed that TLR1/2 is the major TLR mediator for the response to *M.leprae*; [8] this makes it very plausible that any functionally relevant SNP in these *TLR* genes alters the immune response against this pathogen and thus the risk of infection and course of disease. It was shown by Wurfel et al. that the N248S SNP was in strong linkage disequilibrium with 2 *TLR1* SNPs that predispose patients to excessive inflammation during sepsis. [15] A clear relationship was expected between TLR1 functionality (as determined by SNPs) and infection risk. However, we could not find this in our study; the homozygous S248 genotype increases susceptibility to leprosy, and the SN genotype decreases it, but the NN genotype showed no influence on susceptibility.

There are 3 possible explanations for this result. First, N248 diminishes rather than abolishes the function of TLR1, leading to a moderate effect in comparison to a completely nonfunctional TLR1. However, if this were the case we would expect no effect for the heterozygous genotype. Second, the potentially protective effect of the NN genotype might be overcome by the evolutionary pressure of another disease affected by *TLR1* N248S. Third, Krutzik et al. showed that, although no difference in the expression or function of TLR1 and TLR2 was found in the peripheral monocytes of patients with tuberculoid or lepromatous leprosy, the local expression of TLR1 and TLR2 in the lesions of such patients was different, and the expression of TLR1 and TLR2 can be influenced by interleukins. [9] Therefore, local disruption of

TLR1/2 expression and/or function can apparently create a microenvironment in which *M.leprae* can proliferate. This local alteration of TLR1 and TLR2 expression and/or function would be far more disruptive than the *TLR1* SNP N248S, which only diminishes function. Further research is needed to elucidate how *M.leprae* influences the local functioning and/or expression of TLRs.

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Chapter 3

Association between anti-PGL-I IgM and clinical and demographic parameters in leprosy

Ron P. Schuring, F. Johannes Moet, David Pahan, Jan Hendrik Richardus and Linda Oskam. Association between anti-PGL-I IgM and clinical and demographic parameters in leprosy.
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Abstract

Objective. To determine the risk factors and clinical significance of anti-PGL-I seropositivity

Design. A large-scale sero-epidemiological study (COLEP) was carried out in northwest Bangladesh. Blood on filter paper from 1,025 newly diagnosed patients was collected before treatment was started and tested with an anti-PGL-I ELISA; the relation between patient determinants and seropositivity was calculated using logistic regression

Results. The median age was 30 years and the male:female ratio 1.9. Overall, 342 patients (33.4%) were seropositive. The following determinants showed a significant correlation with seropositivity ($P < 0.05$) in multivariate analysis: sex, age, disability grade, bacterial index and classification according to the World Health Organization (WHO) system. The number and extent of clinical signs correlated with seropositivity, except for the presence of satellite lesions. People with or without a BCG vaccination scar had a similar risk to be seropositive.

Conclusion. Serology is a marker for a higher systemic bacterial load and may identify potential infectious sources among patients with few clinical signs. The size of skin lesions was positively correlated with seropositivity. We did not find different levels of seropositivity among patients with one or two skin lesions, neither did we find different levels among patients with or without satellite lesions.

Introduction

Leprosy is a chronic infectious disease, which is still a major public health problem, mainly in Africa, Asia and Latin America. [1] When left untreated, infection with *Mycobacterium leprae* may eventually lead to severe disabilities. Differences in the cellular immune response of the host determine the clinical features, which form a spectrum and vary from one or a few hypopigmented anaesthetic skin lesions to extensive skin involvement and irreversible damage to the peripheral nerve system.

Accurate diagnosis and classification of leprosy patients is important for treatment purposes as correct treatment may prevent disabilities, relapse and continued transmission. Currently, there are two classification systems in use, which are at least partially complementary:

- The classification according to Ridley and Jopling is based on immunological and histopathological features and makes a distinction between tuberculoid (TT) and lepromatous (LL) leprosy. Between these poles there are three borderline groups (BT, BB and BL) and a separate indeterminate group (I). [2]
- The World Health Organization (WHO) designed a less demanding classification system for treatment purposes. The WHO classification system is based on clinical (and when available bacteriological) features and divides leprosy patients into multibacillary patients (MB, 6 or more skin lesions/satellite lesions and/or a positive bacterial index [BI as determined by microscopy]) and paucibacillary patients (PB, up to 5 skin lesions/satellite lesions and a negative BI). [3] In some control programmes PB patients with a single lesion (SLPB) are recorded separately.

In the WHO classification system satellite lesions, small (secondary) lesions in the vicinity of a larger (primary) lesion, are counted as separate lesions. The WHO system does not take into account the large variation in the size of lesions. However, there are theoretical arguments for a relation between lesion size and the proliferation of bacteria. [4] Moreover, the authors learned from discussion with leprosy control staff in various countries that lesion size may influence the classification decision made by doctors and field workers (unpublished observations). This would decrease the power of any statistical analysis based on classification data.

There are currently two tools available for routine control programmes to aid the correct classification of leprosy patients:

- The BI is a logarithmic scale ranking from zero to six, which defines the bacterial load found by microscopy after acid-fast staining of skin smears or biopsies. [5]
- With the development of rapid tools, serology has become an easily applicable method in the field. [6] The presence of antibodies to the *M.leprae*-specific phenolic glycolipid-I (PGL-I) correlates with the bacterial load of a leprosy patient and its detection can aid the classification of confirmed leprosy patients as MB or PB for treatment purposes. [7]

In this study we relate the PGL-I-based serology results of 1,025 newly diagnosed, well characterized leprosy patients from Bangladesh to their detailed clinical and demographic characteristics. Factors determining seropositivity are established as well as the clinical relevance of serology results. This study is part of a prospective (sero-) epidemiological study on contact transmission and chemoprophylaxis in leprosy (COLEP). [8]

Materials and Methods

Patients and samples. This serological study is part of the COLEP study. [8] The patients were from northwest Bangladesh and were detected through passive case detection. They were diagnosed at Danish Bangladesh Leprosy Mission (DBLM) clinics between May 2002 and October 2003. The districts of Nilphamari and Rangpur in northwest Bangladesh have a total population of approximately 4.3 million with 1,505 new leprosy cases detected by the DBLM staff in 2001 (case detection rate 3.5/10,000 population). [9] Patients were classified based on the WHO classification system. [3] A medical doctor confirmed the diagnosis for every patient and treatment was given according to the WHO/DBLM guidelines. Group sizes were set at a maximum of 400 for SLPB and 400 for PB and a minimum of 200 for MB patients; patients with the pure neural form of leprosy were excluded. [8] Eleven patients with a positive disability grade [14] were reclassified from SLPB into PB. Four patients who were initially classified as SLPB (1) or PB (3) were reclassified as MB based on a positive BI. Ridley & Jopling classification is not performed at DBLM.

A single blood sample was obtained from 1,025 of the 1,037 patients enrolled in the COLEP study, consisting of 383 SLPB, 348 PB and 294 MB patients.

From each patient demographic and clinical data were collected. Finger prick blood was collected on 0.37 mm blotting paper (GB002 Schleicher and Schuell, ‘s Hertogenbosch, the Netherlands), air-dried and stored in plastic zip bags with silica gel at –20°C until use.

The study abides by the International Ethical Guidelines for Biomedical Research Involving Human Subjects (Council for International Organizations of Medical Sciences, CIOMS, Geneva, 1993). Ethical clearance was obtained from the Ethical Review Committee of the Bangladesh Medical Research Council and written informed consent was obtained from each patient before inclusion in the study.

Coating of ELISA plates. Serology for the detection of IgM antibodies against *M.leprae* was performed using the ELISA technique previously described [10] with natural tri-saccharide linked to bovine serum albumin via a phenolic ring (NT-P-BSA) as a semi-synthetic analogue of PGL-I. [11] Round-bottomed microtiter plates (NUNC 96 U Invitrogen/Life Technologies, Taastrup, Denmark) were coated with 50 µl/well NT-P-BSA (0.01 µg carbohydrate/ml dilution in 0.1 mol/l ammonium hydrogen carbonate buffer, pH 8.0). Wells to control for non-specific binding were coated with 50 µl of a solution containing 0.082 µg/ml BSA of the same batch that was used for the preparation of NT-P-BSA. Plates were air dried for 2 days at room temperature and stored in sealed plastic bags with silica gel in the dark at room temperature until use (within 6 months).

ELISA. On the day before testing, a 3.17 mm diameter disc was punched from the blood impregnated filter paper card into a polypropylene tube and incubated overnight at 4°C in 25 µl phosphate buffered saline (pH 7.2) containing 0.1% (v/v) Tween 20 (PBST). The next day 183 µl of PBST+10% (v/v) normal goat serum (Gibco Invitrogen/Life Technologies, Auckland, New Zealand; PBST+NGS) was added and incubated for 1 h. This corresponds to an approximately 1:167 dilution of serum.

Before adding the eluted samples, the pre-coated plates were washed with PBST (two times short and two times 2-5 minutes), followed by a blocking step with 100 µl/well PBS+1% (w/v) BSA (Boehringer, Mannheim, Germany) at 37°C for 1h. Next, 50 µl of the sample dilution was added to each well followed by incubation at 37°C for 1 h. Plates were washed as described above and 50 µl/well conjugate (1:10,000 dilution in PBST+NGS of a peroxidase-conjugated goat IgG fraction to human IgM 5FC µ; Capple/Organon Teknika, Turnhout, Belgium) was added and incubated at 37°C for 1 h. After another washing procedure 50 µl/well TMB substrate solution (0.4% (w/v) 3,3',5,5'-tetramethyl-benzidine + 0.4% (w/v) urea hydrogen peroxide in DMSO [all three from Sigma-Aldrich, Steinheim, Germany], diluted 1:10 in 0.1 mol/l

sodium acetate citrate buffer pH 4.0) was added to initiate a colouring reaction. The reaction was stopped by adding 50 µl/well 0.5 N H_2SO_4 when a standard serum reached a net optical density at 450 nm (OD) of 0.600. The status seropositive was given if the net OD was above 0.199. When the OD of the standard serum was either too low (OD < 0.55) or too high (OD > 0.75) the samples were retested. The ELISA performance was monitored using this standard plus a positive and negative control serum sample on each plate.

Bacterial Index. The BI was determined by microscopy on Ziehl-Neelsen stained slit skin smears [12] taken from the earlobe, forehead and a skin lesion; the highest BI was recorded.

Clinical signs. The clinical signs of the patient were recorded as number of skin lesions (hypopigmented and/or anaesthetic skin patches), number of nerves involved (nerves: facial, ulnar, radial cutaneous, median, lateral popliteal and posterior tibial; involved: enlarged, tender or painful) and as number of body areas affected (with a skin lesion and/or nerve involvement) according to the system described by Van Brakel et al., [13] dividing the body into seven body parts, namely head, torso, back and the four extremities. Satellite lesions are recorded separately from the determinant “number of skin lesions”. The size of the largest skin lesion was estimated as being small (< 10 cm diameter), medium (10 to 15 cm diameter) or large (> 15 cm diameter). The clinical data set was based on body charts drawn by the DBLM-staff.

Data analysis. Patient and serological data were stored in Microsoft Access and Excel, respectively. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS for Windows, version 11.0.1, SPSS Inc., Chicago, IL, USA; 2001). Logistic regression was used to identify independent determinants influencing the odds ratio for seropositivity. Determinants associated with seropositivity in univariate analysis ($P < 0.10$) were selected for multivariate analysis. In

multivariate analysis, we tested for statistically significant ($P < 0.05$) interactions between determinants in the final model and for confounding.

Results

Patient characteristics

Blood samples were collected from 1,025 newly diagnosed leprosy patients. The median age was 30 years (range 5-84) and the male:female ratio was 1.9. The distribution of the patients' characteristics is presented in Table 1. The distribution of sex, age, BCG vaccination rate and disabilities differed among the three WHO classification groups. The MB group contained more male and older patients in comparison with the PB and SLPB groups ($P < 0.0001$). The MB group contained fewer BCG vaccinated patients ($P = 0.009$) and more patients with disabilities ($P < 0.0001$). The median age of the BCG vaccinated patients was lower, 25 years (inter quartile range (IQR), 15 to 35) compared with non-vaccinated patients, who had a median age of 32 years (IQR, 20 to 45, $P < 0.0001$). After age and sex adjustment there is an odds ratio of 1.54 for non-vaccinated patients to be classified as MB (exact binomial 95% confidence interval [95% CI] 1.09-2.18).

Factors determining seropositivity

Out of the 1,025 patients, 342 (33.4%; 95% CI 30.5-36.3) were given the status seropositive based on ELISA testing (Table 2).

WHO classification and BI. A strong relation with seropositivity was shown for the determinants WHO classification and BI in both univariate and multivariate analyses (Table 2). In the multivariate

Table 1. Patients' characteristics in relation to WHO classification

Determinants	MB		PB		SLPB		Total		
	no.	%	no.	%	no.	%	no.	%	*P*-value[a]
total	294	28.7	348	34.0	383	37.4	1025	100	
Sex									
Male	230	78.2	221	63.5	222	58.0	673	65.7	
Female	64	21.8	127	36.5	161	42.0	352	34.3	< 0.0001
male:female ratio	3.59		1.74		1.38		1.91		
Age (years)[b]									
5-14	28	9.5	63	18.2	51	13.3	142	13.9	
15-29	78	26.5	118	34.1	150	39.2	346	33.8	
30-44	98	33.3	99	28.6	108	28.2	305	29.8	
45-59	65	22.1	49	14.2	53	13.8	167	16.3	
60-or above	25	8.5	17	4.9	21	5.5	63	6.2	< 0.0001
median age	39		29		28		30		
BCG vaccination[c]									
Yes	58	19.9	100	29.2	113	29.5	271	26.6	
No	233	80.1	243	70.8	270	70.5	746	73.4	0.009
Disability grade[14]									
0	199	67.7	309	89.1	383	100	891	86.9	
1	60	20.4	20	5.7	0	0	80	7.8	
2	35	11.9	19	5.2	0	0	54	5.3	< 0.0001

[a] *P*-value calculated with Pearson Chi-square. [b] For two patients no age information was available. [c] For eight patients no information on the BCG vaccination status was available.

analysis, the odds ratio (OR) for seropositivity was adjusted for differences in sex, age, BCG vaccination and disability distribution. Compared to SLPB patients, MB patients were more likely to be seropositive (adjusted odds ratio (aOR) MB 11.8; 95% CI 7.83-17.8) while PB patients had no difference in risk for seropositivity (aOR PB 1.27; 95% CI 0.86-1.86). Seropositivity increased with the BI: the aOR for patients with a BI 1 or 2 was 6.33 (95% CI 2.42-16.5) and the aOR for patients with a BI higher than 2 was 59.0 (95% CI 25.0-139) compared with BI negative patients.

Sex and age. In univariate analysis, the determinants sex and age were not significantly related with seropositivity. However, after adjustment for the other determinants in the multivariate analyses (model contains determinants: WHO classification, sex, age, BCG vaccination and disability grade) they appeared to be significantly related. Females were more likely to be seropositive than males (aOR 1.46; 95% CI 1.05-2.04) and the positivity prevalence decreased significantly with age (Table 2).

BCG vaccination. In univariate and multivariate analyses with the determinant BCG vaccination, no difference in seropositivity was found between BCG non-vaccinated patients and vaccinated patients (aOR 1.20; 95% CI 0.84- 1.72). In addition, no correlation was found between BCG vaccination and BI positivity (OR BCG non-vaccinated patients 1.24; 95% CI 0.80-1.93).

Disability grade. In univariate analysis, the determinant disability grade had a significant relation with seropositivity; in the multivariate analysis this relation was reduced. Patients with a disability grade 1 and 2 were more likely to be seropositive compared to disability grade 0 patients, but only disability grade 1 showed a significant difference with disability grade 0 patients (aOR 1.79; 95% CI 1.01-3.16). Grouping the disability grade 1 and 2 together resulted in a significant aOR of 1.73 (95% CI 1.09-2.76).

Table 2. Logistic regression analysis to determine risk factors for seropositivity among leprosy patients

Determinants	no.	% sero-positive	Unadjusted OR[a]	95% CI[a]	Adjusted aOR[b]	95% CI
WHO classification						
SLPB	383	16.7	1		1	
PB	348	21.3	1.35	0.93-1.95	1.27	0.86-1.86
MB	294	69.4	11.3	7.84-16.3	11.8	7.83-17.8
			P < 0.0001		*P* < 0.0001	
Bacterial Index[c]						
0	883	25.1	1		1	
1 - 2	23	69.6	6.81	2.76-16.8	6.33	2.42-16.5
> 2	104	94.2	48.6	21.0-112	59.0	25.0-139
			P < 0.0001		*P* < 0.0001	
Sex						
Male	673	34.3	1		1	
Female	352	31.5	0.88	0.67-1.16	1.46	1.05-2.04
			P = 0.369		*P* = 0.027	
Age (years)[d]						
5 - 14	142	31.7	0.90	0.59-1.36	0.92	0.56-1.50
15 - 29	346	34.1	1		1	
30 - 44	305	33.4	0.97	0.70-1.34	0.67	0.45-0.98
45 - 59	167	34.1	1.00	0.68-1.48	0.53	0.33-0.86
60–or above	63	30.2	0.83	0.47-1.49	0.42	0.21-0.86
			P = 0.963		*P* = 0.024	
BCG vaccination[e]						
Yes	271	28.4	1		1	
No	746	35.0	1.36	1.00-1.84	1.20	0.84-1.72
			P = 0.050		*P* = 0.310	
Disability grade[14]						
0	891	29.2	1		1	
1	80	63.8	4.27	2.65-6.89	1.79	1.01-3.16
2	54	57.4	3.27	1.87-5.72	1.66	0.85-3.26
			P < 0.0001		*P* = 0.066	
1 - 2[f]	134	61.2	3.83	2.63-5.58	1.73	1.09-2.76
			P < 0.0001		*P* = 0.020	

[a] OR = odds ratio, 95% CI = 95% confidence interval. [b] Adjusted OR; determinants in the final model: WHO classification, sex, age, BCG vaccination and disability. aOR for Bacterial Index was calculated without the WHO classification determinant. [c] For fifteen patients no BI result was available. [d] For two patients the age was not recorded.
[e] For eight patients BCG data were not available. [f] Analyses were performed with a recoded determinant disability grade (grade 1 and 2 were coded as 1 - 2).

Clinical signs

Detailed clinical data from 996 patients were available for analysis (Table 3).

Satellite lesions. Comparison between patients with satellite lesions and patients without satellite lesions showed no differences in sex, age or BI distribution and no correlation with serology was found in multivariate analysis (model contains: skin lesion (size), skin lesion (number), nerve, body area, sex and age). The correlation of satellite lesions in the univariate analysis was altered from significant to non-significant after adjustment with the determinant skin lesion (number) or with the determinant body area.

Skin lesion size. The size of a skin lesion was found to be a determining factor for seropositivity. The aORs of “medium” and “large” skin lesions were 1.45 (95% CI 0.94-2.23) and 2.37 (95% CI 1.47-3.83), respectively, compared with “small” skin lesions.

Number of skin lesions. The determinants skin lesion (number), nerve and body area showed a positive correlation with seropositivity, in both univariate and multivariate analyses. The seropositivity rate increased significantly with the number of skin lesions: patients with three to five skin lesions had a significantly increased aOR of 2.54 (95% CI 1.42-4.54), while patients with two lesions did not have a significantly different aOR (aOR 0.90; 95% CI 0.54-1.49) compared with patients with one lesion.

Number of nerves and body areas involved. Patients with more than two nerves involved and patients with more than five body areas affected were more likely to be seropositive. Having more than two nerves involved resulted in a significant aOR of 2.01 (95% CI 1.08-3.72) compared with no nerve involvement. When six or seven body areas

were affected the aOR for seropositivity was 2.91 (95% CI 0.99-8.52) compared with one and two body areas.

Table 3. Logistic regression analysis to determine risk factors for seropositivity among clinical signs of leprosy patients.

		%	Unadjusted		Adjusted	
Clinical signs	**no.**	**sero-positive**	**OR[a]**	**95% CI[a]**	**aOR[b]**	**95% CI**
Satellite lesion						
not present	756	29.4	1		1	
present	240	45.0	1.97	1.46-2.65	1.15	0.78-1.70
			P < 0.0001		*P* = 0.489	
Skin lesion (size)[c]						
Small	623	22.6	1		1	
Medium	174	35.1	1.85	1.28-2.65	1.45	0.94-2.23
Large	191	63.9	6.04	4.26-8.58	2.37	1.47-3.83
			P < 0.0001		*P* = 0.002	
Skin lesion (number)						
1	477	17.0	1		1	
2	151	17.2	1.02	0.63-1.65	0.90	0.54-1.49
3 - 5	117	34.2	2.54	1.62-3.99	2.54	1.42-4.54
6 - 15	136	61.0	7.66	5.03-11.6	5.15	2.22-11.9
> 15	115	87.0	32.6	18.0-59.0	10.2	3.41-30.6
			P < 0.0001		*P* = 0.0003	
Nerve						
0	587	24.2	1		1	
1 - 2	274	29.6	1.32	0.95-1.81	1.24	0.85-1.82
> 2	135	79.3	12.0	7.58-18.9	2.01	1.08-3.72
			P < 0.0001		*P* = 0.078	
Body area[d]						
1 - 2	692	18.6	1		1	
3 - 5	176	50.0	4.36	3.07-6.21	0.78	0.37-1.62
6 - 7	128	88.3	32.9	18.6-58.2	2.91	0.99-8.52
			P < 0.0001		*P* = 0.004	

[a] OR = odds ratio, 95% CI = 95% confidence interval. [b] Adjusted OR; Determinants in the final model: skin lesion (size), skin lesion (number), nerve, body area, sex and age. [c] Estimated as small (< 10 cm diameter), medium (10 to 15 cm diameter) and large (> 15 cm diameter). [d] Seven body areas: head, torso, back and the four extremities. [13]

None of the determinants showed any significant interaction. Analyses were repeated with the ELISA cut-off values: 0.149, 0.249 and 0.299 to confirm the conclusions based on the cut-off value 0.199. No significant differences were found (data not shown).

Discussion

Leprosy serology has been studied frequently and many of the factors determining seropositivity are well known, as has been reviewed by Oskam et al. [15] However, these studies were often performed on a limited number of patients. Here we describe a study on more than one thousand patients in which serology is compared to a large variety of clinical and demographic data and in which factors determining seropositivity are established.

The ELISA used was based on the detection of specific antibodies in peripheral blood eluted from blood spots on filter paper. Blood on filter paper was chosen for practical reasons: it is cheap, easy to collect in the field and requires no centrifugation or cold chain. However, blood eluted from blood spots is known to give a slightly lower signal in the ELISA compared with serum. [16]

Patient characteristics

The median age (30) and the male:female ratio (1.9) are in agreement with other reports from this area, [17,18] taking into account that, due to the group size criteria described in the Materials and Methods section, our study population included a higher percentage of MB patients (28.7%) than the actual situation (18.4% in 2002 and 21.9% in 2003). [17]

The MB patient group comprised more males, older patients and more patients with a disability grade > 0 than the PB or SLPB groups. These

differences between MB and PB patients are reported frequently [18-20] and are thus in line with expectations.

In previous studies BCG vaccination was held to be protective against leprosy and was highly associated with the development of tuberculoid leprosy instead of lepromatous leprosy, suggesting that BCG vaccination would protect against lepromatous leprosy. [21,22] We see a similar, but less strong effect in our patient population with regard to the development of PB or MB leprosy: MB patients were less frequently BCG vaccinated than PB and SLPB patients (BCG coverage MB approximately 20%; PB and SLPB, 29%). After age and sex adjustment there is an aOR of 1.54 (95% CI 1.09-2.18) for non-vaccinated patients to be classified as MB, compared to PB-SLPB patients. A lower BI and/or seropositivity among BCG vaccinated patients would have been a supplementary argument for this protective role of BCG and would confirm the hypothesis that BCG vaccination is responsible for a shift in the immune response towards the tuberculoid pole of the spectrum. However, the determinant BCG vaccination did not have any influence on either seropositivity or BI.

Factors determining seropositivity

As expected, a strong correlation was found between serology and the determinants WHO classification and BI. [20,23-25] For the majority of patients who were MB, and particularly those who were skin smear positive, elevated levels of *M.leprae* specific IgM antibodies were found (Table 2).

The prevalence of seropositivity in this study population showed similar age and sex patterns as demonstrated in other studies. [19,25-27] The decline in seropositivity prevalence with increasing age is consistent with the decrease of overall IgM levels with age. It has been

suggested that females have higher innate IgM levels than males, which may be the explanation for the higher seropositivity rate found among females. [28] The alteration in significance for the variable sex on seropositivity in multivariate analysis compared to univariate analysis can be explained by the high number of male MB patients (male:female ratio; 3.59, versus 1.91 total population, table 1). The variable WHO classification confounded the correlation between sex and seropositivity in univariate analysis.

A correlation between disability grade and serology (aOR disability 1 and 2 1.73; 95% CI 1.09-2.76, *P* = 0.020) was found, corresponding with the general trend reviewed by Oskam et al.: [15] PB patients with a disability generally had higher seropositivity rates than PB patients without a disability.

The seroprevalence among MB patients (69%) was rather low compared with other studies in which it varied between 75 and 100%. [15] Comparison between studies is difficult since the classification criteria have changed over the years and our data collection was done using filter paper blood which gives slightly lower titers than serum. [16] Another possible explanation for the relatively low seropositivity in the MB group may be the short detection delay: the DBLM leprosy control programme has been well established in the area since 1977. DBLM [17] and Richardus et al. [29] have reported gradually decreasing percentages of MB cases and disabilities in our study area, which may be caused by a reduction in detection delay due to intensive control efforts. Since the numbers of skin lesions, nerves involved and body areas affected are correlated with both detection delay and seropositivity, a lower number of clinical signs among the MB classified group due to a short detection delay would lead to a lower seropositivity in the MB group. Further study may explore this possibility.

Clinical signs determining seropositivity

For a better understanding of the clinical significance of seropositivity, specific clinical determinants were related to serology results. Based on our results we can make a number of remarks with regard to the WHO classification system as it is currently used:

- There was no serological difference between patients with and without satellite lesions (Table 3). Since there was also no serological difference between patients with one or two skin lesions it can be concluded that there is no serological evidence to distinguish between SLPB and PB with 2 lesions, with and without satellite lesions. This implies that the presence of satellite lesions may be ignored for quantification of skin lesions and that a distinction may be made between PB 1 and 2 lesions on the one hand and PB 3-5 lesions on the other hand, with PB 1 and 2 being equivalent with the current SLPB category.
- The size of a lesion, here subjectively recorded from the largest lesion drawn on the patient information card, may also be a relevant factor for classification. There is a strong indication that the lesion size is a determining factor for seropositivity (Table 3).

The presence of not more than two lesions (regardless of the presence of satellite lesions) and no lesion larger than 10 cm diameter (small sized) may be new criteria for SLPB classification. If a separate SLPB treatment—such as ROM (rifampicin, ofloxacin, minocycline [3])—were used, this insight could have a large impact on the economic aspects of leprosy control. We realize that at the moment this is solely based on serological evidence and more detailed clinical information about response to treatment and risk of impairment will be needed to support our arguments for such an adjustment of WHO classification.

At an individual level seropositive PB patients may have disease that is behaving more like MB disease. It would be interesting to study if seropositive patients would benefit from a longer treatment with regard to relapse and the development of reactions and nerve damage.
The exact number of lesions is less crucial for seropositivity among MB patients, although a difference was seen between patients with up to 15 lesions and patients with more than 15 lesions. The number of nerves and the number of body areas affected seem to be both independent factors for seropositivity.
It may be stated that seropositivity is highly correlated with clinical signs: numbers of skin lesions, nerves involved and body areas affected. All these clinical signs signify the dissemination of the bacterium in the body of the patient, indicating that seropositivity can be used as a marker for a higher systemic bacterial load, and therefore can be used to identify more infectious patients. [30-33]

In conclusion, we have shown that the presence of elevated anti-PGL-I antibody levels is highly correlated with the MB status, BI and the dissemination of clinical signs in a patient. It is clear that serology results reflect the overall systemic bacterial load of a patient. From a serological point of view, it seems reasonable to stop counting satellite lesions as whole lesions, to take skin lesion size into account for clinical decision-making, and consider the possibility to include patients with two skin lesions into the SLPB group. For individual patient management serological testing may give clinicians a better idea about the systemic bacterial load of a patient. The availability of simple serological tests makes this option feasible.

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Chapter 4

Preventing nerve function impairment in leprosy: validation and updating of a prediction rule

Ron P. Schuring, Jan H. Richardus, Ewout W. Steyerberg, David Pahan, William R. Faber, Linda Oskam. Preventing nerve function impairment in leprosy:validation and updating of a prediction rule. PLoS Neglected Tropical Diseases. 2008;2(8):e283. Epub 2008 Aug 27.

Abstract

Background: To validate and update a prediction rule for estimating the risk of leprosy-related nerve function impairment (NFI).
Methodology/Principal Findings: Prospective cohort using routinely collected data, in which we determined the discriminative ability of a previously published rule and an updated rule with a concordance statistic (*c*). Additional risk factors were analyzed with a Cox proportional hazards regression model. The population consisted of 1,037 leprosy patients newly diagnosed between 2002 and 2003 in the health care facilities of the Rural Health Program in Nilphamari and Rangpur districts in northwest Bangladesh. The primary outcome was the time until the start of treatment. An NFI event was defined as the decision to treat NFI with corticosteroids after diagnosis. NFI occurred in 115 patients (13%; 95% confidence interval 11%–16%). The original prediction rule had adequate discriminative ability (*c* = 0.79), but could be improved by substituting one predicting variable: "long-standing nerve function impairment at diagnosis" by "anti-PGL-I antibodies". The adjusted prediction rule was slightly better (*c* = 0.81) and identified more patients with NFI (80%) than the original prediction rule (72%).
Conclusions/Significance: NFI can well be predicted by using the risk variables "leprosy classification" and "anti-PGL-I antibodies". The use of these two variables that do not include NFI offer the possibility of predicting NFI, even before it occurs for the first time. Surveillance beyond the treatment period can be targeted to those most likely to benefit from preventing permanent disabilities.

Author Summary

Leprosy is caused by a bacterium that attacks the peripheral nerves. This may cause nerve function impairment (NFI), resulting in handicaps and disabilities. Therefore, prediction and prevention of NFI is extremely important in the management of leprosy. In 2000, a prediction rule for NFI was published, but circumstances have changed since the study was performed in the 1990s: the leprosy detection delay has shortened and the definition of NFI has changed. The original rule used “leprosy classification” and “NFI present at diagnosis” to predict future NFI. In the current patient population we studied an adjusted rule based on “leprosy classification” and “presence of antibodies”. This adjusted rule predicted NFI more often than the original rule. With the adjusted rule it is now also possible to assess NFI risk before the first nerve damage event takes place. This may help doctors and health workers to improve surveillance for people at high risk. Early detection and treatment can then prevent permanent disabilities.

Introduction

Preventing permanent disabilities due to nerve function impairment (NFI) [1] remains a major concern in leprosy control. *Mycobacterium leprae*, the causative agent of leprosy, infiltrates Schwann cells of peripheral nerve fibers. [2] Subsequently, the nerve fibers can be damaged by accumulation of bacteria and hypersensitivity reactions of the immune system. The decline of nerve function can take place before, during and/or after leprosy treatment. Early detection (within 6 months) and corticosteroid treatment may prevent further decline. [3] With leprosy control becoming less specialized and increasingly integrated into general health care services, there is a need

for simplified procedures at the field level for timely identification and treatment of NFI in leprosy patients. The chances of preventing disability increase when health care workers pay special attention to patients who have a high risk of developing NFI.

To date, several risk factors for NFI have been identified, [4–6] and an NFI prediction rule was formulated based on data from the Bangladesh acute nerve damage study (BANDS). [4] The BANDS prediction rule categorizes patients into NFI risk groups based on their World Health Organization (WHO) classification (ie, paucibacillary [PB] or multibacillary [MB] leprosy) and the presence of long-standing NFI at diagnosis. However, validation of the BANDS prediction rule is needed because i) the definition of NFI has since changed; ii) shorter detection delays have led to a smaller percentage of patients with NFI at diagnosis [7] which may change the contribution of this variable to the prediction rule; iii) a new and simple serological test for anti-phenolic glycolipid I (PGL-I) antibody detection [8,9] has made routine screening feasible; and iv) no study has simultaneously assessed all known potential NFI risk factors, namely sex, age, WHO leprosy classification, long-standing NFI at diagnosis, bacterial load and anti-PGL-I antibodies. [4–6]

We first validated the BANDS NFI prediction rule. Next, we compared the performance of an adjusted NFI prediction rule, taking presence of anti-PGL-I antibodies into account.

Materials and Methods

Patients and procedures. Patients were previously untreated leprosy patients, newly diagnosed at the Rural Health Program (RHP) in northwest Bangladesh in 2002 and 2003. All patients participated in the COLEP trial (ISRCTN 61223447), [10] which studied the effect of chemoprophylaxis in persons who had contact with leprosy patients (n patients=1,037). Patients were classified as PB or MB according to the 1998 WHO classification [11] for treatment purposes. For the

current analysis, patients who had experienced NFI for<6 months at the time of diagnosis (n=162) were excluded as they required immediate treatment with corticosteroids, which influences the future occurrence of NFI, the primary outcome event of the study. Eleven patients were excluded because essential data were missing. This leaves a study population of 864 patients (538 males, 326 females; median age 34 years, range 5–84 years). Follow-up ended in September 2006 (median follow-up time 46 months). Patient information was prospectively recorded on standardised forms by the RHP staff.

The primary outcome was the time until the start of treatment. An NFI event was defined as the decision to treat NFI with corticosteroids after diagnosis. The decision was based on the guidelines described in the Rural Health Program (RHP, formerly DBLM) treatment protocol, [12] which states that a full dose course of prednisolone (starting with 40 mg/day and tapering off over 16 weeks for adults) should be given in case of i) nerve function reduction by ≥2 points in sensory and/or motor function tests of the ulnar, median, and/or posterior tibial nerves; ii) corneal anaesthesia; iii) a nerve tenderness score of 2; or iv) mixed mild symptoms of neuritis (ie, tenderness, sensory, and motor function scores of 1). The level of tenderness was defined as mild (score =1) if palpation of the nerve causes some pain, but does not cause the patient to jump or cry out and defined as severe (score =2) if touching the nerve causes the patient to jump or cry out. A low dose course of prednisolone (starting with 20 mg/day and tapering off over eight weeks for adults) is given for i) cutaneous neuritis; or ii) a mild skin reaction in a patch near or overlying a facial nerve. Thus, the criteria to treat NFI with prednisolone include all leprosy reactional and silent neuritis events. In both the BANDS and the current study, sensory testing was performed with the Watson ball-point pen test, [13] motor function was assessed according to Medical Research Council grading, [14] and changes in nerve function were evaluated by a physiotechnician trained in nerve function assessment.

Patients were under monthly surveillance during standard multidrug treatment (MDT): 6 months for PB patients, 12 months for MB patients. In the original BANDS study [4] recommendations for extended surveillance were formulated, stating that for the low-risk group—PB patients without long-standing NFI at diagnosis—routinely performed surveillance for NFI during MDT is sufficient and health education should be provided so that patients are able to recognise and report NFI after completion of MDT. Medium-risk group patients—PB patients with and MB patients without longstanding NFI at diagnosis—require 12 months of surveillance and health education, implying that extended surveillance is only necessary for PB patients, who receive 6 months of MDT. For the high-risk group—MB patients with long-standing NFI at diagnosis—24 months of surveillance is recommended, resulting in 12 months of surveillance in addition to the routine follow-up during MB MDT.

The bacterial load was determined by microscopy on Ziehl-Neelsen stained slit skin smears [15] taken from the earlobe, forehead and a skin lesion. The bacterial load was positive if any bacteria were detected in one of the smears.

The presence of IgM antibodies against *M.leprae* was determined at diagnosis with a previously described enzyme-linked immunosorbent assay (ELISA), [16] using dried blood on filter paper. Briefly, the terminal trisaccharide of phenolic glycolipid I (PGL-I) linked to bovine serum albumin via a phenolic ring (NT-P-BSA, kindly provided by Prof. T. Fujiwara, University of Nara, Japan) was used as a semisynthetic analogue. [17] The titer of IgM antibodies against *M.leprae* was expressed as net optical density (OD): the absorbance of NT-P-BSA minus that of BSA-coated wells at 450 nm. The status ''seropositive'' was assigned if the net OD was ≥0.20.

Ethical implications. This study uses data and samples that are routinely collected by the Rural Health Program from all leprosy patients

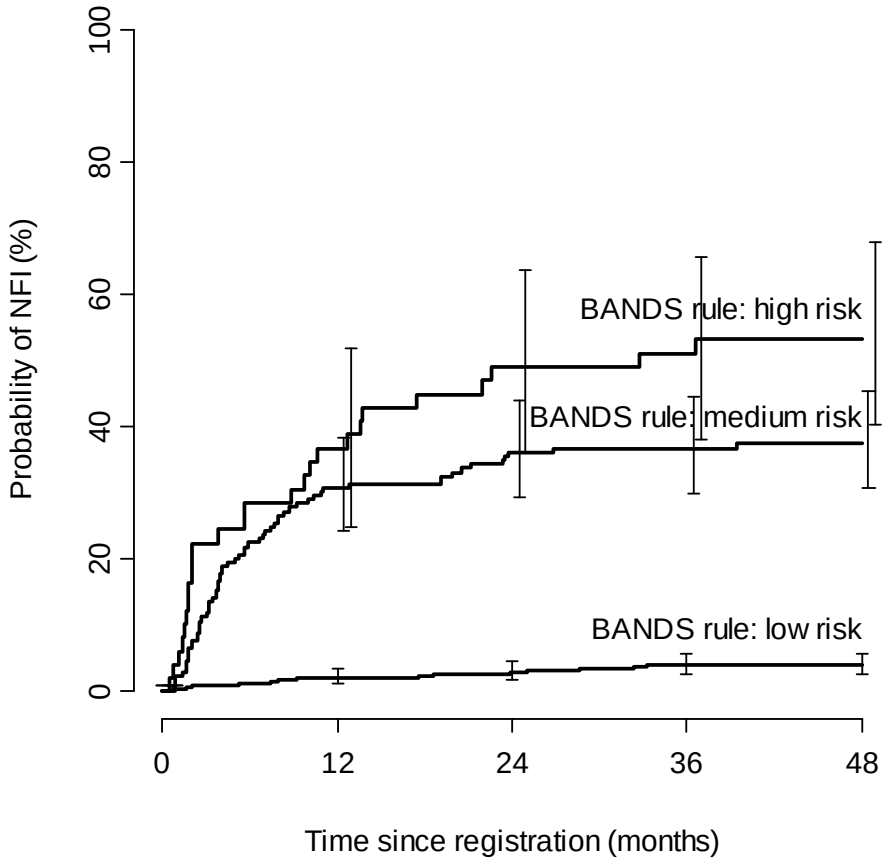
before, during and after treatment and when patients undergo leprosy reactions. All patients included here gave written informed consent to participate in the COLEP trial (ISRCTN 61223447), [10] a study approved by the Bangladesh Medical Research Council (BMRC/ERC/2001-2004/799). By giving written informed consent to participate in COLEP and accepting treatment they agreed that their data could be used anonymously for research.

Statistical analysis. Kaplan-Meier survival curves were used to determine the cumulative incidence of NFI for the risk groups defined by the prediction rules. Discriminative ability was expressed as a concordance (*c*) statistic (range 0.5–1.0). [18] Cox proportional hazards regression was used to identify independent variables that influenced the hazard ratio for NFI. The results are expressed as rate ratios or hazard ratios. Variables associated with NFI in univariate analyses ($p<0.10$) were selected for multivariable analysis in which stepwise backward selection was used to lessen the number of predictors, inclusion at $p<0.05$. Interactions between variables were tested but not included because they had limited predictive effects. The total number of monthly surveillances was calculated by multiplying the number in a risk group with the recommended surveillance period. The formula for routine surveillances was [(n PB*6)+(n MB*12)], and for surveillance based on the prediction rule the formula was [(n low risk*6)+(n medium risk*12)+(n high risk*24)]. The number of surveillances needed to detect 1 case is the total number of surveillances/NFI cases found. Data analyses were performed with SPSS for Windows (version 14.0 SPSS Inc., Chicago, IL) and R software (version 2.3.1 www.r-project.org).

Results

NFI occurred in 115 of 864 patients (13%; 95% confidence interval [CI] 11–16%).

The BANDS prediction rule defines NFI risk groups according to the WHO leprosy classification (PB/MB) and longstanding NFI at diagnosis. The low-risk group, comprised of PB patients without longstanding NFI at diagnosis, had a cumulative NFI incidence of 4.0% (95% CI 2.8–5.9% [Figure 1]),

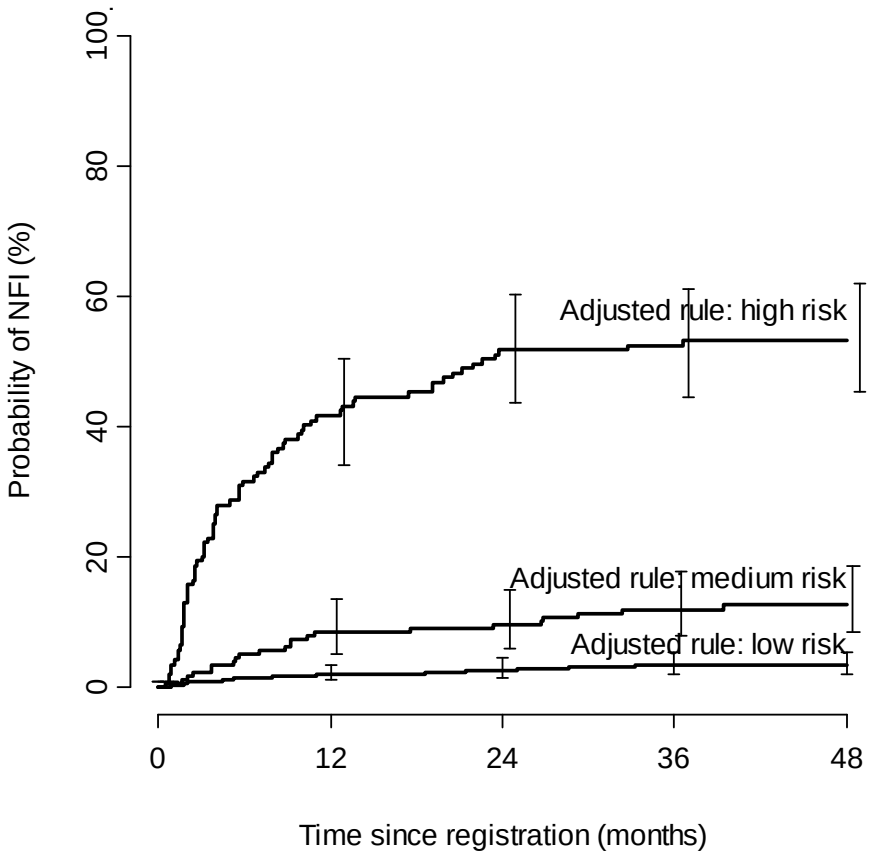


Numbers at risk

Low risk	646	632	627	592	219
Medium risk	169	117	108	92	29
High risk	49	31	25	21	3

Figure 1. Cumulative incidence of NFI for risk groups defined by the BANDS prediction rule, using WHO leprosy classification and longstanding NFI at diagnosis as predictive variables. *NFI=nerve function impairment, BANDS=Bangladesh acute nerve damage study.*

the medium-risk group—PB patients with and MB patients without longstanding NFI at diagnosis—of 37% (95% CI 30–45%) and the high-risk group—MB patients with longstanding NFI at diagnosis—of 53% (95% CI 40–68%). The cumulative incidences of NFI between the medium- and high-risk groups did not differ significantly.



Numbers at risk

Low risk	549	538	534	511	198
Medium risk	176	161	159	133	39
High risk	139	81	67	61	14

Figure 2. Cumulative incidence of NFI for risk groups defined by the adjusted prediction rule, using WHO leprosy classification and presence of anti-PGL-I antibodies as predictive variables. *NFI=nerve function impairment, PGL-I=phenolic glycolipid I.*

Substituting “long-standing NFI at diagnosis” with “anti-PGL-I antibodies” resulted in risk groups with cumulative incidences similar to those observed in the original BANDS study (Figure 2). [4] With the adjusted prediction rule the low-risk group—seronegative PB patients—had a cumulative incidence of NFI of 3.5% (95% CI 2.2–5.4%), the medium-risk group—seropositive PB patients and seronegative MB patients—of 13% (95% CI 8.5–19%), and the high-risk group—seropositive MB patients—of 53% (95% CI 45–62%). The cumulative incidences of NFI differed significantly between low-, medium-, and high-risk groups. The cumulative incidence of this medium-risk group is much lower than the 37% in the medium-risk group defined by the BANDS prediction rule.

Statistical analyses (Table 1) evaluated the association of NFI with sex, age, WHO classification, long-standing NFI at diagnosis, bacterial load, and anti-PGL-I antibodies. All variables but age were univariately associated with NFI ($p<0.05$). A multivariable analysis indicated that “WHO classification” and “anti-PGL-I antibodies” were significantly associated with NFI ($p<0.0001$). MB patients were at an increased risk of NFI (HR 8.0, 95% CI 5.0–13.0) compared to PB patients, and seropositive patients had an increased hazard risk of 2.9 (95% CI 1.8–4.6) compared to seronegative patients. When adjusted for WHO classification, the variables sex, age, bacterial load, and longstanding NFI at diagnosis were not significantly associated with NFI anymore.

Table 1. Cox proportional hazards regression analysis, determination of NFI risk factors.

			Univariate		Multivariable (full model)		Multivariable (selected)	
Variables	**Number**	**NFI event**	HR[a]	95% CI	HR[a]	95% CI	HR[a]	95% CI
All patients	864	115						
Sex								
Male	538	87	1		1			
Female	326	28	0.5	0.3-0.8	0.8	0.5-1.2		
Age (years)								
<15	136	11	0.6	0.3-1.2	0.7	0.3-1.3		
15-29	294	39	1		1			
30-39	161	25	1.2	0.7-2.0	1.1	0.6-1.8		
≥ 40	273	40	1.1	0.7-1.7	0.9	0.6-1.3		
WHO leprosy classification								
PB	669	29	1		1		1	
MB	195	86	13.4	8.8-21	7.5	4.4-13.0	8.0	5.0-13.0
Longstanding NFI at diagnosis								
No	792	86	1		1			
Yes	72	29	4.4	2.9-6.8	1.3	0.9-2.1		
Bacterial load[b]								
Negative	759	66	1		1			
Positive	91	48	8.2	5.7-12.0	1.0	0.6-1.6		
Anti-PGL-I serology								
Negative	605	31	1		1		1	
Positive	259	84	7.5	5.0-11.3	2.7	1.6-4.5	2.9	1.8-4.6

[a] HR=hazard ratio. [b] data missing for 14 patients. *CI=confidence interval, NFI=nerve function impairment.*

The observed *c* statistic for the BANDS prediction rule in our study was 0.79. The *c* statistic for the adjusted prediction rule was 0.81, showing a better discriminative ability. Table 2 shows the number of patients that would be classified differently with the adjusted prediction rule compared to the BANDS prediction rule. The adjusted prediction rule would place 115 of the low-risk group patients in the medium-risk group and 97 of the medium-risk group patients in the high-risk group; only 18 patients from the medium-risk group and seven patients from the high-risk group would be placed in a lower risk group.

Seventy-six (76/115, 66%) NFI events occurred while patients were undergoing routine surveillance. For the remaining 39 NFI events, additional surveillance would have been necessary for early detection. Extended surveillance using the BANDS prediction rule [4] led to the detection of an additional seven patients with NFI for a total of 83 (83/115, 72%: 726 extra visits needed). Using the adjusted prediction rule, the number of additional detected patients with NFI increased to 16, for a total of 92 (92/115, 80%: 2388 extra visits needed). With routine surveillance, 83.6 visits led to the detection of 1 case, for the BANDS prediction rule this was 85.3, and for the adjusted prediction rule 95.0.

Table 2. Agreement between NFI risk groups according to the BANDS prediction rule and the adjusted prediction rule.

	Adjusted rule			
BANDS rule	Low risk	Medium risk	High risk	**Total**
Low risk	531 (18)	115 (8)	0	646 (26) 4.0%
Medium risk	18 (1)	54 (13)	97 (49)	169 (63) 37.3%
High risk	0	7 (1)	42 (25)	49 (26) 53.1%
Total	549 (19)	176 (22)	139 (74)	
	3.5%	12.5%	53.2%	

Table shows number of patients per risk group and (number of patients with NFI event). Totals show number of patients, (number of patients with NFI event) and percentage of NFI events in that particular group.
NFI=nerve function impairment, BANDS=Bangladesh acute nerve damage study.

Discussion

Predicting NFI is important for identifying new leprosy patients that are at risk for nerve damage and, consequently, permanent disability. We describe an adjusted NFI prediction rule that replaces the variable “longstanding NFI at diagnosis” with “anti-PGL-I antibodies”.

The adjusted prediction rule was better able to identify patients at risk of developing NFI after diagnosis.

The original BANDS prediction rule for NFI is based on WHO leprosy classification and long-standing NFI at diagnosis. [4] A Kaplan-Meier survival analysis showed that the medium- and high-risk groups had similar survival curves (Figure 1), indicating that the BANDS prediction rule could not differentiate between these two groups. One explanation may be that the definition of NFI has changed since the BANDS study: a new NFI category, with less serious events that require a low dose course of prednisolone, was added to original NFI events that require a full dose course. [12] This leads to more patients being identified at an early stage of NFI. In addition, a smaller percentage of long-standing NFI (>6 months) and a higher percentage of recent NFI (<6 months), due to shorter detection delays, may have changed the contribution of longstanding NFI at diagnosis.

Presence of anti-PGL-I antibodies against *M.leprae* are a well-known risk factor for NFI. [5] In-depth analysis of all known risk factors for NFI in the current patient cohort showed that NFI is best predicted by “WHO classification” and “anti-PGL-I antibodies” (Table 1).
We adjusted the BANDS prediction rule by replacing “long-standing NFI at diagnosis” by “anti-PGL-I antibodies”. The adjusted rule was able to differentiate between three risk groups with significantly different cumulative incidences of NFI (Figure 2); the *c* statistic increased from 0.79 to 0.81. Unfortunately, we could not validate the adjusted prediction rule on the original BANDS cohort because no serology data were available.

The adjusted prediction rule distinguished three risk groups comparable to those in the BANDS study (Figure 2). [4] Therefore, the surveillance recommendations that were based on the BANDS study [4] can be maintained (see Materials and methods). When replacing the

BANDS prediction rule with the adjusted prediction rule 212 patients were reassigned to a higher risk group and 25 patients to a lower risk group (Table 2), suggesting that the adjusted prediction rule has considerable implications for patient care. The reassignment of these patients to a higher risk group is warranted because they have a higher-than-average risk to develop NFI: 7% for patients moving from the low to the medium risk group and 51% for patients moving from the medium to the high risk group. The adjusted prediction rule can thus be used to identify a substantially higher number of new NFI cases than either routine or BANDS rule based surveillance and offers increased opportunity to prevent nerve damage in leprosy. However, the number of visits needed to detect one case is higher than with alternative strategies. We consider this operationally feasible and medically justifiable in view of the serious consequences of NFI, including life-long disability.

WHO classification is a good predictor of future NFI [6] but it rather crudely divides leprosy patients into two groups (PB and MB). The presence of anti-PGL-I antibodies is known to correlate with the bacterial load, [16] and thus offers a further refinement of the WHO classification into patients with high and low bacterial loads. This may explain the added predictive value of the presence of antibodies. In contrast to the BANDS rule the adjusted rule uses two variables that do not include NFI. This offers the possibility of predicting NFI before it actually occurs.

We expect that the adjusted NFI prediction rule will be relevant in other settings, since the predicting variables are well defined and easily determined, but it should be validated externally. We believe that the adjusted prediction rule can be applied in current health services, since it fulfils the need for simplified guidelines and diagnostic protocols. Contrary to the BANDS prediction rule, the adjusted rule does not rely on a specialist physiotechnician for the prediction. However, this person

is needed to document the baseline nerve status and for surveillance during follow up examinations. Recently, a simple anti-PGL-I field test was developed that gives results within ten minutes, [8,9] making routine testing feasible. Thus, leprosy diagnosis and NFI prediction can be accomplished during a single consultation. Additional benefits of the anti-PGL-I test are that it assists with the classification and aids diagnosis of leprosy patients with doubtful clinical signs. [8,9,16]

With the adjusted prediction rule, the necessity to continue surveillance beyond the treatment period can be determined. New leprosy patients can be assigned to an NFI risk group, and appropriate surveillance can be planned. Nerve damage can thus be successfully prevented despite the fact that leprosy control has been integrated into general health services.

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Chapter 5

Protective effect of the combination BCG vaccination and rifampicin prophylaxis in leprosy prevention

Ron P. Schuring, Jan Hendrik Richardus, David Pahan, Linda Oskam. Protective effect of the combination BCG vaccination and rifampicin prophylaxis in leprosy prevention.

In press Vaccine

Abstract

BCG vaccination and rifampicin chemoprophylaxis are both strategies for leprosy prevention. While the combined effect is unknown, the combination may give the desired push to halt leprosy transmission. Secondary analysis was done on results from a single centre, double blind, cluster randomized, placebo controlled trial. Individually, BCG and rifampicin showed to protect against leprosy (57% [95% CI 24-75%] and 58% [95% CI 30-74%], respectively). The combined strategies showed a protective effect of 80% (95% CI 50-92%). This is the first time that the additive effect of BCG and rifampicin are shown; the combined strategies can possibly lower leprosy incidence.

Introduction

Leprosy is an infectious disease caused by *Mycobacterium leprae*, which still persists in Asia, South-America and Africa, despite the availability of effective and free antimycobacterial treatment. In the past years emphasis in leprosy has been on the availability and accessibility of control activities, which include diagnosis, treatment with multidrug therapy (MDT), patient and family counseling, community education, prevention of disabilities/impairments, rehabilitation and referral for complications. [1] While these control activities need to be continued and where possible strengthened, innovative approaches are necessary to further lower leprosy incidence. The research community is actively involved in the development of prevention strategies, with the eventual aim to halt transmission of *M.leprae*. Methods under study are, among others, immunoprophylaxis and chemoprophylaxis. [2]

Bacille Calmette-Guérin (BCG) vaccination, originally developed for tuberculosis, is currently the only candidate for immunoprophylaxis in leprosy. It is part of the World Health Organization's (WHO) Expanded Program of Immunization and is known to decrease the risk of leprosy. [3,4] Protection against leprosy by neonatal BCG vaccination can persist over long periods of time, [5] but the positive impact of BCG revaccination for people in contact with leprosy patients is disputable. [4,6,7]

Chemoprophylactic regimens in leprosy have been studied as well. Two recent studies showed that rifampicin chemoprophylaxis is protective against leprosy. [8,9] In a randomized controlled trial by Moet et al. (2008) a single dose of rifampicin gave a 57% reduction in leprosy incidence during the first two years. [8] Bakker et al. (2005) showed 75% reduction after 33.5 months with two doses of rifampicin supplied to the complete population of three small islands. [9] No reduction,

however, was seen in a neighbouring island population where only spatially defined contacts of leprosy patients received rifampicin. [9] Both studies found that the protective effect was strongest in the contact groups furthest away from the index patients.

Both strategies are known to prevent leprosy, but the effect of the combination of these interventions has not yet been determined. By studying the protective effect of BCG vaccination given in infancy, in combination with rifampicin prophylaxis given to contacts of leprosy patients, we aim to establish evidence for the combined effect of these preventive strategies.

Materials and Methods

Study population, intake and follow-up. The study population consisted of inhabitants of the Nilphamari and Rangpur districts in northwest Bangladesh who participated in the COLEP trial (ISRCTN 61223447). The trial is a single centre, double blind, placebo controlled, cluster randomized trial to determine the effectiveness of rifampicin to prevent leprosy in close contacts of leprosy patients. The protocol and primary outcome have been published elsewhere. [8,10] Ethical approval for the COLEP study was granted by the Ethical Review Committee of the Bangladesh Medical Research Council in Dhaka (reference no.: BMRC/ERC/2001-2004/799).

The intake started in June 2002 and was completed by the end of December 2003. The study population consisted of 21,711 contacts and 1,037 index patients. For the current analysis an additional exclusion criterion was missing BCG data, resulting in 21,526 contacts and 1,028 index patients. Contacts were categorized according to their physical and genetic distance to the index patient. A person could only be included in the contact group of one patient.

The primary outcome of the trial was the development of clinical leprosy. Leprosy patients were classified as paucibacillary (PB) or multibacillary (MB) according to the 1998 WHO leprosy classification for treatment purposes. Contacts were asked to return to the clinic if they suspected to have signs or symptoms of leprosy; in addition they were actively followed up after 24 and 48 months. Leprosy diagnosis was confirmed by a leprosy control officer and a medical officer with minimum of 5 years' experience in the diagnosis of leprosy at referral centre level.

Chemo- and immunoprophylactic interventions. At intake—that is, after the index patient had received the second supervised dose of MDT—all eligible contacts of a patient received a single dose of either rifampicin or placebo under direct supervision of a staff member. The dosage schedules were 600 mg for adults weighing 35 kg and over, 450 mg for adults weighing less than 35 kg and for children older than 9 years, and 300 mg for children aged 5 to 9 years. BCG vaccination status was assessed by examining both upper arms for the presence of a BCG scar. The vaccination program in Bangladesh is according to WHO recommendations with a dose of 0.05 ml scheduled within the first year after birth.

Statistical methods. Statistical analyses were done using SAS software, version 9.1 and SPSS software, version 16.0. We used techniques for the analysis of complex survey samples to account for the clustering at the level of the index patient in the sample. Bivariate associations were investigated using the SAS procedure "proc surveyfreq" and the Rao Scott chi square test instead of the Pearson chi square test. We also used the SAS procedure "proc surveylogistic" instead of the ordinary logistic procedure. We report odds ratios, but because of the low prevalence of the outcome these are comparable with relative risks. The protective effect of BCG was defined as 100*(1 – OR). [11] A significance level of 5% was used in all tests. For

multivariate analysis the risk variables age, sex, and physical distance to and classification of index patient were considered. The BCG protective effect against development of MB leprosy was analysed using logistic regression among the index patients with adjustment for the variables age, sex and geographical district.

Results

BCG frequency among contacts of leprosy patients. The BCG frequency among the contacts of leprosy patients was 40% and well balanced over both arms of the trial for all characteristics except for age (Table 1). The population under 20 years of age had a distinctly higher BCG coverage (60%) than people aged 20 years or older (25%); this difference was the same between the two study arms of the trial.

Table 1. BCG frequency among contacts of leprosy patients at intake, by variable category and stratified for intervention: placebo and rifampicin.

	Placebo group		**Rifampicin group**	
Variable	no.	% BCG	no.	% BCG
Total	10776	40	10750	40
Age (in years)				
< 20	4601	60	4606	59
≥ 20	6175	25	6144	25
Sex				
male	5134	41	5116	41
female	5642	39	5634	38
Physical distance				
shares kitchen only				
or kitchen and house	1756	41	1749	41
other	9020	40	9001	40
Genetic distance				
closely related[a]	1707	40	1629	40
not closely related[b]	9069	40	9121	40
Index patient				
multibacillary	3128	40	2881	42
paucibacillary	7648	40	7869	39

[a] Parent, child or sibling. [b] Other than parent, child or sibling

BCG frequency among index patients. The BCG frequency among the index patients differed for leprosy type: 20% of the MB and 29% of the PB patients were vaccinated, showing a statistically significant association of BCG with leprosy type; the adjusted OR for being vaccinated was 0.70 (96% CI 0.49-0.98) in MB patients, compared to PB patients (data not shown, adjusted for age, sex and geographical district).

Protective effect of BCG against leprosy. Moet et al. (2008) showed that chemoprophylaxis with rifampicin given to contacts of newly diagnosed leprosy patients was effective at preventing the development of clinical leprosy during the first two years. The effect was maintained, but no difference was seen between the treatment arms of the trial beyond two years. [8] Table 2 shows the univariate effect of BCG vaccination on leprosy incidence during the first two years by variable category and stratified by intervention. In both trial arms, BCG had approximately the same overall protective effect (57% and 52% for placebo and rifampicin arm, respectively), although statistical significance was only present in the placebo arm. BCG vaccination appears to be effective (95% CI < 1) in male contacts and in contacts with more physical- or genetic distance to the index patient in both arms of the study. In addition, in the placebo arm, BCG vaccination appears to be effective (95% CI < 1) in contacts aged 20 years and older and in contacts of PB patients. The same overall protective effect for BCG was seen in multivariate analysis; 56% (95% CI 23-75%) in the placebo arm and 53% (95% CI 0-82%) in the rifampicin arm. Furthermore, the BCG effect on leprosy incidence persisted after four years of follow-up, the protective effect of BCG vaccination was 48% in the placebo arm (95% CI 19-67%) and 41% in the rifampicin arm (95% CI 0-69%) (data not shown).

Table 2. Effect of BCG vaccination on the incidence of leprosy after two years of follow-up by variable category and stratified for intervention: placebo and rifampicin.

	BCG		No BCG				
Variable	Leprosy	No leprosy	Leprosy	No leprosy	**OR 95% CI univariate**		**Protective effect BCG (%) [100*(1-OR)]**
PLACEBO arm							
Total	15	4291	52	6418	0.43	0.25-0.75	57 [c]
Age (in years)							
<20	10	2734	14	1843	0.48	0.21-1.09	52
≥20	5	1557	38	4575	0.39	0.16-0.97	61 [c]
Sex							
male	8	2112	29	2985	0.39	0.18-0.83	61 [c]
female	7	2179	23	3433	0.48	0.21-1.10	52
Physical distance							
shares kitchen only or kitchen and house	5	715	13	1023	0.55	0.20-1.53	45
other	10	3576	39	5395	0.39	0.20-0.76	61 [c]
Genetic distance							
closely related[a]	4	672	14	1017	0.43	0.15-1.26	57
not closely related[b]	11	3619	38	5401	0.43	0.23-0.83	57 [c]
Index patient							
multibacillary	6	1250	15	1857	0.59	0.26-1.37	41
paucibacillary	9	3041	37	4561	0.37	0.18-0.75	63 [c]
RIFAMPICIN arm							
Total	7	4259	22	6462	0.48	0.19-1.20	52 [c]
Age (in years)							
<20	4	2724	7	1871	0.39	0.10-1.61	61
≥20	3	1535	15	4591	0.60	0.17-2.07	40
Sex							
male	2	2107	15	2992	0.19	0.04-0.84	81 [c]
female	5	2152	7	3470	1.15	0.32-4.13	0
Physical distance							
shares kitchen only or kitchen and house	5	703	8	1033	0.92	0.29-2.96	8
other	2	3556	14	5429	0.22	0.05-0.96	78 [c]
Genetic distance							
closely related[a]	5	641	8	975	0.95	0.27-3.30	5
not closely related[b]	2	3618	14	5487	0.22	0.05-0.96	78 [c]
Index patient							
multibacillary	2	1196	8	1675	0.35	0.04-2.83	65
paucibacillary	5	3063	14	4787	0.56	0.21-1.47	44

[a] Parent, child or sibling. [b] Other than parent, child or sibling. [c] significant $p<0.05$. (data not shown, adjusted for age, sex, physical distance to and classification of index patient).

Additive protective effect of BCG and rifampicin against leprosy. In table 3 the effect of the combination of BCG and rifampicin chemoprophylaxis is shown after two years of follow-up. In both univariate and multivariate analysis, a protective effect was seen in contacts who received either rifampicin or BCG, compared to contacts with no intervention (BCG adjusted protective effect was 57% [95% CI 24-75%]; for rifampicin 58% [95% CI 30-74%]). In contacts receiving both interventions—BCG and rifampicin—the protective effect was 80% (95% CI 50-92%).

Table 3. Additive effect of BCG and rifampicin intervention on incidence of leprosy after two years of follow-up by variable category.

			OR 95% CI				Protective effect (%)
Intervention	**Leprosy**	**No leprosy**	Univariate		Multivariate[a]		**[100*(1-OR)]**
none	52	6418	1		1		
rifampicin only	22	6462	0.42	0.26-0.69	0.42	0.26-0.70	58
BCG only	15	4291	0.43	0.25-0.75	0.43	0.25-0.76	57
rifampicin and BCG	7	4259	0.20	0.08-0.49	0.20	0.08-0.50	80

[a] adjusted for age, sex, physical distance to and classification of index patient.

Discussion

This study shows that BCG vaccination in infancy halves the risk of getting leprosy in a high-risk population consisting of contacts of newly diagnosed leprosy patients. For the first time we show here that this effect is additive to the effect of the chemoprophylactic intervention with rifampicin. These strategies may therefore be combined with the aim to lower the incidence of leprosy.

The strength of this study is its robust design, being a single centre, double blind, placebo controlled, cluster randomized trial with a

large number of participants included in a short period of time. [10] When interpreting the results, it should be considered that the study was conducted in a high leprosy endemic area and that the results may be different in low leprosy endemic areas, although we see no particular reason to assume that this will be the case. Further research in different geographic and endemic areas is necessary to confirm this. The results confirm previous observations that BCG vaccination protects against leprosy. [3,4] It is, however, the first time that the combination of immuno- and chemoprophylactic strategies for leprosy control is studied and therefore we cannot compare our results with others. Yet the additive effect of both strategies was a rational outcome because they differ in their protective approach.

In tuberculosis prevention, the merit of BCG vaccination has been discussed continually and new vaccines are being developed with higher protection rates and fewer side effects. [12] The protectiveness against leprosy of this new generation of vaccines has yet to be determined, while it is well known that BCG protects against leprosy. The replacement of BCG by newer, more TB-specific vaccines may thus be detrimental for leprosy control.

We described previously that in our study population chemoprophylaxis with rifampicin had its main protective effect in those contacts groups with the lowest risk profile based on the intake data. [13] Analysis of the protective effect of BCG showed a similar pattern: contacts of a PB index patient and more physical and genetic distance to the index patient seem to benefit the most. A possible explanation for these findings could be that the prophylactic interventions used here—be it immunoprophylaxis with BCG or chemoprophylaxis with a single dose of rifampicin—are not sufficient for more heavily *M.leprae* infected contacts. Such contacts will be found more frequently in the higher risk groups such as physically or genetically close contacts, and contacts of MB patients. Higher infection pressure, higher bacterial load and/or

genetic host factors could cause this effect. There was one exception: males are known to be at high risk than females. [13] In contrast, males benefitted more from the BCG vaccination. Since other risk factors, such as the above stated infection pressure and genetic host factors, are randomly distributed between the sexes, the most logical explanation for this differential risk pattern seems to be subtle differences in immune responses between males and females. Finally, our study also showed that in the index patients a statistically significant lower BCG frequency was observed among the MB patients, compared to the PB patients. This may imply that BCG protects against the development of MB leprosy, an observation that was in agreement with the meta-analysis by Setia et al. [4]

This is the first time that the additive effect of immunoprophylaxis by routine infancy BCG vaccination and chemoprophylaxis with rifampicin given to close contacts of newly diagnosed leprosy patients is demonstrated. Rifampicin and BCG vaccination are effective as a combined strategy to lower leprosy incidence. Monitoring of close contacts however, particularly at household level and blood relatives, remains necessary even when both immuno- and chemoprophylaxis are supplied.

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Chapter 6

Association of anti-PGL-I serology with leprosy, results form a large prospective cohort

Ron P. Schuring, Jan Hendrik Richardus, Caspar Looman, David Pahan, Paul R Klatser, Linda Oskam. Association of anti-PGL-I serology with leprosy, results form a large prospective cohort.

submitted for publication

Abstract

Objective. To examine the potential of anti-PGL-I IgM serology to identify high risk groups for leprosy.

Methods. Single centre, cluster randomized, placebo-controlled trial to determine the chemoprophylactic effect of single dose rifampicin to prevent leprosy in contacts of newly detected leprosy patients. Analysis included 18,761 contacts followed for four years at two-yearly intervals.

Results. The presence of anti-PGL-I IgM at intake was statistically significantly associated with the future development of multibacillary (MB) leprosy (HR 6.0, CI 95% 2.15-16.5), but not with paucibacillary (PB) leprosy (HR 0.5, CI 95% 0.15-1.48). This association was stronger for contacts that became seropositive (seroconverted) at first follow-up (HR for MB leprosy 15.6, CI 95% 3.73-65). Chemoprophylaxis suppressed the development of clinical disease for at least two years, but did not suppress seroconversion.

Conclusions. Seropositivity and seroconversion of leprosy contacts are associated with MB leprosy disease. Due to the low incidence of leprosy in general, the predictive value remains low, hence further research is needed to find additional markers for the detection of leprosy infection and for the diagnosis of especially pre-clinical and PB leprosy.

Introduction

Leprosy is an infectious disease caused by *Mycobacterium leprae*, and still persists in Asia, South-America, and Africa despite the availability of effective treatment. Emphasis in leprosy control is on early detection and treatment of patients to avoid nerve damage and resulting physical disabilities and to reduce the transmission of *M.leprae*. [1] The leprosy research community is actively developing tests for early diagnosis and prevention strategies with the aim to reduce leprosy incidence. We hypothesize that anti-phenolic glycolipid-I (PGL-I) IgM serology can help focusing leprosy control activities on high risk groups for leprosy.

Contacts of leprosy patients have an increased risk of developing leprosy compared to the general population [2] and the use in this group of either chemoprophylaxis, [3-5] immunoprophylaxis, [6] or a combination of both [7] reduces the incidence of leprosy and may also have an impact on transmission. By further refining the definition of "high risk" for contacts, the effectiveness of prophylactic treatment may be further enhanced.

Douglas et al. (2004) studied the presence of anti-PGL-I IgM in contacts of multibacillary (MB) leprosy patients in a long-term prospective study and found that serology can identify contacts at high risk of developing leprosy: seropositivity gave a seven-fold increased risk. [8] The main shortcoming of this study however, was that contacts of paucibacillary (PB) leprosy patients were excluded, thereby overestimating the results, since contacts of MB patients have in general a higher risk of developing leprosy. Therefore, confirmation of these results is required for contacts of MB patients together with expansion of the investigations to contacts of PB patients. Two other prospective studies also found increased risks for leprosy among contacts positive

for anti-PGL-I antibodies. Bakker et al. (2006) reported an almost four-fold higher risk [9] and Goulart et al., [10] found a six-fold higher risk for leprosy per se. In all three studies it was suggested that chemoprophylaxis may be suitable for the high risk group “seropositive for anti-PGL-I IgM” to prevent the development of clinical disease.

The data from a large randomized controlled trial of chemoprophylaxis with a single dose of rifampicin [11] were used to determine the risk of developing leprosy for seropositive and seronegative contacts of leprosy patients with or without a chemoprophylactic intervention and to establish the association of serology with clinical leprosy.

Materials and Methods

Study population. The study population consisted of participants of the COLEP trial (ISRCTN 61223447), which is a single centre, double blind (for intervention and serology testing), placebo controlled, cluster randomized field trial in Bangladesh to determine the effectiveness of single dose rifampicin chemoprophylaxis to prevent leprosy among contacts of leprosy patients. [5,11] Approval was granted by the Ethical Review Committee of Bangladesh Medical Research Council (BMRC/ERC/2001-2004/799). All participants gave written informed consent.

In short, for this part of the study we included 18,761 contacts of 1,037 new leprosy patients (index patients) that were registered at the Rural Health Program (RHP) in the Nilphamari and Rangpur districts in northwest Bangladesh. The intake of the contacts started in June 2002 and was completed by the end of December 2003. Contacts were categorized according to their physical and genetic distance to the index patient. Exclusion criteria for the contacts were: refusing informed

consent; being pregnant; receiving tuberculosis or leprosy treatment at intake; suffering from (previously undiagnosed) leprosy at intake; younger than 5 years of age; suffering from liver disease or jaundice; or residing temporarily in the area. A person could only be included in the contact group of one patient. Finger prick blood samples for anti-PGL-I antibodies were taken from all index patients during intake and from all contacts during intake and follow-up. All samples were collected on Schleicher & Schuell blotting paper GB 002, air dried and stored at –20°C until transport to the Netherlands.

Intervention. After the index patient had received the second dose of multidrug treatment (4 weeks after the first dose), all contacts of the patient received blindly either rifampicin or placebo under direct supervision of a staff member. The following dosage schedule was used: adults weighing ≥35 kg: 600 mg; adults weighing <35 kg and children >9 years: 450 mg; and children 5-9 years: 300 mg.

Follow-up. The primary outcome was the development of clinical leprosy. Contacts were asked to return to the clinic if they had signs or symptoms of leprosy; in addition, they were actively followed up after 24 and 48 months. If leprosy was diagnosed, the date of official registration was recorded. For each newly found leprosy patient, the disease was confirmed by a leprosy control officer and a medical officer with minimum 5 years experience in the diagnosis of leprosy at referral centre level. Microscopy on slit skin smears was also performed. All patients were classified as either paucibacillary (PB) or multibacillary (MB) according to the 1998 WHO classification for treatment purposes (12).

Serology. The presence of IgM antibodies against *M.leprae* was determined with a previously described enzyme-linked immunosorbent assay (ELISA). [13] A terminal trisaccharide of PGL-I linked to bovine serum albumin (BSA) via a phenolic ring (NT-P-BSA) was used as a

semi-synthetic analogue. [14] The level of IgM antibodies against *M.leprae* was expressed as net optical density (OD): the absorbance of NT-P-BSA minus that of BSA-coated wells at 450 nm. Tests were performed without knowledge of individual leprosy risk. Three control sera were tested on each plate: a standard four times, a negative and positive control sera two times. Standard serum was used to reduce day-to-day variation and if needed samples were retested.
The following definitions for serology results were used in the analysis:

- The status "seropositive at intake" was assigned if the net OD was ≥0.200 at intake.
- "Seroconversion" was defined as seropositive at the first follow-up (24 months), but seronegative at intake.

Statistical analysis. Data were analyzed using SPSS for Windows version 16. The dataset was not a random sample, but consisted of groups around the index patients. To correct for the dependency of observations caused by this design, we calculated the over-dispersion parameters for each outcome separately (leprosy per se, MB and PB). [15] None of these over-dispersion parameters was significant, so we concluded that clustering did not influence the significance of the results. When analyzing MB or PB cases specifically we censored the cases of the other type at the moment of incidence. Associations were investigated using the SPSS procedure "GENLIN", with a Poisson distribution and link log and an offset variable "ln(time)". A significance level of 5% was used in all tests. For multivariate analysis the risk variables age, sex, BCG scar, intervention, physical distance to and classification of the index patient were considered. We converted the probabilities of having developed leprosy during the follow-up period of two years to incidence rates at one year assuming a constant hazard during the period (rate=ln (leprosy/(number at risk*2)). To obtain confidence intervals for rates we applied standard errors (sqrt 1/leprosy) around the log (rate).

Results

Study population follow-up. Originally, 21,711 contacts were included in COLEP. [5] Missing data on serology at intake was an additional exclusion criterion for the present analyses, resulting in 18,761 contacts at intake and 81 new patients after 24 months and 50 new patients after 48 months.

Association between serological status at intake and leprosy incidence—placebo arm. The univariate association of serology and leprosy incidence among contacts who did not receive chemoprophylaxis is shown in table 1 (under placebo arm). In the placebo arm, a total of 579 out of 9351 contacts were seropositive at intake, giving a seroprevalence of 6.2%. Among these 579 seropositive contacts, a total of six new patients were found after 4 years of follow-up — 4 with MB and 2 with PB leprosy. The MB incidence rate among contacts seropositive at intake is higher than seronegative contacts, 6.9 versus 0.9/1000 person years at risk (PYAR) (IR 7.5, CI95% 2.3-25), whereas the PB incidence rate is lower, 3.5 versus 7.4/1000 PYAR (IR 0.5, CI95% 0.1-11.9). Since seropositivity was positively correlated with the development of MB leprosy but negatively with PB leprosy, the association with leprosy per se was non-informative and thus omitted. Of the 4 new MB patients found among the seropositive contacts at intake, 3 were found after two years and 1 additional patient after four years, giving MB incidence rates of 5.2 and 1.7/1000 PYAR, respectively. This indicates that the serological status at intake has a higher association with MB at the shorter 2-year term than at the longer 4-year term.

Association between serological status at intake and leprosy incidence—rifampicin arm. The univariate association of serology and leprosy incidence among contacts who received chemoprophylaxis is also shown in table 1—rifampicin arm. In the rifampicin arm, a total of

536 out of 9,410 contacts were seropositive at intake (5.7%). One new MB patient was found among these seropositive contacts (incidence 1.9/1000 PYAR).

Table 1. Cases of leprosy among contacts by anti-PGL-I serological status at intake during four years' follow-up.

anti-PGL-I serology at intake	number at risk	leprosy inc. after 2 years		leprosy inc. after 4 years					MB incidence rate/10.000 PYAR (95% CI)[a]
		PB	MB	PB	MB	total	PB inc.	MB inc.	
Both arms									
positive	1115	2	4	1	1	8	2.7	4.5	5.3 (1.9-14)
negative	17646	69	6	39	9	123	6.1	0.9	1
Placebo arm									
positive	579	1	3	1	1	6	3.5	6.9	7.5 (2.3-25)
negative	8772	49	4	16	4	73	7.4	0.9	1
Rifampicin arm									
positive	536	1	1	0	0	2	1.9	1.9	2.4 (0.3-19)
negative	8874	20	2	23	5	50	4.8	0.8	1

[a] PYAR= person years at risk; 95% CI= 95% confidence interval.
PB= paucibacillary leprosy; MB= Multibacillary leprosy; inc.= incidence.

Effect of chemoprophylaxis on seroconversion and leprosy incidence. An additional serological test was performed at first follow-up (after 24 months), enabling analysis of the effect of rifampicin on leprosy seroconversion and incidence (table 2). Chemoprophylaxis suppresses the development of clinical disease for at least two years. A reduction of incidence was seen in the rifampicin arm after the first two years of follow-up: the placebo arm had 57 new cases versus 24 in the rifampicin arm. The incidence however, was similar in the third and fourth year of follow-up with the placebo arm having 22 new cases and the rifampicin arm 27. Detailed analysis is reported elsewhere. [5] Interestingly, chemoprophylaxis with rifampicin did not suppress seroconversion. Despite the decline in incidence of clinical disease in the first two years, the number of contacts that seroconverted during this

period is nearly equal (placebo 4.4% versus rifampicin arm 4.0%, p=0.263).

Table 2. Cases of leprosy among contacts by anti-PGL-I serological status at first follow up during four years' follow-up.

anti-PGL-I serology after 2 years follow up[c]	number at risk	leprosy inc. after 2 years[b] PB	MB	leprosy inc. after 4 years PB	MB	total	PB inc.	MB inc.	MB incidence rate/10.000 PYAR (95% CI)[a]
Both arms (-2561)									
seroconverted	632	2	2	0	4	4	nd	6.3	18.2 (4.9-68)
(still) negative	14453	60	3	32	5	37	2.2	0.3	1
Placebo arm (-1272)									
seroconverted	328	2	2	0	2	2	nd	6.1	21.7(3.1-154)
(still) negative	7172	43	1	13	2	15	1.8	0.3	1
Rifampicin arm (-1289)									
seroconverted	304	0	0	0	2	2	nd	6.6	15.9 (2.7-95)
(still) negative	7281	17	2	19	3	22	2.6	0.4	1

[a] PYAR= person years at risk; 95% CI= 95% confidence interval. [b] data not used for prospective analysis. [c] data from contacts who were seronegative at intake.
PB= paucibacillary leprosy; MB= Multibacillary leprosy; inc.=incidence.

Association between seroconversion and leprosy incidence—placebo arm.

Of the 7,500 seronegative contacts at intake, 328 (4.4%) became seropositive (seroconverted) after 2 years follow up (Table 2). Among these seroconverted contacts, no PB and 2 MB leprosy patients were found. The MB incidence rate among the seroconverted contacts is higher compared to contacts who remained seronegative: 6.1 versus 0.3/1000 PYAR (IR 21.7, 95% CI 3.1-154).

Association between seroconversion and leprosy incidence—rifampicin arm. Of the 7,585 seronegative contacts at intake, 304 (4.0%) seroconverted. Among these seroconverted contacts, no PB and 2 MB leprosy patients were found. The MB incidence rate among the

seroconverted contacts is higher compared to contacts that remained seronegative: 6.6 versus 0.4/1000 PYAR (IR 15.9, 95% CI 2.7-95).

Difference in association between seropositivity at intake or seroconversion and leprosy incidence. Notably, contacts who seroconverted during follow-up had a higher association with the development of MB leprosy than contacts who were seropositive at intake. The relative risks (RR_{MB}) for serology at intake were 7.5 and 2.4 for the placebo and rifampicin arms, respectively; whereas these risks for the contacts who seroconverted between intake and 2 years were 21.7 and 15.9 (see Tables 1 and 2). Despite the protective effect of rifampicin on leprosy incidence in the first two years, similar percentages of MB patients were seropositive prior to diagnosis in the placebo and rifampicin arms: 6/12 (50%) in the placebo arm versus 3/8 (38%) in the rifampicin arm ($p = 0.670$). Moreover, the MB incidence rates for the period 2-4 years, when rifampicin did not protect anymore, were similar: 6.1 versus 0.3/1000 PYAR for seroconverted versus still seronegative contacts in the placebo arm and 6.6 versus 0.4/1000 PYAR in the rifampicin arm ($p = 1.000$).

Association between seropositivity and leprosy incidence—whole study population. Chemoprophylaxis did not show interaction in the association of serological status and leprosy. Hence, no stratification for the intervention was required and therefore “intervention” was treated as a variable in the analysis. The multivariate association of serological status and leprosy incidence is shown in table 3. Overall, 1,115 out of 18,761 contacts (5.9%) were seropositive at intake. Serological status was associated with MB leprosy: contacts that were seropositive at intake had a six times higher risk of developing MB leprosy than seronegative contacts (adjusted 95% CI 2.15-16.5). Contrary to this significant predictive effect ($p < 0.001$), we found a non-significant effect for PB leprosy ($p = 0.11$). Contacts who seroconverted had a 16 times higher risk of developing MB leprosy than seronegative contacts

(adjusted 95% CI 3.73-65.4)(data not shown). The association between seroconversion and PB incidence could not be determined as no new PB patients were found among the contacts that had seroconverted.

Discussion

Our results show that the presence of anti-PGL-I antibodies is associated with the future development of MB leprosy—seropositive contacts at intake and especially those who had seroconverted at the 2-year follow-up had an increased risk of developing MB leprosy. The chemoprophylactic intervention reduced the number of cases with leprosy disease at 2-year follow-up, but not the number of seroconversions during this period.

The strength of this study is its robust design, being a single centre, double blind, placebo controlled, cluster randomized trial with a large number of participants included in a short period of time. [11] When interpreting the results, it should be considered that the study was conducted in a high leprosy endemic area and that the results may be different in low leprosy endemic areas, although we see no particular reason to assume that this will be the case. Further research in different geographic and endemic areas is necessary to confirm this. Our results confirm previous observations of a positive association of anti-PGL-I antibodies with leprosy incidence. [8-10] In these studies it was suggested that chemoprophylaxis may be suitable for the high risk group “seropositive for anti-PGL-I IgM” to prevent the development of leprosy disease.

Our results indicate that anti-PGL-I IgM detection is not predictive for PB leprosy or leprosy per se. Presence of anti-PGL-I IgM has a strong association with MB leprosy, but since overall numbers are low, the serological test lacks predictive value. However, the results

Table 3. Association between serology at intake and leprosy incidence of contacts after 4 years of follow-up—stratified for classification.

Variables	Category	aHR[a] for leprosy per se	95% CI
Sex	Female	1	
	Male	1.8	1.28 - 2.60
BCG	Yes	1	
	No	2.0	1.34 - 2.92
Intervention	Rifampicin	1	
	Placebo	1.5	1.09 - 2.20
Serology[b]	Negative	1	
	Positive	1.1	0.5 - 2.28
Index patient	PB	1	
	MB	1.4	0.98 - 2.03
Physical distance	not close	1	
	Close	2.1	1.42 - 3.03
		aHR[a] for MB leprosy	**95% CI**
Sex	Female	1	
	Male	5.4	1.80 - 16.3
BCG	Yes	1	
	No	3.2	1.08 - 9.74
Intervention	Rifampicin	1	
	Placebo	1.5	0.61 - 3.62
Serology[b]	Negative	1	
	Positive	6.0	2.15 - 16.5
Index patient	PB	1	
	MB	3.7	1.55 - 9.06
Physical distance	not close	1	
	Close	7.6	3.09 - 18.6
		aHR[a] for PB leprosy	**95% CI**
Sex	Female	1	
	Male	1.5	1.06 - 2.30
BCG	Yes	1	
	No	1.8	1.20 - 2.78
Intervention	Rifampicin	1	
	Placebo	1.6	1.07 - 2.29
Serology[b]	Negative	1	
	Positive	0.5	0.15 - 1.48
Index patient	PB	1	
	MB	1.2	0.77 - 1.75
Physical distance	not close	1	
	Close	1.5	0.99 - 2.40

[a] aHR= adjusted hazard ratio; adjusted for variables: sex, BCG, intervention, serology, index patient and physical distance. 95% CI = 95% confidence interval. [b] anti-PGL-I IgM serology results at intake, positive was an optical density greater than 0.199nm.

from this cohort did indicate that of all 19 MB patients, 8 (42%) were seropositive prior to diagnosis. Therefore, serology may contribute to a preventive strategy in which MB disease is targeted specifically. A potential benefit of such a strategy could be the interruption of transmission: MB leprosy is considered as the most infectious form of the disease and MB patients can be expected to transmit bacteria before their clinical diagnosis and treatment. An intervention that prevents MB disease among seropositive contacts could have a disruptive effect on the transmission of *M.leprae* and subsequently reduce the risk for the community. Such MB-specific intervention, however, still needs to be designed and evaluated. Moreover, because a substantial number of new patients—including MB cases—come from the seronegative contacts and other low risk groups, [5] it remains uncertain whether the above approach would have sufficient impact to halt transmission. A way to enhance the predictive value of a tests for leprosy is to identify additional markers for the development of a prognostic test that can predict all forms of leprosy. [16,17] A combination of a serological assay with an assay based on cell-mediated immunity against *M.leprae* may allow detection of both PB and MB and possibly pre-clinical leprosy as well. [17]

The number of contacts that seroconverted in the first two years was nearly equal in both arms of the trial. The group that seroconverted consisted of two subgroups: (i) contacts that were already infected but still seronegative at intake; and (ii) contacts that were newly infected and became seropositive between intake and first follow-up. Chemoprophylaxis suppresses the development of clinical disease in the first two years after its provision but apparently not seroconversion. This suggests that rifampicin did not prevent new infections after it was provided and that it could not influence the course of infection in those already infected but seronegative at intake.

In conclusion, anti-PGL-I antibody detection and particularly seroconversion can contribute to the early identification of high risk groups for MB leprosy, but anti-PGL-I antibody detection is not efficient as a stand-alone test for the prediction of leprosy. It may add to the predictive value of a prognostic test, but further research is needed to find additional markers for the detection of leprosy infection to enable a prognostic or diagnostic test for (especially pre-clinical and PB) leprosy.

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Chapter 7

General discussion

All studies presented in this thesis are part of the COLEP study, which is a large double-blind, placebo-controlled chemoprophylaxis trial, performed in Northwest Bangladesh. The study area, Nilphamari and Rangpur districts, had recorded prevalence rates of 3.0 and 1.3 per 10,000 population, respectively, at the beginning of the COLEP study (2002). [1] Surveys done for the COLEP study revealed that the actively found prevalence in the general population was six times higher than the registered prevalence. [2] The field work was conducted by the Rural Health Program (formerly DBLM), which has long-time experience in the study area, both in clinical leprosy services and research. The robust design of the study, the high prevalence in the study area and the experienced field staff, made high quality research possible.
This thesis presents five studies addressing topics related to the current research themes as postulated by the research community and WHO. [3,4]

Theme 1: Prevention and management of nerve function impairment (NFI) and reaction

"*Effective management of leprosy complications, including reactions and neuritis, can prevent or minimize the development of further disability. The disease and its associated deformities are responsible for social stigma and discrimination against patients and their families in many societies*" [3]

As stated by Scollard et al. (2006 [5]), leprosy reactions and the accompanying NFI can be seen as medical emergencies. The acute events of inflammatory response are often a reason to seek medical help. Even when diagnosed with leprosy and during and after MDT, reactions can still occur and potentially damage the nerves in an irreversible manner. Most reactions are recorded within the first year of treatment and decrease in number every sequential year.

NFI may lead to the hallmark deformities of leprosy. Daily activities will be a challenge, and stigma and shame may cause the leprosy patient (and family) to become isolated from his or her social environment. The fear of such a prospect has been related to delay in self reporting, not only increasing the risk of NFI but also the transmission of the bacterium.
From the above follows that prevention and management of NFI and reactions is an important research theme. With leprosy control becoming more and more integrated into general health care services and becoming less specialized, there is a need for simplified procedures at the field level for timely identification and treatment of NFI in leprosy patients.

Much progress has been made by studies like TRIPOD, BANDS, and INFIR mentioned in the introduction. NFI treatment consists of treatment with corticosteroids, which may prevent NFI during reactions and even may result in some recovery of nerve function loss if given early—within 6 months after the event. [6-8] The chances of preventing disabilities increase when health care workers pay special attention to patients who have a high risk of developing NFI. To date, several risk factors for NFI have been identified, [9-11] and an NFI prediction rule was formulated based on data from the BANDS study. [9]

Prediction of NFI

Chapter 4 describes an adjustment on the BANDS NFI prediction rule: the variable “longstanding NFI at diagnosis” is replaced with “anti-PGL-I antibodies”. The adjusted prediction rule was better able to identify patients at risk of developing NFI after diagnosis, using “WHO classification” and “anti-PGL-I antibodies” as risk indicators.
The adjusted prediction rule can identify a substantially higher number of new NFI cases than either routine or BANDS rule-based surveillance

and offers increased opportunity to prevent nerve damage in leprosy. However, the number of visits needed to detect one case is higher than with alternative strategies. We consider this operationally feasible and medically justifiable in view of the serious consequences of NFI, including life-long disability.

In contrast to the BANDS rule, the adjusted rule uses two variables that do not include NFI. This offers the possibility of predicting NFI before it actually occurs. We believe that the adjusted prediction rule can be applied in current health services, since it fulfils the need for simplified guidelines and diagnostic protocols. With the adjusted prediction rule, the necessity to continue surveillance beyond the treatment period can be determined. New leprosy patients can be assigned to an NFI risk group, and appropriate surveillance can be planned. Nerve damage can thus be successfully prevented despite the fact that leprosy control has been integrated into general health services.

Involvement of host genetics

It is probably impossible to select a single genetic prognostic marker for leprosy because of the diversity and interdependence of the immunological mechanisms involved. [12] The availability of the whole human genome sequence, allowing gene comparison and genome wide-scans, may further increase our understanding of host immunology, potentially leading towards a multiple marker test, which may include one or more genetic markers.

Host genetics are associated with the occurrence of reactions: chapter 2 clearly shows that the function-diminishing SNP *TLR1* N248 is strongly associated with ENL reactions. In addition, we found for the PARK_e01(-2599) SNP that all patients with ENL reaction had the TT genotype (unpublished results).

Although, it may not be easy to interpret the relationship of a few SNPs and an infectious disease, our results do illustrate the impact of host

genetics for the development of reactions and even leprosy susceptibility.

In conclusion, there is a definite need for improving the quality of NFI assessment and management. We believe that the adjusted prediction rule including anti-PGL-I antibodies instead of longstanding NFI at diagnosis can improve patient management in general health services. Also, studies on host genetics may one day provide markers to identify NFI risk and potentially provide insight into the mechanisms leading to nerve damage.

Theme 2: Improved chemotherapy

"The current treatment of leprosy based on WHO's recommended multidrug therapy (MDT) for MB and PB leprosy is unlikely to see major changes during the next 10 years or so. However, the longer term role of MDT will be dependent on M.leprae *remaining sensitive to the component drugs particularly rifampicin"* [13]*. Judicious use of MDT is thus extremely important.*

The WHO technical advisory group [4] noted that the current MDT regimen is still complicated, with the risk that patients fail to take their daily and monthly doses for the (relatively long) treatment period. Moreover, available resources should be used in an optimal manner. Accurate diagnosis and classification of leprosy patients is important for treatment purposes as correct treatment may prevent disabilities, relapse and continued transmission.

Classification for treatment purposes

After its publication in 1966 [14], the standard way to classify leprosy was according to the Ridley and Jopling scale. Starting from 1982 onwards, the WHO has step-wise designed a classification system for treatment purposes, dividing patients into two groups (PB and MB) with matching drug regiments. Initially the WHO classification was based on the Ridley and Jopling scale and microscopy, but nowadays a classification based on skin lesion counting only is promoted. In the WHO classification, “satellite lesions”, small secondary lesions in the vicinity of a larger primary lesion, may be counted as separate lesions. The WHO classification system does not take into account the large variation in the size of lesions.

There are currently two tools available for routine control programmes to help the correct classification of leprosy patients, 1) microscopy on acid-fast stained skin smears or on biopsies can be used to determine the bacterial index (BI). 2) Anti-phenolic glycolipid-I (PGL-I) antibody detection by serology may be used instead of microscopy, since the presence of antibodies to the *M.leprae*-specific PGL-I correlates with the bacterial load. Although microcopy may deliver a definite “proof” of *M.leprae* infection when positive, the need for laboratory facilities makes it demanding. For individual patient management serological testing may give clinicians a better idea about the systemic bacterial load of a patient. The availability of simple serological tests makes it more field applicable. Unfortunately, the implementation of anti-PGL-I serology field test for routine leprosy control is not very likely, since the focus of classification procedures is very much on reducing complexity and costs.

Skin lesions in classification

In view of the integration of specialized leprosy services into general health care systems, lesion counting will become more and more important. Clear and standardized classification rules while help health workers to prescribe chemotherapy. Chapter 3 critically evaluates classification using a large number of patient characteristics using seropositivity as a proxy parameter for bacterial load and pays special attention to the group of patients with single lesion leprosy. The apparent association of skin lesion size with anti-PGL-I antibodies implies that size does matter: patients with larger lesions are more seropositive. Lesion size may thus be a valuable addition for classification. In contrast, the presence of satellite lesions did not influence anti-PGL-I seropositivity, and we therefore suggest that they should not be counted as separate lesions.

Importance of correct classification

Currently, trials are ongoing to evaluate uniform MDT for all leprosy patients—with both PB and MB patients receiving MB MDT for six months meaning clofazimine as an extra drug for PB patient and only six months instead of twelve months treatment for MB patients [4]. Although it is uncertain if this uniform regiment is going to be implemented, its implementation will not make the need for correct classification obsolete: correct classification remains important for instance to determine risk factors for transmission and reactions.

The WHO classification determines the chemotherapy regimen for a patient, so correct classification is important for the judicious use of MDT. Additional, the uniform interpretation of classification guidelines is needed in order to interpret and compare the epidemiological records:

the WHO strategy guide of 2006-2010 [3] states that the MB rate may be an additional indicator for case detection. Besides, the association between WHO classification and reactions/NFI is clearly demonstrated in chapter 4 and can be used to define risk groups, underscoring the need for correct classification. Therefore, the skin lesion counting system remains an important aspect of the classification of leprosy and subsequent treatment regimens.

Theme 3: Operational research to improve sustainability and integration of leprosy services

"A prime component of the WHO strategy is to ensure that leprosy control activities are available and accessible to all affected individuals at their nearest health facility" [3]

This theme is not discussed, since it is outside the scope of this thesis.

Theme 4: Diagnostics to identify individuals at high risk of developing leprosy

"Surveillance of the disease will be one of the most important activities to be conducted under low endemic situations. In addition, innovative approaches need to be developed based on a 'population at-risk' approach which will help to reduce the disease burden further in the community" [13]

Accurate identification of risk factors in combination with a test for infection could help control activities for monitoring purposes and may justify extended monitoring, intervention and/or treatment of smaller, well-defined groups that are at high risk to develop leprosy.

The latest review by Bakker et al. [15] gives a good overview of risk factors found in cohort studies. In addition, a number of research projects are investigating biomarkers and genetic markers that influence the risk for disease.

Before going into detail, one should consider that leprosy has a low incidence: the number of subclinically infected individuals is estimated to be larger, [16,17] although it remains uncertain to what degree. The diversity of clinical symptoms and extent of disease as well as the variable course of infection and disease—from spontaneous clearance via self-healing to full-blown lepromatous leprosy—are a result of the diverse immunological mechanisms active at different stages of infection and disease. The challenge is thus to identify appropriate (sets of) biomarkers that will allow identification of those persons that are prone to develop clinical disease.

The low incidence of clinical disease makes a "*population at-risk approach"* necessary, since prevalence directly influences the cost per case ratio. So, when applying a biomarker test to define risk groups, the frequency of detecting a real case within that risk group will determine the cost per case for the biomarker test. Thus, it will be extremely important that any diagnostic or predictive test offers a clear advantage over easily obtainable information, such as demographic or contact information. It may be worthwhile to consider a combination of risk factors into a decision model and even to develop a non-tech model based on risk factors that can be determined by interviewing as an alternative for those control programs lacking resources to perform laboratory-based tests.

Another aspect in leprosy detection is the role of stigma, which contributes to delay of diagnosis. The fear of social stigma and discrimination against patients and their families will lead people to refrain from participating in surveys to determine their risk status. Leprosy is a disease with a very low incidence, even in so-called "high risk" groups. Labeling someone as "high risk" may have a negative

impact on that person's quality of life, despite the very real possibility that the person will never develop leprosy. It will therefore be of prime importance to develop a tool with high positive and negative predictive value that can accurately identify high risk groups and so enable health systems to focus their resources.

Host genetic and immunological risk factors

In chapter 2 it is shown that host genetics can increase the risk of leprosy and reactions. In the COLEP population, we found that a SNP in the *TLR* gene had a minor association with leprosy and a strong association with reactions.

Chapter 6, describes the potential of an anti-PGL-I antibody serology test. It shows the association between seropositivity and future development of leprosy, especially MB disease, but the predictive value remains low. Furthermore, a significant proportion of persons who did develop leprosy were seronegative at intake or follow-up. It is well known that a substantial number of (PB) patients are seronegative for anti-PGL-I antibodies. This indicates that an anti-PGL-I antibody test will never detect all patients and it remains to be seen whether other tests detecting antibodies will perform any better.

A combination of a serological assay with an assay based on cell-mediated immunity against *M.leprae* might allow detection of both PB and MB and maybe even preclinical leprosy. Geluk et al. (2009 [18]) reported cell-mediated immune (CMI) responses against five *M.leprae* antigens in 59% of the PGL-I seronegative household contacts of BL/LL patients, indicating a serologically undetected but potentially *M.leprae* infected group. Besides the detection of anti-PGL-I antibodies and host genetic factors a CMI test may be used for a multiple marker test for leprosy.

In low-endemic areas, the relative importance of transmission under high-risk groups increases and may justify an intervention in these groups. Serology and host genetic factors may be used to identify risk groups, ideally combined with CMI markers in a multiple marker test, however future research will be needed for such a test.

Theme 5: A test for infection

"There is a need for […] development of epidemiological tools to monitor completeness of case detection and for novel tests for exposure to infection" [12]

The presence of *M.leprae*-specific antibodies is an indication of the presence of the bacterium. Although not all current or future patients are seropositive, seropositivity is associated with the systemic bacterial load and MB disease (Chapters 3 and 6). Ideally, as mentioned above, an assay should include markers for cellular and humoral immune responses in order to identify both responses against *M.leprae*.

Once a reliable test for infection is available, one needs to determine the feasibility of implementation. Of prime importance are the test characteristics, both in terms of sensitivity, specificity and predictive value as well as technical applicability: the test should be robust and easy to perform. With a test that fulfils these criteria one can then detect infected persons. The next step is to decide what intervention should be applied for subclinical infection. Study results from COLEP as well as from a chemoprophylaxis trial in Indonesia [19,20] indicate that one/two dose(s) of rifampicin chemoprophylaxis may not be sufficient to cure subclinical infection. Yet another question is the impact that such early interventions would have on prevalence and incidence of disease; with mathematical modelling one should be able to predict the potential impact.

It is very likely that the number of subclinically infected individuals is much larger than those who develop clinical disease, and that most will never develop clinical disease—the seroprevalence among healthy contacts included in the COLEP study was 6%. Even with a perfect test, it will be demanding to commit sufficient resources to the monitoring of "positive" persons and/or have a justified intervention that will protect against future development of leprosy without many side-effects and increasing the risk of drug resistance in the population. The intervention with a single dose of rifampicin done in the COLEP study did not prevent new infections after it was provided and it could not influence the course of infection in those already infected but seronegative at intake. Further research will be needed to determine the optimal treatment needed for subclinical infected persons.

Theme 6: Understanding transmission

"The mode of transmission of the leprosy bacillus remains uncertain, but most investigators believe that M.leprae *is spread from person to person, primarily as a nasal droplet infection"* [3]

Understanding transmission is an ongoing research theme, and is crucial for control and reduction of leprosy. At the introduction of MDT, WHO postulated that early diagnosis and treatment of all individuals with clinical signs would lead to reduced transmission and ultimately elimination of the disease. However, until recently the incidence of leprosy was stable and there is no prove that MDT treatment lowered or even interrupted transmission. This suggests that subclinically infected persons may also play a role in reducing transmission. The disappearance of leprosy from the European continent—even before treatment was available—illustrates that breaking the chain of transmission is possible.

It is generally assumed that multibacillary patients are the most infectious. But, at an individual level, seropositive PB patients may have disease that is behaving more like MB disease. Chapter 3, describes that seropositivity can be used as a marker for more extensive disease, since seropositivity is highly correlated with the extend of clinical signs, like numbers of skin lesions, nerves involved and body areas affected. These clinical signs signify the dissemination of the bacterium in the body of the patient, indicating that seropositivity can be used as a marker for a higher systemic bacterial load, and therefore can be used to identify more infectious patients.

Chapter 6 shows that seropositivity and especially seroconversion has a high association with the future development of MB leprosy. From all 19 MB patients, 8 (42%) were seropositive prior to diagnosis. However, a substantial number of new patients—including MB cases—come from the seronegative contacts and other low risk groups, like non-households and non-relatives. [19] Despite this limitation, serology may contribute to a preventive strategy in which MB disease is targeted specifically. A potential benefit of such a strategy could be the interruption of transmission: MB leprosy is considered as the most infectious form of the disease and MB patients can be expected to transmit bacteria before their clinical diagnosis and treatment.

In order to understanding transmission one could include serological monitoring to identify those at higher risk to transmit the disease. However, for identifying infection serology alone is insufficient, since not all (sub)clinical infections are detected with serology. Future research to understand transmission would benefit form a test for infection, but such a test has still to be developed.

Theme 7: Understanding the development of a protective immune response

"Leprosy provides an excellent opportunity to investigate mechanisms of innate and adaptive immunity in humans" [21]

It is assumed that after infection clearance of the bacteria or self healing are far more likely than the development of disease. However, many aspects of the development of a protective immune response are not known. The overall immune response is a complex interaction between innate, cell-mediated and humoral immune responses.

The humoral immune reponse, as indicated by (anti-PGL-I IgM) antibodies, does not protect against leprosy. Monitoring a combination of serological and cell-mediated immunological markers could give insight in the balance between the two immune responses. In chapter 6, seroconversion had a strong association with the development of MB disease and not with PB disease, indicating that, along with other markers, monitoring high risk groups or patients may reveal a pattern of immunological changes leading towards disease or disease alterations like reactions.

Host genetic involvement has been proposed to attribute to the different course of infection and of disease. SNP association studies provide small pieces of information that may help to understand the complex involvement and interaction of various aspects of the immune system in this process. Chapter 2 describes the association between an SNP in *TLR1*, which is part of the innate immune system, and leprosy. A clear relationship was expected between TLR1 functionality, as determined by SNP N248S) and the infection risk. [22] However, results were not that straightforward: homozygous S248 increases and SN decreases the risk of leprosy, but NN showed no influence. So, how

M.leprae influences the local functioning and/or expression of TLRs remains unclear.

There is still limited understanding of the development of a protective immune response in leprosy and further research is needed. The availability of the whole human genome sequence, allowing gene comparison and genome wide-scans, may further increase our understanding of host immunology.

Theme 8: Development of effective, safe, acceptable and inexpensive interventions

"There is a lack of effective tools to reduce the incidence of leprosy" [4]

As MDT in combination with early detection of clinical disease has been shown to be insufficient to lower the incidence of leprosy, the research community has been involved in developing novel early diagnostic tools as well as prophylactic regimens. The primary research question of the COLEP study is the impact that single dose chemoprophylaxis with rifampicin on leprosy incidence in close contacts of leprosy patients. The study also found that 40% of the participants were vaccinated with BCG. Bacille Calmette-Guérin (BCG) vaccination, originally developed for tuberculosis (TB), has a high global coverage since the start of the WHO Expanded Program of Immunization. It protects against the most severe forms of childhood TB and is currently the only candidate for immunoprophylaxis in leprosy. The well-known protection of BCG against development of leprosy and its potentially ameliorating role in the course of disease may have contributed to the reduction of incidence. [23]

The potential for chemoprophylaxis to reduce transmission of leprosy has been studied first for dapsone and more recently for

rifampicin. [24] Two recent studies with rifampicin chemoprophylaxis showed that a single/double dose of rifampicin is protective against the development of clinical leprosy. [19,20]

Chapter 5 describes the effect of the combination of both immuno- (BCG) and chemoprohylaxis (rifampicin) among contacts of patients. In this high-risk study population, BCG vaccination halves the risk of developing leprosy. Moreover, a similar, additive effect of chemoprophylactic intervention with rifampicin was shown. The combination of these strategies showed a protective effect of 80% and may be successful in reducing the incidence of leprosy.

However, another observation was that both BCG and rifampicin prophylaxis were most effective in the contact groups with the initially lowest risk, such as those groups with more physical and genetic distance to the index patient. This indicates that more extensive regimens are needed to prevent leprosy among close contacts, particularly at household level and blood relatives.

The merit of BCG vaccination for TB prevention has been debated continually and second generation vaccines are being developed with higher protection rates and fewer side effects. [25] The protectiveness of these more TB specific vaccines against leprosy is questionable and the replacement of BCG by newer, more TB-specific vaccines may thus be disadvantageous for leprosy control.

The development of effective, safe, acceptable and inexpensive interventions are needed in order to reduce the incidence of leprosy. Immunoprophylaxis with BCG and chemoprophylaxis with rifampicin are both effective strategies, with BCG now having a high global coverage—at least for the time-being. While rifampicin and BCG are effective as a combination strategy, monitoring of close contacts remains necessary even when both immuno- and chemoprophylaxis are supplied. Future

research will be needed to determine the preventive effect of chemoprophylaxis in the long term and to find an intervention appropriate for all sub-clinical infected persons.

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Summary

This thesis focuses on determinants for the development and course of leprosy. Data were collected as part of the COLEP study—a large, double-blind, placebo-controlled chemoprophylaxis trial, performed in Northwest Bangladesh.

Introduction. Leprosy is a chronic infectious disease that usually affects the skin and peripheral nerves. The subsequent nerve function impairment may progress into the hallmark handicaps and disabilities, despite the availability of effective treatment. For several years the WHO has developed strategies to reduce the incidence and burden of leprosy and sustain the health services in all endemic countries. In chapter 1 gives an overview of aspects of the pathogen *Mycobacterium leprae*, its effect on humans, disease characteristics, progress in disease control, and concludes with a summary of the current research needs. These research needs are discussed per theme in the last chapter using the results from the other chapters when relevant.

Susceptibility. Chapter 2 addresses the influence of host genetics on susceptibility to leprosy and leprosy reactions. Here a polymorphism in a key innate immunity receptor, Toll-like receptor 1 (*TLR1*) N248S, was studied that has been shown elsewhere to diminish TLR1 signaling and subsequent leprosy disease. An alteration in the TLR1 function, or at least in a *TLR1* N248S–linked trait, may affect the progression from infection to disease as well as the disease course and the risk of debilitating reactional episodes in the COLEP study population.

Patient characteristics. Chapter 3 describes patient characteristics and especially their association with the presence of *M.leprae* specific anti-PGL-I antibodies. It concludes that serology is a marker for a higher

systemic bacterial load and gives some recommendations to modify the current skin lesion counting system used for classification en treatment.

Leprosy reactions. Leprosy reactions and the accompanying nerve function impairment (NFI) are acute medical emergencies that may occur before, during, and after diagnosis and treatment. The ability to predict and prevent NFI is therefore of utmost importance in the management of patients. In chapter 4 a previously published prediction rule for NFI in leprosy patients is validated and updated. With the described adjusted rule, NFI risk can now be assessed prior to the first event and targeted surveillance can be improved in order to prevent permanent disabilities.

Prevention. For prevention of leprosy both BCG vaccination and rifampicin chemoprophylaxis are effective strategies. While the combined effect is unknown, the combination may give the desired push to halt leprosy transmission. Chapter 5 describes the protective additive effect of the combination BCG vaccination and rifampicin chemoprophylaxis.

Identifying risk groups. Interventions should be targeted at high risk populations. In chapter 6 the potential of serology, detecting *M.leprae* specific anti-PGL-I antibodies, to identify risk groups is described. Seropositivity and seroconversion of leprosy contacts are shown to be associated with MB leprosy.

Discussion. In chapter 7 the results are discussed per research theme as defined by the World Health Organization and its Technical Advisory Group.

Conclusions. The main conclusions drawn are:

- Serological monitoring may help to identify those at higher risk to develop MB leprosy.
- Serology and host genetic factors, ideally combined with markers for cell-mediated immunity in a multiple marker test, may be used to identify leprosy risk groups.
- Determination of lesion size may be a valuable addition to the WHO skin lesion counting system for classification purposes.
- Serology in combination with WHO classification can identify leprosy patients prone to develop NFI. This can improve patient management in general health services.
- Chemoprophylaxis with rifampicin and vaccination with BCG are highly effective as a combination strategy to prevent leprosy, but monitoring of close contacts remains necessary even when both immuno- and chemoprophylaxis are supplied.

Samenvatting

Dit proefschrift behandelt de determinanten voor de ontwikkeling en het verloop van lepra. De gegevens maken deel uit van de COLEP studie — een grote, dubbelblinde, placebo-gecontroleerde chemoprofylaxe trial, welke plaatsvond in Noordwest Bangladesh.

Introductie. Lepra is een chronische infectieziekte die meestal de huid en perifere zenuwen aantast. De zenuwfunctie kan hierdoor verminderen wat kan leiden tot de voor lepra kenmerkende beschadigingen en handicaps. Er is een effectieve behandeling voor lepra beschikbaar. De Wereldgezondheidsorganisatie (WHO) past al enige jaren verschillende strategieën toe om de incidentie en de gevolgen van lepra te reduceren, en tegelijkertijd het duurzaam maken van de (lepra)gezondheidszorg in alle endemische landen. In hoofdstuk 1 wordt een overzicht gegeven over verschillende aspecten van de ziekteverwekker *Mycobacterium leprae*, zoals het effect op mensen, ziektekarakteristieken en vooruitgang in ziektebestrijding. Het hoofdstuk sluit af met een opsomming van de huidige onderzoekprioriteiten en -thema's. Deze onderzoekthema's worden bediscussieerd in het laatste hoofdstuk, de discussie, op basis van de resultaten uit de hoofdstukken 2 tot en met 6.

Susceptibiliteit. Hoofdstuk 2 behandelt de invloed van verschillen in het menselijk genoom op de gevoeligheid voor lepra en leprareacties. Het hoofdstuk beschrijft de mutatie N248S in de Toll-like receptor 1 (*TLR1*), een belangrijke component van de aangeboren immuniteit. Van deze mutatie is eerder aangetoond is dat hij de TLR1 signaleringsfunctie kan verminderen, wat mogelijk invloed heeft op (het verloop van) lepra. Hoodstuk 2 toont aan dat een wijziging in de TLR1 functie, of een *TLR1* N248S–gelinkt kenmerk, invloed kan hebben op de ontwikkeling van infectie naar ziekte, het verloop van de ziekte en leprareacties.

Patiëntkenmerken. Hoofdstuk 3 beschrijft patiëntkenmerken en vooral de associatie van deze kenmerken met *M.leprae* specifieke anti-PGL-I antilichamen. Serologie is een indicator voor een hogere (systemische) bacteriële lading en een uitgebreider ziektebeeld. Ook worden er in dit hoofdstuk aanbevelingen gedaan voor het aanpassen van het huidige WHO telsysteem van huidlaesies welke gebruikt wordt voor het bepalen van classificatie en behandeling.

Leprareacties. Leprareacties en de bijbehorende zenuwfunctie-vermindering gelden als medische noodgevallen die plaats kunnen vinden voor, tijdens en na de diagnose en behandeling van een patiënt. Voor de patiëntenzorg is het erg belangrijk om deze episodes te kunnen voorspellen en voorkómen. In hoofdstuk 4 is een voorspellend model geëvalueerd en aangepast. Met de aanpassingen kan het model nu het risico op leprareacties bepalen voordat de eerste reactie plaatsvindt en hierdoor kan gerichte surveillance plaatsvinden om permanent functieverlies te voorkomen.

Preventie. Zowel vaccinatie met BCG als rifampicine chemoprofylaxe zijn effectieve strategieën voor preventie van lepra. Echter het effect van de combinatie van deze strategieën was niet bekend, terwijl het mogelijk is dat juist de combinatie kan zorgen voor de gewenste doorbraak in de bestrijding van de transmissie van lepra. In hoofdstuk 5 wordt het beschermend effect van de combinatie BCG vaccinatie en rifampicine chemoprofylaxe beschreven en wordt aangetoond dat de combinatie een complementair beschermend effect had.

Identificeren van risicogroepen. Interventies dienen plaats te vinden in groepen met een hoog risicoprofiel. In hoofdstuk 6 wordt bekeken of serologie, die *M.leprae* specifieke anti-PGL-I antilichamen detecteert, geschikt is om risicogroepen te identificeren. Het blijkt dat seropositiviteit en seroconversie geassocieerd zijn met het ontwikkelen van multibacillaire (MB) lepra onder contacten van leprapatiënten.

Discussie. In hoofdstuk 7 worden de resultaten bediscussieerd per onderzoeksthema als gedefinieerd door de WHO en de WHO Technical Advisory Group.

Conclusies. De belangrijkste conclusies zijn:

- Het bijhouden van het serologisch profiel kan helpen om groepen te identificeren met een verhoogd risico op MB lepra.
- Serologische en gastheergenetische factoren, zo mogelijk gecombineerd in een test met indicators van de cellulaire afweer, kunnen gebruikt worden om groepen met risico op lepra te identificeren.
- Het in acht nemen van de grootte van huidlaesies zal een waardevolle aanvulling zijn op het huidige WHO huidlaesie telsysteem dat gebruikt wordt voor classificatie en behandeling.
- Serologie in combinatie met WHO classificatie kan gebruikt worden om patiënten te identificeren met een verhoogde kans op zenuwfunctievermindering. Dit kan ook gebruikt worden in de (niet-lepraspecifieke) gezondheidszorg om de patiëntenzorg te verbeteren.
- Chemoprofylaxe met rifampicine en vaccinatie met BCG zijn erg effectief als combinatiestrategie om lepra te voorkomen; surveillance voor naaste contacten blijft echter nodig zelfs als beiden worden toegepast.

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Ron

Curriculum vitae

Ron Philip Schuring was born on the June 2nd, 1979 in Delft, the Netherlands. After finishing the Lower General Secondary Education (MAVO) in 1995 he went to the senior secondary vocational education (MBO) and straight on to the higher professional education (HBO), both focussed on chemical laboratory techniques. He did his internship at the former ID-Lelystad (nowadays known as the “Animal Science Group of Wageningen UR”, but it is still located at Lelystad) with the task to select and produce recombinant Llama antibodies, to be used in an immune-supporting diet for newborn pigs. Now a “Bachelor of Science in Biochemistry” he left the Netherlands in the winter season 2001-2 to work in Tirol, Austria, as a waiter in a “hutte”.

Refreshed, he went on to seek a career, and found it at the “Koninklijk Instituut voor de Tropen” at the Biomedical Research department in Amsterdam. Initially hired as a lab technician for the COLEP trial, he faced the task to perform a standardized serology test on about 60,000 samples. In the following years he stepped up to become a scientist and in the meantime finished yet another education, this time his MSc in Epidemiology at the Erasmus Medical Centre in 2008.