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PhD thesis, Leiden University, The Netherlands

Author: Anouk van Hooij
Cover design: Anouk van Hooij
Lay-out: Anouk van Hooij
Printed by: Universitair Facilitair Bedrijf

The research presented in this thesis was supported by the Netherlands Leprosy Relief Foundation (NLR) and the Leprosy Research Initiative (LRI), both together with the Turing Foundation, The European & Developing Countries Clinical Trials Partnership (EDCTP), the Q.M. Gastmann-Wichers Foundation, the Order of Malta-Grants-for-Leprosy-Research (MALTALEP), the Leiden University Fund (LUF), and the New York Community Trust (NYCT).

Printing of this thesis was financially supported by the Q.M. Gastmann-Wichers Foundation.

Development of immunodiagnostic tests for leprosy: from biomarker discovery to application in endemic areas

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op woensdag 17 november 2021
klokke 15.00 uur

door

Anouk van Hooij

geboren te Roosedaal en Nispen
in 1992

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Chapter

General introduction

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Leprosy, still not eliminated

Leprosy is one of the oldest recorded human diseases (1). *Mycobacterium leprae (M. leprae)*, the causative agent of leprosy, was spread by human migration throughout the world and most likely originated in the Far East (2). More recently, a new mycobacterial species causing leprosy, *M. lepromatosis,* was also identified (3, 4). These pathogens mainly affect the peripheral nerves leading to various degrees of neuropathy which can result in severe lifelong disabilities. In the mid-1980s the introduction of multidrug therapy (MDT) significantly reduced the prevalence of the disease, from 5.4 million cases at that time to 202,185 newly reported cases in 2019 (5). Of the new cases, 80% are accounted for by Brazil, India and Indonesia (6). However, the number of new cases has remained fairly stable in the past decade, not showing a relevant decline anymore (7). A possible reason is that the initial huge drop in prevalence resulted in a reduction of leprosy control activities, especially since the World Health Organization (WHO) declared that leprosy was eliminated as a public health problem in 2000 (8). The prevalence of leprosy at global level is indeed less than 1 per 10,000, but several hot spots in endemic countries have remained (8, 9). The reduced knowledge to recognize the signs and symptoms of leprosy frequently leads to delayed diagnosis, resulting in large numbers of undetected cases (10). This is not only the situation in endemic countries, especially in non-endemic countries the possibility of symptoms being signs of leprosy is often overlooked. For example, in the United Kingdom in 80% of the cases leprosy was not suspected at the first visit resulting in an average diagnostic delay of 1.8 years (11). As migration rates are increasing, leprosy cases will again emerge in parts of the world where leprosy is not prevalent anymore in the native population. This situation urges to raise awareness that leprosy is a communicable disease that despite all the efforts is still prevalent today.

Clinical presentation of leprosy

Recognition of the often subtle clinical signs is of major importance for leprosy diagnosis. A case of leprosy can be defined as an individual that has one of the three cardinal signs (12):

- Definite loss of sensation in a hypopigmented or reddish skin patch
- Thickened or enlarged peripheral nerves with a loss of sensation and/or weakness in the muscles supplied by the nerve
- The presence of acid-fast bacilli in slit-skin smears (SSS). The acid-fast bacilli, resistant to decolorization by acids during laboratory staining procedures, in SSS are counted using a microscope and expressed as the bacteriological index (BI). The BI indicates the extent of bacterial load on a logarithmic scale.

These cardinal signs are based on clinical symptoms, SSS and histopathology. However, several dermatological diseases (i.e. psoriasis, sarcoidosis, dermal leishmaniasis) show similar clinical manifestations (13), resulting in the possibility of leprosy being overlooked by clinicians. As severe disabilities, like the loss of digits, can be prevented by early and adequate treatment (MDT), diagnosis of leprosy at an early stage is vital. As a result, leprosy disease heavily impacts the affected individuals and their family, not only their economic but also their social status as leprosy still leads to social stigma (14). Prevention of these disabilities can therefore have great impact.

Another factor that complicates the diagnosis of leprosy is the spectral presentation, which is determined by the host's immune response. The spectrum spans from T helper-2 (Th2) immunity concomitant with large numbers of bacteria as well as antibodies against *M. leprae* antigens in multibacillary (MB) leprosy, to paucibacillary (PB) leprosy characterized by strong pro-inflammatory, T helper-1 (Th1) as well as helper 17 (Th17) immunity leading to bacterial control (15, 16). Besides adaptive immunity, cells of the innate immune systems also display a polar response to *M. leprae*. Macrophages of PB patients can restrict bacterial dissemination via an IL-15 induced antimicrobial program, which is dependent on vitamin D (17). In MB patients, IL-10 induces a scavenger receptor program which enables *M. leprae* to persist in macrophages. The phagocytosis of mycobacteria and oxidized lipids is increased in these macrophages, which leads to foam cell formation (18, 19). Both innate and adaptive immune responses of the host thus contribute to the spectral presentation of leprosy.

The WHO classification of this spectrum is based on the number of lesions (PB <5 lesions or MB >5 lesions) and determines the treatment regimen (20). MB cases must be treated for 12 months with MDT (Dapsone, Rifampicin and Clofazimine) and PB cases for 6 months (Dapsone and Rifampicin). In case SSS are available any patient with a positive skin smear is classified as MB, irrespective of the clinical picture

A more detailed five-part classification system based on histopathology was developed by Ridley and Jopling (21). On one end of the spectrum tuberculoid leprosy (TT) is defined as an individual with a single lesion that has well-developed epithelioid granulomas and rare acid-fast bacilli. At the other end lepromatous leprosy (LL) is defined as an individual with multiple lesions in which foamy macrophages and large numbers of bacilli can be identified. The majority of patients, however, present with the borderline phenotypes; borderline tuberculoid (BT), mid-borderline (BB) and borderline lepromatous (BL). In these phenotypes the bacterial load correlates with the histological features, BT being more closely related to TT patients and BL to LL patients. The two classification systems coexist as the WHO classification can be applied in resource-limited settings, but if biopsy diagnosis

and microscopic classification is available the Ridley-Jopling classification is preferred. In general, BT/TT leprosy corresponds to PB leprosy and BL/LL leprosy to MB leprosy (Figure 1).

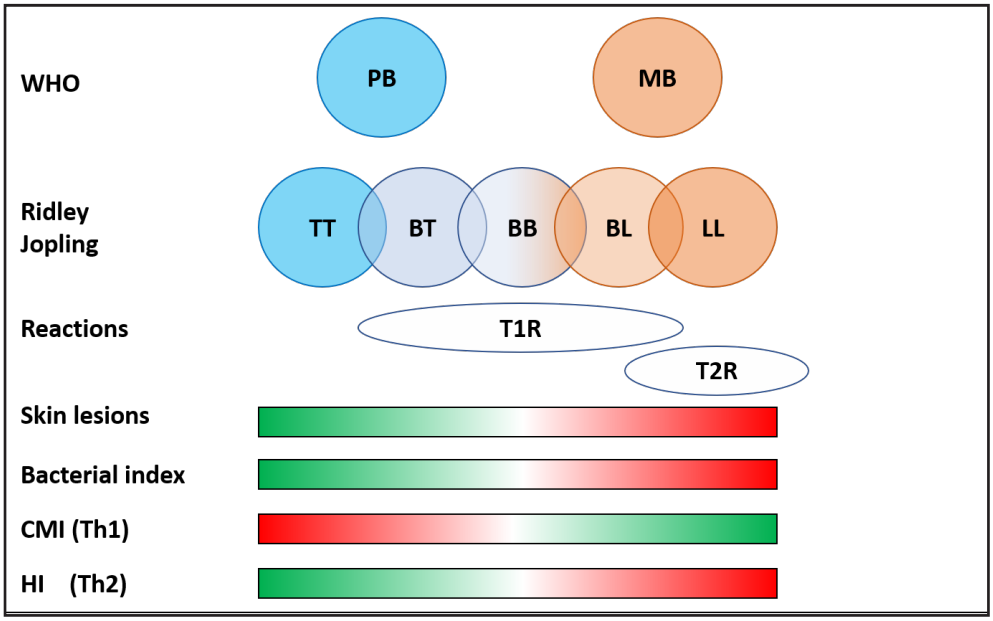

Figure 1: The leprosy spectrum. Leprosy is a spectral disease ranging from patients with few lesions, low bacterial loads and strong Th1-cell-mediated responses (Cell-mediated immunity (CMI)) to patients with multiple lesions, high bacterial loads and predominantly Th2 antibody responses (Humoral immunity (HI)). Two classification systems are used to describe this spectrum, the WHO classification categorizes patients as multibacillary (MB) or paucibacillary (PB). The Ridley-Jopling classification categorizes patients in five groups: Lepromatous leprosy (LL), borderline lepromatous (BL), mid-borderline (BB), borderline tuberculoid (BT) or tuberculoid leprosy (TT). Characteristics of both classification systems are shown as a gradient ranging from low (green) to high (red). Type 1 leprosy reactions (T1R) most frequently occur in borderline leprosy patients, whereas type 2 leprosy reactions (T2R) only occur in BL/LL patients.

The borderline states are immunologically unstable and susceptible to the occurrence of leprosy reactions. These are exacerbated inflammatory episodes that affect the peripheral nerves and are the main cause of leprosy-associated disabilities (22). Two types of reactions can develop, type 1 (T1R; reversal reaction) or type 2 reactions (T2R; erythema nodosum leprosum) (figure 1). T1R are delayed hypersensitivity reactions associated with the development of *M. leprae antigenic* determinants and an increase in cell-mediated immune responses in the lesions (23). In relation to the leprosy spectrum, T1R can cause a clinical conversion from the MB to the PB side (18). T2R are induced by antigen-antibody complexes to *M. leprae* antigenic determinants. Disposition of these immune complexes in the circulation and in tissues is manifested by the infiltration of neutrophils. T2R predominantly occur in BL/LL patients with high bacillary loads and have systemic effects, including high fever and oedema (24).

The diverse clinical presentation together with the similarity of symptoms to other dermatological diseases complicate the diagnosis of leprosy. Moreover, it can take up to 20 years after *M. leprae* infection before clinical symptoms develop (25, 26), leaving these infected individuals unnoticed for many years as the diagnosis relies on clinical symptoms only. Diagnostic tools that can confirm leprosy diagnosis and/or identify *M. leprae* infected individuals in a pre-clinical stage could therefore be of great help to initiate either therapeutic or prophylactic treatment. However, to achieve elimination of leprosy it is vital to not only treat adequately but also prevent transmission (27).

Transmission of *M. leprae*

The rather stable number of new cases reported yearly, with 7.6% being children in 2018 (7), indicates that the transmission of *M. leprae* is still ongoing. Although the exact mode of *M. leprae* transmission is not completely understood, it is assumed that *M. leprae* is mostly transmitted from person to person via a respiratory route. Contacts closest to leprosy patients have the highest risk of acquiring the infection, especially from patients with high bacillary loads (28-30). In combination with the observed presence of *M. leprae* DNA in nasal swabs (NS) of contacts (31), human to human transmission through aerosols is the most likely route of transmission. The long incubation period of *M. leprae* (25, 26) and the fact that *M. leprae* cannot be cultured in vitro (32, 33) complicates the unravelling of *M. leprae* transmission. The main questions regarding *M. leprae* transmission are (34):

1. What is the route of entry/exit of *M. leprae* in humans? How *M. leprae* migrates in humans from the port of entry to the site of initial lesion and subsequently to the point of exit is unknown.

2. Do asymptomatic carriers of *M. leprae* contribute to transmission? It is unknown to what extent undiagnosed patients, patient contacts and other inviduals living in endemic areas spread the bacterium. The presence of *M. leprae* DNA in NS of healthy household contacts of patients (31) supports a role for asymptomatic carriers in transmission.

3. Do animal and environmental reservoirs contribute to transmission? Recent reports demonstrated the presence of *M. leprae* in water and soil (35, 36) as well as in amoeba (37) pointing towards an environmental reservoir for *M. leprae*. Evidence for zoonotic leprosy has been found in the Southern United States and Brazil, where armadillos and humans were infected with the same *M. leprae* strain (38). Frequent consumption of or contact with armadillos also increased the risk

for *M. leprae* infection (39). Moreover, *M. leprae* DNA has been detected in non-human primates (40) and in red squirrels (41), also in areas where leprosy in humans is already absent for centuries ndicating that animals can serve as a reservoir for *M. leprae*. To what extent these animal and environmental reservoirs contribute to the perpetuating transmission in humans needs to be elucidated.

A vaccine that effectively prevents transmission is not available. Bacillus Calmette-Guerin (BCG) is routinely administered, which is a vaccine for tuberculosis (TB) included in neonatal immunization schemes in many parts of the world. BCG reduces the risk of leprosy when administered at birth (42). The pooled reduction risk is 55%, but effectiveness was variable between studies (42) and the immune response to BCG wanes over time (43). Several trials have examined BCG revaccination in endemic populations of individuals vaccinated at birth, with varying results (44-47). Until a vaccine that can interrupt transmission is available, research on transmission patterns is vital to gain insight in *M. leprae* transmission.

Examination of the transmission patterns by molecular epidemiology is difficult due to the low degree of genetic diversity of *M. leprae* (33). It is estimated that *M. leprae* has approximately one single nucleotide polymorphism (SNP) per 28,000 basepairs (48), which is much lower than for instance *Mycobacterium tuberculosis* (*M. tuberculosis,* the causative agent of TB) with 1 SNP per 200 basepairs (49). Two methods for strain typing of *M. leprae* have been described:

- Variable number tandem repeat (VNTR) typing: Typing based on polymorphic DNA regions known as short tandem repeats. VNTRs differ in the number of repeat sequences due to mutations occurring during DNA replication (50). Based on this number of repeats in certain loci *M. leprae* strain typing can be performed using PCR to assess transmission patterns (51).

- SNP typing: Strain typing based on polymorphic SNP sites. Comparing multiple *M. leprae* strains from different sites globally identified 4 SNP types and 16 subtypes (48, 52). The 16 subtypes showed a strong geographical association and reflected human migration routes.

The emergence of whole genome sequencing techniques allowed more extensive analysis of *M. leprae* strains (1, 2), enabling further characterization and identification of new lineages beyond the earlier identified 16 subtypes (2, 53, 54). As *M. leprae* cannot be grown in culture, for all of these methods sufficient *M. leprae* DNA needs to be present, which is especially difficult in patients' contacts where few bacilli are present but also in patients with low bacillary loads (PB) (2, 53). Complete mapping of *M. leprae* transmission patterns based on molecular epidemiology therefore remains difficult.

Risk factors

Despite the continuous transmission of *M. leprae* in endemic areas, the majority of exposed individuals (approximately 90% (55)) is naturally immune to infection. Several risk factors contribute to the establishment of *M. leprae* infection or disease, a particularly important factor being **genetic predisposition**. Before Gerhard Armauer Hansen discovered *M. leprae* to be the cause of leprosy in 1873 (56), leprosy was believed to be a hereditary disease (57). The host genetic background contributes more to leprosy susceptibility than bacterial variability (33). Advancement in molecular techniques boosted the number of host genes and variants identified as leprosy risk factors (i.e. in host defense pathways), which are extensively reviewed by Fava *et al.* (58). Even though all close contacts of leprosy patients are exposed to *M. leprae* for prolonged periods of time, **genetic relationship** significantly affected the risk to develop leprosy independent of physical distance (59, 60). On the other hand, close **physical distance** is still a risk factor for individuals that are not genetically related to the leprosy patient**. Classification (MB or PB) of the index patient** and **age of the contacts** also influence the leprosy risk (59). For age a bimodal distribution was observed, with an increased risk for higher age groups. **Poverty related factors,** such as low education level, food shortage and poor hygiene (61) showed to be significant risk factors as well. A combination of the mentioned risk factors is most likely required to develop clinical leprosy as a result of *M. leprae* exposure.

Development of diagnostic tests for leprosy

Although several risk factors are described, it is still not possible to predict who will be infected with *M. leprae* after exposure and who will develop disease. Together with the gaps in knowledge on *M. leprae* transmission and the difficulty of diagnosing leprosy, especially at an early stage, this allows the transmission to perpetuate. New control strategies are required, of which a diagnostic test that can identify *M. leprae* infected individuals at risk of developing leprosy would reduce the new case detection rate significantly an thus expectedly reduce transmission (62). Efforts to develop such a diagnostic test are continuously ongoing and can broadly be divided in two categories. One category aims to develop diagnostic tests that identify the **presence of the *M. leprae* bacillus**, whereas the other category is aiming for tests assessing the **host response to *M. leprae***.

Pathogen detection

The presence of acid-fast bacilli in SSS is one of the three cardinal signs of leprosy. However, this detection method has low sensitivity and cannot differentiate *M. leprae*

from other mycobacteria. Nucleic acid-based methods have therefore been developed, detecting a repetitive sequence named RLEP, which is highly specific for *M. leprae* (63). The first report of RLEP PCR in 1989 used gel-based visualization and enabled the identification of bacilli isolated from armadillo livers, mouse footpads and human biopsies, with a detection sensitivity of 100 bacilli greatly improving sensitivity compared to microscopy (64). Current procedures for DNA extraction also allow the detection of *M. leprae* DNA in other specimens such as SSS, nerve biopsies, NS, blood, environmental samples and even archeological samples (65-69). The PCR technique is also the basis for the earlier described strain typing (*section transmission*). In contrast to conventional PCR, quantitative PCR (qPCR) allowed quantification of the *M. leprae* bacilli. DNA based PCR assays can, however, not discriminate between viable and dead *M. leprae* and requires quantification of the RNA target 16S rRNA by reverse transcriptase PCR (RT-PCR), as 16S rRNA levels declined during MDT treatment (70, 71). (RT)-qPCR can thus be used to monitor treatment efficacy, but has also been applied for diagnostic purposes especially focusing on the detection of PB patients. Different studies examined the use of RLEP qPCR to detect *M. leprae* DNA in skin biopsies of leprosy patients, showing a correlation with the BI, identifying the majority of MB patients, and sensitivities from 44% to 74.5% in PB patients (72-74). SSS (75) or NS (31, 76) showed a similar pattern as obtained with biopsies for MB as well as PB patients. These samples can also be collected in contacts of leprosy patients, who in contrast to patients do not have lesions. Both in SSS (75) and NS (31, 76) *M. leprae* DNA was detected in household contacts, indicative of infection (SSS) or colonization (NS). Presence of *M. leprae* DNA in NS of contacts was, however, not determinant of later disease onset (31). Moreover, comparable bacterial DNA levels in SSS of contacts and PB patients were observed, indicating limited value in predicting progression from infection to disease (75).

In conclusion, pathogen detection using nucleic acid-based methods is more sensitive than classic microscopy, but it remains difficult to detect patients with very low bacillary loads. A clear link between the presence of *M. leprae* in NS and SSS of contacts and disease development has not been shown.

Host response

An alternative to pathogen detection is a diagnostic test based on the host immune response, known to play a role in disease outcome after *M. leprae* infection (16). Upon identification of *M. leprae*-specific antigens, antibody responses to these antigens have been examined. One of the first identified (77) and most widely evaluated antigens is phenolic glycolipid-I (PGL-I), a cell wall component unique to *M. leprae*. An enzyme-linked immunosorbent assay (ELISA) detecting PGL-I specific antibodies showed a highly specific response in *M. leprae* infected individuals, proving the detection of αPGL-I-specific IgM antibodies very useful for diagnostics (78-82).

A meta-analysis performed in 2019 (83) on 39 studies utilizing ELISA showed a sensitivity of 63.8% (95% CI 55.0-71.8) and a specificity 91.0% (95% CI 86.9-93.9). Another meta-analysis evaluated the use of PGL-I antibodies in contacts as a predictive biomarker for progression to leprosy and showed less than 50% sensitivity for the individual studies (84). Thus, the detection of αPGL-I antibodies only is not sufficient to identify all leprosy patients, in concordance with the fact that most PB patients show either low titers or no antibody response (83, 84). Antibody responses to other *M. leprae*-specific antigens such as lipoarabinomannan (85), fusion protein LID-1 (86-88), major membrane protein II (89), and several recombinant *M. leprae* proteins (85, 90, 91) have also been examined. These antibody responses showed a similar pattern as the PGL-I response, with higher levels and positive responses observed in MB patients.

Instead of tests based on humoral immunity, tests based on cell-mediated immunity (CMI; Th1), associated with the PB side of the leprosy spectrum as well as contacts, are already implemented in TB care (92). For TB diagnostics, the interferon gamma release assay (IGRA) uses the hallmark cytokine of Th1 responses, IFN-γ, as readout. The production of IFN-γ in response to the highly specific *M. tuberculosis* antigens ESAT-6 and CFP-10 enables the discrimination of (latently) infected individuals from non-infected individuals (93, 94). However, this assay does not discriminate active from latent disease nor indicates progression from infection to disease. Hence, the IGRA is most useful as a diagnostic in low endemic countries, where infection rates are low (95). The advantage of the IGRA test for TB diagnostics is the lack of cross-reactivity with BCG and the majority of non-tuberculous mycobacteria (96). A second screening test, which is also based on CMI, is the tuberculin skin test (TST), in which a mixture of TB proteins is intradermally administered. If an individual is previously exposed to *M. tuberculosis* a delayed hypersensitivity reaction occurs within 48-72 hours, causing an induration at the site of administration which is the readout of this assay (93). In contrast to the IGRA test, the TST is subject to cross-reactivity with BCG and other mycobacteria (97). Although less specific, the TST test is not as costly as the IGRA and is therefore often applied in resource-limited settings.

As leprosy diagnostic tool, the lepromin skin test uses a similar concept as the TST, intradermally inoculating a suspension of heat-killed *M. leprae* and evaluating the induration at the site of inoculation after 3-4 weeks. The lepromin skin test is not indicative of infection, but of the immunologic capability of an individual in response to *M. leprae* and is also highly cross-reactive (98). This test is useful for classification of leprosy as it indicates the extent of CMI of an individual in the disease progress, ranging from no to weak reaction in LL patients to strong reactions in TT patients (98).

The identification of the ESAT-6 (99, 100) and CFP-10 (101, 102) homologues in *M. leprae* enabled the assessment of the IFN- γ response to these antigens, similar to the IGRA for TB. Both *M. leprae* antigens induced T-cell dependent IFN-γ production in leprosy patients, also reflecting the leprosy spectrum with an absence of response at the lepromatous pole of the spectrum. *M. leprae/M. tuberculosis*-non-exposed healthy individuals did not show a response, but both antigens were recognized by TB patients and healthy individuals from areas where leprosy and TB are endemic (100, 101). IFN-γ responses to *M. leprae* ESAT-6 and CFP-10 are thus indicative of exposure, but the observed cross-reactivity impeded the use of this test in areas where both TB and leprosy are endemic. Unfortunately, these are exactly the areas where a leprosy diagnostic tool is most urgently needed.

To minimize the possibility of cross-reaction with other mycobacterial species several *M. leprae* unique proteins, as an alternative to ESAT-6 and CFP-10, were identified by comparative genomics of the *M. leprae* genome to the genomes of other mycobacteria (33). These proteins were evaluated for their ability to induce T-cell dependent IFN-γ production (103-106). In one study, five antigens expressed as recombinant proteins induced significant T-cell responses in PB patients and healthy individuals exposed to *M. leprae*, but not in TB patients or endemic controls (103). With the knowledge that *M. leprae* peptides induced more specific responses than *M. leprae* proteins (107), but are HLA-restricted with different responses per region due to differences in genetic background (108), the T-cell response to 50 synthetic peptides spanning the sequence of the earlier identified proteins (103) was examined. A combined T-cell response to 4 peptides was identified, detecting both PB patients and household contacts (frequently exposed to *M. leprae*). To examine the applicability of these proteins/peptides in different populations, T-cell responses at five different sites were compared (109), revealing that T cells of endemic controls could also respond to these proteins and to a lesser extent to peptides. To identify to what extent the level of leprosy endemicity influenced the cellular immunity to *M. leprae* unique antigens, endemic controls with different degrees of exposure to *M. leprae* were evaluated in a subsequent study (110). Whole blood was stimulated with a panel of *M. leprae* antigens measuring multiple cytokines instead of IFN-γ only. The more practical 24-hours whole blood assay (WBA), instead of the commonly used 6 days stimulation with *M. leprae* whole cell sonicate (WCS) or recombinant proteins (111, 112), induced weak levels of IFN- γ (109) but other cytokines could be sensitively detected (113). Two recombinant proteins ML0840 and ML2478 inducedhigh IFN-γ responses in endemic controls from high-prevalence areas, but this response was absent in controls from areas where leprosy is not endemic anymore (110). This was in contrast to *M. leprae* WCS, which mounted comparable IFN-γ responses in all control groups. Although useful for exposure, the IFN-γ response did not differ between patients and contacts from the same endemic area.

Of the additionally assessed cytokines IP-10 showed a similar pattern as IFN-γ indicative of exposure, whereas IL-1β, MCP-1 (CCL2) and MIP-1β (CCL4) in response to *M. leprae* WCS and ML2478 did discriminate patients from endemic controls, potentially identifying pathogenic immune responses to *M. leprae* (110). This study provided the first identification of cellular biomarkers other than IFN-γ for discrimination of leprosy patients from endemic controls. Moreover, the two recombinant proteins that proved useful in this study (ML0840 and ML2478) also induced IFN-γ responses in *M. leprae*-infected mice (114) and armadillos (115). Evaluating host cytokine profiles in response to both WCS and ML0840/ML2478 in WBAs thus enables discovery of potential new biomarkers for leprosy diagnosis.

Lateral flow assays

Numerous efforts have been conducted to either detect *M. leprae* or the host response to *M. leprae* in infected individuals using sophisticated and high-end equipment and expensive reagents. Well-equipped laboratory facilities are, however, not available in all health centers in leprosy endemic areas, which are predominantly located in low- or middle income countries. Newly developed diagnostic tests should therefore require little training and laboratory equipment to be applicable in these areas. Lateral flow assays (LFAs) are common low-cost, user-friendly and rapid tests to detect any type of biomarker, well-suited for applications in remote and resource limited settings (116).

LF strips are usually composed of a sample pad, conjugate release pad, membrane with immobilized antibodies and an absorbent pad (Figure 2). The sample is applied to the sample pad and migrates to the conjugate release pad, which contains colored or fluorescent reporter particles coated with antibodies specific to the target analyte. Target analytes bound to the reporter conjugate migrate through the membrane and to the test (T) line, containing a complementary antibody that recognizes the same analyte. Reporters passing the T-line will bind to the flow control (FC) line. The absorbent pad prevents backflow and maintains the capillary force of the strip material. The readout of the strip is based on the difference in intensity between the T and FC line and can be assessed by eye or using a dedicated reader depending on the used label.

LFAs using colloidal gold as a visible label have been described for leprosy diagnostics, either detecting PGL-I specific IgM (117) or NDO-LID (a conjugate of synthetic PGL-I and LID-1) specific antibodies (118). The qualitative outcome of these tests does not require the use of a reader and these tests were useful to detect the majority of MB patients. Up-converting phosphor (UCP) has been applied to a variety of analytes (119-121) and UCP-LFAs

detecting αPGL-I IgM, IFN-γ, IL-10, IP-10 and CCL4 have been developed and evaluated in TB and leprosy patients (122-125). The phosphorescent reporter is excited with infrared light to generate a visible light. This process is called up-conversion and does not occur in nature, thereby avoiding autofluorescence issues. Moreover, UCP particles do not bleach, allow permanent excitation (126), and LF strips can be stored as a permanent record also for re-analysis. The Ratio of the observed fluorescent intensity at the T and FC line is a quantitative test outcome corresponding to the concentration of the analyte present. The UCP-LFA format enables user-The Ratio of the observed fluorescent intensity at the T and FC line is a quantitative test outcome corresponding to the concentration of the analyte present. The UCP-LFA format enables user-friendly quantitative evaluation of both cellular and humoral biomarkers rendering this format promising for field-applicable leprosy diagnostics.

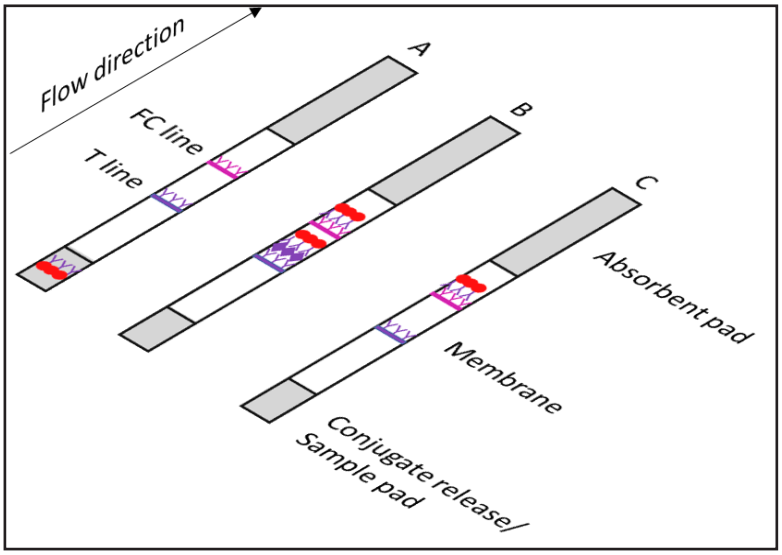

Figure 2: Schematic representation of lateral flow assays. Lateral flow assays (LFAs) generally consist of a sample pad, conjugate release pad, membrane and absorbent pad. On the sample pad/conjugate release pad colored or fluorescent particles conjugated (red dot) to an antibody (Y) specific for the target analyte are incorporated. The test (T) line contains antibodies complementary to those on the reporter, recognizing the same target but a different epitope. Reporters that pass the T-line can bind to the flow control (FC) line, through binding the FC-part of the antibodies conjugated to the reporter. (A) LF strip before addition of sample. Reporter particles conjugated to the antibodies are located in the conjugate release/sample pad. (B) LF strip after application of a sample that contains the analyte of interest. Reporters are detected at both the T and FC control line. (C) LF strip after application of a sample that does not contain the analyte of interest. Reporters are detected only at the FC line.

Longitudinal follow-up of household contacts in Bangladesh

Based on the observed positive effect of single dose rifampicin (SDR) (127) and BCG revaccination (46) on new case detection rate, in 2012 a cluster randomized controlled vaccination trial started in Bangladesh to examine the effect of combined chemoprophylaxis (SDR) and immunoprophylaxis (BCG) on clinical outcome (128). Blood was collected to examine the host immune responses and gene expression profiles in contacts of newly diagnosed leprosy patients (128). Additionally, contacts without intervention and endemic controls were included in this study. Including large numbers of new cases and long-term follow-up of their contacts is necessary to identify correlates of disease progression and predictive correlates, due to the long incubation time (typically two-five years) and relatively low incidence of leprosy compared to for instance TB. This study, as one of its kind, met these criteria and whole blood was collected and stimulated for 24-hours with *M. leprae* WCS and ML0840/ML2478 (WBA; section host response). Blood was also collected for RNA isolation and subsequent gene expression profiling. This set-up provided a huge biobank of samples (>6500) for biomarker discovery (2012 – 2021), including rare, longitudinal samples of leprosy contacts that developed disease during the course of the study. WBA samples from this biobank have been applied in this thesis for the identification and validation of biomarkers.

Thesis outline

The lack of a diagnostic test that can sensitively detect leprosy patients across the leprosy spectrum, as well as identify *M. leprae* infected individuals that are prone to develop disease, sustains diagnostic delay leading to irreversible, leprosy-associated handicaps and enables the perpetuating transmission of *M. leprae*. A diagnostic tool is urgently needed to pinpoint individuals requiring either prophylactic or therapeutic treatment. This thesis focusses on the identification of biomarkers for leprosy diagnostics and the subsequent development and evaluation of tests for these biomarkers based on the UCP-LFA technology, applicable to remote and resource-limited settings. The exploratory and developmental phase has been performed using the described biobank with samples from Bangladesh, whereas the evaluation phase has been performed in multiple areas with varying leprosy endemicity (Figure 3).

Chapters 2-4 focus on **biomarker discovery and the development of diagnostic tests** to improve the detection of leprosy patients, particularly patients with low bacillary loads.

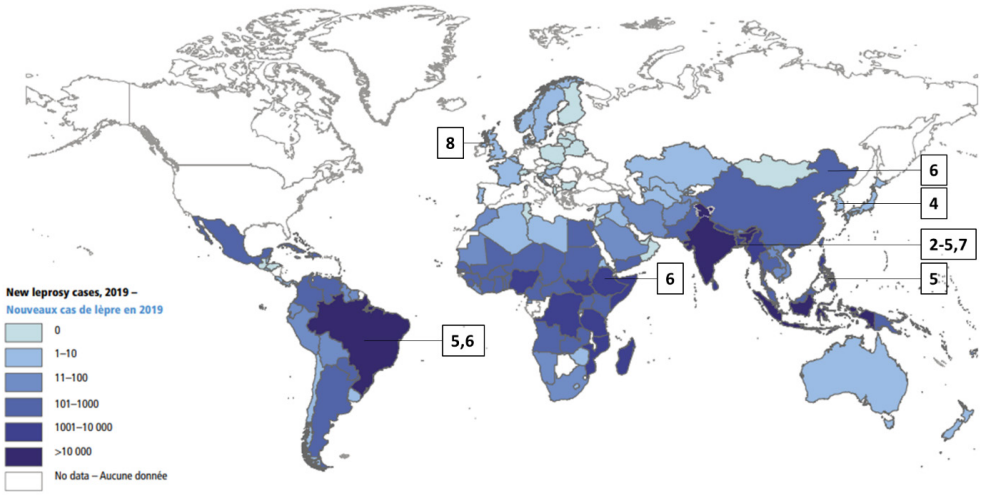

Figure 3: Geographical distribution of cohorts tested in this thesis and the corresponding number of new leprosy cases in 2019. Indication per chapter from which country the samples originated and the corresponding number of new cases reported per country. Bangladesh (chapter 2-5,7), Brazil (chapter 5,6) China (chapter 6), Ethiopia (chapter 6), Philippines (chapter 5), South Korea (chapter 4) and the United Kingdom (chapter 8). Adapted from (5).

In **chapter 2** the diagnostic accuracy of UCP-LFAs for αPGL-I IgM, IP-10, IL-10 and CCL4 was assessed in WBAs stimulated with *M. leprae* WCS and ML0840/ML2478. These four markers comprised innate, adaptive cellular as well as humoral immunity potentially covering the spectral pathology of leprosy. Detected levels of these four markers in leprosy patients, (BCG-vaccinated) contacts and endemic controls were used to successfully design biomarker profiles for *M. leprae* infection, disease and leprosy classification.

In **chapter 3** extensive proteomic profiling using multiplex-bead-arrays was performed in order to identify new biomarkers in addition to αPGL-I IgM, IP-10, IL-10 and CCL4. Using a funnel approach, the identified biomarkers in the first sample set were validated in a second sample set of WBAs. Biomarkers validated in the second sample set were subsequently tested in a set of plasma samples to assess applicability in point-of-care settings. The identified biomarkers in plasma (αPGL-I IgM, IP-10, CRP, APOA1 and S100A12) were applied to improved UCP-LFAs requiring no pre-incubation before flowing the samples, in contrast to the format used in chapter 2. This decreases the assay time from 2 hours to 15-30 minutes.

In **chapter 4** a multi-biomarker test (MBT) strip was developed, enabling the simultaneous measurement of multiple markers on a single test strip. The five-biomarker profile identified in chapter 3 was implemented in the MBT strip and evaluated in both plasma, serum as well as fingerstick blood samples to assess the applicability of the biomarker

signature in these different biosamples.

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Chapters 5-8 cover **the evaluation of developed UCP-LFAs** in different leprosy endemic areas, contacts exposed to high levels of *M. leprae* bacilli and naturally *M. leprae* infected red squirrels. In **chapter 5** the UCP-LFA detecting αPGL-I IgM was compared to ELISA and a LFA assay using NDO-LID as a target antigen. Samples from three leprosy endemic areas (Bangladesh, Brazil and Philippines) including patients with different bacterial loads per cohort were evaluated in all three tests to compare the different test formats.

In **chapter 6** UCP-LFAs for IP-10, CRP, αPGL-I IgM and CCL4 were evaluated in patients and controls from three countries with different leprosy endemicity (Brazil, China and Ethiopia).

In **chapter 7** levels of the biomarkers identified in chapter 3 were measured in WBA samples from a study tracing contacts from patients with high bacillary loads. For these contacts the presence of *M. leprae* DNA in NS and SSS and the correlation of the biomarkers with the detection of bacterial DNA was determined.

In **chapter 8** the applicability of the αPGL-I IgM UCP-LFA for diagnostics in red squirrels was examined. The presence of the αPGL-I antibodies was assessed longitudinally in red squirrels from the British isles that were followed up and screened for signs and symptoms of leprosy and the presence of *M. leprae* DNA.

In **chapter 9** the general findings of the performed studies will be discussed, focusing on the identified biomarkers for leprosy and *M. leprae* infection and the future implications of this research in leprosy control activities.

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2

Chapter

Quantitative lateral flow strip assays as user-friendly tools to detect biomarker profiles for leprosy

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Sci Rep. 2016 Sep 29;6:34260

Abstract

Leprosy is a debilitating, infectious disease caused by *Mycobacterium leprae*. Despite the availability of multidrug therapy, transmission is unremitting. Thus, early identification of *M. leprae* infection is essential to reduce transmission. The immune response to *M. leprae* is determined by host genetics, resulting in paucibacillary (PB) and multibacillary (MB) leprosy associated with dominant cellular or humoral immunity, respectively. This spectral pathology of leprosy compels detection of immunity to *M. leprae* to be based on multiple, diverse biomarkers.

In this study we have applied quantitative user friendly lateral flow assays (LFAs) for four immune markers (anti-PGL-I antibodies, IL-10, CCL4 and IP-10) for whole blood samples from a longitudinal BCG vaccination field-trial in Bangladesh.

Different biomarker profiles, in contrast to single markers, distinguished *M. leprae* infected from non-infected test groups, patients from household contacts (HHC) and endemic controls (EC), or MB from PB patients. The test protocol presented in this study merging detection of innate, adaptive cellular as well as humoral immunity, thus provides a convenient tool to measure specific biomarker profiles for *M. leprae* infection and leprosy utilizing a field-friendly technology.

Introduction

Leprosy, a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*) ranking second as the most pathogenic mycobacterial infectious disease after tuberculosis (TB), is still considered a major threat in developing countries (1). The condition is characterized by skin lesions and damage to peripheral nerves, the hallmark of leprosy pathology often resulting in severe, life-long disabilities and associated stigma (2,3). Despite the remarkable decrease in prevalence following introduction of multidrug therapy, it remains challenging to further reduce transmission as substantiated by the stable global annual incidence around 200,000 new cases for the past 10 years (4). This continued transmission is largely due to *M. leprae* infected individuals lacking clinical symptoms (5). In addition, identification of host-derived biomarkers for progression to disease is complicated by the low incidence and long incubation time requiring extensive, longitudinal studies. Furthermore, although molecular techniques to elicit strain differences within the leprosy bacillus are important diagnostic tools to enhance our understanding of the epidemiology of leprosy, differentiate between relapse and re-infection (6-10), these pathogen-derived profiles are not suitable to indicate development of leprosy in infected, asymptomatic individuals. These hurdles contributed to the current lack of tests for detection of asymptomatic *M. leprae* infection and diagnosis of early stage leprosy (11). As clinical resistance to commonly used antibiotics in leprosy treatment is increasingly occurring (12, 13), such tests should be highly specific to prevent redundant use of antibiotics.

Clinical manifestations closely parallel cellular immunity to *M. leprae* such that leprosy presents as a characteristic spectrum ranging from tuberculoid (TT) or paucibacillary (PB) leprosy to lepromatous (LL) or multibacillary (MB) leprosy (14). TT patients in general show strong T helper 1 cell (Th1) immunity with exacerbated levels of pro-inflammatory cytokines and develop localized granulomatous disease with bacilli scarcely detectable in their lesions. At the opposite pole of the spectrum are LL patients who predominantly generate Th2 and anti-inflammatory cytokines such as interleukin-10 (IL-10) resulting in disseminating, progressive infections (15). In between these two opposite poles of the leprosy spectrum, borderline states of leprosy [borderline tuberculoid (BT), borderline (BB) and borderline lepromatous (BL)] are positioned. Due to the diverse disease spectrum, detection of *M. leprae* infection in diagnostic tests requires multiple, diverse biomarkers specific for both cellular and humoral mediated immunity. In previous studies we have shown that IFN--inducible protein 10 (IP-10) in response to a *M. leprae*-specific antigen (ML2478) correlates with *M. leprae* exposure and thereby the risk of infection and its subsequent transmission (16). Additionally, we demonstrated that chemokine (C-C motif) ligand 4 (CCL4) a component of the innate immunity, can be used to identify

pathogenic immunityagainst *M. leprae* since it was increased in patients, partly in household contacts but not in endemic controls (16). IL-10, on the other hand, is associated with suppression of Th1 cells in leprosy (17-19). Moreover, most lepromatous patients with high bacillary loads produce antibodies against the *M. leprae* specific phenolic glycolipid I (PGL-I) (20, 21), which are hardly detected in PB (22). Hence, sensitive tests that can simultaneously quantitate multiple analytes in one sample provide apt tools to characterize different clinical leprosy types. In particular, tests based on multicomponent host biomarker profiles that can identify *M. leprae* infected individuals (yet) without clinical symptoms of leprosy, will be useful for guidance of prophylactic treatment, thereby contributing to reduction of *M. leprae* transmission as well as prevention of disabilities.

Inherent to the situation in leprosy endemic areas is the absence of sophisticated laboratories. It is therefore imperative that new diagnostic tests are facilitated for application in the field. Up-converting phosphor lateral flow assays (UCP-LFAs) have previously shown to be robust, low-complexity assays, representing a field-friendly alternative for common laboratory-based ELISAs (23, 24), applicable for detection of multiple pathogens including food-borne pathogenic strains and potential biowarfare/ bioterrorism agents (25-27) . Field evaluation of UCP-LFAs for detection of IL-10, IP-10, CCL4 and anti-PGL-I IgM demonstrated high correlation with ELISAs using samples from cohorts of limited numbers of leprosy- or TB patients (28, 29).

In the current study UCP-LFAs were applied to a more extensive (five-fold) sample size compared to our previous studies, derived from a randomized BCG vaccination field trial in Bangladesh (30). Six test groups were included: MB patients, PB patients, healthy household contacts (HHC), HHC vaccinated with Bacillus Calmette-Guérin (BCG) (HHC&BCG), HHC who developed leprosy after BCG vaccination (new cases; NC) and endemic controls (EC) from the same area without known contact with leprosy patients. This extended cohort study allowed exploratory identification of biomarker profiles for *M. leprae* infection, leprosy disease per se, the type of leprosy and BCG vaccination as determined with UCP-LFAs for the above indicated targets.

Materials and methods

Study participants

Participants were recruited on a voluntary basis between January 2013 and December 2014 in leprosy endemic areas in Bangladesh as described previously (30). Leprosy was diagnosed based on clinical, bacteriological and histological observations and classified by skin smears according to Ridley and Jopling (14). Clinical and demographic data was

collected in a database. Participants were classified into six test groups; MB patients, PB patients, HHC, HHC&BCG, NC and EC. Control individuals from the same leprosy endemic area (EC) were examined for the absence of clinical signs and symptoms of leprosy and TB; staff of leprosy- or TB clinics were excluded.

Test group selection

A randomized sample selection was taken from 1110 participants (30). Individuals were randomly assigned for sample inclusion using the RAND formula (Excel 2010), aiming for a 50/50 male/female ratio and a 1:1:1 ratio of three age groups: 0-14, 15-29, and 30+ (Supplementary Table S1). In total 242 individuals were selected; MB patients (n=34), PB patients (n=45), HHC (n=54), HHC&BCG (n=50), EC (n=51) and NC (n=8; PB=7, MB=1). Patient characteristics are shown in Supplementary Table S2.

Leprosy prevalence

During this study the prevalence in the four districts (Nilphamari, Rongpur, Ponchagor and Thakurganch) was 0.82 per 10,000 with a new case detection rate of 0.98 per 10,000 (monthly report of Rural Health Program of 4 districts of Nilphamari, Bangladesh).

Whole blood assay (WBA)

Upon recruitment venous, heparinized blood (4 ml) was used directly in whole blood assays (WBA), using microtubes pre-coated with *M. leprae* whole cell sonicate (designated WCS), ML2478/ ML0840 recombinant proteins (designated Mlep) (16) or without antigen stimulus (designated Nil) (30). After 24 hour incubation at 37°C materials were frozen at -20°C, shipped on dry ice to the LUMC and stored at -80°C until analysis by ELISA or UCP-LFA (24).

PGL-I and *M. leprae* whole cell sonicate (WCS)

Synthesized disaccharide epitope (3,6-di-O-methyl-β-D-glucopyranosyl(1 -4)2,3-di-O-methylrhamnopyranoside), similar to *M. leprae* specific PGL-I glycolipid, coupled to human serum albumin (synthetic PGL-I; designated ND-O-HSA) and *M. leprae* whole cell sonicate (WCS) generated with support from the NIH/NIAID Leprosy Contract N01-AI-25469 were obtained through the Biodefense and Emerging Infections Research Resources Repository (http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx)(47).

PGL-I ELISA

IgM antibodies against *M. leprae* PGL-I were detected as previously described (19).

Absorbance of horseradish peroxidase (HRP) was determined at a wavelength of 450 nm.

ELISA for IL-10, IP-10 and CCL4

IP-10 (851.870.015, Diaclone Research, Besancon, France), IL-10 (851.540.015, Diaclone Research, Besancon, France) and CCL4 (DY271-05, R&D systems, Minneapolis, USA) ELISA kits were used. ELISA testing was performed according to the manufacturer's protocol using coating antibody clones B-S10, B-C50 and #24006 and detection antibodies B-T10, BC-55 and BAF271 respectively for IL-10, IP-10 and CCL4. HRP absorbance was determined at wavelength of 450 nm.

UCP-LFA for IL-10, IP-10 and CCL4

UCP-LFAs for CCL4, IL-10 and IP-10 were prepared and performed as described previously (24, 28, 29). The same antibody pairs as used for ELISAs were applied, with the non-biotinylated variant of the detection antibodies (non-biotinylated CCL4: AF-271-NA). Briefly, mixtures of 100 ng cytokine-specific UCP reporter conjugate and diluted serum sample (1:4 for IL-10, 1:30 for IP-10 and 1:300 for CCL4) were incubated for 60 min on a thermoshaker at 37 °C and 900 rpm. The mixture was then applied to cytokine-specific LF strips (containing a Test line with an antibody complementary to the antibody on the UCP particles) and immunochromatography was allowed to continue until strips were dry. LF strips were scanned in a Packard FluoroCount microtiterplate reader adapted for measurement of the UCP label (980 nm IR excitation, 550 nm emission). Results are displayed as the ratio value between Test and Flow-Control signal based on relative fluorescence units (RFUs) measured at the respective lines (48). Ratio values were translated to concentration based on standard curves for each immunemarker. Lower limit of detection was 32 pg/ml for IL-10 and 316 pg/ml for IP-10 and CCL4.

To determine test positivity, similar wholeblood samples from a set of healthy, non-endemic control individuals (NEC) were analysed and UCP-LFA thresholds were calculated based on the average value of all NEC samples (Supplementary Table S3).

UCP-LFA for anti-PGL-I antibody

For detection of anti-PGL-I IgM antibodies, the same protocol as used for cytokine detection was applied utilizing 100-fold diluted serum and IgM-specific UCP conjugate ($UCP^{\alpha IgM}$). Only unstimulated samples were analysed as the level of antibody levels does not change upon antigen stimulation. The threshold for positivity of 0.29 was determined by computing receiver operating characteristic (ROC) curves.

Ethics

This study was performed according to the Helsinki Declaration as described previously (30). The national Research Ethics Committee (Bangladesh Medical Research Council) has approved the study protocol (Ref no. BMRC/NREC/2010-2013/1534).Participants were informed about the study-objectives, the samples and their right to refuse to take part or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. All patients received treatment according to national guidelines.

ROC curves

Graphpad Prism version 6.02 for Windows (GraphPad Software, San Diego CA, USA www.graphpad.com) was used to plot ROC curves and calculate the area under curve (AUC); for IP-10, IL-10, and CCL4 the concentrations (pg/ml) were applied, whereas for anti-PGL-I IgM the OD_{450} corrected for background (ELISA) and ratio value (UCP-LFAs) was used.

Statistical analysis

Differences in cytokine or antibody levels between test groups, as determined with UCP-LFA, were analysed with the One-way ANOVA for non-parametric distribution (Kruskall-Wallis) and Dunn's correction for multiple testing using GraphPad Prism. For IP-10, IL-10 and CCL4 the concentrations (pg/ml) and for anti-PGL-I IgM the UCP-LFA ratio values were utilized. The statistical significance level used was $p \leq 0.05$.

Results

Performance of the UCP-LFA versus ELISA

Whole blood samples (n=726) from all individuals were analysed using ELISA, as well as the field-friendly UCP-LFAs for IL-10, IP-10, CCL4 and anti-PGL-I antibodies. Comparison of UCP-LFA and ELISA results demonstrated significant correlation for all four biomarkers ($p<0.0001$), confirming earlier observations (28, 29).

The diagnostic performance of the UCP-LFA in comparison to ELISA was further assessed through AUCs for the two most distinct phenotypes: MB patients (n=34) and EC (n=51). IL-10, IP-10 and CCL4 levels were determined in Nil, WCS and Mlep samples, as well as anti-PGL-I IgM levels (Figure 1). The IL-10 and IP-10 UCP-LFAs outperformed the corresponding ELISAs, whereas the CCL4 and anti-PGL-I IgM tests performed equally. For discrimination of MB patients from EC, the proposed diagnostic field-tool UCP-LFA provides an equally well or even better alternative for the conventional ELISAs.

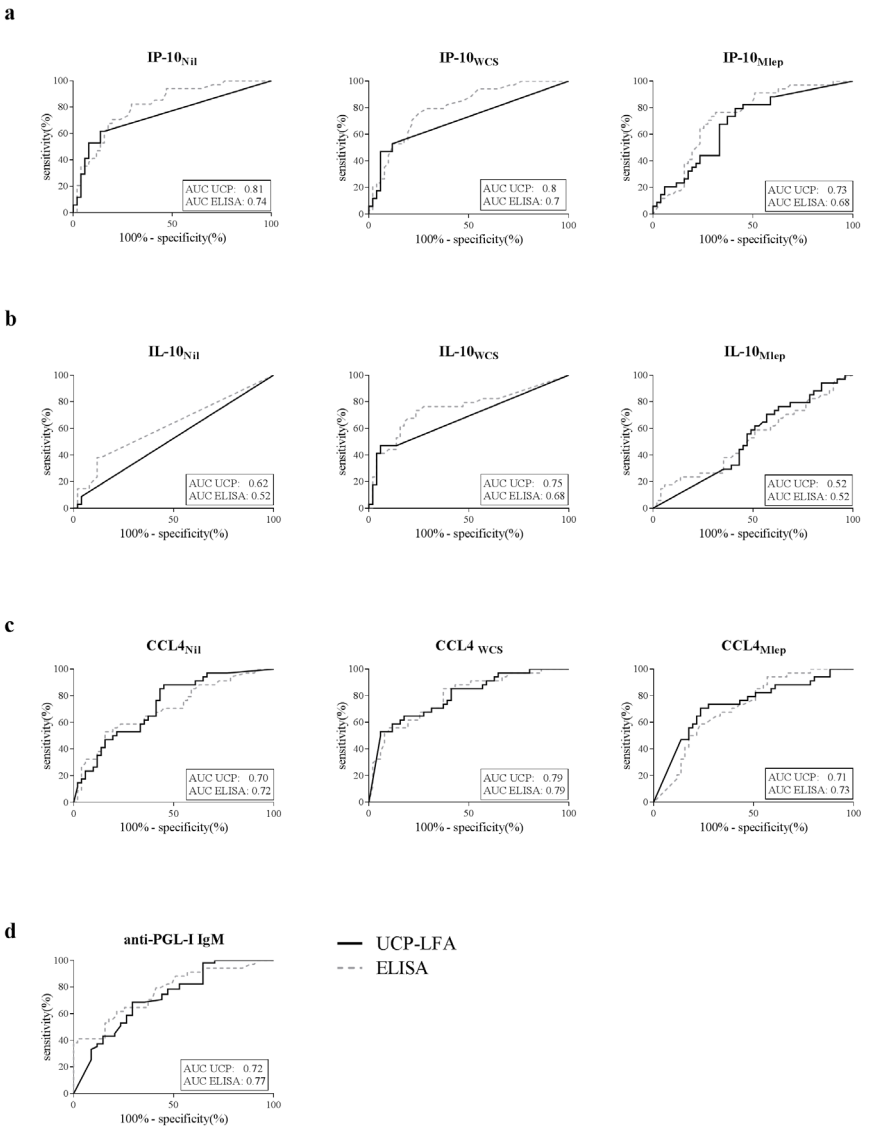

Figure 1: Discriminatory capacity of ELISA and UCP-LFA. To compare the ability of ELISA (dotted line) and UCP-LFA (solid line) to discriminate between individuals with or without disease ROC curves were computed using data of MB patients and EC. Areas under the curve (AUCs) were compared for all 10 conditions tested, shown in the lower right corner of each graph. (a) ROC curves for IP-10 stimulated and unstimulated samples based on concentration in pg/ml, showing an improved AUC for the UCP-LFA for IP-10_{Nil} and IP-10_{WCS}. (b) ROC curves for IL-10 stimulated and unstimulated samples based on concentration in pg/ml, showing an improved (IL-10_{Nil}) or equal AUC for UCP-LFA. (c) ROC curves for CCL4 stimulated and unstimulated samples based on concentration in pg/ml, showing comparable values for UCP-LFA and ELISA. (d) ROC curves for anti-PGL-I IgM in unstimulated samples based on ratio, showing comparable AUCs for ELISA and UCP-LFA.

M. leprae specific responses based on single analyte UCP-LFA measurements

In order to put the quantitative test results obtained with the four single UCP-LFAs in the context of their biomarker potential, we assessed each analyte/ stimulus combination by comparing median group levels. As indicated by the AUCs in Figure 1, MB patients can be distinguished from EC based on IP-10 and CCL4 (irrespective of stimulus), anti-PGL-I IgM and IL-$10_{WCS,}$ therefore also showing significantly different median levels (Figure 2, Table 1). Moreover, median levels of anti-PGL-I IgM and IP-10_{Nil} differed between MB patients and (BCG-vaccinated) HHC, whereas median levels of IP-10_{WCS} and $CCL4_{WCS}$ only distinguished the non-vaccinated HHC from MB patients.

BCG vaccination therefore affects the immune response in HHC, as reflected by the significant difference in IP-10_{WCS} levels between HHC and HHC&BCG ($p=0.018$) (Figure 2B). Furthermore, IL-10_{WCS} levels differed between HHC and EC, while median levels of IP-10_{WCS} and $CCL4_{WCS}$ differed between HHC&BCG and EC as well. PB patients and EC showed significantly different median $CCL4_{Nil}$ and $CCL4_{WCS}$ levels (Figure 2C), as well as borderline significant different levels for IL-10_{WCS} ($p=0.07$) and IP-10_{WCS} ($p=0.06$).

The ability of each analyte/ stimulus combination to distinguish between two groups is summarized in Table 1, thereby reviewing the biomarker potential of the four individual host immune markers. Remarkably, the levels of IP-10 ($p<0.0001$), IL-10 ($p=0.003$) and CCL4 ($p<0.0001$) in WCS stimulated samples were more significantly different for MB and EC than anti-PGL-I IgM levels ($p=0.0042$). Moreover, anti-PGL-I IgM levels could not be used to discriminate PB patients or (BCG-vaccinated) HHC from EC, which clearly demonstrates the added value of IP-10, IL-10 and CCL4 in leprosy diagnostics.

Table 1: Discriminatory biomarkers with potential for leprosy diagnostics

	MB vs HHC	MB vs HHC&BCG	MB vs EC	PB vs EC	HHC vs EC	HHC&BCG vs EC	HHC&BCG vs HHC
anti-PGL-I IgM	++	++	++	-	-	-	-
IP-10	++	++	++	-	-	-	-
IP-10 WCS	++++	-	++++	-	-	++	+
IP-10 *M.leprae* proteins	-	-	+	-	-	-	-
IL-10	-	-	-	-	-	-	-
IL-10 WCS	-	-	+++	-	++	+	-
IL-10 *M.leprae* proteins	-	-	-	-	-	-	-
CCL4	-	-	+	+	-	-	-
CCL4 WCS	++	-	++++	+	-	++	-
CCL4 *M.leprae* proteins	-	-	+	-	-	-	-

Differences in IP-10, IL-10, CCL4 and anti-PGL-I IgM levels between various test groups detected by UCP-LFA are provided . Each row represents one of the 10 different analyte/ stimulus combinations measured. Each column shows the potential to distinguish the test groups indicated, only displaying the groups for which significant differences were observed: - $p \geq 0.05$ indicates inability to distinguish test groups, + : $p \leq 0.05$, ++ : $p \leq 0.01$, +++ : $p \leq 0.001$, ++++ : $p \leq 0.0001$ indicating increasing capacity to distinguish test groups. Using one or multiple analyte/ stimulus combination MB patients could be distinguished from (BCG-vaccinated) HHC and EC, whereas PB patients and BCG-vaccinated HHC could be distinguished from EC.

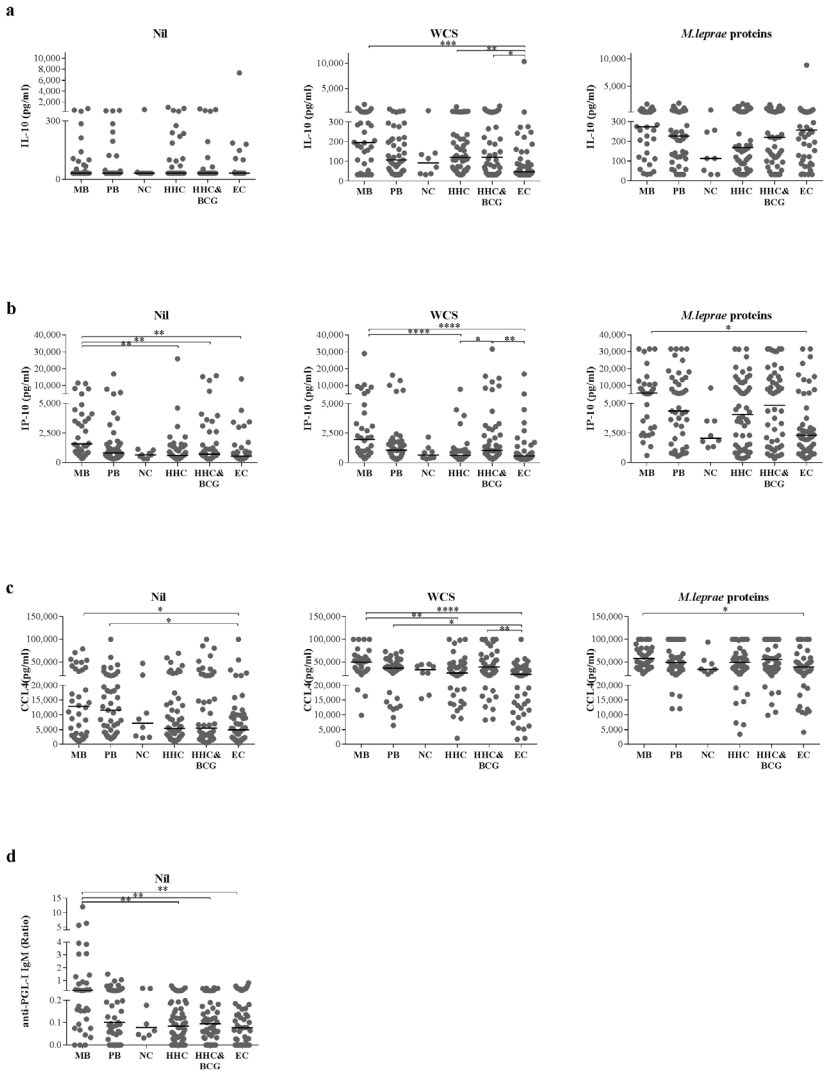

Figure 2: Identification of *M. leprae* specific IL-10, IP-10, CCL4 and anti-PGL-I IgM antibodies by UCP-LFA. (a) IL-10 concentrations (pg/ml) measured per group per stimulus show that MB patients, HHC and BCG-vaccinated HHC significantly differ from EC upon WCS stimulation. (b) IP-10 concentrations (pg/ml) measured per group per stimulus show that MB patients significantly differ from EC in both stimulated and unstimulated samples, from HHC in unstimulated and WCS stimulated samples and from BCG vaccinated HHC in unstimulated samples. BCG vaccinated HHC significantly differ from HHC and EC upon WCS stimulation. (c) CCL4 concentrations (pg/ml) measured per group, per stimulus show that MB patients significantly differ from EC in both stimulated and unstimulated samples and from HHC in WCS stimulated samples. PB patients significantly differ from EC in unstimulated and WCS stimulated samples and BCG vaccinated HHC significantly differ from EC in WCS stimulated samples. (d) anti-PGL-I IgM ratio measured per groups shows that MB patients have significantly higher levels of anti-PGL-I IgM compared to HHC, BCG vaccinated HHC and EC. P-values: *: $p \leq 0.05$, **: $p \leq 0.01$, *** : $p \leq 0.001$, **** : $p \leq 0.0001$.

Biomarker signatures to specify *M. leprae* infection, leprosy or disease classification

Diagnostic tests that allow detection of *M. leprae* infection, leprosy per se and leprosy classification would be of great benefit to the general healthcare in leprosy endemic areas. The four host immune markers allowed distinction between two groups (Table 1). However, to distinguish *M. leprae* infected from non-infected individuals or patients from healthy contacts, we compared host immune markers for multiple groups (Figure 3).

First, in order to combine immune markers into multicomponent host biomarker profiles, positive UCP-LFA results for each analyte/ stimulus combination were collectively specified (Figure 3; Supplementary Table S3). Second, analyte/ stimulus combinations were selected such that they optimally distinguished individuals with a specified disease- or infection state (Figure 3), considering all HHC as *M. leprae* infected. This resulted in three specific profiles:

I. To indicate *M. leprae* infection we selected single test results obtained with IP-10_{Mlep}, $CCL4_{WCS}$ and IL-10_{WCS} UCP-LFAs as these analyte/ stimulus combinations individually showed the least positive test results for EC compared to the *M. leprae* infected test groups (MB, PB, HHC and HHC&BCG) (Figure 3). The combination of IP-10_{Mlep}, $CCL4_{WCS}$ and IL-10_{WCS} indeed was more frequently positive for MB/PB patients and (BCG-vaccinated) HHC than EC (Figure 4A). Moreover, AUCs confirmed discrimination between non-infected and *M. leprae* infected test groups based on this multicomponent host immune profile (AUCs: 0.84 (MB vs. EC), 0.75 (PB vs. EC), 0.7 (HHC vs. EC) and 0.71 (HHC&BCG vs. EC) (Supplementary table S4A).

II. To detect leprosy patients from healthy, though possibly *M. leprae* infected individuals, $CCL4_{WCS}$ and IP-10_{WCS} were selected as immune markers since these single tests were more frequently positive in patients (MB and PB) compared to contacts (HCC and HCC&BCG) and are therefore associated with pathogenic immunity to *M. leprae*. The combination of $CCL4_{WCS}$ and IP-10_{WCS} indeed demonstrated a positive test result more often in patients than in HHC or EC (Figure 4B), whereas the related AUCs were ≥ 0.66 thus confirming leprosy disease-specificity (Supplementary table S4B).

III. For classification of leprosy a signature consisting of anti-PGL-I IgM, IL-10_{WCS} and IP-10_{Nil} was applied, as each of these markers individually showed more positive test results in MB patients compared to PB patients (Figure 3). This profile proved to be specific for MB patients (Figure 4C) and thereby allowed the differentiation of MB and PB patients (AUC=0.73, Supplementary table S4C).

a

	Groups to differentiate	Potential Profile
Infection	MB, PB, HHC, HHC&BCG vs. EC	$IP10_{Mlep}$, $CCL4_{WCS}$, $IL10_{WCS}$
Disease per se	MB, PB vs. HHC, HHC&BCG, EC	$IP10_{WCS}$, $CCL4_{WCS}$
Disease classification	MB vs. PB	anti-PGL-I IgM, $IL10_{WCS}$, $IP10_{Nil}$

b

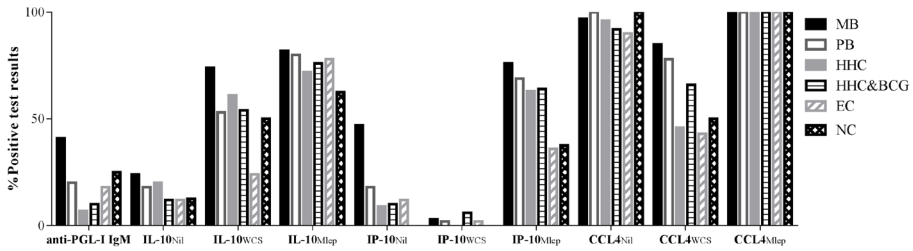

Figure 3: Positive test results per analyte/ stimulus combination used to construct potential biomarker profiles. The groups that should be differentiated to indicate *M. leprae* infection, disease per se and disease classification are shown. The potential profiles indicated are based on the percentage of positive individuals of these particular groups. The cut-off for positivity was based on values for NEC (Supplementary Table S3) per analyte/ stimulus combination the percentage of individuals with a positive test result per group is shown. Based on these data the optimal analyte/ stimulus combination to differentiate either infected from non-infected groups, patients and non-patients groups or MB and PB patients were selected to construct the potential profiles described.

Ideally only one multicomponent host biomarker profile for diagnosis of *M. leprae* infection, leprosy per se and leprosy classification would be more suitable for field-use. In this exploratory study, a 4 marker profile of IL-10_{WCS}, IP-10_{Mlep}, $CCL4_{WCS}$ and anti-PGL-I IgM was selected for this purpose, enabling distinction of infected and non-infected individuals by IL-10_{WCS}, IP-10_{Mlep} and $CCL4_{WCS}$, MB and PB patients from HHC and EC by $CCL4_{WCS}$ and MB from PB patients by anti-PGL-I IgM and IL-10_{WCS} (Figure 4D; Supplementary Table S4D). However, to distinguish MB from PB patients or PB patients from HHC profile III for leprosy classification showed a higher AUC compared to the 4 marker profile (0.73 vs. 0.65 and 0.66 vs. 0.62 respectively, Supplementary Table S4). These data indicate the importance of distinct phase-specific profiles, the application of which will depend on the nature of the diagnosis to be made.

Nonetheless, application of the 4 marker profile demonstrated the influence of multicomponent host biomarker profiles on test accuracy, showing increased AUCs compared to individual markers (Supplementary Figure S1). The added value of using various analytes indicates the potential of multicomponent host biomarker profiles for leprosy diagnostics to detect *M. leprae* infection, leprosy disease or disease classification.

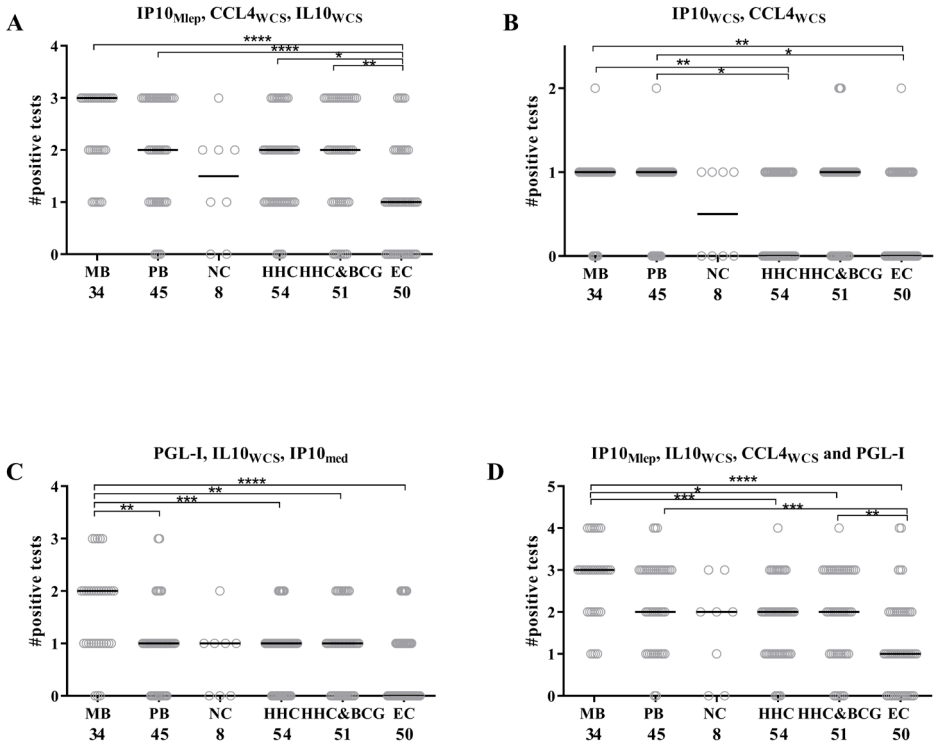

Figure 4: Potential of biomarker profiles to indicate *M. leprae* infection, disease per se and disease classification. The amount of positive test results per group is shown. (a) IP-10_{Mlep}, $CCL4_{WCS}$ and IL-10_{WCS} significantly differed in MB/PB patients and (BCG-vaccinated) HHC from EC, showing more positive test results in the groups that are exposed to *M. leprae* and thereby indicating *M .leprae* infection. (b) $CCL4_{WCS}$ and IP-10_{WCS} enabled the distinction between patients and HHC, thereby indicating the pathogenic immune responses to *M. leprae* in patients. (c) Anti-PGL-I IgM, IL-10_{WCS} and IP-10_{Nil} showed more positive test results in MB patients thereby enabling the distinction between MB and PB patients. (d) A four marker profile of IL-10_{WCS}, IP-10_{Mlep}, $CCL4_{WCS}$ and anti-PGL-I IgM shows the majority of significant differences observed in A, B and C. P-values: *: $p \leq 0.05$, **: $p \leq 0.01$, *** : $p \leq 0.001$, **** : $p \leq 0.0001$.

Discussion

The obvious incessant transmission of *M. leprae* has brought about increased focus in leprosy research on discovery of biomarkers to improve diagnosis. Nevertheless, thus far only few biomarkers for leprosy are recommended by expert panels (11). Consequently, there is a growing need for new and sensitive diagnostic tools based on specific biomarkers which should, ideally, allow straightforward translation into field-friendly tests.

In this exploratory study, we aimed to provide several multicomponent host immune-biomarker profiles which distinguish between distinct stages of *M. leprae* infection.

In this process we also emphasized the challenges that need to be tackled to allow application of these biomarkers in the field. As high-tech laboratories are often lacking in leprosy endemic areas, we examined the diagnostic potential of earlier developed field-friendly UCP-LFAs for detection of anti-PGL-I IgM antibodies and cyto/chemokines IP-10, IL-10 and CCL4 (24, 28, 31), in an extensive cohort in Bangladesh.

We demonstrated the biomarker potential of IP-10, IL-10, CCL4 and anti-PGL-I IgM measured by UCP-LFAs in whole blood, either in response to *M. leprae* specific stimuli or without stimulus. Moreover, multicomponent host biomarker profiles including selected analyte/ stimulus combinations could indicate *M. leprae* infection, leprosy per se or be used for classification of leprosy subtypes. A biomarker profile of IP-10_{Mlep}, $CCL4_{WCS}$ and IL-10_{WCS} was highly indicative of *M. leprae* infection, consistent with our previous finding that the IP-10 response to *M. leprae* specific proteins indicates exposure to *M. leprae* (16,32).

Leprosy per se, on the other hand, was indicated by $CCL4_{WCS}$ and IP-10_{WCS}, showing the potential to identify pathogenic immunity against *M. leprae* and confirming earlier observations on CCL4 (16). As current diagnostic assays for leprosy are antibody-based and only facilitate the diagnosis of MB cases (33-35), inclusion of the host immune markers CCL4 and IP-10 in the profile shows promise for diagnosis of PB patients and indicates the importance of measuring cellular markers simultaneously with humoral markers.

For leprosy classification, the combination of anti-PGL-I IgM, IL-10_{WCS} and IP-10_{Nil} was indicative for MB patients, enabling the distinction between MB and PB patients. Although IL-10 and particularly anti-PGL-I IgM have been identified as characteristic markers for MB leprosy (36, 37), we also identified IP-10 as a, seemingly counterintuitive, host immune marker for patients at this side of the spectrum who usually display decreased pro-inflammatory immunity. However, since T-cells are not the exclusive source of IP-10 (38), IP-10 may still be produced in MB patients by monocytes and neutrophils (39), as described for HIV-infected TB patients (40).

To detect *M. leprae* infection, leprosy per se, as well as leprosy classification simultaneously with only one biomarker profile, IL-10_{WCS}, IP-10_{Mlep}, $CCL4_{WCS}$ and anti-PGL-I IgM demonstrated the most optimal 4 marker profile performance. However, it performed less optimal for the distinct stages of *M. leprae* infection than the phase-specific profiles. Other cyto-/ chemokines to identify pathogenic immunity to *M. leprae* (e.g. MCP-1 and IL-1β (16)), leprosy classification (e.g. CCL17 and CCL18 (41)) or general mycobacterial infection (EN-RAGE (42, 43)) could therefore be included to achieve more optimal diagnostic accuracy (44) as distinct phase-specific profiles. In a multiplex UCP-LFA format multicomponent host immune biomarker profiles can be measured in one

single test. This format therefore provides a field-friendly diagnostic tool, facilitating the diagnosis of leprosy based on biomarker signatures.

Of note is the observation that CCL4 levels in response to *M. leprae* WCS were elevated for HHC who received BCG vaccination compared to those who did not. Thus, BCG vaccination may also cause increased pro-inflammatory immune responses which renders contacts more prone to development of over-reactive, pathogenic immunity to *M. leprae*. Indeed, in a recent vaccination study an unexpectedly high proportion of HHC presented with PB leprosy after BCG vaccination supporting this idea (45). In this respect, this vaccination study also shows the importance of immunomonitoring individuals at high risk to identify and treat patients at an early stage. In addition, since BCG vaccination or boost is a well-accepted prophylaxis against leprosy in contacts of newly diagnosed patients (46), it is relevant to distinguish BCG-induced immunity in healthy contacts from early stage leprosy in these individuals. To efficiently monitor contacts for this purpose, the different stages of infection and disease of leprosy should be covered in diagnostics tools. Through simultaneous measurement of all analytes of interest on a single lateral flow strip, this format allows assessment of multicomponent host biomarker profiles using a unique field-friendly technology (24, 29, 31). Thereby, the UCP-LFA format not only provides diagnostic tools for leprosy but similarly holds promise for TB diagnosis (28) and immunomonitoring of other chronic diseases (31).

Acknowledgements

The authors gratefully acknowledge all patients and blood donors. LUMC, Erasmus MC and TLMI,B are part of the IDEAL (***I***nitiative for ***D***iagnostic and ***E***pidemiological ***A***ssays for ***L***eprosy) Consortium. We are indebted to staff of the Rural Health Program and dr. Ruth Butlin (The Leprosy Mission International Bangladesh, Nilphamari, Bangladesh) for recruitment of study participants and sample collection.

Funding Statement

Recruitment of the study participants in Bangladesh was supported by grants from the Order of Malta-Grants-for-Leprosy-Research (MALTALEP), the Netherlands Leprosy Relief Foundation (NLR; ILEP#: 7020273 and 703.15.07) together with the German Leprosy and TB Relief Association (GLRA) and the Turing Foundation, the Novartis Foundation and the Leprosy Research Initiative (LRI). Development of the UCP-LFA was supported by the Q.M. Gastmann-Wichers Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Financial Interests

The authors declare that they have no conflict of interest.

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Supplementary material

Tables

Supplementary Table S1: Test group selection

Test group	Gender	Age group		
		0-14	15-29	30+
EC	F	10	10	10
EC	M	10	10	10
HHC&BCG	F	10	10	10
HHC&BCG	M	10	10	10
HHC	F	10	10	10
HHC	M	10	10	10
MB	F	2	1	4
MB	M	0	4	20
PB	F	2	8	11
PB	M	0	15	15

Samples were randomly selected using a 50/50 ratio of males and females and a 1:1:1 ratio of three age groups (0-14 yrs; 15-29 yrs; 30 yrs or older) within each group. For the MB and PB patient groups samples could not be evenly distributed for age and gender, due to the limited number of samples present.

Supplementary Table S2: Patient characteristics according to Ridley-Jopling classification. The bacterial index (BI) is indicated for all MB patients.

	BI	Classification
MB1	5	BL
MB2	5	LL
MB3	4	BL
MB4	4	BL
MB5	4	LL
MB6	4	LL
MB7	3	BL
MB8	3	BT
MB9	1	BT
MB10	0	BL
MB11	0	BT
MB12	0	BT
MB13	0	BT
MB14	0	BT
MB15	0	BT
MB16	0	BT
MB17	0	BT
MB18	0	BT
MB19	0	BT
MB20	0	BT
MB21	0	BT
MB22	0	BT
MB23	0	BT
MB24	0	BT
MB25	0	BT
MB26	0	BT
MB27	0	BT
MB28	0	BT
MB29	0	BT
MB30	0	BT
MB31	0	BT
MB32	0	BT
MB33	0	BT
MB34	0	BT

	Classification
PB1	TT
PB2	TT
PB3	TT
PB4	TT
PB5	BT
PB6	BT
PB7	BT
PB8	BT
PB9	BT
PB10	BT
PB11	BT
PB12	BT
PB13	BT
PB14	BT
PB15	BT
PB16	BT
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PB37	BT
PB38	BT
PB39	BT
PB40	BT
PB41	BT
PB42	BT
PB43	BT
PB44	BT
PB45	BT

Supplementary Table S3: Cut-off values for IL-10, IP-10 and CCL4

	IL-10 Nil	IL-10 WCS	IL-10 Mlep	IP-10 Nil	IP-10 WCS	IP-10 Mlep	CCL4 Nil	CCL4 WCS	CCL4 Mlep
NEC1	32	32	32	316	379	450	316	11621	1379
NEC2	32	32	32	316	1935	519	316	12732	566
NEC3	32	32	32	379	14771	790	316	8374	460
NEC4	32	32	32	1481	992	2015	316	2838	316
Threefold Average	96	96	96	1869	13557	2830	948	26673	680
Cut-off	100	100	100	1900	14000	2800	950	27000	700

IP-10, IL-10 and CCL4 concentrations (pg/ml) determined by UCP-LFA of 24h whole blood cultures of non-endemic healthy control individuals (NEC; n=4) without stimulus (Nil), in response to M. leprae whole cell sonicate (WCS) or M. leprae recombinant proteins [ML2478 and ML0840(Mlep)]. The cut-off for a positive test result was set as three times the average value of the four NEC in unstimulated samples, WCS and Mlep stimulated samples.

Supplementary Table S4: Areas under the curve for each biomarker profile

a

AUC	*M. leprae* Infection ($IP10_{Mlep}$ $CCL4_{WCS}$ $IL10_{WCS}$)					
	MB	PB	NC	HHC	HHC&BCG	EC
MB		ns	0.76	0.7	0.63	0.84
PB	ns		ns	ns	ns	0.75
NC	0.76	ns		ns	ns	ns
HHC	0.7	ns	ns		ns	0.7
HHC&BCG	0.63	ns	ns	ns		0.71
EC	0.84	0.75	ns	0.7	0.71	

b

AUC	Leprosy ($IP10_{WCS}$ $CCL4_{WCS}$)					
	MB	PB	NC	HHC	HHC&BCG	EC
MB		ns	ns	0.7	ns	0.71
PB	ns		ns	0.66	ns	0.67
NC	ns	ns		ns	ns	ns
HHC	0.7	0.66	ns		0.61	ns
HHC&BCG	ns	ns	ns	0.61		0.62
EC	0.71	0.67	ns	ns	0.62	

c

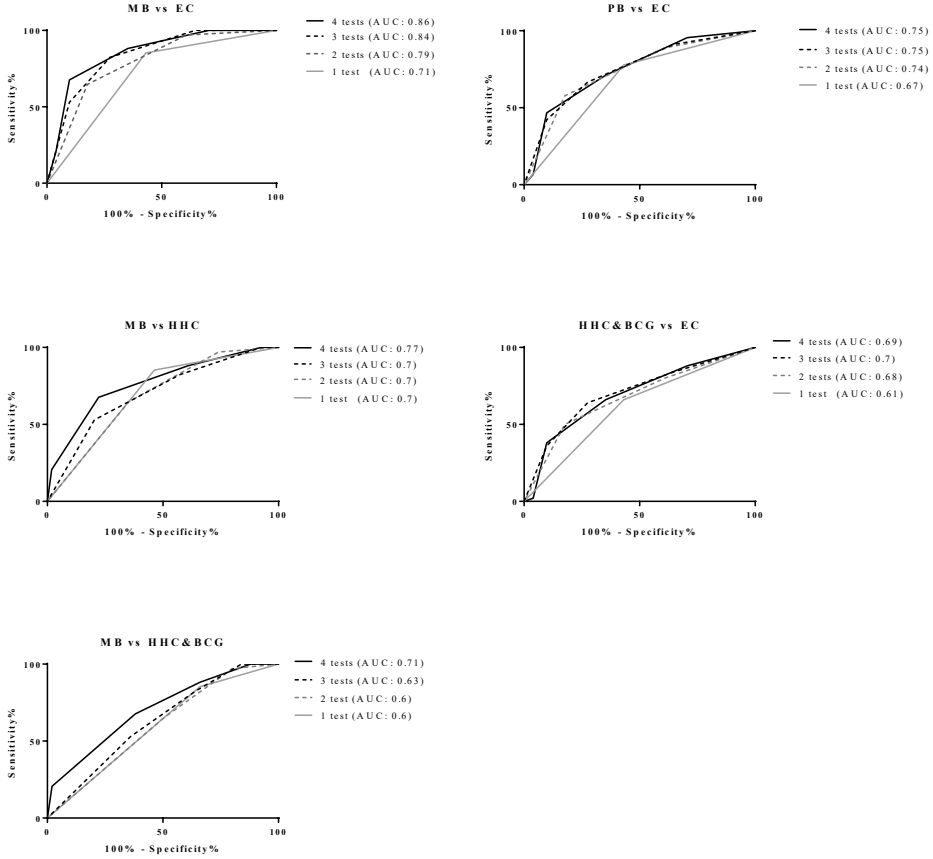
AUC	Leprosy Classification (PGL-I $IL10_{WCS}$ $IP10_{med}$)					
	MB	PB	NC	HHC	HHC&BCG	EC
MB		0.73	0.77	0.77	0.73	0.82
PB	0.73		ns	ns	ns	0.64
NC	0.77	ns		ns	ns	ns
HHC	0.77	ns	ns		ns	0.61
HHC&BCG	0.73	ns	ns	ns		0.63
EC	0.82	0.64	ns	0.61	0.63	

d

AUC	Four marker profile ($IP10_{MIep}$ $IL10_{WCS}$ $CCL4_{WCS}$ PGL-I)					
	MB	PB	NC	HHC	HHC&BCG	EC
MB		0.65	0.77	0.77	0.7	0.86
PB	0.65		ns	0.62	ns	0.75
NC	0.77	ns		ns	ns	ns
HHC	0.77	0.62	ns		ns	0.67
HHC&BCG	0.7	ns	ns	ns		0.69
EC	0.86	0.75	ns	0.67	0.69	

Areas under the curve (AUC) were determined using Graphpad Prism version 6.02 for Windows (GraphPad Software, San Diego CA, USAwww.graphpad.com) for M. leprae infection (a), leprosy (b), leprosy classification (c) and the four marker profile (d), displaying only AUCs that showed a significant p-value (ns=non-significant). AUCs were determined for each combination of groups. (MB: multibacillary patients; PB: paucibacillary patients; NC=new cases who developed leprosy after BCG vaccination; HHC: healthy household contacts; HHC&BCG: BCG-vaccinated HHC; EC: endemic controls). (a) Combined test results for $IP\text{-}10_{MIep}$, $CCL4_{WCS}$ and $IL10_{WCS;}$ (b) Combined test results for $IP\text{-}10_{WCS}$, and $CCL4_{WCS\ ;}$ (c) Combined test results for anti-PGL-I IgM $IP\text{-}10_{Med}$, and $IL10_{WCS;}$ (d) Four marker profile of $IL\text{-}10_{WCS}$, $IP\text{-}10_{MIep}$, $CCL4_{WCS}$ and anti-PGL-I IgM, showing the potential of this profile to indicate M. leprae infection, leprosy per se and leprosy classification.

Figures



Supplementary Figure S1: Influence of multicomponent host biomarker profiles on test accuracy. ROC curves and the corresponding areas under the curve (AUC) using UCP-LFAs for 1, 2, 3 or 4 marker profiles ($CCL4_{WCS}$, $IP\text{-}10_{Mlep}$, $IL\text{-}10_{WCS}$ and anti-PGL-I IgM). Only the groups that showed significant differences in test results based on this 4 marker profile are shown. The AUC for the 4 marker profile test was increased compared to the AUC for 1 marker profile, showing improved diagnostic accuracy (44). 1 marker profile = $CCL4_{WCS}$ (the most sensitive test condition for detecting patients); 2 marker profile = $CCL4_{WCS}$ and $IP\text{-}10_{Mlep}$; 3 marker profile = $CCL4_{WCS,}$ $IP\text{-}10_{Mlep}$ and $IL\text{-}10_{WCS}$; 4 marker profile = $CCL4_{WCS,}$ $IP\text{-}10_{Mlep}$, $IL\text{-}10_{WCS}$ and anti-PGL-I IgM

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Chapter

Application of new host biomarker profiles in quantitative point-of-care tests facilitates leprosy diagnosis in the field

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EBioMedicine. 2019 Sep;47:301-308

Abstract

Background: Transmission of *Mycobacterium leprae*, the pathogen causing leprosy, is still persistent. To facilitate timely (prophylactic) treatment and reduce transmission it is vital to both early diagnose leprosy, and identify infected individuals lacking clinical symptoms. However, leprosy-specific biomarkers are limited, particularly for paucibacillary disease. Therefore, our objective was to identify new biomarkers for leprosy and assess their applicability in point-of-care (POC) tests.

Methods: Using multiplex-bead-arrays, 60 host-proteins were measured in a cross-sectional approach in 24-hours whole blood assays (WBAs) collected in Bangladesh (79 patients; 54 contacts; 51 endemic controls (EC)). Next, 17 promising biomarkers were validated in WBAs of a separate cohort (55 patients; 27 EC). Finally, in a third cohort (36 patients; 20 EC), five candidate markers detectable in plasma were assessed for application in POC tests.

Findings: This study identified three new biomarkers for leprosy (ApoA1, IL-1Ra, S100A12), and confirmed five previously described biomarkers (CCL4, CRP, IL-10, IP-10, αPGL-I IgM). Overnight stimulation in WBAs provided increased specificity for leprosy and was required for IL-10, IL-1Ra and CCL4. The remaining five biomarkers were directly detectable in plasma, hence suitable for rapid POC tests. Indeed, lateral flow assays (LFAs) utilizing this five-marker profile detected both multi- and paucibacillary leprosy patients with variable immune responses.

Interpretation: Application of novel host-biomarker profiles to rapid, quantitative LFAs improves leprosy diagnosis and allows POC testing in low-resource settings. This platform can thus aid diagnosis and classification of leprosy and also provides a tool to detect *M.leprae* infection in large-scale contact screening in the field.

Introduction

Despite decades of control programs using multidrug therapy (MDT), leprosy still poses a public health problem in low and middle income countries affecting the poorest, most vulnerable people in their productive stage of life (1). This does not only have impact on affected individuals, but also imposes a significant social and financial burden on society (2). Key to leprosy control is the reduction of transmission of *Mycobacterium leprae (M.leprae),* the causative agent of leprosy, to breach the number of new cases which has stagnated around 200,000 annually for over a decade (2). Development of methods and tools to early diagnose disease and detect infection to direct (prophylactic) treatment in leprosy healthcare programs therefore has a high priority on the leprosy research agenda.

Current diagnosis of leprosy relies on clinical symptoms requiring well-trained clinicians. However, due to decreased clinical expertise for leprosy in the field (3), delayed diagnosis occurs frequently which increases the risk of severe disabilities. *M.leprae* infected individuals lacking clinical symptoms who are at risk of developing leprosy disease are even more difficult to identify. A diagnostic test detecting leprosy disease as well as *M.leprae* infection would be a valuable tool for health care workers.

Leprosy is a spectral disease for which the clinical outcome after *M.leprae* infection is determined by host factors. The spectrum spans from anti-inflammatory T helper-2 (Th2) immunity concomitant with large numbers of bacteria as well as antibodies against *M.leprae* antigens in multibacillary (MB) leprosy, to paucibacillary (PB) leprosy characterised by strong pro-inflammatory, T helper-1 (Th1) as well as T helper 17 (Th17) immunity (4). The pro-inflammatory response in PB patients leads to bacterial control, but also to collateral damage in the form of destruction of the body's own cells by the vigorous T cell response, mimicking autoimmunity.

Since antibodies against *M.leprae* phenolic glycolipid I (PGL-I) indicate infection and are associated with bacillary load (5) rapid diagnostic tests detecting anti-PGL-I antibodies have been developed (5, 6). However, these are still not yet widely implemented in the field due to limited availability. Moreover, to capture the different clinical outcomes of *M.leprae* infection across the leprosy disease spectrum we have shown that both cellular and humoral markers should be included in diagnostic tests: biomarker profiles including cellular and/or inflammatory biomarkers such as CCL4, IL-10, IP-10, CRP combined with *M.leprae* specific anti-PGL-I antibodies, increased sensitivity for leprosy (7, 8). In this respect, IL-10 discriminated disease and infection from healthy status, whereas CCL4 was particularly informative for PB patients. On one hand, for classification and confirmation of leprosy diagnosis 24 hour incubation with *M. leprae* antigens in WBAs represents a specific

approach, similar to the application of the Quantiferon® test for TB diagnosis (9). On the other hand, a triage for rapid identification of infection/disease (e.g. in large-scale contact screening efforts) must rely on biomarkers detectable in samples directly, without stimulation. To allow improved diagnosis and classification of leprosy patients as well as detection of infection by triage, we thus used a funnel approach assessing additional host proteins for their diagnostic performance in both rapid tests and 24 hour WBAs, including cytokines, chemokines and growth factors (CCGF). First, we applied high throughput multiplex bead arrays (WBAs) and ELISAs (WBA and plasma) of samples from leprosy patients, household contacts (HC) and endemic controls (EC) from Bangladesh. Appropriate biomarkers were subsequently validated in low complexity, quantitative up converting phosphor lateral flow assays (UCP-LFAs) (7).

Materials and methods

Study Setting

During this study the prevalence in the four districts (Nilphamari, Rangpur, Panchagar and Thakurgaon; population 8,190,035) was 0.9 per 10,000 and the new case detection rate 1.18 per 10,000 (Rural health program, the leprosy mission Bangladesh, yearly district activity report 2018).

Study participants

Participants were recruited on a voluntary basis between January 2013 and 2018 in leprosy endemic areas in Bangladesh as described previously (10). Leprosy was diagnosed based on clinical and bacteriological observations and classified as MB or PB as described by the WHO (11). Clinical and demographic data were collected in a database. As a reference group healthy individuals without known contact to leprosy patients in their village or at work from the same area (EC) were assessed for the absence of clinical signs and symptoms of leprosy and TB at intake, and after 2 and 4 years. Samples were collected from 8 villages spread randomly across the study area (2 representative villages for each of the 4 districts).
Inclusion/exclusion criteria. Patients of the Rural Health program and their contacts who were willing to participate were included in the study (10). Contacts were either living in the same house (household members) or in a house on the same compound, sharing the same kitchen or direct neighbors (first neighbors). The following exclusion criteria were applied to patients: refusal of examination of contacts, suffering from the pure neural form of leprosy, residing only temporarily in the study area, new patients found during contact examination of the index case, living less than 100 m away from a patient already included in the study or first and second degree relatives of a patient already included in the study. Contacts who refused informed consent were also excluded, as well as any woman

indicating to be pregnant, any person on TB or leprosy treatment, children below 5 years of age, contacts known to suffer from liver disease or jaundice, residing temporarily in the area, suffering from leprosy at the initial survey (these patients were referred to the clinic for leprosy treatment) and contacts who were already enrolled in the contact group of another patient. Staff of leprosy or TB clinics were excluded as EC.

Study Cohorts

Three different cohorts were tested: a discovery cohort, including age and gender matched (7) leprosy patients (n=79; 34 MB; 45 PB), HC (n=54) and EC (n=51) from Bangladesh for biomarker discovery; two validation cohorts, cohort I for biomarker validation in WBA including leprosy patients (n=55; 27 MB; 28 PB) and EC (n=27) and cohort II for biomarker validation in plasma consisting of leprosy patients (n=36; 21 MB; 15 PB) and EC (n=20). For age and gender matching a 50/50 male/female ratio and a 1:1:1 ratio of three age groups (0-14, 15-29 and 30+) was aimed at (7).

Samples

For discovery cohort and validation cohort I WBA samples, 4 ml venous blood was drawn and 1 ml applied directly to a microtube pre-coated with 10 µg *M.leprae* whole cell sonicate (WCS), 10 µg ML2478 and 10 µg ML0840 recombinant proteins (combined designated as Mlep) (3) or without stimulus (Med). Pre-coating of the tubes was done by lyophilizing the material. After 24 hours incubation at 37°C the microtube was frozen at -20°C, shipped to the LUMC and stored at -80°C until further analysis. For validation cohort II, plasma was collected as described previously (12).

Ethics

This study was performed according to the Helsinki Declaration (2008 revision) and the study protocol was approved by the National Research Ethics Committee (Bangladesh Medical Research Council) (Ref no. BMRC/NREC/2010-2013/1534). Participants were informed about the study objectives, the samples and their right to refuse to take part or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. All patients received treatment according to national guidelines.

Multiplex bead arrays (MBA)

BCA-1 (CXCL13), CCL17, CTACK (CCL27), sCD40L, EGF, ENA-78 (CXCL5), Eotaxin (CCL11), FGF, Flt3L, Fraktalkine (CX3CL1), G-CSF, GM-CSF, GRO, I309, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-

1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-16, IL-17A, IL17F, IL-20, IL-21, IL-22, IL-23, IL-27, IL28A, IL-33, IP-10, MCP-1 (CCL2), MCP-3 (CCL7), MDC (CCL22), MIP-1α (CCL3), MIP-1β (CCL4), PDGF-AA, PDGF-AB/BB, RANTES (CCL5), SCF, SDF-1, TGF-α, TNF-α, TNF-β, TPO, TRAIL, TSLP and VEGF were measured in the discovery cohort using the Milliplex magnetic bead kit (Merck, USA) as described previously (13).

ELISAs

Validation cohort I was assessed by ELISA for ApoA1, CCL4, CFH, CRP, CCL27, CXCL9, IL-1Ra, IL-19, IL-32, MMP9, PDGF-BB, PTX3, S100A12, SAA1 (R&D systems, Minneapolis, USA), IP-10 and IL-10 (Diaclone Research, Besancon, France) and TTR (Abcam, Cambridge, UK). To detect anti-PGL-I IgM the ELISA was performed as previously described (5). Validation cohort II was assessed by ELISA for anti-PGL-I IgM, ApoA1, CCL4, CRP, IL-1Ra, IP-10 and S100A12.

Lateral flow assays (LFA)

LFAs for IP-10, CRP and αPGL-I IgM strips were produced as described earlier (3). ApoA1 and S100A12 strips were produced similarly with 200 ng goat-anti-S100A12 pAb (AF1052; R&D systems, Minneapolis, USA) and Goat-anti-ApoA1 pAb (AF3664; R&D systems, Minneapolis, USA) on the test lines. The respective flow control lines comprised 100 ng Goat-anti-Rabbit or Rabbit-anti-Goat antibody. Conjugates of UCP particles were applied to the sample/conjugate pad at a density of 200 ng per 4 mm. UCP conjugates were prepared according to a previously described protocol (14) with Rabbit-anti-ApoA1 (Clone # 2083A; R&D systems, Minneapolis, USA) or goat-anti-S100A12 pAb (AF1052; R&D systems, Minneapolis, USA) at a concentration of 50 µg antibody per mg UCP. 10 µl, 1 µl, 0.1 µl and 0,01 µl plasma was diluted in high salt lateral flow (HSLF) buffer (100 mM HEPES pH 7.5, 270 mM NaCl, 1% (w/v) BSA, 0.5% (v/v) Tween-20). 50 ul of diluted sample was added to microtiter plate wells before target-specific LF strips were placed in the corresponding wells. Immunochromatography was allowed to continue for at least 30 min until dry.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, San Diego,CA, USA; http://www.graphpad.com), www.graphpad.com)SPSS Statistics 24 (http://www.spss.com.hk) and R Version 3.3.0 (R, Vienna, Austria; http://www.R-project.org). Hierarchical clustering of the CCGF based on absolute correlation difference and average linkage was performed using the global test (15). Log2 fold changes were calculated for MB, PB and HC compared to EC. Volcano plots were computed using R,

by plotting the log2 fold change against the -log10(p-value) of each marker (p-values calculated by global test). Radar plots showing the log2 fold change were generated using Excel 2016. Receiver operating characteristic (ROC) curves were computed in Graphpad Prism and the respective area under the curve (AUC) was calculated. Cut-offs were determined by calculating the Youden's index (16). To determine the optimal classification method three approaches (logistic regression, random forest classification and classification tree) were computed using Orange data mining version 3.3.9 (17), comparing the AUC after 10-fold stratified cross-validation for each method.

Results

To obtain new biomarkers for leprosy with high potential for user-friendly POC applications, we applied a funnel approach using discovery and validation cohorts (Figure 1). First, in a discovery cohort of MB (n=34) and PB (n=45) patients, HC (n=54) and EC (n=51), 60 CCGFs were measured in WBA supernatant using high throughput multiplex bead arrays (Figure 2;Supplementary Table S1-4): in 24 hour whole blood samples without stimulus IL-1Ra, CCL27 and CCL4 identified both MB and PB patients. IL-6, IL-10 and IP-10 levels were significantly different from EC in MB patients only, whereas CCL22, PDGF-AA and PDGF-BB identified PB patients (Figure 2A, left column). In samples stimulated with *M. leprae* WCS IL-10 and GCSF levels were higher in both leprosy patients and their contacts.

Elevated levels of IP-10 were observed in both MB and PB patients, whereas PDGF-BB, CCL4 and CCL27 levels were significantly higher for MB patients and IL-1Ra and PDGF-AA for PB patients in response to WCS (Figure 2A, middle column). In response to 2 *M.leprae* specific proteins (Mlep) CCL27 was identified as a marker for both types of leprosy disease, IP-10 and BCA-1 for MB leprosy only (Figure 2A, right column). Thus, in this discovery cohort IP-10, IL-1Ra, CCL4, CCL27 and PDGF-BB enabled the distinction of leprosy patients from EC irrespective of leprosy classification (Figure 2B) and were used for further evaluation by ELISAs in validation cohort I consisting of 27 MB patients, 28 PB patients and 27 EC. The WCS-induced levels of IL-10 and GCSF, discriminating both patients and HC from EC significantly, correlated in the discovery cohort. Therefore, only IL-10 was included as a marker for infection as these data confirm previous reports on IL-10 as an infection marker (7). Additionally, 11 markers with potential for diagnosis of mycobacterial diseases in earlier reports (8, 18-22) (not available in the multiplex bead assay) were also included (Figure 1;Supplementary Table S1). AUCs were calculated to assess the potential of the markers tested to discriminate the test groups from EC, demonstrating significance for S100A12, CRP, ApoA1, IL-10 in response to *M.leprae* specific proteins and CCL4 in response to *M. leprae* WCS for both leprosy types. Furthermore, MB patients could be discriminated from EC based on αPGL-I IgM and IP-10 as well, whereas for PB patients this was feasible

based on IL-10$_{WCS}$, CCL4$_{Med}$/CCL4$_{Mlep}$ and IL-1Ra (Figure 3A). Thus, this validation cohort confirmed diagnostic potential for leprosy of 8 markers.

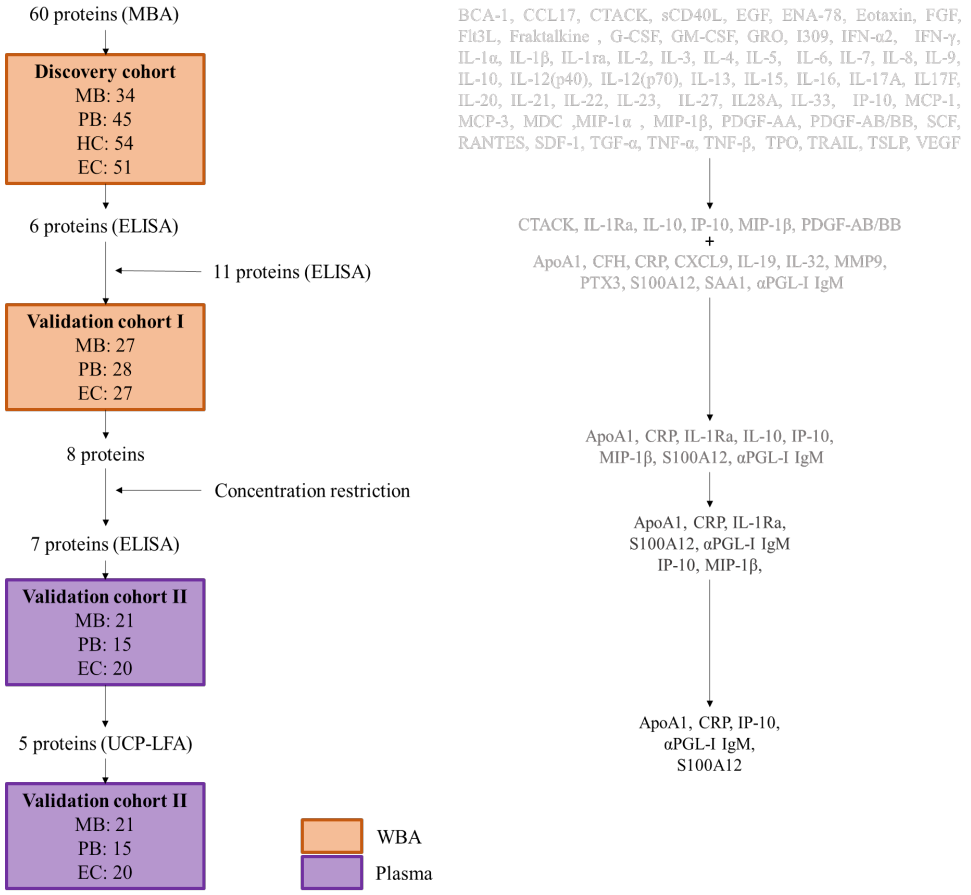

Figure 1: Funnel approach workflow. Three different cohorts including samples originating from Bangladesh (multibacillary (MB) and paucibacillary (PB) leprosy patients, household contacts (HC), healthy endemic controls (EC)) were used. Both whole blood assays (WBA) samples (orange; unstimulated and stimulated with *M. leprae* whole cell sonicate or *M. leprae* specific proteins (ML0840, ML2478)) and plasma samples (purple) were analyzed using multiplex bead assays (MBA), ELISA or up-converting phosphor lateral flow assays (UCP-LFA). The markers tested in each step are displayed in the right column.

Fingerstick blood (FSB) is an easy to use sample, requiring no phlebotomist or overnight stimulation, making it suitable for rapid testing using field friendly LFAs. As a proxy for FSB (3) we here tested plasma samples from Bangladeshi leprosy patients and EC (validation cohort II) for the seven markers that were significantly different in unstimulated WBA samples (Figure 3A). Since stimulation is required for detection of IL-10 we did not further include this marker for analysis of plasma samples. Without antigen stimulation, anti-PGL-I IgM antibodies, IP-10, CRP and S100A12 remained valuable markers in plasma for

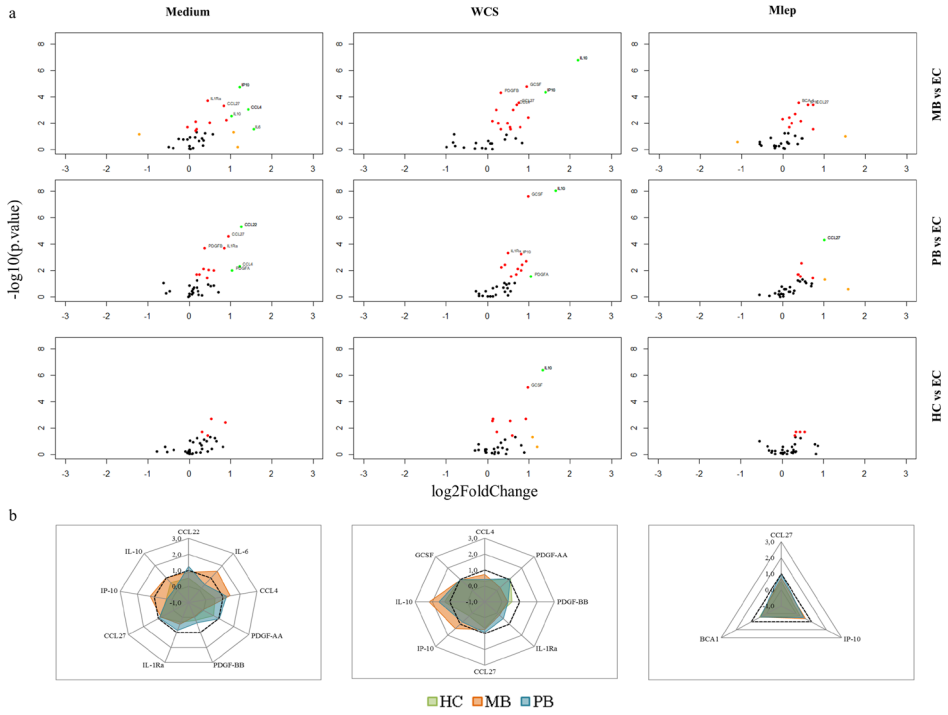

Figure 2: Production of cytokines, chemokines and growth factors (CCGFs) in leprosy patients and household contacts compared to endemic controls. 60 CCGFs were detected in whole blood assay (WBA) supernatant of multibacillary (MB) and paucibacillary (PB) leprosy patients, household contacts (HC) and endemic controls (EC). (a) Volcano plots show the log2 fold change compared to EC (x-axis) and the -log10(p-value) (y-axis) in unstimulated WBA supernatant (Medium; left column), in response to *M.leprae* whole cell sonicate (WCS; middle column) and two specific *M.leprae* proteins (Mlep; ML0840, ML2478; right column). The markers in either of the three groups with both a log2 fold change of 1 (-1) and a p-value <0,05 or markers with a p-value <0,001 are indicated (P-value < 0,05 = red dot, log2 fold change of 1(-1) = orange dot, P-value < 0,05 & log2 fold change of 1(-1) = green dot). (b) Summary of the markers indicated in the volcano plots per stimulus(Medium = left, WCS = middle and Mlep = right). Radar plots show the log2 FC of the markers indicated in the volcano plots for MB (orange), PB (blue) and HC (green) compared to the levels in EC. Dotted lines indicated a log2 FC of 1.

MB patients and ApoA1 for both MB and PB (Figure 3B), whereas IL-1Ra and CCL4 levels could not be detected in these plasma samples. To assess the potential of the five-marker plasma signature for POC/field applications, quantitative UCP-LFAs specific for ApoA1, CRP, IP-10, αPGL-IgM and S100A12 were tested in validation cohort II. Data obtained by the UCP-LFAs are in line with the ELISA data for plasma samples, with ApoA1 being the most optimal marker to discriminate PB patients from EC, whereas the other four markers are elevated especially in MB patients, but also discriminate some PB patients from EC (Figure 4). To optimally identify leprosy across the disease spectrum, cut-offs were determined comparing patients irrespective of leprosy type to EC (Supplementary Table S5).

Based on the cut-off values, the number of positive tests was determined per individual resulting in a five-marker signature. A sum of positive test results is a practical way to apply biomarker signatures in the field. This signature (AUC: 0.93, $p<0.0001$) identified 86% of the leprosy patients, with a specificity of 90% (cut-off > 2 tests positive). Moreover, in contrast to single markers, the five-marker signature showed similar AUCs for MB (AUC:0.94) and PB (AUC:0.91) patients (Figure 4B).

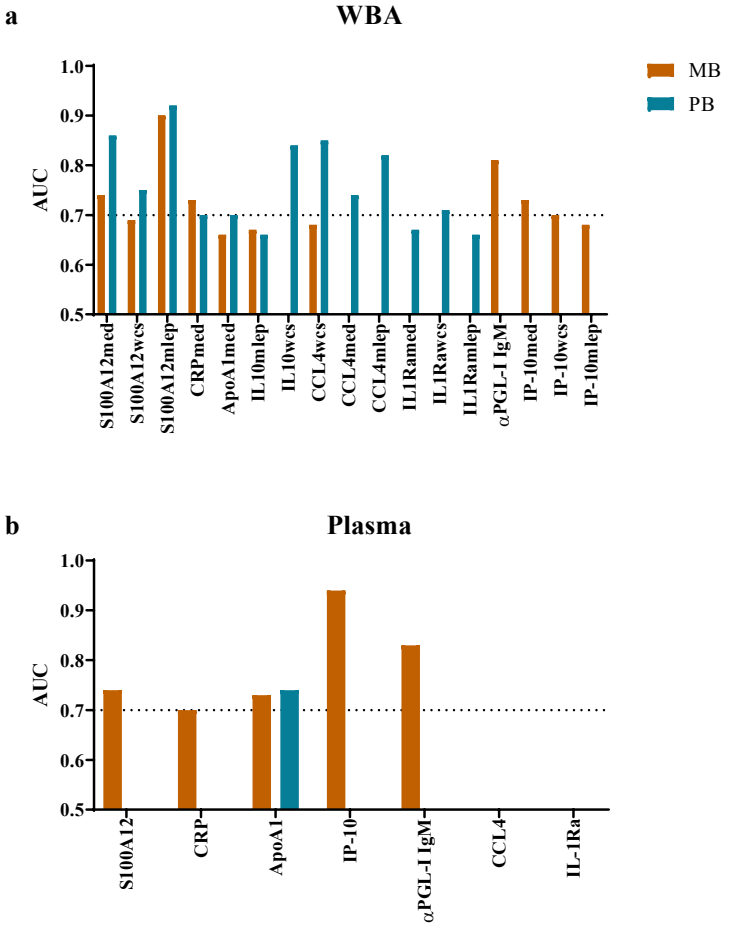

Figure 3: Biomarkers validated by ELISA in whole blood assay supernatant and plasma of leprosy patients. Markers showing significant areas under the curve (AUC) for multibacillary (MB; orange) and/or paucibacillary (PB; blue) leprosy patients in unstimulated whole blood assay (WBA) supernatant (med), in response to *M.leprae* whole cell sonicate (WCS) and two specific *M.leprae* proteins (Mlep) (A) or plasma samples (B). Biomarkers levels were compared to those of endemic controls. Values for AUC can range from 0.5 to 1, the dotted line indicates an AUC of 0.7.

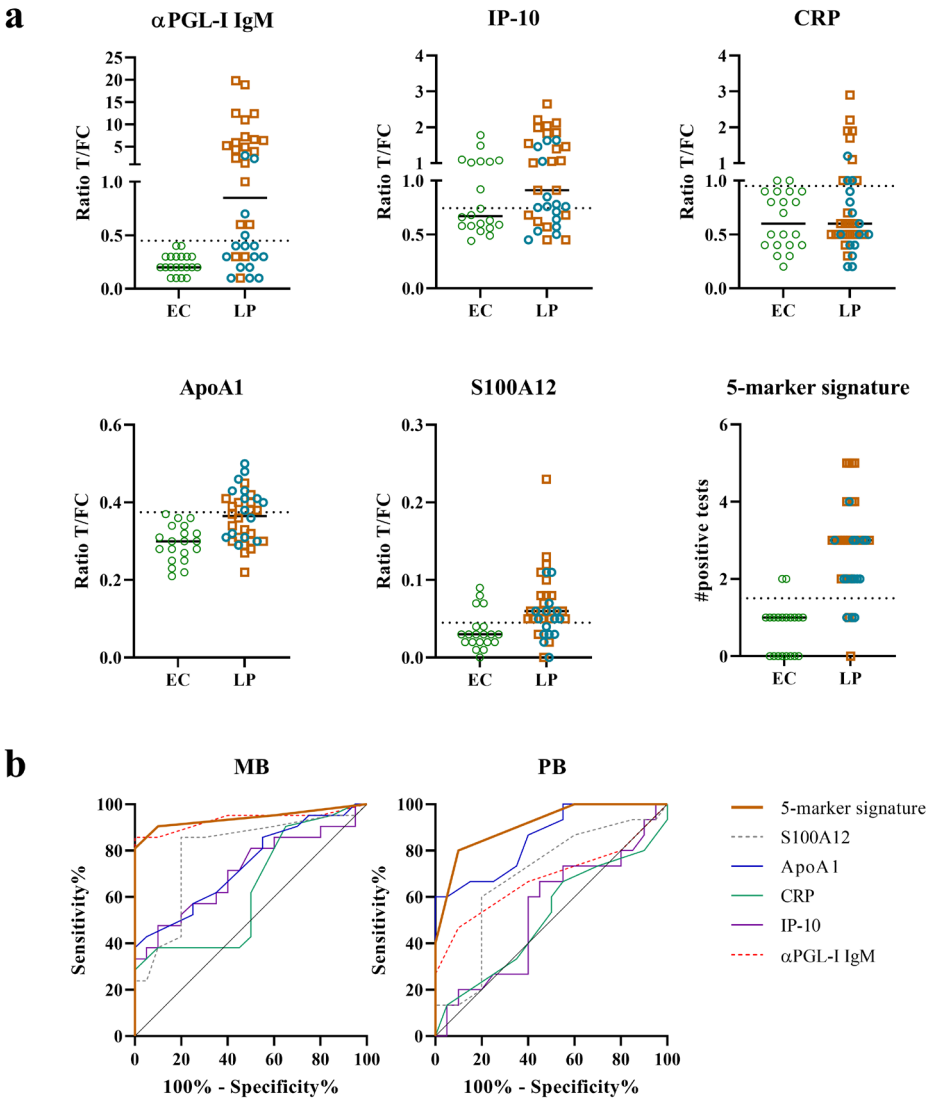

Figure 4: five-marker plasma signature assessed by up-converting phosphor lateral flow assays (UCP-LFA). Levels of αPGL-I IgM, IP-10, CRP, ApoA1 and S100A12 were measured by UCP-LFAs comparing 36 leprosy patients (LP, orange squares = multibacillary (MB) patients and blue dots = paucibacillary (PB) patients) to 20 endemic controls (EC = green dots). (a) Ratio values for the 5 markers tested were calculated by dividing the relative fluorescence units (RFU) from the test line (T) by the RFU from the flow controls (FC). The dotted line indicates the cut-off value for each markers as calculated by the Youden's index. Values above the cut-off line are considered a positive test result, the sum of all positive tests results in the values displayed for the five-marker signature. Cut-offs are shown in supplementary Table S5. (b) receiver operating characteristic curves (ROC) for MB and PB patients compared to EC showing all 5 markers tested (αPGL-I IgM (red), IP-10 (purple), CRP (green), ApoA1 (blue), S100A12 (grey)) and the five-marker signature (orange).

Additionally, three different classification methods (logistic regression, classification tree and random forest classification) were applied to the two validation cohorts to assess the performance of the POC five-marker signature. In general, ten-fold stratified cross-validation showed the most optimal AUC and classification accuracy for the classification tree algorithm (Supplementary Table S6). The cross-validated sensitivity and specificity of this algorithm for WBA and plasma as assessed by ELISA was comparable to that assessed by UCP-LFA, indicating that the signature can also be accurately detected in POC-tests (Supplementary Figure S1). Moreover, cross-validated signatures showed only a minor decrease in sensitivity (12%)/specificity(16%) compared to the POC signature, indicating the robustness of this signature. The here described "funnel- approach" thus identified biomarker signatures, applicable to either WBAs and plasma, that sensitively detect MB as well as PB leprosy patients.

Discussion

Tools that detect disease at an early state and identify *M.leprae* infection are eminent to interrupt transmission. Previous reports showed that the combined detection of humoral markers capturing MB leprosy and cellular markers detecting PB, significantly improved the detection of leprosy patients (7, 8). However, PB patients and HC could not be distinguished as these markers showed similar responses for these cellular markers, especially in highly endemic areas (7, 8). In this study, using a wide array of CCGFs, five markers differentiated PB patients from HC (Supplementary Table S2-4), whereas 18 makers were different in PB patients compared to EC in WBA samples. These included markers previously tested in the UCP-LFA format such as CCL4, CRP and IL-10 (7, 8), as well as the newly identified markers ApoA1, IL-1Ra and S100A12.

Apolipoprotein A1 (ApoA1) is a negative acute phase protein which is suggested to bind to stimulated T-cells thereby inhibiting contact-mediated activation of monocytes (23) and reported to be decreased during inflammation (24) and active tuberculosis (18). Indeed, in WBA samples both MB and PB patients showed decreased levels of anti-inflammatory ApoA1. IL-1Ra (Interleukin-1 receptor antagonist) also exerts anti-inflammatory functions by binding to the IL-1 receptor, thereby inhibiting the function of the proinflammatory IL-1α and IL-1β. *M.leprae* can induce high levels of IL-1Ra in monocytes, and high expression of IL-1Ra in skin lesions was associated with increased susceptibility to leprosy irrespective of polarity (25). Both MB and PB patients showed elevated levels of IL-1Ra in WBA samples, supporting the use of IL-1Ra as a biomarker in leprosy diagnostics. S100A12 (calgranulin C) can induce proinflammatory cytokines and serum levels have been shown to correlate with disease activity in inflammatory disorders (26). Interestingly, S100A12 has antimicrobial properties exerting direct effects on both *M.leprae* and *M.tuberculosis*

and was more strongly expressed in skin lesions of PB leprosy patients (27). Serum levels did, however, not significantly differ between MB and PB patients (21) in line with the data observed in this study. In response to *M.leprae* specific proteins, S100A12 showed the optimal AUC of all the markers tested in WBAs, both for MB and PB patients. For MB leprosy this study also confirmed the use of IP-10 as a biomarker in line with our previous studies (7, 8).

In view of point-of-care (POC) test applicability (i.e. direct analysis of clinicals samples without antigen stimulation), biomarker levels were also assessed in plasma samples as a proxy for FSB collectable without venipuncture (3). A plasma biomarker signature including αPGL-I IgM, IP-10, S100A12, ApoA1, CRP accurately detected leprosy patients irrespective of type with high sensitivity (97%) and specificity (100%) in the UCP-LFAs; indicating the diagnostic value of this signature in leprosy as it identifies both patients with high and low bacillary loads. The future detection of this signature in FSB by rapid POC testing can be useful for screening purposes in a triage approach: a FSB-based multi-biomarker LF strip rules out individuals who lack host biomarkers associated with leprosy, and individuals requiring further testing are selected for overnight incubation of whole blood with *M.leprae* specific antigens (7). In the 24 hour stimulated WBA samples a larger selection of (stimulated) discriminatory markers were identified, especially to detect PB patients. The levels of biomarkers in WBAs can thus be used for multiple applications, besides contact screening i.e. to help in classification of leprosy patients in referral hospitals or for monitoring of the development of complications such as leprosy reactions (12, 28).

PB patients and HC show similar immune responses and often have undetectable loads of *M.leprae* bacilli. The infection status of HC is, however, largely unknown. Reports from Brazil and India indicate the presence of *M.leprae* DNA in nasal swabs and skins slit smears of HC ranging from 8.8% to 49% (29, 30) or 21%, respectively (31). Therefore, elaborate host immune profiling of HC stratified by *M.leprae* DNA presence in nasal swabs or slit skin slides may aid in identifying biomarkers associated with *M.leprae* exposure or infection without clinical symptoms. αPGL-I IgM levels have been measured in HC in order to predict the development of leprosy disease, but has so far proven insufficient for early detection of leprosy or onset of disease (32, 33). Longitudinal monitoring of the host biomarkers described in this study can provide more insight into the predictive capacity of this biomarker signature. Moreover, validation of this signature in different populations in leprosy endemic areas and validation in FSB is required for large numbers of samples before multi-biomarker testing can be implemented in leprosy healthcare. Translation of biomarkers into clinical practice is still challenging as evidenced by the low percentage of discovered biomarkers

validated for routine clinical practice (34). Identifying markers in three independent cohorts using a funnel approach ensure that the strongest biomarkers remain.

Application of biomarker signatures in rapid POC tests can not only facilitate leprosy diagnosis and classification but also aid decision making on which individuals are candidate for prophylactic treatment. Contacts of leprosy patients are 4 to 9 times more at risk of developing leprosy than the general population (35). Therefore, these individuals are targeted for post-exposure prophylaxis. Large scale contact screening trials to select *M. leprae* infected individuals for post-exposure prophylaxis with single dose rifampicin (SDR) according to WHO guidelines (28) for leprosy control will thus contribute to decrease transmission and thereby prevent leprosy-associated irreversible nerve damage. Moreover, the quantitative LF test data enable the assessment of SDR efficacy and dosage regimens in infected individuals, as well as monitoring of treatment in leprosy patients. Importantly, the biomarker signature identified in this study, including novel biomarkers, accurately detected patients across the leprosy spectrum and, importantly, was compatible with low-complexity lateral flow tests. Implementation of these host biomarker-based field tests can thus provide the urgently needed diagnostic tool for leprosy applicable in low-resource settings.

Acknowledgements

The authors gratefully acknowledge all patients and blood donors. LUMC, EMC and TLMI,B are part of the IDEAL (***I***nitiative for ***D***iagnostic and ***E***pidemiological ***A***ssays for ***L***eprosy) Consortium. We thank the staff of the Rural Health Program, The Leprosy Mission International Bangladesh, Nilphamari, Bangladesh for recruitment of study participants and sample collection.

Funding statement

This study was supported by the Order of Malta-Grants-for-Leprosy-Research (MALTALEP), the Q.M. Gastmann-Wichers Foundation and the Leprosy Research Initiative (LRI) together with the Turing Foundation (ILEP#: 703.15.07). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Declaration of interests

The authors declare that they have no conflict of interest.

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Supplementary material

Tables

Supplementary Table S1: Overview of cytokines, chemokines and growth factors tested per cohort

	Discovery	Validation I	Validation II		Discovery	Validation I	Validation II
1	BCA-1 (CXCL13)			37	IL-21		
2	CCL17			38	IL-22		
3	CTACK (CCL27)	CTACK (CCL27)		39	IL-23		
4	sCD40L			40	IL-27		
5	EGF			41	IL28A		
6	ENA-78 (CXCL5)			42	IL-33		
7	Eotaxin (CCL11)			43	IP-10	IP-10	IP-10
8	FGF			44	MCP-1 (CCL2)		
9	Flt3L			45	MCP-3 (CCL7)		
10	Fraktalkine (CX3CL1)			46	MDC (CCL22)		
11	G-CSF			47	MIP-1α (CCL3)		
12	GM-CSF			48	MIP-1β (CCL4)	MIP-1β (CCL4)	MIP-1β (CCL4)
13	GRO			49	PDGF-AA		
14	I309			50	PDGF-AB/BB	PDGF-AB/BB	
15	IFN-α2			51	RANTES (CCL5)		
16	IFN-γ			52	SCF		
17	IL-1α			53	SDF-1		
18	IL-1β			54	TGF-α		
19	IL-1ra	IL-1ra	IL-1ra	55	TNF-α		
20	IL-2			56	TNF-β		
21	IL-3			57	TPO		
22	IL-4			58	TRAIL		
23	IL-5			59	TSLP		
24	IL-6			60	VEGF		
25	IL-7			61		ApoA1	ApoA1
26	IL-8			62		CFH	
27	IL-9			63		CRP	CRP
28	IL-10	IL-10		64		CXCL9	
29	IL-12(p40)			65		IL-19	
30	IL-12(p70)			66		IL-32	
31	IL-13			67		MMP9	
32	IL-15			68		PTX3	
33	IL-16			69		S100A12	S100A12
34	IL-17A			70		SAA1	
35	IL17F			71		αPGL-I IgM	αPGL-I IgM
36	IL-20						

Supplementary Table S2: p-values (obtained by global test analysis) for 60 cytokines, chemokines and growth factors in unstimulated whole blood assay supernatants

	MB			PB		HC
	vs. PB	vs. HC	vs. EC	vs. HC	vs. EC	vs. EC
EGF	0.33	0.97	0.67	0.34	0.36	0.93
FGF2	0.69	0.23	0.61	0.06	0.3	0.61
Eotaxin	0.53	0.14	0.07	0.16	0.09	0.6
TGFα	0.23	0.03	0.2	0.11	0.37	0.83
GCSF	0.76	0.11	0.04	0.2	0.06	0.64
Flt3L	0.6	0.15	0.52	0.42	0.89	0.32
GMCSF	0.43	0.83	0.87	0.1	0.14	0.97
Fraktalkine	0.83	0.77	0.84	0.98	0.95	0.99
IFNα2	0.91	0.07	0.12	0.11	0.14	0.97
IFNγ	0.3	0.005	0.05	0.05	0.16	0.7
GRO	0.51	0.65	0.16	0.3	0.008	0.08
IL10	0.8	0.28	0.003	0.64	0.02	0.06
MCP3	0.83	0.37	0.05	0.37	0.04	0.12
IL12p40	0.92	0.28	0.63	0.37	0.72	0.61
CCL22	0.11	0.04	0.006	0.08	4.84E-06	0.002
IL12p70	0.77	0.54	0.31	0.76	0.5	0.74
PDGFAA	0.28	0.63	0.18	0.54	0.01	0.1
IL13	0.98	0.56	0.93	0.63	0.82	0.47
PDGFBB	0.36	0.44	0.02	0.09	0.0002	0.06
IL15	0.83	0.91	0.21	0.99	0.4	0.61
sCD40L	0.125	0.69	0.36	0.4	0.43	0.99
IL17A	0.16	0.21	0.8	0.75	0.12	0.2
IL1Ra	0.79	0.02	0.0002	0.05	0.0002	0.02
IL1α	0.19	0.1	0.008	0.83	0.2	0.27
IL1β	0.67	0.44	0.18	0.69	0.37	0.6
IL2	0.86	0.92	0.51	0.96	0.64	0.62
IL4	0.54	0.65	0.78	0.82	0.32	0.47
IL6	0.35	0.04	0.03	0.21	0.12	0.71
IL7	0.3	0.82	0.03	0.42	0.26	0.09
IL8	0.43	0.59	0.23	0.78	0.4	0.21
Ip10	0.04	6.48E-05	1.86E-05	0.12	0.02	0.38
MCP-1	0.93	0.34	0.99	0.29	0.9	0.37
MIP1α	0.95	0.11	0.77	0.04	0.5	0.27
CCL4	0.26	0.003	0.0009	0.02	0.005	0.37
RANTES	0.75	0.85	0.18	0.78	0.13	0.23
TNFα	0.79	0.36	0.009	0.46	0.01	0.05
TNFβ	0.19	0.38	0.3	0.87	0.64	0.81
VEGF	0.27	0.03	0.06	0.23	0.21	0.81
BCA-1	0.15	0.09	0.15	0.78	0.8	0.96
I309	0.92	0.25	0.12	0.28	0.14	0.72
IL23	0.38	0.32	0.27	0.86	0.79	0.96
CCL27	0.91	0.46	0.0005	0.29	2.73E-05	0.004
ENA78	0.1	0.04	0.12	0.59	0.99	0.61
IL28A	0.82	0.02	0.54	0.0004	0.62	0.1
IL16	0.09	0.39	0.76	0.22	0.009	0.14
CCL17	0.68	0.85	0.07	0.72	0.45	0.04
TPO	0.9	0.21	0.63	0.03	0.55	0.45
SDF1	0.57	0.37	0.21	0.38	0.07	0.27
SCF	0.37	0.13	0.29	0.18	0.73	0.37

Each column represents a comparison between 2 test groups as indicated by the top two rows.

Supplementary Table S3: p-values (obtained by global test analysis) of 60 cytokines, chemokines and growth factors in whole blood assay supernatant after 24 hour stimulation with Mycobacterium leprae whole cell sonicate

	MB			PB		HC
	vs. PB	vs. HC	vs. EC	vs. HC	vs. EC	vs. EC
EGF	0.56	0.56	0.74	0.91	0.2	0.17
FGF2	0.86	0.4	0.74	0.35	0.76	0.56
Eotaxin	0.18	0.02	0.07	0.1	0.23	0.73
TGFα	0.93	0.42	0.02	0.31	0.004	0.08
GCSF	0.77	0.29	1.72E-05	0.21	2.43E-08	8.81E-06
Flt3L	0.4	0.3	0.89	0.74	0.29	0.16
GMCSF	0.25	0.92	0.67	0.16	0.23	0.95
Fraktalkine	0.74	0.91	0.64	0.76	0.81	0.54
IFNα2	0.7	0.32	0.19	0.59	0.3	0.56
IFNγ	0.62	0.25	0.08	0.48	0.12	0.41
GRO	0.35	0.49	0.69	0.54	0.82	0.73
IL10	0.28	0.08	1.70E-07	0.26	8.92E-09	4.08E-07
MCP3	0.24	0.27	0.03	0.98	0.43	0.32
IL12p40	0.47	0.27	0.32	0.71	0.78	0.93
CCL22	0.65	0.62	0.14	0.96	0.03	0.02
IL12p70	0.77	0.75	0.8	0.98	0.96	0.94
PDGFAA	0.61	0.73	0.01	0.94	0.03	0.05
IL13	0.45	0.99	0.94	0.48	0.72	0.73
PDGFBB	0.08	0.13	4.84E-05	0.84	0.006	0.003
IL15	0.7	0.17	0.69	0.49	0.99	0.5
sCD40L	0.12	0.97	0.13	0.11	0.91	0.18
IL17A	0.33	0.47	0.91	0.7	0.2	0.36
IL1Ra	0.37	0.21	0.001	0.45	0.0005	0.003
IL1α	0.4	0.21	0.01	0.66	0.09	0.27
IL1β	0.11	0.09	0.02	0.68	0.18	0.29
IL2	0.21	0.07	0.004	0.7	0.21	0.42
IL4	0.59	0.52	0.67	0.8	0.59	0.66
IL6	0.98	0.49	0.36	0.19	0.02	0.41
IL7	0.99	0.65	0.34	0.67	0.37	0.73
IL8	0.07	0.13	0.22	0.85	0.87	0.65
Ip10	0.13	0.0002	4.43E-05	0.009	0.0006	0.81
MCP-1	0.71	0.67	0.44	0.79	0.24	0.15
MIP1α	0.16	0.98	0.85	0.02	0.01	0.81
CCL4	0.16	0.01	0.0004	0.14	0.004	0.13
RANTES	0.62	0.83	0.55	0.76	0.35	0.58
TNFα	0.36	0.46	0.001	0.99	0.008	0.002
TNFβ	0.61	0.44	0.36	0.83	0.38	0.5
VEGF	0.07	0.02	0.17	0.39	0.91	0.54
BCA-1	0.18	0.09	0.007	0.68	0.09	0.2
I309	0.15	0.46	0.29	0.57	0.9	0.47
IL23	0.46	0.58	0.31	0.89	0.75	0.67
CCL27	0.43	0.04	0.0003	0.18	0.002	0.05
ENA78	0.98	0.28	0.87	0.24	0.97	0.17
IL28A	0.13	0.006	0.32	0.04	0.4	0.002
IL16	0.09	0.26	0.81	0.29	0.1	0.37
CCL17	0.51	0.54	0.03	0.73	0.39	0.04
TPO	0.74	0.24	0.55	0.02	0.62	0.5
SDF1	0.51	0.4	0.25	0.64	0.18	0.32
SCF	0.25	0.1	0.25	0.29	0.999	0.31

Each column represents a comparison between 2 test groups as indicated by the top two rows.

Supplementary Table S4: p-values (obtained by global test analysis) of 60 cytokines, chemokines and growth factors in whole blood assay supernatant after 24 hour stimulation with Mycobacterium leprae specific proteins (ML0840 and ML2478)

	MB			PB		HC
	vs. PB	vs. HC	vs. EC	vs. HC	vs. EC	vs. EC
EGF	0.81	0.67	0.59	0.46	0.3	0.66
FGF2	0.9	0.43	0.85	0.26	0.62	0.56
Eotaxin	0.52	0.06	0.26	0.06	0.27	0.53
TGFα	0.96	0.45	0.09	0.4	0.06	0.27
GCSF	0.6	0.03	0.06	0.1	0.26	0.47
Flt3L	0.54	0.13	0.57	0.47	0.77	0.26
GMCSF	0.31	0.7	0.37	0.12	0.71	0.1
Fraktalkine	0.93	0.72	0.74	0.8	0.75	0.92
IFNα2	0.83	0.17	0.39	0.32	0.56	0.71
IFNγ	0.71	0.07	0.03	0.31	0.09	0.55
GRO	0.66	0.82	0.55	0.8	0.16	0.23
IL10	0.8	0.09	0.06	0.16	0.19	0.69
MCP3	0.77	0.89	0.02	0.79	0.07	0.02
IL12p40	0.63	0.02	0.1	0.1	0.28	0.63
CCL22	0.45	0.75	0.15	0.74	0.02	0.02
IL12p70	0.8	0.97	0.58	0.8	0.48	0.67
PDGFAA	0.76	0.97	0.13	0.72	0.05	0.17
IL13	0.87	0.73	0.85	0.97	0.92	0.92
PDGFBB	0.88	0.17	0.005	0.26	0.003	0.05
IL15	0.42	0.44	0.65	0.14	0.73	0.28
sCD40L	0.29	0.65	0.34	0.19	0.67	0.43
IL17A	0.41	0.48	0.71	0.75	0.43	0.58
IL1Ra	0.27	0.07	0.004	0.63	0.07	0.18
IL1α	0.91	0.29	0.27	0.62	0.53	0.95
IL1β	0.18	0.05	0.34	0.54	0.65	0.25
IL2	0.34	0.72	0.38	0.22	0.1	0.6
IL4	0.34	0.7	0.56	0.47	0.59	0.94
IL6	0.65	0.23	0.31	0.35	0.35	0.93
IL7	0.83	0.11	0.36	0.13	0.37	0.49
IL8	0.7	0.79	0.77	0.96	0.87	0.9
Ip10	0.32	0.02	0.0004	0.32	0.05	0.45
MCP-1	0.64	0.75	0.91	0.83	0.85	0.72
MIP1α	0.65	0.86	0.56	0.37	0.18	0.6
CCL4	0.02	0.01	0.002	0.62	0.44	0.81
RANTES	0.72	0.54	0.27	0.19	0.13	0.89
TNFα	0.5	0.09	0.28	0.51	0.74	0.7
TNFβ	0.24	0.76	0.5	0.45	0.48	0.97
VEGF	0.1	0.04	0.14	0.7	0.98	0.7
BCA-1	0.24	0.2	0.0003	0.84	0.02	0.06
I309	0.78	0.02	0.01	0.07	0.04	0.81
IL23	0.72	0.52	0.33	0.74	0.5	0.76
CCL27	1	0.16	0.0004	0.1	4.91E-05	0.02
ENA78	0.81	0.87	0.78	0.57	0.42	0.81
IL28A	0.71	0.41	0.54	0.37	0.67	0.65
IL16	0.36	0.9	0.51	0.24	0.03	0.27
CCL17	0.17	0.28	0.007	0.56	0.54	0.04
TPO	0.69	0.21	0.5	0.04	0.61	0.54
SDF1	0.41	0.28	0.15	0.49	0.08	0.24
SCF	0.54	0.14	0.48	0.07	0.85	0.1

Each column represents a comparison between 2 test groups as indicated by the top two rows.

Supplementary Table S5: Areas under the curve (AUC) for the 5 markers and 5-marker profile as determined using UCP-LFAs

	AUC	cut-off	sens%	95% CI	spec%	95% CI	Youden's index
αPGL-I IgM	**0.83**	>0.45	61	45 to 75	100	84 to 100	0.61
IP-10	0.64	>0.75	67	50 to 80	60	39 to 78	0.27
CRP	0.57	>0.95	31	18 to 47	90	70 to 98	0.21
ApoA1	**0.79**	>0.38	47	32 to 63	100	84 to 100	0.47
S100A12	**0.75**	>0.045	75	59 to 86	80	58 to 92	0.55
5-marker profile	**0.93**	>1.5	86	71 to 94	90	70 to 98	0.76

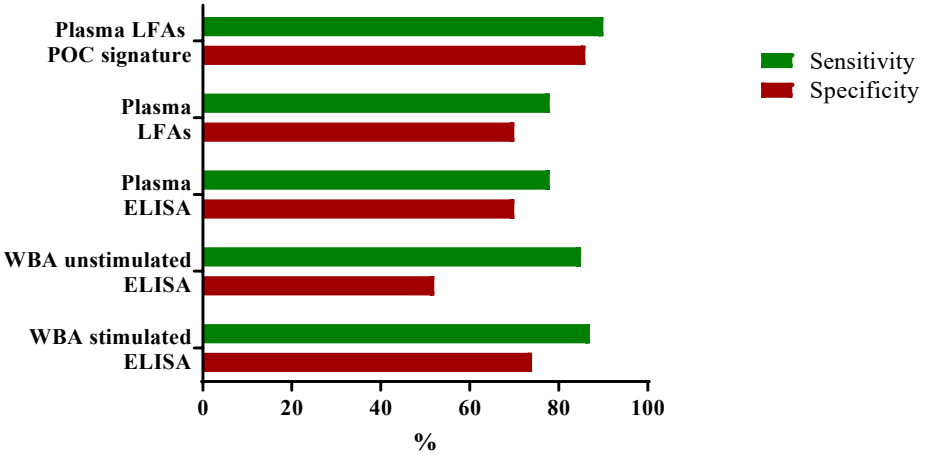
For each marker, a ROC curve was computed comparing leprosy patients to EC and the AUC was calculated. Cut-offs were determined based on the Youden's index and the accompanying sensitivity and specificity is shown. The higher the Youden's index, the better the trade-off between sensitivity and specificity is.

Supplementary Table S6: Classification models

		AUC	CA
WBA stimulated ELISA	Classification tree	0.822	0.829
	Random Forest Classification	0.758	0.78
	Logistic Regression	0.785	0.805
WBA unstimulated ELISA	Classification tree	0.68	0.744
	Random Forest Classification	0.635	0.707
	Logistic Regression	0.697	0.744
Plasma ELISA	Classification tree	0.737	0.75
	Random Forest Classification	0.717	0.75
	Logistic Regression	0.658	0.679
Plasma LFA	Classification tree	0.804	0.839
	Random Forest Classification	0.796	0.839
	Logistic Regression	0.596	0.661

Stratified 10-fold cross validation of 3 different classification methods (Classification tree, Random Forest Classification and Logistic regression) was performed by Orange data mining software (https://orange.biolab.si/). The area under the curve (AUC) and classification accuracy (CA) indicate the strength of the model ranging from 0 to 1, with 1 being the perfect value (perfect distinction and classification of two groups). These models were computed for Validation cohort I and II. For validation cohort I two analyses were performed: for unstimulated and stimulated (*M. leprae* whole cell sonicate and 2 *M. leprae* specific proteins) whole blood assays (WBA all) or unstimulated WBA only (WBA Medium). For validation cohort II consisting of plasma samples, models were calculated using data from ELISAs (plasma) and lateral flow assays (Plasma LFA).

Figures



Supplementary Figure S1: Sensitivity and specificity of biomarker signatures in whole blood assay supernatant and plasma. Decision tree algorithms were computed for the markers with significant AUCs (Figure 3) for validation cohort I and II. For validation cohort I two classification trees were computed, for both unstimulated and stimulated (*M. leprae* whole cell sonicate and 2 *M. leprae* specific proteins) whole blood assays (WBA stimulated) or unstimulated WBA only (WBA unstimulated) using data generated by ELISA. For validation cohort II consisting of plasma samples classification trees were computed for the results based on ELISA (plasma ELISA) and lateral flow assays (Plasma LFAs). The 10-fold stratified cross-validation sensitivity (green) and specificity (red) was compared to the point-of-care signature (POC) signature, which was determined by the sum of positive test results.

4

Chapter

Prototype multi-biomarker test for point-of-care leprosy diagnostics

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iScience. 2020 Dec 29;24(1):102006

Abstract

To end the decade-long, obstinately stagnant number of new leprosy cases, there is an urgent need for field-applicable diagnostic tools that detect infection with *Mycobacterium leprae*, leprosy's etiologic agent. Since immunity against *M. leprae* is characterized by humoral- and cellular markers, we developed a lateral flow test measuring multiple host proteins based on six previously identified biomarkers for various leprosy phenotypes. This multi-biomarker test (MBT) demonstrated feasibility of quantitative detection of six host serum proteins simultaneously, jointly allowing discrimination of multi- and paucibacillary leprosy patients from control individuals in high- and low leprosy endemic areas. Pilot-testing of fingerstick blood showed similar MBT-performance in point-of-care settings as observed for plasma and serum.

Thus, this newly developed prototype MBT measures six biomarkers covering immunity against *M. leprae* across the leprosy spectrum. The MBT thereby provides the basis for immunodiagnostic POC tests for leprosy with potential for other (infectious) diseases as well.

Introduction

For over a decade the annual number of newly detected leprosy cases has stagnated around 200,000 including children (1). This indicates that transmission of the causative agent of leprosy, *Mycobacterium leprae (M. leprae)*, is still ongoing. Leprosy can be effectively cured by multidrug therapy (MDT) and early identification and treatment of leprosy patients prevents irreversible nerve damage correlated with advanced stages of the disease (2). Prevention of disability reduces health as well as socioeconomic burden on leprosy-affected individuals, as their visible handicaps can lead to loss of income or unemployment due to social stigma and exclusion. Currently, leprosy diagnosis is based on clinical symptoms requiring well-trained clinicians. As a result of the declaration by the WHO in 2000 that the global target of leprosy elimination had been reached (3), leprosy control activities received considerably less attention and leprosy care was integrated in general health care programs. This leads to diminished leprosy expertise amongst clinicians which currently results in frequent missed- or delayed diagnosis (4). Undiagnosed patients and *M. leprae* infected individuals (yet) without clinical symptoms are likely to contribute significantly to the ongoing transmission(5), which is emphasized by the fact that 75% of the new leprosy cases in high endemic areas cannot be directly attributed to known index cases (6, 7). Implementation of diagnostic tests specific for *M. leprae* infection in contact- and population surveys will allow the identification of *M. leprae* infected individuals as target for post-exposure prophylaxis (PEP), as well as detection of early-stage leprosy for timely treatment (8, 9). Such diagnostic tests are not yet available (9, 10). Moreover, in order to implement novel tools in leprosy endemic areas, which are often resource-limited settings, diagnostic tests need to be available in a user- and field-friendly, rapid test format.

Leprosy has a wide spectrum of clinical manifestations which are closely related to the host' immune response against *M. leprae*. In multibacillary (MB) patients (individuals with high bacillary loads) IgM antibody responses to phenolic glycolipid-I (PGL-I), a cell wall component of *M. leprae,* are frequently detected (11). In paucibacillary (PB) leprosy, this antibody response is generally absent, but instead biomarkers of (Th1-)cell-mediated immunity are observed (12, 13). Examining the anti-*M. leprae* antibody response only, is therefore not sufficient to identify patients at both sides of the leprosy spectrum, but requires detection of multiple biomarkers specific for humoral as well as cellular immunity (14). Recently, we identified host biomarkers associated with leprosy in *M. leprae* antigen-stimulated whole blood assays (WBA) and plasma from a leprosy endemic population in Bangladesh. A host biomarker signature of αPGL-I IgM, IP-10, CRP, ApoA1 and S100A12 was identified, covering both the humoral- and cellular pole of the immunopathologic

leprosy spectrum (13, 15). High αPGL-I IgM, IP-10, CRP levels, relative to controls, were associated with MB leprosy, whereas ApoA1 and S100A12 levels were critical for identification of both patients groups. For PB patients, ApoA1 was identified as the most important biomarker (13). ApoA1 and S100A12 levels also differentiated highly-exposed contacts from endemic controls, identifying potentially *M. leprae*-infected individuals (16). In addition, CCL4 showed added diagnostic value in overnight stimulated WBA samples, particularly for PB leprosy patients (13) and was also associated with *M. leprae* infection among household contacts (16).

Utilizing the unique up-converting reporter particles (UCP), individual lateral flow (LF) test strips for separate detection of each of the five identified biomarkers were previously developed and applied to several cohorts from different geographic regions (12, 13, 17). UCP-LF is virtually background-free as the up- conversion upon excitation with infrared light does not occur in nature. This prevents autofluorescence with other assay components, providing a rapid and highly sensitive point-of-care (POC) test format (18-20). In contrast to most POC tests (21), the results generated by UCP-LF tests are quantitative. This allows cross-sectional comparison of test groups as well as intra-individual longitudinal monitoring at POC level.

Aiming at user- and field-friendly test applications, we developed a multi-biomarker test (MBT) strip that allows simultaneous, detection of these six biomarkers on one strip rather than separate strips for each biomarker. To demonstrate feasibility of this MBT to identify leprosy patients and *M. leprae* infected individuals, we analyzed banked plasma and serum samples of leprosy patients from a highly endemic area in Bangladesh as well as an area in South Korea that has reached the WHO elimination target (registered prevalence of less than 1 case per 10,000 population) in 1984 (22) but still reports new (import) leprosy cases annually (1). Finally, we pilot-tested the MBT in Bangladesh collecting and directly testing fingerstick blood (FSB) samples from leprosy patients and their contacts in the field to assess POC application of the MBT.

Materials and methods

Study participants

Leprosy was diagnosed based on clinical, histological and bacteriological observations and classified as MB or PB as described by the WHO (1) and the bacteriological index (BI) was determined (23). In Bangladesh 63 leprosy patients, 15 household contacts (HC) and 20 endemic controls (EC) were recruited between January 2013 and 2019 in leprosy endemic areas in Bangladesh as part of the MALTALEP/IDEAL trial (16, 23). Staff of leprosy-

or TB clinics were excluded as EC (Supplementary Table S1). In South Korea participants included 25 leprosy patients, 25 frequent contacts of the patients (HC: 88% family contact, 12% office contact), 24 individuals with a history of other dermatological diseases (ODD) and 24 healthy controls from the same area (C) (Supplementary Table S1). ODD showed symptoms similar to leprosy, including patients with psoriasis vulgaris, eczema, fungal infections and sarcoidosis.

Ethics

This study was performed according to the Helsinki Declaration (2008 revision) and the study protocol was approved by the National Research Ethics Committee (Bangladesh Medical Research Council) (Ref no. BMRC/NREC/2010-2013/1534). Participants were informed about the study objectives, the samples and their right to refuse to take part or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. All patients received treatment according to national guidelines.

Leprosy prevalence

The prevalence in the four Bangladeshi districts (Nilphamari, Rangpur, Panchagar and Thakurgaon) was 0.9 per 10,000 and the new case detection rate 1.18 per 10,000 (Rural health program, the leprosy mission Bangladesh, yearly district activity report 2018). The leprosy prevalence in South Korea was 0.025 per 10,000 (2018 (1)).

Samples

Plasma and whole blood assay samples were collected in Bangladesh, shipped to the LUMC on dry ice and stored at -80 ^{0}C until further testing (24). For the WBA, 4ml venous blood was drawn and 1ml was applied directly to a microtube precoated with 10 µg *M. leprae* whole cell sonicate (WCS) or without stimulus (Med). After 24 h incubation at 37 ₋C the microtube was frozen at –20 ₋C. Serum samples from South Korea were collected and stored at -80 ^{0}C until testing by local health care workers. An extensive standard operating procedure and a quality control sample were provided to limit procedural differences. FSB was collected using disposable 50 µl Minivette® collection tubes (Heparin coated; Sarstedt) and directly mixed with 455 µl high salt finger stick (HSFS) buffer supplemented with 1% (v/v) Triton X-100 (HSFS;100mM Tris pH 8, 270mM NaCl, 1% (w/v) BSA). FSB was tested directly after collection in a reference center for leprosy patients in Bangladesh (The Leprosy Mission International, Bangladesh, Nilphamari Hospital).

Multi-biomarker test (MBT) production

MBT strips were assembled by mounting 10 mm glass fiber sample/conjugate pad (Glass Fiber Conjugate Pad #8964, Ahlstrom), 25 mm laminated nitrocellulose membrane (Sartorius UniSart CN95) and 10 mm absorbent pad (High Purity Cotton Grade #320, Ahlstrom) on a 36 mm plastic backing card; all strip materials were obtained from Kenosha (Amstelveen, the Netherlands). Sample/conjugate and absorbent pad overlap respectively 3 and 6 mm with the nitrocellulose. Nitrocellulose was pre-striped such that each MBT strip contained 6 Test (T) lines with respective Flow Control (FC) lines, providing capture zones for the six biomarkers. The six pairs of capture lines (T and FC) were distributed evenly over the 50 mm wide MBT strip starting at 14 mm from the base of the MBT strip. Capture lines were located in a linear array of slanted lines, such that liquid only passed single capture lines, with T and FC lines separated by 3 mm and a 5mm distance between individual T and FC pairs. Sample flow direction and scanning direction of the reader are perpendicular. Each Test (T_n) line comprised 300 ng capture molecules (Figure 1): T_1 = ND-O-HSA, obtained through the Biodefense and Emerging Infections Research Resources Repository (https://www.beiresources.org/) (25); T_2 = mouse-anti-IP-10 mAb (BC-55, Diaclone Research, Besancon, France); T_3 = mouse-anti-CRP mAb (C5, Labned.com, Amstelveen, Netherlands); T_4 = mouse-anti-CCL4 mAb (MAB271, R&D systems, Minneapolis, USA);T_5 = goat-anti-S100A12 pAb (AF1052; R&D systems); and T_6 = Goat-anti-ApoA1 pAb (AF3664; R&D systems). The respective Flow Control (FC_n) lines comprised 37.5 ng Goat-anti-Mouse ($FC_{2,3}$), 75 ng Goat-anti-Rabbit (FC_6) or Rabbit-anti-Goat ($FC_{1,4,5}$) antibody per 6 mm (Sigma-Aldrich, Saint-Louis, United States). Individual UCP conjugates (26) were prepared with mouse-anti-IP-10 (BC-50; Diaclone Research), mouse-anti-CRP (CRP135; Labned.com), goat-anti-CCL4 (AF-271-NA; R&D systems), goat-anti-human IgM (I0759; Sigma-aldrich), goat-anti-S100A12 pAb (AF1052; R&D systems, Minneapolis, USA) and Rabbit-anti-ApoA1 (Clone # 2083A; R&D systems) at a concentration of 50 µg antibody per mg UCP. Sodium yttrium fluoride upconverting nanomaterials (200 nm, $NaYF_4$:Yb^{3+},Er^{3+}) functionalized with polyacrylic acid were obtained from Intelligent Material Solutions Inc. UCP reporter conjugates were applied to the sample/conjugate pad at a density of 400 ng per 7 mm. After assembly a quality control was performed, testing the MBT strips with recombinant proteins and standardized sample.

MBT assay

Banked plasma and serum samples were thawed and 50 µl was mixed with 455 µl HSFS buffer to obtain a 10-fold dilution similar to the dilution of the FSB sample upon collection. A 1000-fold dilution was prepared by adding 5 µl of the 10-fold dilution with 495 µl HSFS buffer (1000-fold dilution). The diluted samples (500 µl) were added to a channel in a

disposable tray, immunochromatography was initiated by placing the MBT strip into the channel and allowed to continue for at least 30 min. In South Korea this procedure was performed by local health care workers without prior training. Air dried MBT strips were scanned with a portable reader (ESEQuant *LFR* reader with 980 nm excitation and 550 nm emission; QIAGEN Lake Constance GmbH, Stockach, Germany). The strip holder was in-house adapted to fit the MBT which requires scanning perpendicular to the sample flow. Test results are displayed as the Ratio (R) value of the signal (peak area) from individual T_n lines normalized to the respective FC_n signal measured at the respective lines as determined by LF-Studio (ver. 3.3.8; QIAGEN Lake Constance GmbH) (Figure 1).

Data analysis

The R-value corresponds to the level of the biomarker present in the sample. Two different scores based on the MBT readout were evaluated.

i) **NUM-score**: Based on R-values, the optimal cut-offs to discriminate leprosy patients from their respective controls were determined using the Youden's index (27) per biomarker (Supplementary Table S1). Qualitative stratification of the biomarker result as positive or negative based on these cut-offs was used to calculate the number of positive biomarkers in the MBT per individual.

ii) **ALGO-score**: The ALGO-score was calculated using the median R-values of the individual biomarkers for the leprosy patients. First, scores were classified for each biomarker in three groups based on the association with disease as strong, intermediate or not associated (Supplementary Table S1). Strong association: $R \geq 2x$ median of patient group, intermediate association: median of patient group $\leq R < 2x$ median of patient group, no association: $R <$ median of patient group. Second, the ALGO-score was set as 2x the number of strong biomarkers ($2x\ n_{strong}$), plus the number of intermediate biomarkers ($n_{intermediate}$), minus the number of biomarkers not associated with disease (n_{not}) . Note that for ApoA1, the 1000-fold dilution resulted in an inverse correlation of the Ratio values with the biomarker level a consequence of a distinct but reproducible high dose hook effect.

Statistical analysis was performed using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego,CA, USA; http://www.graphpad.com). Receiver operating characteristic (ROC) curves were computed in Graphpad Prism and the respective area under the curve (AUC) was calculated. Group differences were determined using Mann-Whitney U test. The statistical significance level used was $p \leq 0.05$.

Results

The MBT format

MBT strips comprising six Test lines (biomarkers) with their respective Flow Control lines were produced using the sequence of biomarkers as indicated in Figure 1A. For this study, a predefined five-biomarker signature for leprosy (αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 (13)) was incorporated in the MBT strip format. As we envisage use of MBT strips both as POC tests and as user-friendly rapid tests for overnight stimulated whole blood samples, CCL4 as a biomarker for PB leprosy (12, 13, 17) was included as the sixth biomarker. The read-out provided by the luminescent reporter technology (UCP) is indicated as the R-value, for each biomarker individually. The R-value is a relative value that quantifies the difference between the signal intensity of the Test line and Flow Control. For each biomarker, this relative value can be converted to concentrations by generating a standard curve using known (recombinant) biomarker concentrations.

Application of the MBT to representative samples of EC and clinically diagnosed MB and PB patients clearly showed the difference in peak height and concomitant R-values between the plasma samples of the MB patient and the EC, as well as the WBA sample of the PB patient (Figure 1A). Importantly, overnight stimulation with *M. leprae* antigens, as demonstrated for the PB patient, increased CCL4 levels which were undetectable in unstimulated samples/sera (13). Moreover, the dichotomy in αPGL-I IgM between MB and PB unambiguously confirmed the presence of antibodies in the former leprosy type and absence in the latter.

The MBT format thus enabled simultaneous, quantitative detection of six biomarkers in one test (Figure 1A), thereby representing a unique feature for user-friendly lateral-flow assays. To explore scoring procedures for the read-out, we defined and evaluated the NUM-score and ALGO-score. The NUM-score is based on the sum of the number of positive biomarkers detected in the MBT providing a quick and easy-to-interpret readout. This required determination of a cut-off R-value to discriminate leprosy patients from controls, which was done using the Youden's index (27) for each individual biomarker. The ALGO-score was based on an algorithm that tentatively indicates an association of the R-values with disease (Figure 1B). Using the biomarker median R-value of the patient group, individual R-values were classified as strongly, intermediately or not associated with disease. Fig. 1B illustrates both scoring methods, showing an MB and PB sample with higher NUM- and ALGO-scores as compared to the EC.

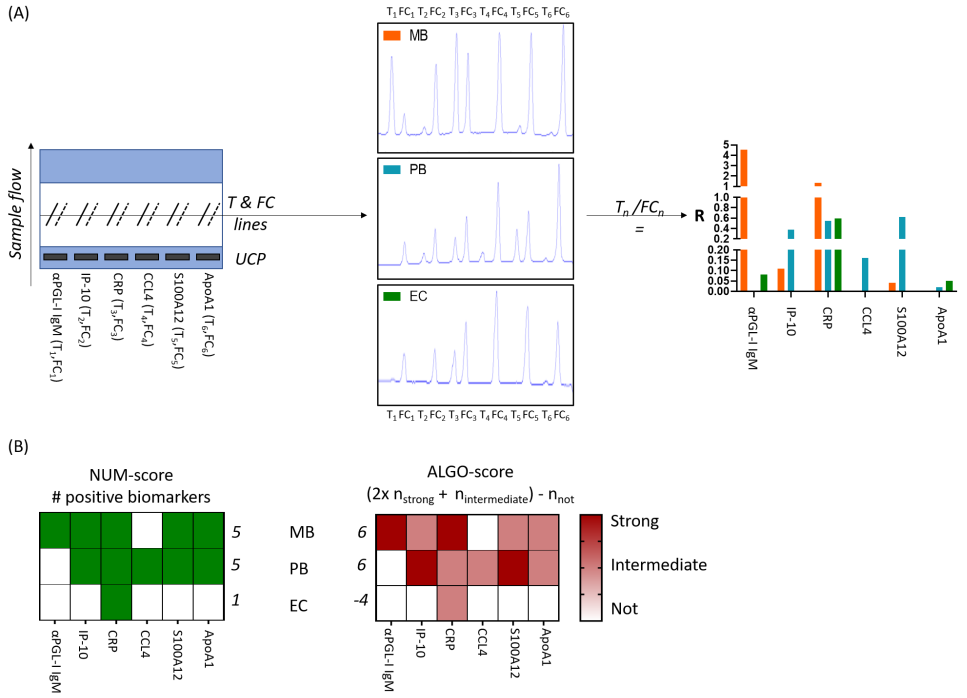

Figure 1: MBT schematic overview and scoring. (A) The MBT strip consists of 12 parallel lines, of which six are test lines (T_n) and six are flow controls (FC_n). Each T_n and FC_n pair measures a single biomarker: αPGL-I IgM, IP-10, CRP, CCL4, S100A12, ApoA1. The upconverting phosphor (UCP) particles are incorporated in the sample pad. The strip is read using a portable reader perpendicular to the sample flow. The test readout is a pattern of peaks, showing the signal of each of the 12 lines resulting in a Ratio value (R) per biomarker (T_n/FC_n). R-values for each biomarker are displayed for plasma samples of a multibacillary patient (MB; orange) and an endemic control (EC; green) and an *M. leprae* antigen-stimulated whole blood assay sample of a paucibacillary patient (PB; blue). (B) Two scores were calculated using the R-values, the NUM-score and the ALGO-score. The NUM-score is the sum of positive biomarkers per individual (green; R-value above the cut-off). The ALGO-score is based on an algorithm that contributes higher weights to R-values associated with disease and was calculated using the median R-values of the individual biomarkers for the leprosy patients.

MBT evaluation in two cohorts with varying leprosy endemicity

To further explore the MBT performance, MBT strips were applied to banked plasma samples from Bangladesh and banked sera from South Korea.

Bangladesh (high endemic area): Since the biomarkers studied here are generally not present in the same concentration range in blood, the optimal sample dilution per biomarker (10-fold and 1000-fold) was first determined. Results indicated that the biomarkers present in high concentrations (ApoA1, CRP and αPGL-I IgM) based on previously obtained ELISA data (13), distinguished leprosy patients from controls effectively using 1000-fold dilutions, whereas for detection of IP-10 and S100A12, 10-fold

dilutions were required (Supplementary Figure S1). CCL4 could not be detected in unstimulated plasma samples, in line with what we have observed previously (13). For the other five individual biomarkers, AUCs observed in this cohort were comparable to results from an earlier study (13) using the same plasma samples but with multiple singleplex UCP-LF strips (Supplementary Figure S2).

The NUM-score as determined previously with singleplex UCP-LF strips accurately distinguished leprosy patients from EC (AUC:0.93) (13). Application of the NUM-score to the MBT results showed a similar AUC (AUC: 0.9: $p<0.0001$; Figure 2A). This score thus performed equally well for MBT as for singleplex strips, signifying the potential of this MBT read-out to identify leprosy patients in endemic areas.

South Korea (non-endemic): The MBT was also evaluated using serum samples from a South Korean cohort (Supplementary Figure S3). Application of the NUM-score to the MBT data significantly discriminated leprosy patients from healthy controls living in that area (AUC: 0.88; $p<0.0001$; Figure 2B). Furthermore, leprosy patients showed significantly higher NUM-scores than contacts of leprosy patients or patients with other dermatological diseases (ODD) (HC: $p=0.0079$; ODD: $p=0.003$; Figure 2B). These data indicate the applicability of the MBT to identify leprosy patients also in a non-endemic area.

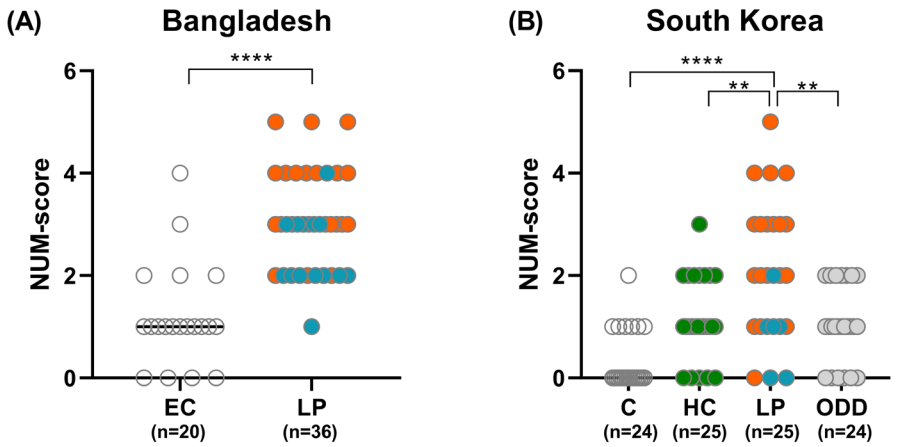

Figure 2: The MBT NUM-score identifies leprosy patients. The levels of αPGL-I IgM, IP-10, CCL4, CRP, S100A12 and ApoA1 were assessed by the MBT strip. The NUM-score, indicating the number of positive biomarkers based on the Ratio value was calculated per individual (*y*-axis). (A) NUM-scores observed in the cohort from Bangladesh (plasma) comparing leprosy patients (LP) to healthy endemic controls (EC). (B) NUM-scores in the South Korean cohort comparing LP to healthy controls (C), household contacts (HC) or patients with other dermatological diseases (ODD). Group differences were determined using Mann-Whitney U test, the statistical significance level used was $p \leq 0.05$. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Multibacillary leprosy patients are indicated with orange dots, paucibacillary patients with blue dots, HC with green dots, ODD patients with grey dots and healthy controls with white dots.

Although the NUM-score performed equally well in both cohorts to differentiate leprosy patients from healthy controls, it transformed the quantified MBT readout to a qualitative result (positive or negative) per biomarker. Apart from αPGL-I IgM, the other five MBT biomarkers are also present in unexposed, healthy individuals but the R-values observed differed between patients and controls. Thus, to evaluate the difference in biomarkers between test groups we stratified R-values as strongly, intermediately or not associated with disease, based on the median R-value per biomarker determined for the leprosy patients group in each country (Supplementary Table S1). This showed that the earlier observed pattern for MB patients of high αPGL-I IgM, CRP and IP-10 R-values (13) was confirmed in both the Bangladeshi and South Korean cohort. Similarly, MBT data of both cohorts showed that the ApoA1 R-values in PB patients differed from those in healthy controls (Figure 3). Interestingly, contacts of leprosy patients in South Korea showed ApoA1 R-values similar to PB patients. This indicates not only the potential of this biomarker for discriminating PB from ODD, but also to detect *M. leprae* exposure/infection. An overview of the biomarkers differentiating controls from MB and PB patients, HC and ODD indicates that assessing a combination of biomarkers is essential to allow proper interpretation of the MBT outcome (Figure 3C).

To reflect the effect of the observed patterns in individual biomarker R-values in the MBT results, we assessed the second scoring method. This ALGO-score showed a clear gradient from MB to PB patients and healthy controls in Bangladesh (Figure 4). For the South Korean cohort, however, the ALGO-score of PB patients did not differ from the scores observed in controls and contacts. Interestingly, MB patients clearly showed the highest ALGO-scores, and scores ≥5 were uniquely observed in this patient group (Figure 4B). This observation implicates that the ALGO-score is associated with bacterial load in leprosy patients.

In summary, the MBT accurately detected multiple biomarkers using a single test strip and allowed detailed assessment of biomarkers in blood samples. Two scoring methods were explored to interpret the MBT results, the easy-to-use NUM-score to indicate the number of positive biomarkers and the more quantitative ALGO-score reflecting the number of biomarkers per individual displaying R-values strongly, intermediately or not associated with disease. Application of these scoring methods facilitates interpretation of the quantitative MBT readout to identify leprosy patients and *M. leprae* infected individuals.

MBT evaluation in fingerstick blood

A pilot-test to evaluate the use of FSB combined with the MBT was performed in Bangladesh aiming at future POC application. All 42 FSB samples were collected from patients and contacts visiting the field-hospital on the same day. Analysis of the MBT

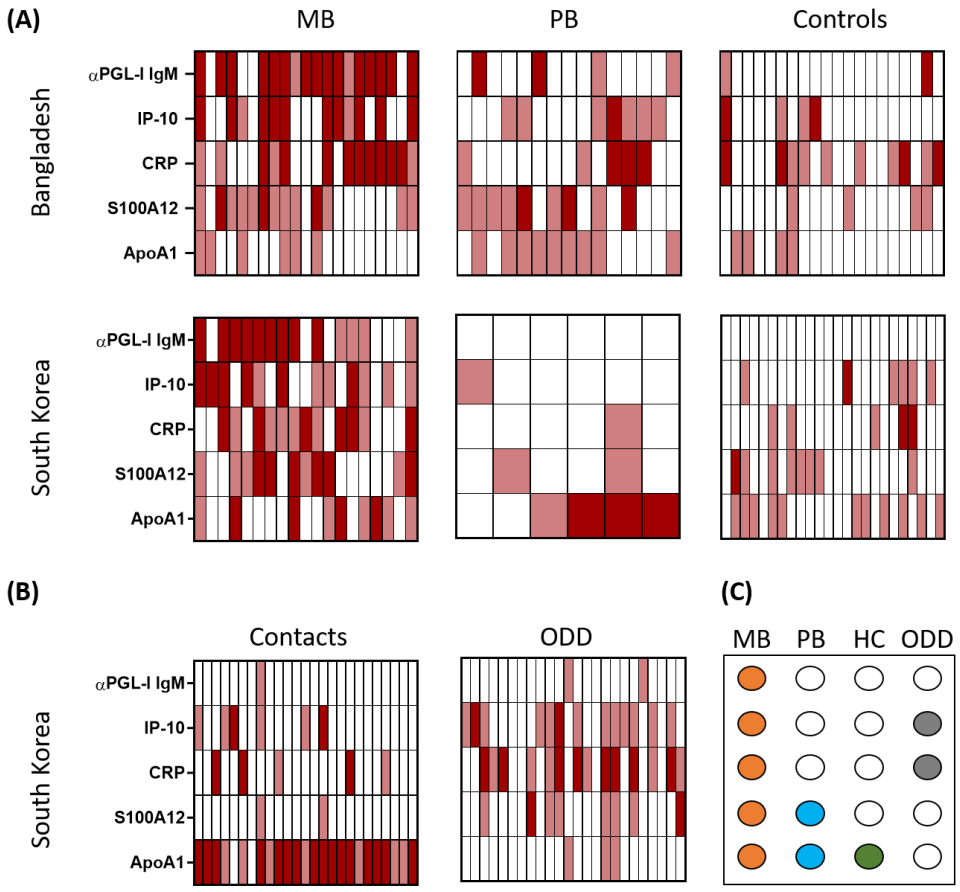

Figure 3: Stratification of biomarker levels using the MBT readout. Heatmap indicating per individual the αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 R-values classified in three groups based on the association with disease as strong (dark red), intermediate (pink) or not associated (white) in the Bangladeshi and South Korean cohort. Strong association: R ≥ 2x median of patient group, intermediate association: median of patient group ≤ R < 2x median of patient group, no association: R < median of patient group (Supplementary Table S1). (A) R-value classification of biomarkers in multibacillary (MB) patients (Bangladesh: 21; South Korea: 19), paucibacillary (PB) patients (Bangladesh: 15; South Korea: 6) and healthy controls (Bangladesh: 20; South Korea: 24). (B) R-value classification of biomarkers in household contacts (HC; n=25) and patients with other dermatological diseases (ODD; n=25) (South Korean cohort). (C) Dots indicating which biomarker showed a different pattern relative to controls in MB (orange) and PB (blue) patients, HC (green) and ODD (grey).

strips by portable UCP-reader showed that all biomarkers could be clearly detected in FSB enabling the determination of R-values. R-values similar to those in plasma/sera samples were obtained (Supplementary Figure S4), demonstrating that hemoglobin formed by hemolysis did not hamper the UCP-signal. Importantly, like in sera and plasma samples, higher NUM-scores were more frequently observed in FSB of leprosy patients compared

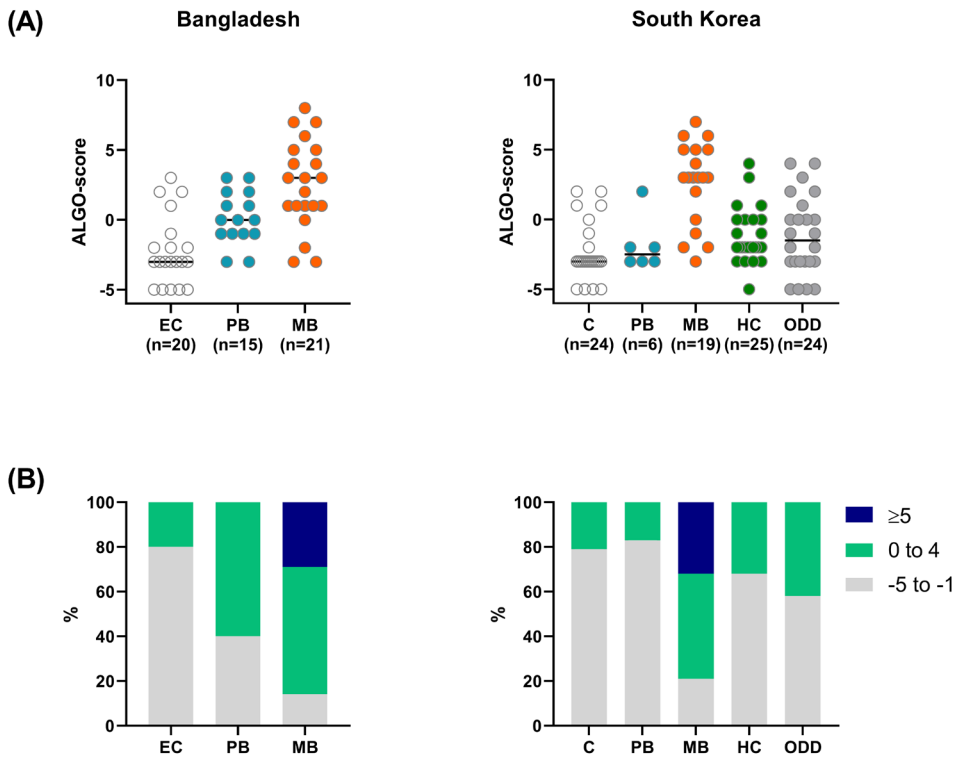

Figure 4: The MBT ALGO-score reflects disease severity and bacterial load. The ALGO-score is based on an algorithm that contributes higher weights to R-values associated with disease (Figure 1B). Based on the median R-value of the patient group, for each biomarker R-values were classified as strongly, intermediately or not associated with disease. The ALGO-score was set as 2x the number of strong biomarkers (2x n_{strong}), plus the number of intermediate biomarkers ($n_{intermediate}$), minus the number of biomarkers not associated with disease (n_{not}) ((2*nbiomarker^{strong}+nbiomarker^{intermediate})-nbiomarker^{not}). (A) ALGO-scores observed in the Bangladeshi and South Korean cohorts per test group. Untreated multibacillary (MB) leprosy patients (orange), untreated paucibacillary (PB) patients (blue), household contacts (HC;green), patients with other dermatological diseases (ODD; grey) and healthy controls ((E)C;white). (B) percentage (%) of individuals per test group with ALGO-scores ranging from -5 to -1 (grey), from 0 to 4 (green) or ALGO-scores ≥ 5 (blue) for the Bangladeshi plasma (left) and South Korean serum (right) cohort.

to HC (Supplementary Figure S4). Hence, application of the MBT using low invasive FSB samples is technically feasible at low resource settings providing potential for MBT use at POC.

Discussion

This study provides proof of concept for the use of the MBT platform, thereby representing, to the best of our knowledge, the first demonstration of a diagnostic tool simultaneously and quantitatively detecting multiple host biomarkers with a user-friendly test easily

applicable with FSB in the field. Leprosy is ideally suited as a model disease to test this platform due to the close parallel between the ability of the host to establish effective immunity to *M. leprae* and the inter-individual variability in clinical manifestations, ranging from self-limited (PB) disease with a predominant Th1 response to disseminated (MB) disease characterized by extensive anti-*M.leprae* antibody titers (14, 28, 29). This study showed POC testing of a biomarker signature covering humoral- and cellular immune responses against *M. leprae* (13). The combination of six biomarkers in this new strip format in a single MBT device avoids running six individual tests and as such is a major step forward towards POC near-patient applications. Moreover, the procedure is less prone to error as the automated reader will immediately provide the MBT result, this would be much more complicated when running the six individual tests in sequence. The six-marker MBT strip provided similar test results as previously obtained with individual UCP-LF strips for each of the biomarkers separately (13). This clearly demonstrated technical feasibility of this new diagnostic platform.

Besides, enabling detailed evaluation of six biomarkers individually the MBT allows combined analysis of multiple biomarkers as part of a biomarker signature. To allow for scoring, two methods were explored which are independent of each other. The NUM-score, indicating the number of biomarkers with a value above the biomarker-specific cut-off, allowed discrimination of leprosy patients from their contacts and healthy individuals. The ALGO-score represents a more direct quantitative score linking the relative biomarker R-values with leprosy disease. Irrespective of leprosy endemicity, MB patients showed the highest ALGO-scores, confirming the association of these MBT-implemented biomarkers with disease severity and bacterial load (13). Both scores are an example of the ample possibilities to analyze the MBT readout. As described previously, standard curves can also be generated to convert the quantifiable R-values to absolute concentrations (30, 31).

Selection of a suitable scoring method depends on the aim of the study. The NUM-score provides a quick interpretation of the test result suitable for large-scale screening studies, for instance to identify *M. leprae*-infected individuals that contribute to the perpetuating transmission. HC of MB patients are at the highest risk of acquiring *M. leprae* infection (32-34) and thus represent candidates for preventive drug administration in multiple studies (35-39) to prevent progress to leprosy disease as well as decrease transmission. Since June 2018 the WHO guidelines for leprosy control have included single dose rifampicin (SDR) as PEP for leprosy prevention (40). The MBT could aid in the identification of *M. leprae* infected individuals eligible for PEP to allow a more efficient and better targeted drug administration approach.

For personalized diagnostics and monitoring of the treatment response, the more detailed evaluation by the ALGO-score could be informative. On the other hand, the MBT can be useful as adjunct diagnostic for patients presenting with symptoms suggestive of leprosy in both leprosy endemic and non-endemic countries. Identification of PB patients lacking anti-*M. leprae* antibodies is challenging using the currently available diagnostic methods leading to delayed or misdiagnosis. In Bangladesh the MBT result separated PB patients clearly from endemic controls, although in the low endemicity setting in South Korea the current biomarker signature could not distinguish the small-sized cohort of PB patients (n=6). Separate evaluation of biomarkers, however, indicated that ApoA1 differed significantly in these PB patients from controls, corroborating the potential of ApoA1 as a biomarker for PB leprosy (13). *M. leprae* exposed HC in South Korea showed a similar ApoA1 response as the PB patients, as observed previously in Bangladesh (16). In contrast to our findings in Bangladesh (16), R-values of S100A12 in contacts and PB patients were similar to those of healthy controls in South Korea. Leprosy is no longer endemic in this country, it has to be taken into account that the frequency of exposure to *M. leprae* as well as other environmental pathogens, can definitely influence biomarker levels, stressing the importance of quantitative measurements.

We acknowledge several limitations to the findings in this study: the leprosy patients of which fingerstick blood was collected included patients treated with MDT, with varying treatment duration. As this study aimed at developing a test platform rather than evaluation of a biomarker signature relatively small sample sizes were tested. For the same reason, direct comparison of fingerstick blood and plasma/serum was not included, and intra-individual differences in biomarker R-values in these different samples could therefore not be determined. For global application including identification of PB patients, the currently implemented biomarker signature will need fine-tuning and evaluation in large cohorts is warranted. The flexible MBT format allows replacement of biomarkers upon identification of additional candidate markers. To identify new biomarkers, especially for PB leprosy, the scope can be widened from the broadly studied immune markers to metabolic markers, which contribute to leprosy pathogenesis as well (41-43). New techniques (44, 45) to identify disease markers in HC developing PB leprosy in high throughput fashion are currently explored by us.

An important advantage of the MBT is its field-applicability, ensuring implementation in low-resource settings. Furthermore, its flexible format also enables the application of the MBT to other diseases for which diagnosis will benefit from the quantitative detection of multiple biomarkers simultaneously. Serum biomarker signatures have been described for example for tuberculosis (46, 47), rheumatoid arthritis (48) and inflammatory bowel

disease (49). More recently, it was also described for COVID-19 patients that cytokines play an important role in determining the outcome of infection besides SARS CoV-2-specific antibodies (50, 51).

In this study, we demonstrated the technical feasibility and applications of the MBT platform for leprosy diagnostics by successfully implementing host biomarkers covering a well-defined biomarker signature for leprosy, on one MBT strip. Moreover, the MBT was not only compatible with plasma and serum but allowed POC testing with FSB samples. Thus, the MBT format represents a step forward in the development of the urgently needed immunodiagnostic POC test for detection of *M. leprae* infection and early stage leprosy.

Acknowledgements

The authors gratefully acknowledge all patients and blood donors. LUMC, EMC and TLMI,B are part of the IDEAL (***I***nitiative for ***D***iagnostic and ***E***pidemiological ***A***ssays for ***L***eprosy) Consortium. We thank the staff of the Rural Health Program, The Leprosy Mission International Bangladesh, Nilphamari, Bangladesh for recruitment of study participants and sample collection. We gratefully thank Sang-Nae Cho (Yonsei University College of Medicine, Seoul, Korea) for stimulating discussions.

Funding statement

This study was supported by the Order of Malta-Grants-for-Leprosy-Research (MALTALEP), the Q.M. Gastmann-Wichers Foundation (AG) and the Leprosy Research Initiative (LRI) together with the Turing Foundation (ILEP#: 703.15.07). Formats of the in this study evaluated diagnostic platform were assessed in parallel studies aimed at user- and field-friendly diagnostics for active tuberculosis: EDCTP funded projects, AETBC (IP_2009_32040) and Screen-TB (DRIA2014-311).

Declaration of interests

The authors declare to have no conflicts of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supplementary material

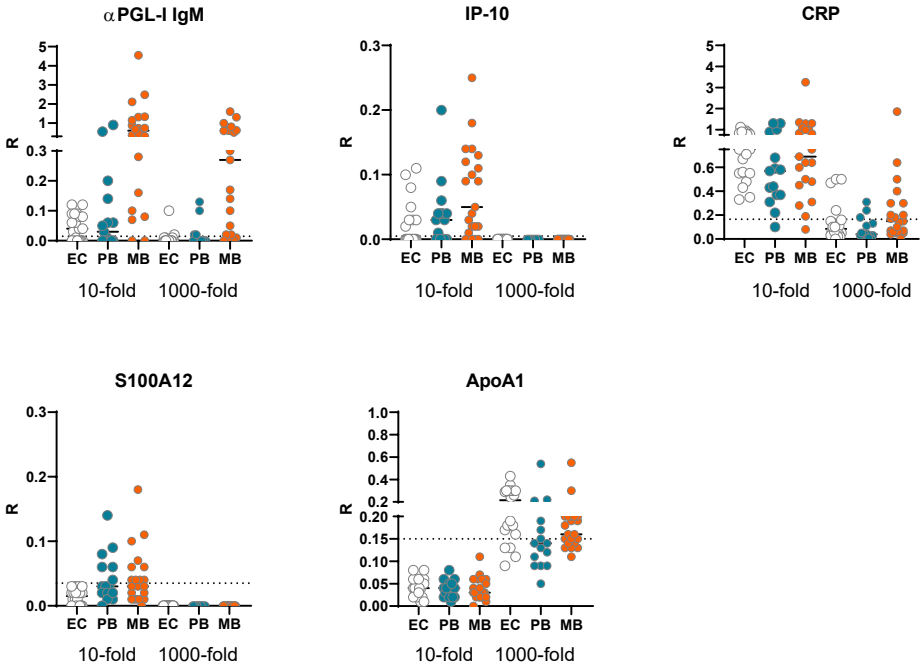
Tables

Supplementary Table S1: Study cohorts related to Figures 2 -4

	Group	N	BI (Mean)	Age range (years)	Male/Female (%)	MDT
Bangladesh (plasma)	*MB*	21	2.9+	18 to 60	91/9	0 (0%)
	PB	15	0	19 to 56	71/29	0 (0%)
	EC	20	NA	20 to 41	40/60	NA
	Biomarker characteristics	**αPGL-I IgM**	**IP-10**	**CRP**	**S100A12**	**ApoA1**
	Sample dilution	1000-fold	10-fold	1000-fold	10-fold	1000-fold
	AUC	0.75	0.7	0.52	0.78	0.69
	Cut-off	>0,015	>0,005	>0,165	>0,035	<0,23
	Sensitivity(%)	58	75	36	44	92
	Specificity(%)	90	60	80	100	50
	Median Ratio of patients	0.02	0.04	0.085	0.03	0.15
	Not	<0.02	<0.04	<0.085	<0.03	>0.15
	Intermediate	0.02-0.04	0.04-0.08	0.085-0.17	0.03-0.06	0.15-0.075
	Strong	>0.04	>0.08	>0.17	>0.06	<0.075
South-Korea (Serum)	**Group**	**N**	**BI (Mean)**	**Age range (years)**	**Male/Female (%)**	**MDT**
	MB	19	5.2+	21 to 82	63/37	0 (0%)
	PB	6	0	23 to 68	100/0	0 (0%)
	HC	25	NA	12 to 85	44/56	NA
	C	24	NA	24 to 95	71/29	NA
	ODD	24	NA	18 to 84	71/29	NA
	Biomarker characteristics	**αPGL-I IgM**	**IP-10**	**CRP**	**S100A12**	**ApoA1**
	Sample dilution	1000-fold	10-fold	1000-fold	10-fold	1000-fold
	AUC	0.77	0.6	0.67	0.6	0.54
	Cut-off	>0.03	>0.65	>0.205	>0.33	<0.105
	Sensitivity(%)	52	44	56	24	36
	Specificity(%)	100	92	79	96	100
	Median Ratio of patients	0.04	0.46	0.24	0.16	0.15
	Not	<0.04	<0.46	<0.24	<0.16	>0.15
	Intermediate	0.04-0.08	0.46-0.92	0.24-0.48	0.16-0.32	0.15-0.075
	Strong	>0.08	>0.92	>0.48	>0.32	<0.075
Bangladesh (FSB)	**Group**	**N**	**BI (Mean)**	**Age range (years)**	**Male/Female (%)**	**MDT**
	MB	11	1.4+	22 to 65	64/36	10 (91%)
	PB	16	0	17 to 70	38/62	2 (12,5%)
	HC	15	NA	17 to 60	53/47	NA
	Biomarker characteristics	**αPGL-I IgM**	**IP-10**	**CRP**	**S100A12**	**ApoA1**
	Sample dilution	1000-fold	10-fold	1000-fold	10-fold	1000-fold
	AUC	0.55	0.64	0.56	0.6	0.54
	Cut-off	>0.005	>0.175	> 0.375	> 1.06	< 0.225

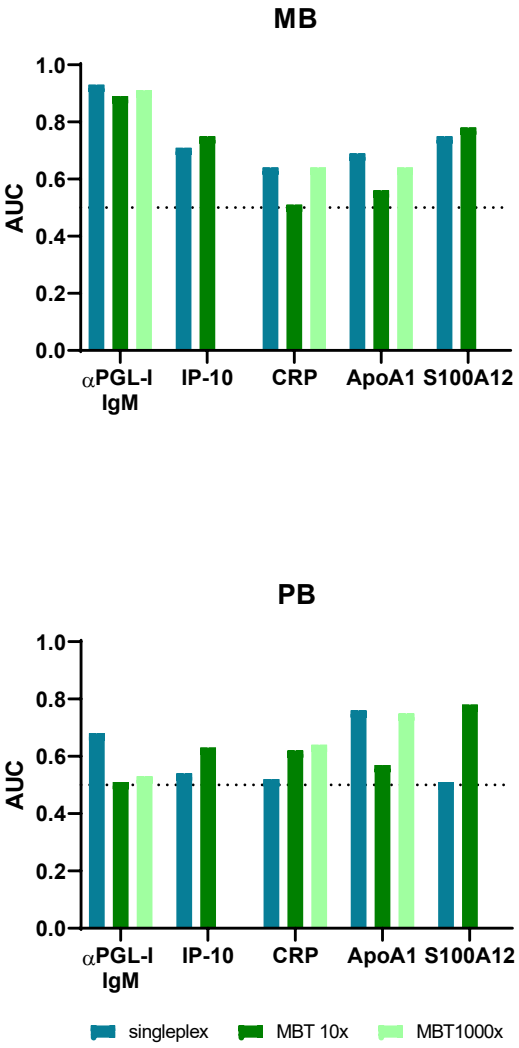
The percentages of males and females, individuals receiving multidrug therapy (MDT), the mean bacteriological index (BI) and the age range of the multibacillary (MB) and paucibacillary (PB) patients, household contacts (HC), healthy (endemic) controls ((E)C) and patients with other dermatological diseases (ODD) in the study cohorts from Bangladesh (plasma and fingerstick blood (FSB)) and South Korea (serum). Ratio (R) values for αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 determined by the MBT strip in these samples were used to calculate the optimal cut-off (as determined by Youden's index) to discriminate leprosy patients from controls applying the indicated dilution. The MBT readout (R-value) was also stratified based on the association with disease as strongly, intermediately or not associated. These categories are based on the patient median (Median). Strong association: R ≥ 2x median of patient group, intermediate association: median of patient group ≤ R < 2x median of patient group, no association: R < median of patient group. AUC: Area under the curve (as determined by computing receiver operating characteristic curves for leprosy patients compared to controls) . NA = not applicable

Figures



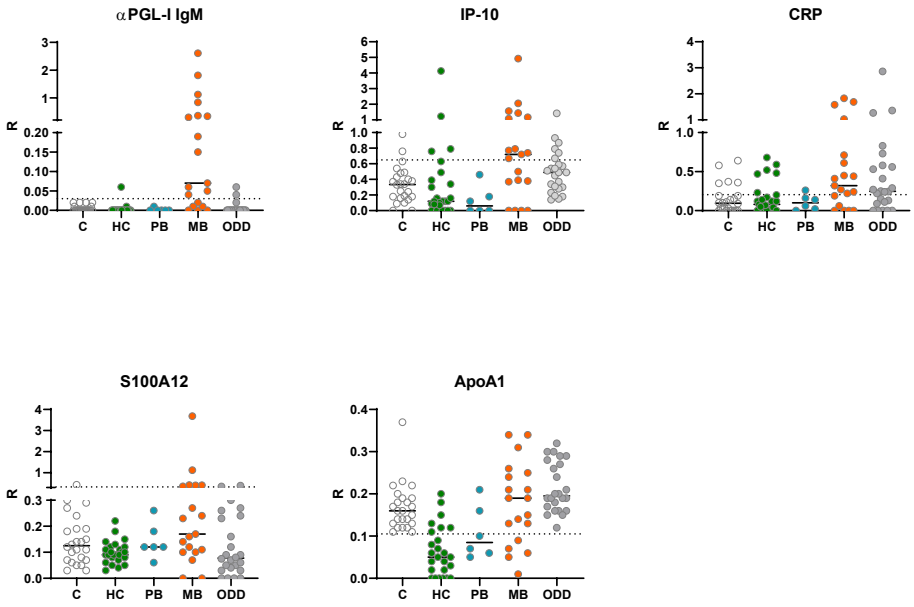
Supplementary Figure S1: Levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 measured by multibiomarker test (MBT) strips (Bangladesh cohort) related to Figures 2 -4.

The levels of αPGL-I IgM, IP-10, CCL4, CRP, S100A12 and ApoA1 were assessed by applying 10- and 1000-fold diluted plasma samples of leprosy patients (n=36; multibacillary (MB) = 21, paucibacillary (PB) =15) and endemic controls (EC; n=20) to MBT strips. CCL4 was not detected in these plasma samples, a graph was therefore not included. Ratio values (R) for each biomarker were calculated by dividing the peak area of the test line by the peak area of the flow control line (*y*-axis). The dashed line indicates the optimal study cut-off value to discriminate leprosy patients from controls, determined by the Youden's index for the optimal dilution (10-fold: IP-10, S100A12; 1000-fold αPGL-I IgM, CRP, ApoA1.



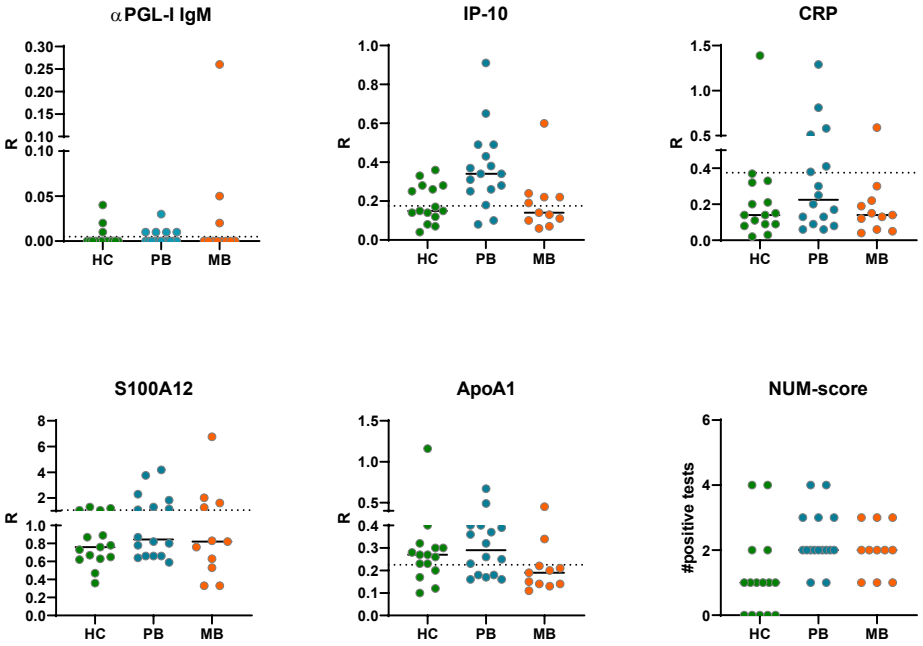
Supplementary Figure S2: Areas under the curve (AUCs) for αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 related to Figure 2.

Receiver operating characteristic (ROC) curves were computed and the respective AUC was calculated. AUCs obtained using either UCP-LFA strips specific for a single biomarker (singleplex) or multibiomarker test (MBT) strips were determined as a measure for discrimination between leprosy patients and endemic controls (EC). αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 were assessed in plasma samples of leprosy patients (MB=21; PB=15) and EC (n=20). The AUCs (*y*-axis) were calculated based on the Ratio values for each marker for singleplex strips (blue) or MBT strips in the 10-fold (dark green) and 1000-fold (mint green) dilution. Singleplex data was described previously, samples were diluted 10-fold (IP-10), 100-fold (αPGL-I IgM and S100A12), 1000-fold (CRP) and 10000-fold (ApoA1). The dashed line at 0.5 indicates a non-discriminatory AUC.



Supplementary Figure S3: Levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 measured by multibiomarker test (MBT) strips (South Korea cohort) related to Figures 2-4.

The levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 were assessed in serum samples of leprosy patients (n=25; multibacillary (MB) = 19; paucibacillary (PB) = 6), household contacts (HC; n=25), healthy controls (C; n=24) and patients with other dermatological diseases (ODD; n=24) from South Korea using MBT strips. Results of the optimal dilution per biomarker are shown, 10-fold for IP-10 and S100A12 and 1000-fold for CRP, ApoA1 and αPGL-I IgM. Ratio values (R) (*y*-axis) were calculated by dividing the peak area of the test line by the peak area of the flow control line. The dashed line indicates the optimal study cut-off value to discriminate leprosy patients from controls, determined by the Youden's index.



Supplementary Figure S4: Fingerstick blood (FSB) levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 measured by MBT strips.

The levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 were assessed in FSB samples (two dilutions) of leprosy patients from Bangladesh (Leprosy; n=27) and household contacts (HC; n=15) using MBT strips. Patients treated for several months when FSB samples were taken (91% MB (n=10), 12,5% PB (n=2)) were included. Results of the optimal dilution per biomarker are shown, 10-fold for IP-10 and S100A12 and 1000-fold for CRP, ApoA1 and αPGL-I IgM. Ratio values (R) (*y*-axis) were calculated by dividing the peak area of the test line by the peak area of the flow control line. MBT NUM-scores were determined for HC, PB and MB patients. The dashed line indicates the optimal study cut-off value to discriminate leprosy patients from controls, determined by the Youden's index.

5 Chapter

Field-friendly serological tests for determination of *M. leprae*-specific antibodies

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Sci Rep. 2017 Aug 21;7(1):8868.

Abstract

Early detection of leprosy is key to reduce the ongoing transmission. Antibodies directed against *M. leprae* PGL-I represent a useful biomarker for detecting multibacillary (MB) patients. Since efficient leprosy diagnosis requires field-friendly test conditions, we evaluated two rapid lateral flow assays (LFA) for detection of *Mycobacterium leprae*-specific antibodies: the visual immunogold OnSite Leprosy Ab Rapid test [Gold-LFA] and the quantitative, luminescent up-converting phosphor anti-PGL-I test [UCP-LFA]. Test performance was assessed in independent cohorts originating from three endemic areas.

In the Philippine cohort comprising patients with high bacillary indices (BI; average:4,9), 94%(n=161) of MB patients were identified by UCP-LFA and 78%(n=133) by Gold-LFA. In the Bangladeshi cohort, including mainly MB patients with low BI (average:1), 41%(n=14) and 44%(n=15) were detected by UCP-LFA and Gold-LFA, respectively. In the third cohort of schoolchildren from a leprosy hyperendemic region in Brazil, both tests detected 28%(n=17) seropositivity.

Both rapid tests corresponded well with BI($p<0.0001$), with a fairly higher sensitivity obtained with the UCP-LFA assay. However, due to the spectral character of leprosy, additional, cellular biomarkers are required to detect patients with low BIs. Therefore, the UCP-LFA platform, which allows multiplexing with differential biomarkers, offers more cutting-edge potential for diagnosis across the whole leprosy spectrum.

Introduction

Leprosy, an infectious disease caused by *Mycobacterium leprae* (*M. leprae*), still poses a major health threat in developing countries. The availability of effective multi-drug therapy (MDT) has decreased the global disease burden significantly, however, the annual new case detection rate has remained virtually stable during the past decade which undeniably points towards the continuation of bacterial transmission. Mis- or delayed diagnosis frequently occurs as leprosy diagnosis still relies on clinical symptoms (1). These symptoms typically take 2-6 years, but also up to 20 years, to become manifest (2). Moreover, as the majority of people have sufficient natural immunity to (myco)bacterial infection and will not progress to disease (3), a small proportion (1-5%) of *M. leprae* infected individuals will actually develop clinical symptoms. During subclinical *M. leprae* infection the host, without being aware of the infectious state, may transmit the bacteria, allowing transmission to continue, especially among close contacts of the infected individuals(4, 5) . Diagnostic tests for early detection of leprosy, allowing adequate treatment of early-stage leprosy and *M. leprae* infection, could therefore make significant differences in transmission and clinical outcomes.

Leprosy presents as a spectral disease, ranging from a dominant cellular phenotype with the ability to mount a cellular response that leads to effective killing of *M. leprae*, to an immune response characterized mostly by humoral immunity against *M. leprae* (6). Within the leprosy spectrum five disease types can be identified according to the Ridley Joplin classification (7): tuberculoid (TT), borderline tuberculoid (BT), borderline (BB), borderline lepromatous (BL) and lepromatous leprosy (LL). Alternatively, the WHO classification is based on the number of skin lesions and nerve involvement and classifies leprosy as multibacillary (MB; > 5 lesions) or paucibacillary (PB; ≤ 5 lesions) (2) in which PB is predominantly associated with the cellular phenotype and MB with the humoral immune response.

A useful biomarker for leprosy, predominantly for MB patients, is the level of IgM antibodies directed against the *M. leprae*-specific phenolic glycolipid I (PGL-I) (8, 9). Moreover, upon effective treatment of leprosy IgM levels drop and can therefore be used to monitor efficacy of leprosy treatment (10).

Leprosy endemic areas are often short of sophisticated laboratories which makes it imperative to develop diagnostic tests for the detection of PGL-I antibodies suitable for field settings. The aim of this study was to evaluate two recently designed field-friendly lateral flow assays (LFAs) , the OnSite Leprosy Ab Rapid test (11) and the in-house developed PGL-I up-converting phosphor (UCP)-LFA (12). The OnSite Leprosy Ab Rapid

test is an immunochromatographic LFA, detecting IgM antibodies against PGL-I and IgG antibodies to LID-1. The latter fusion protein, encoded by the genes for ML0405 and ML2331, has been shown to be a useful diagnostic marker for MB leprosy (13, 14). UCP-LFAs have previously demonstrated applicability for detection and monitoring of a variety of analytes (15-17), including cellular biomarkers for *Mycobacterium tuberculosis* (18, 19). Therefore, we developed user-friendly UCP-LFAs for the detection of IgM antibodies against PGL-I, which proved robust in multiple (field) studies (10, 12, 20). The luminescent UCP-label enables quantitative determination of IgM levels using portable readers, whereas the colloidal gold label of the OnSite Leprosy Ab Rapid test (Gold-LFA) generates qualitative results which are visually inspected. Test performance of these two different field-friendly LFA formats was assessed using identical sera of 3 cohorts derived from different leprosy endemic areas in the Philippines, Bangladesh and Brazil.

Materials and methods

Study participants

Cohort 1

Leprosy patients (LL/BL (n=127), BT/TT (n=24) and LL patients (n=44; longitudinal samples of 9 patients), were diagnosed at the Cebu Skin Clinic and Leonard Wood Memorial (LWM) Center for Leprosy Research, Cebu, Philippines based on histological findings and clinical observations determined by experienced leprologists and a leprosy pathologist as previously described (8, 13). Patients were categorized according to Ridley-Jopling classification (7) and bacterial indices (BI) were determined. For some patients, serum was also obtained at specified intervals during treatment (at 1, 2, 3, 6, 9 and 12 months), and up to 2 years after the start of treatment (13). Samples were collected from March 2007 until February 2012. All leprosy patient sera were collected at initial diagnosis prior to multidrug therapy (MDT). As a control group, non-BCG-vaccinated, U.S.-born healthy individuals with no known exposure to either tuberculosis or leprosy were included and designated as nonendemic controls (NEC; n=5).

Cohort 2

Participants were recruited on a voluntary basis between January 2013 and December 2014 in leprosy endemic areas in Bangladesh as described previously (21). Leprosy was diagnosed based on clinical, bacteriological and histological observations as previously described (22). Clinical and demographic data were collected in a dedicated database. Participants were classified into five test groups: MB patients (n=34; BL/LL=8, BT=26), PB patients (n=45; BT= 41, TT=4), healthy household contacts (HHC; n=54) and BCG

vaccinated HHC (HHC&BCG; n=50) selected as previously described (20). Control individuals without clinical disease symptoms from the same leprosy endemic area (endemic controls, EC; n=51) were examined for the absence of clinical signs and symptoms of leprosy and tuberculosis. Staff of leprosy- or TB clinics were excluded as EC.

Cohort 3

Serum samples of schoolchildren were collected in the state of Pará, Brazil (n=60), considered hyperendemic for leprosy (new case detection >4.0 per 10,000 population) as previously described (23). Serum samples from all sources were coded to protect donor identities and were obtained with informed consent and/or with permission from the respective institutional review boards of each country.

Synthetic PGL-I

The disaccharide epitope (3,6-di-*O*-methyl-β-D-glucopyranosyl(1→4)2,3-di-*O*-methylrhamnopyranoside) of *M. leprae* specific native PGL-I glycolipid was synthesized and coupled to human serum albumin (synthetic PGL-I; designated ND-O-HSA, approximately 40 disaccharides per molecule) (24). It was obtained through the Biodefense and Emerging Infections Research Resources Repository. (http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx).

PGL-I ELISA

Antibodies (IgM, IgG, IgA) against *M. leprae* PGL-I were detected by ELISA. Briefly, 200 ng ND-O-HSA was coated per well in 50 µl in 0.1M Na_2CO_3/$NaHCO_3$ buffer (pH 9.6) at 4 °C overnight. After blocking with 200 µl PBS/1%BSA/0.05% Tween 80 per well for 1 hour, 50 µl of 1:400 diluted sample was added and incubated for 2 hours at room temperature. Then, 50 µl of a 1:8000 dilution of anti-human IgG/IgM/IgA-HRP, (Dako P0212) in 0.05%Tween 20/PBS was incubated for 2 hours. In between each step the wells were washed 3 times with PBS/0,05% Tween 20. 50 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) was added and the color reaction was stopped using H_2SO_4 after 10-15 minutes. Absorbance was determined at a wavelength of 450 nm. Samples with an optical density at 450 nm (OD_{450}) after correction for background OD (0,1%BSA in coating buffer) above 0.200 were considered positive. This threshold was determined by a threefold multiplication of an average NEC value.

The OnSite Leprosy Ab Rapid test

The OnSite Leprosy Ab Rapid test (11) was purchased from CTK Biotech (San Diego,

CA) and used according to the manufacturer's instructions. Test band intensity was independently scored ranging from 0.5 to 4 by two independent individuals, in case of differences between operators the highest rated score was used (11). The OnSite Leprosy Ab Rapid test is referred to as the Gold-LFA in this study.

PGL-I UCP-LFA

Anti-PGL-I IgM antibodies were detected as previously described (20) using 100-fold diluted serum and an IgM-specific UCP conjugate ($UCP^{\alpha IgM}$). LF strips were scanned in a Packard FluoroCount microtiterplate reader adapted for measurement of the UCP label (980 nm IR excitation, 550 nm emission). Results are displayed as the ratio value between Test and Flow-Control signal based on relative fluorescence units (RFUs) measured at the respective lines. A threshold for positivity of 0.29 was determined by computing receiver operating characteristic (ROC) curves (20).

Ethics

This study was performed according to the Helsinki Declaration as described previously (21). The national Research Ethics Committee has approved the study protocol (Ref no. BMRC/NREC/2010-2013/1534 (Bangladesh) and Ethical Appreciation Certificate Nº 26765414.0.0000.0018 (Brazil)). Participants were informed about the study-objectives, the samples and their right to refuse to take part or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. Serum samples from the Philippines and NEC serum samples are part of a pre-existing collection at Colorado State University (JSS), and thus considered exempt from human subjects research. All patients received treatment according to national guidelines.

Statistical analysis

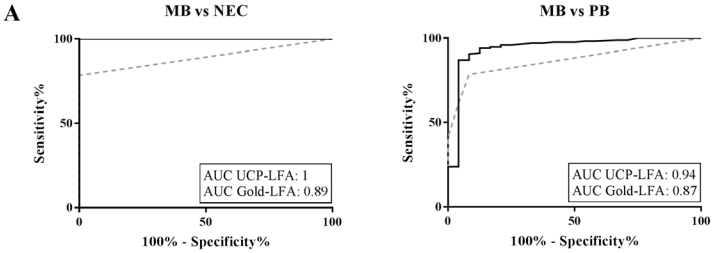
Graphpad Prism version 7.00 for Windows (GraphPad Software, San Diego CA, USA) was used to perform Mann-Whitney U tests, plot ROC curves and calculate the area under curve (AUC).

Results

Performance of UCP-LFA and Gold-LFA in selected, polar leprosy patients

To analyze the performance of the UCP-LFA and the Gold-LFA, we first analyzed a selection of Philippine polar leprosy patients (cohort 1, n=200). MB patients (n=171) could be adequately distinguished from nonendemic controls (NEC; n=5) using the UCP-LFA

(AUC:1; cut-off:0.29, sensitivity: 94%, specificity: 100%) or the Gold-LFA (AUC:0.89; cut-off:0.5, sensitivity: 78%, specificity: 100%) (Figure 1A). In the PGL-I ELISA, 145 out of 171 MB patients (85%) were positive (Figure 1B). The UCP-LFA identified 161 (94%) MB patients as positive, whereas for 133 (78%) MB patients a positive test band was observed in the Gold-LFA. Furthermore, all 5 NEC included as negative controls were confirmed negative in both Gold-LFA and UCP-LFA.



B

Cohort 1	ELISA	UCP-LFA (LUMC)	Gold-LFA (CTK)
Target	ND-O-HSA	ND-O-HSA	NDO-LID
Measurement	Reader	Reader	Visual
	positive (n)	positive (n)	positive (n)
Patients total (n=195)	146 (75%)	165 (85%)	135 (69%)
MB (n=171)	145 (85%)	161 (94%)	133 (78%)
PB (n=24)	1 (4%)	4 (17%)	2 (8%)
NEC (n=5)	0 (0%)	0 (0%)	0 (0%)

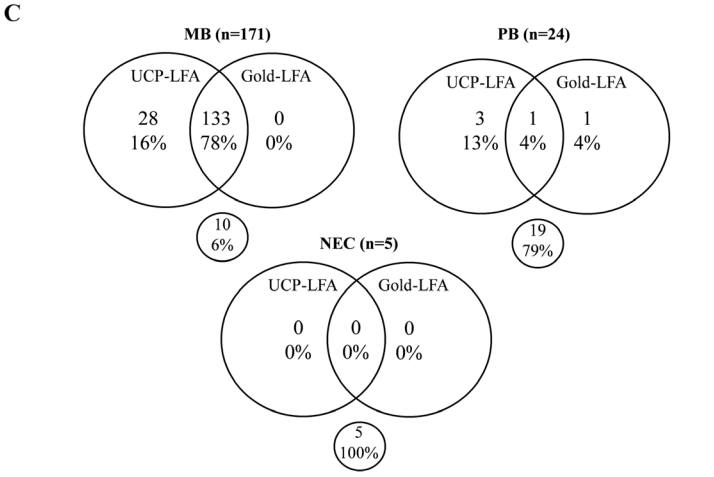


Figure 1: Test performance of UCP-LFA and Gold-LFA in Philippine leprosy patients. Test performance was assessed using serum of 171 multibacillary (MB) and 24 paucibacillary (PB) patients. Non-endemic controls were included as negative control. (**A**) Receiver operating characteristics (ROC) curves showing the distinction between MB patients and NEC (left panel) and MB and PB patients (right panel) for the UCP-LFA (solid line) and Gold-LFA (dotted line). Areas under curve (AUCs) are displayed for both tests. (**B**) Number and percentage of positive individuals per test (ELISA, UCP-LFA, Gold-LFA) are shown for each test group. (**C**) Venn diagrams showing the concordance in positive individuals between UCP-LFA and Gold-LFA per test group.

Of the 24 PB patients, one (4%) was positive in the PGL-I ELISA, 2 (8%) in the Gold-LFA and 4 (17%) in the UCP-LFA (Figure 1B). With respect to disease classification both tests distinguished MB and PB patients very well, providing an AUC of 0.94 for the UCP-LFA (sensitivity: 94%; specificity: 81%) and 0.87 for the Gold-LFA (sensitivity: 78%; specificity: 94%) (Figure 1A).

Assessment of test concordance showed that the majority (n=133; 78%) of MB patients were detected by both LFAs, whereas 28 (16%) were identified by UCP-LFA only (Figure 1C; supplementary Figure S2). Of these 28 individuals, 22 (79%) showed ELISA values around the cut-off (0.1-0.5), indicating sensitive detection of low PGL-I positives. Out of 24 PB patients one was positive in both tests, one in the Gold-LFA only, 3 in the UCP-LFA only and 19 were negative in both tests.

In summary, although some samples showed discordant results between the 2 tests (Supplementary Figure S2) in the Philippine cohort both LF-based tests identified the majority of MB patients (≥78%) and could distinguish MB patients from PB patients or NEC.

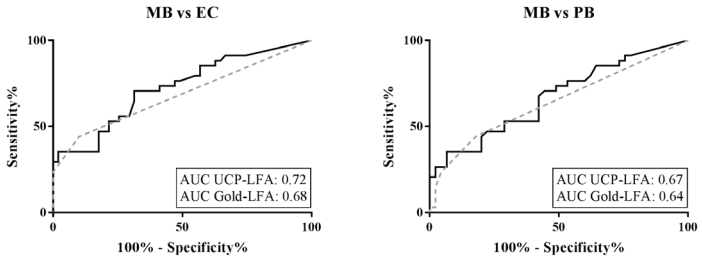
Test performance of UCP-LFA and Gold-LFA in a Bangladeshi cohort

Since both LFAs performed well in cohort 1, which was selected to include polar types of leprosy, we next evaluated test performance in an unbiased population characterized by representation of less polar forms of leprosy and MB patients with low BIs, consisting of leprosy patients, healthy household contacts (HHC) and endemic controls (EC) from Bangladesh (Cohort 2). For EC (n=51), PGL-I seropositivity was detected, 8 (16%) in the PGL-I ELISA, 9 (18%) in UCP-LFA and 5 (10%) in the Gold-LFA, resulting in AUCs for MB diagnosis of 0.72 and 0.68 for the PGL-I UCP-LFA and Gold-LFA, respectively (Figure 2A, B). For classification of leprosy into MB or PB, AUCs were comparable, 0.67 for the UCP-LFA and 0.64 for the Gold-LFA (Figure 2A).

14 out of 34 MB patients (41%), the majority of whom had a low or negative BI (supplementary Figure S1), were positive in the PGL-I ELISA, which was similarly reflected in the UCP-LFA (n=14; 41%) and the Gold-LFA (n=15; 44%) (Figure 2B). As in the PGL-I ELISA, seropositivity was also observed in both HHC groups (with or without BCG vaccination). Besides only a low number in all test groups in cohort 2 being seropositive in either of the 2 tests, the PGL-I UCP-LFA and Gold-LFA also detected different seropositive HHC (8 seropositive in UCP-LFA only and 2 in Gold-LFA only; Figure 2C; supplementary Figure S1), indicating that detection of different antibodies in these two test formats can identify different individuals. In patients, 7 MB and 9 PB patients were seropositive in either the

UCP-LFA or Gold-LFA, of whom 75% (n=12) showed a weakly positive test response (Supplementary Figure S2, Figure 2C).

A



B

Cohort 2	ELISA	UCP-LFA (LUMC)	Gold-LFA (CTK)
Target	ND-O-HSA	ND-O-HSA	NDO-LID
Measurement	Reader	Reader	Visual
	positive (n)	positive (n)	positive (n)
Patients total (n=79)	20 (25%)	23 (29%)	23 (29%)
MB (n=34)	14 (41%)	14 (41%)	15 (44%)
PB (n=45)	6 (13%)	9 (20%)	8 (18%)
HC (n=54)	7 (13%)	4 (7%)	1 (2%)
HC&BCG (n=50)	3 (6%)	5 (10%)	2 (4%)
EC (n=51)	8 (16%)	9 (18%)	5 (10%)

C

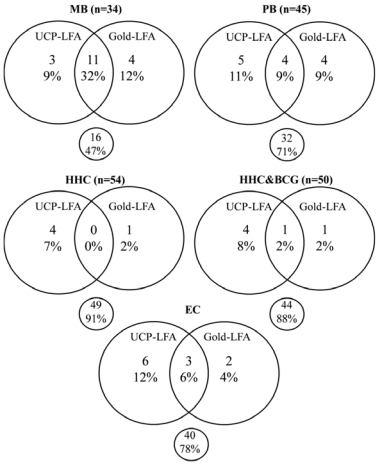


Figure 2: Test performance of UCP-LFA and Gold-LFA in a Bangladeshi cohort. Test performance was assessed using serum of 34 multibacillary (MB), 45 paucibacillary (PB) patients, 104 healthy household contacts (BGC-vaccinated [n=50], non-vaccinated [n=54]) and 51 endemic controls (EC). (**A**) Receiver operating characteristics (ROC) curves showing the distinction between MB patients and EC (left panel) and MB and PB patients (right panel) for the UCP-LFA (solid line) and Gold-LFA (dotted line). Areas under curve (AUCs) are displayed for both tests. (**B**) Number and percentage of positive individuals per test (ELISA, UCP-LFA, Gold-LFA) are shown for each test group. (**C**) Venn diagrams showing the concordance in positive individuals between UCP-LFA and Gold-LFA per test group.

Application of UCP-LFA and Gold-LFA in a hyperendemic region in Brazil

Samples of cohort 3 (n=60) were collected in a region considered hyperendemic for leprosy in Brazil. Although none of the individuals showed clinical signs of leprosy, both the UCP-LFA and Gold-LFA detected antibodies to *M. leprae* specific antigens in 17 individuals (28%), whereas the conventional ELISA detected antibodies in 11 individuals (18%). All individuals seropositive by ELISA were also detected by the UCP-LFA and Gold-LFA. Moreover, the majority of individuals detected by UCP-LFA and Gold-LFA showed concordant results (n=15), 2 individuals were detected by UCP-LFA only and 2 others by Gold -LFA only (supplementary Figure S1) indicating similar test performance for the 2 field-friendly assays. Moreover, we evaluated both test in TB patients, which were all negative showing the lack of cross-reactivity at antibody level with other mycobacterial infections (Supplementary Table S1).

Correlation of lateral flow tests with Bacterial Index

Antibodies against PGL-I and LID-1 are reported to predominantly identify MB patients. Consistent with this the majority of MB patients was identified in cohort 1 (MB patients with high bacterial index (BI; average: 4,9; range: 3,2-6)), whereas in cohort 2 (MB patients with a low or negative BI (average: 1; range:0-5)) more than half of the MB patients was not seropositive in either of the tests (UCP-LFA:56%; Gold-LFA:59%).

To examine the correlation between the test results and BI, leprosy patients from both cohorts (n=198) were stratified by BI (BI^+ [n=145], BI^- [n=53]). In ELISA, 125 (86%) of BI^+ patients showed a positive anti-PGL-I titer, in the UCP-LFA 136 (94%) and in the Gold-LFA 114 (78%) (Figure 3). Of the 53 BI^- patients 6 (11%) showed a positive anti-PGL-I titer in ELISA, 9 (17%) in the UCP-LFA and 10 (19%) in the Gold-LFA. In all tests, significantly higher positive values were observed in the BI positive group ($p < 0.0001$), indicating strong correlation with BI and implying the need of other (cellular) biomarkers to diagnose patients with low BI.

Discussion

User-friendly tools to facilitate the diagnosis of leprosy or/*M. leprae* infection are urgently needed to tackle the ongoing transmission. In this study we have compared the UCP-LFA and the OnSite Leprosy Ab Rapid test (Gold-LFA), two user-friendly tools for the identification of *M. leprae* specific humoral immune responses. The UCP-LFA detects IgM antibodies directed against PGL-I, whereas the Gold-LFA additionally identifies IgG antibodies directed against LID-1. In a head-to-head comparison of these two LFA tests, the UCP-LFA identified similar (cohort 2 [41%] and 3 [28%]) or more (cohort 1 [94%]) MB

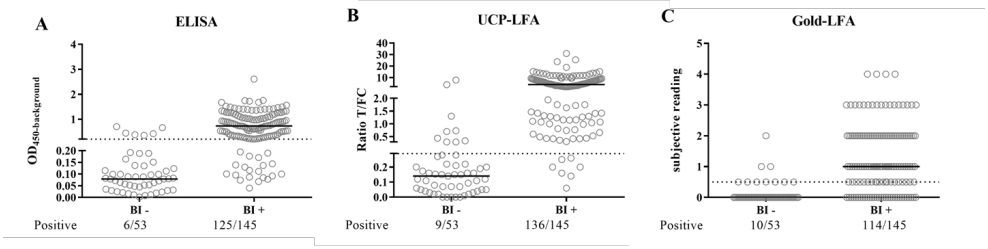


Figure 3: Test correspondence with bacterial index. Leprosy patients of which the BI was assessed were stratified by bacterial index (BI negative [n=53] and positive [n=145]) to evaluate the correlation between BI and LF test results. (**A**) ELISA data stratified by BI, the cut-off of OD_{450}-background (= 0.2) is visualized by the dotted line. 125 BI^+ patients and 6 BI^- patients showed a positive result in ELISA. Values significantly differed between BI^- and BI^+ patients ($p<0.0001$) (**B**) UCP-LFA data stratified by BI, the positive cut-off of ratio 0.29 is visualized by the dotted line. 136 BI^+ patients and 9 BI^- negative patients showed a positive result in the UCP-LFA. Values significantly differed between BI^- and BI^+ patients ($p<0.0001$) (**C**) Gold-LFA data stratified by BI, the cut-off for a positively scored samples (observed test band >0.5) is visualized by the dotted line. 114 BI^+ patients and 10 BI^- patients were scored as positive in the Gold-LFA. Values significantly differed between $BI^-$and BI^+ patients ($p<0.0001$).

patients than the Gold-LFA (cohort 1 [78%], cohort 2 [44%], cohort 3 [28%]). Especially in MB patients with a positive BI the UCP-LFA turned out to be more sensitive than the Gold-LFA, identifying 94% of these patients and outperforming the conventional PGL-I ELISA. Despite the similarity in test results between both tests, a number of individuals were detected exclusively by either the UCP-LFA (n=55) or the Gold-LFA (n=15). On one hand, this discrepancy can be explained by the more sensitive quantitative detection of anti-PGL-I IgM in the UCP-LFA due to the use of a luminescence label in contrast to a visual, gold label (25). On the other hand, though less frequently occurring, seropositivity due to the presence of LID-1- specific anti-IgG may detect other samples only in the Gold-LFA. Furthermore, since leprosy patients may present only anti-PGL-I IgG and not IgM (26) the inclusion of detection of IgG specific for PGL-I in diagnostic tests for leprosy should also be considered.

An essential difference between the two antibody tests is the interpretation of test results, whereas the UCP-LFA results are objectively measured by a calibrated reader, the Gold-LFA test relies on somewhat more subjective visual evaluation by operators. Especially in the field operator differences should be taken into account, and re-examination by a second individual is therefore vital. This should be done within 10-15 minutes after the test is performed (11) to acquire reliable results. In contrast, the UCP-LFA strips can be permanently stored and sent to a reference lab for re-analysis.

The use of a reader (Smart Reader) to analyse immunogold LF strips similar to the Gold-LFA test for detection of *M. leprae*-specific antibodies has been described (27, 28) and

demonstrated that results by visual interpretation and results read by Smart Reader agreed moderately (κ index: 0,55) (29). However, haemolysis of red blood cells when using the immunogold label may hamper accurate measurements and visualisation of low positives, contrary to the UCP-LFA format which is a virtually background-free reporter technology (25).

In Bangladesh the majority of leprosy patients develop PB leprosy (2), which were mostly negative in both LF tests used in this study as well as the standard ELISA for detection of antibodies against PGL-I. Moreover, PB patients cannot be distinguished from HHC or EC due to the seropositivity observed in these groups. Therefore, to identify not only the BI^+ MB patients and increase specificity it is imperative to include detection of cellular markers in field friendly diagnostic assays for leprosy. Incorporation of the cellular markers IP-10, CCL4 and IL-10 into the same UCP-LFA format improved the assay sensitivity for detection of MB as well as PB patients in Bangladesh (20).

The ability to simultaneously detect both humoral and cellular biomarkers of *M. leprae* infection in a single test, as proved feasible for the UCP-LF format (10), can cover the entire spectrum of leprosy, therefore enabling more comprehensive diagnosis of leprosy patients.

Acknowledgements

We thank the staff of the RHP in Nilphamari, Bangladesh (Korshed Alam) and the Cebu skin clinic, Philippines (Marivic F. Balagon) for patient recruitment in the context of previous studies and our collaborators in the LRI project, Jan Hendrik Richardus and Renate Richardus, for critical discussions.

Funding statement

This study was supported with grants from the Order of Malta-Grants-for-Leprosy-Research (MALTALEP), the Netherlands Leprosy Relief Foundation (NLR; ILEP#: 7.01.02.48, 7.02.02.73 and 7.03.15.07) together with the German Leprosy and TB Relief Association (GLRA) and the Turing Foundation, the Leprosy Research Initiative (LRI), the Heiser Program for Research in Leprosy of The New York Community Trust (grants P15-000827 and P16-000796), the J. William Fulbright Scholar to Brazil award 2015-2016 (to JSS), CNPq (308980/2015-8 and 428964/2016-8), CAPES PROAMAZONIA (3288/2013) (to CS) and the Q.M. Gastmann-Wichers Foundation (to AG).

Competing financial interests

The authors declare to have no financial/commercial conflicts of interests.

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Supplementary material

Tables

Supplementary Table S1: Evaluation of ELISA, UCP-LFA and Gold-LFA in patients with active tuberculosis.

	ELISA	UCP-LFA	Gold-LFA
TB1	0.024	0.09	0
TB2	0.05	0.06	0
TB3	0.055	0.24	0
TB4	0.042	0.00	0
TB5	0.082	0.11	0
TB6	0.046	0.10	0
TB7	0.071	0.11	0
TB8	0.061	0.24	0
TB9	0.055	0.20	0
TB10	0.024	0.04	0
TB11	0.025	0.11	0
TB12	0.009	0.03	0
TB13	0.005	0.10	0
TB14	0.022	0.14	0
TB15	0.09	0.16	0
TB16	0.004	0.01	0
TB17	0.033	0.13	0
TB18	0.034	0.08	0

Figures

Supplementary Figure S1: Heatmap of ELISA, UCP-LFA and Gold-LFA test results per cohort. Heatmap showing the positive test results (green) for ELISA (OD450-background >0.200), UCP-LFA (ratio >0.29) and Gold-LFA (visual score >0.5) for the Philippine, Bangladeshi and Brazilian cohort. The bacterial index (BI) is shown if assessed (BI+ = green; BI- = orange). Values range from 0-2,605 for ELISA, 0-31 for the UCP-LFA and from 0-4 for the Gold -LFA. MB = multibacillary, PB = paucibacillary, NEC = non-endemic controls, HHC = healthy household contacts, HHC&BCG = BCG-vaccinated healthy household contacts, EC = endemic controls.

Supplementary Figure S1 can be viewed via this link: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-017-07803-7/MediaObjects/41598_2017_7803_MOESM1_ESM.pdf

Supplementary Figure S2: Heatmap of UCP-LFA and Gold-LFA nonconcordant test results in leprosy patients. Heatmap showing test results of leprosy patients (MB and PB) from the Philippine and Bangladeshi cohort with a positive result in either the UCP-LFA or Gold-LFA. Positive test results were divided into two groups, weak positives (light green; UCP-LFA : 0,29-0,4 ; Gold-LFA : 0,5) and strong positives (dark green; UCP-LFA ≥ 0,4; Gold-LFA ≥1). MB = multibacillary, PB = paucibacillary.

6 Chapter

Evaluation of immunodiagnostic tests for leprosy in Brazil, China and Ethiopia

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Sci Rep. 2018 Dec 18;8(1):17920

Abstract

Leprosy remains persistently endemic in several low- or middle income countries. Transmission is still ongoing as indicated by the unabated rate of leprosy new case detection, illustrating the insufficiency of current prevention methods. Therefore, low-complexity tools suitable for large scale screening efforts to specifically detect *M. leprae* infection and diagnose disease are required. Previously, we showed that combined detection of cellular and humoral markers, using field-friendly lateral flow assays (LFAs), increased diagnostic potential for detecting leprosy in Bangladesh compared to antibody serology alone.

In the current study we assessed the diagnostic performance of similar LFAs in three other geographical settings in Asia, Africa and South-America with different leprosy endemicity. Levels of anti-PGL-I IgM antibody (humoral immunity), IP-10, CCL4 and CRP (cellular immunity) were measured in blood collected from leprosy patients, household contacts and healthy controls from each area. Combined detection of these biomarkers significantly improved the diagnostic potential, particularly for paucibacillary leprosy in all three regions, in line with data obtained in Bangladesh. These data hold promise for the use of low-complexity, multibiomarker LFAs as universal tools for more accurate detection of *M. leprae* infection and different phenotypes of clinical leprosy.

Introduction

Leprosy is a debilitating, infectious disease caused by *Mycobacterium leprae* (*M. leprae*) causing skin and nerve damage often leading to lifelong handicaps. The continued transmission of *M. leprae* accounts for approximately 200,000 new cases each year. Pockets of high endemicity where intense transmission is witnessed are still present (1). Leprosy diagnosis mainly relies on detection of clinical symptoms (2), which can take up to 20 years to manifest (1). Moreover, the majority of infected individuals will never progress to disease but instead develop adequate immunity to eventually clear *M. leprae* or remain asymptomatically infected (3). However, individuals from the latter group may still be accountable for transmission of *M. leprae* bacteria, particularly to close contacts. To reach worldwide elimination of leprosy abrogation of transmission of *M. leprae* is a top priority for leprosy research. Approaches that support detection of *M. leprae* infected individuals without clinical symptoms are therefore vital to achieve that goal.

Due to this inter-individual variability in immunity against *M. leprae*, diagnostic tests merely detecting antibodies against *M. leprae* specific antigens such as phenolic glycolipid I (PGL-I) or the LID-1 protein (4-8) are not adequate as stand-alone tests to detect (early) disease since antibody detection tests identify mainly multibacillary (MB) leprosy patients with high bacillary loads (BI: bacillary index) which only cover part of the leprosy disease spectrum. On the other part of the spectrum, paucibacillary (PB) leprosy displays a dominant cellular phenotype showing restricted anti-*M. leprae* antibody production (9). The MB/PB classification endorsed by WHO is based on the number of skin lesions and nerve involvement (1). Alternatively, the Ridley-Jopling classification system (10) identifies five disease types: tuberculoid (TT), borderline tuberculoid (BT), borderline (BB), borderline lepromatous (BL) and lepromatous leprosy (LL).

In 2016, 59% of the new cases worldwide were diagnosed with MB leprosy with ratios of MB and PB patients varying per endemic region (11). Since PB cases are generally not detected using serological tools for anti *M. leprae* antibody detection, additional biomarkers are needed to identify the remaining 41% of PB leprosy patients. Moreover, as not all PGL-I seropositive individuals will develop disease, new diagnostic tests should be based on disease- and infection specific biomarkers allowing the distinction between individuals requiring therapeutic or prophylactic therapy, respectively. Tests based on signatures combining humoral- and cellular biomarkers may help to guide administration of postexposure prophylaxis (PEP), a currently introduced strategy aimed at reduction of transmission by *M. leprae* infected individuals without clinical symptoms of leprosy (12, 13).

In Bangladesh we previously demonstrated, using a field-friendly lateral flow assay (LFA) (7, 14, 15), that combined detection of a humoral immune-marker (*M. leprae* PGL-I specific IgM antibodies) with additional cellular immune-markers (IP-10, CCL4 and IL-10) significantly improved distinction between *M. leprae* infected and non-infected individuals (7). In this setting the BI of most leprosy patients was less than 1 which generally corresponds with the absence of anti-PGL-I antibodies (6, 16). The detection of additional cellular markers increased the sensitivity of the assay for these individuals with 39% compared to the LFA based on antibody detection alone(7).

In the current study, whole blood samples of leprosy patients, their household contacts (HHC) and endemic controls (EC) were collected in Asia, Africa and South-America to evaluate the diagnostic potential of the previously used LFAs in Bangladesh applying detection of IP-10, CCL4 and PGL-I specific antibodies (7). Additionally, a new UCP-LFA for detection of C-reactive protein (CRP) was developed and evaluated in these cohorts as CRP, an acute phase protein produced by the liver in response to inflammation, is elevated in LL/BL leprosy patients (17) and active tuberculosis (TB) (18, 19).

The Asian cohort originated from Guizhou, the province with the second highest leprosy prevalence in China, a country with overall low leprosy endemicity (20). Patients originated from the Qianxinan and the Guiyang prefecture, with a prevalence of 0.085/10,000 and 0.011/10,000, respectively and mostly MB patients (MB/PB ratio: 8.2). The South American cohort was recruited in the state of Pará, Brazil, a region hyperendemic for leprosy with an annual new case detection rate of 35.34 per 10,000 with an MB to PB patient ratio of 1.932. Active transmission is ongoing in this area, evidenced by the high number of children amongst new cases (6.4% in 2013) (21, 22). The African cohort was collected in Kokosa Woreda (Oromia region) in Ethiopia with a prevalence of 0.32/10,000 and 5.9-fold more MB than PB patients (23). Thus, this study describes the evaluation of multiple UCP- LFAs for leprosy in low (China), medium (Ethiopia) and hyperendemic (Brazil) settings.

Materials and methods

Study cohorts

Leprosy patients were diagnosed based on histological findings and clinical observations determined by experienced leprologists and a leprosy pathologist as previously described (4, 24). Patients were categorized according to WHO classification (MB/PB) and Ridley-Jopling classification and bacterial indices (BI) were determined. All leprosy patient whole blood was collected at initial diagnosis prior to multidrug therapy (MDT).

Brazil

Leprosy patients were diagnosed at URE Marcello Candia, Marituba, Pará. From January 2016 until June 2017 samples were collected from 97 leprosy patients (LL/BL:30, BT/TT:41, other: 26 (BB/Indeterminate (I):6, NA:20)), 103 healthy household contacts (HHC) and 237 endemic controls (EC). The EC group consisted of school children that were screened for signs of leprosy but were not diagnosed with the disease.

China

Leprosy patients were diagnosed at Guizhou Provincial Center for Disease Control and Prevention and samples were collected from April 2014 until April 2017 from 62 leprosy patients (LL/BL: 47, BT/TT: 10, other: 5 (BB/I:3, NA:2), 87 HHC and 56 EC. EC were not known to have any prior contact with leprosy or tuberculosis patients.

Ethiopia

Patients were collected in Kokosa Woreda (West Arsi zone, Oromia region) in Ethiopia from December 2016 until August 2017. Samples from 24 patients (LL/BL:17, BT/TT:4, neural leprosy: 3), 24 HHC and 25 EC were collected.

Whole Blood Assays (WBAs)

Upon recruitment venous, heparinized blood (4 ml) was added within 3 hours to vials pre-coated with *M. leprae* whole cell sonicate (designated WCS), ML2478/ML0840 recombinant proteins (designated Mlep) (25) or without antigen stimulus (designated Nil) (7, 26). After 24 hour incubation at 37 °C, materials were frozen at -20°C. Before analysis by UCP-LFA (14) WBA vials were thawed and supernatants removed after centrifugation.

PGL-I and *M. leprae* whole cell sonicate (WCS)

The synthetic disaccharide epitope (3,6-di-O-methyl-β -D-glucopyranosyl(1 →4)2,3-di-O-methylrhamnopyranoside), identical to that found on the *M. leprae* specific PGL-I glycolipid, was coupled to human serum albumin (to produce synthetic PGL-I; designated ND-O-HSA) (27) and *M. leprae* whole cell sonicate (WCS) generated with support from the NIH/NIAID Leprosy Contract N01-AI-25469 were obtained through the Biodefense and Emerging Infections Research Resources Repository (http://www.beiresources.org/TBVTRMResearchMaterials/ tabid/1431/Default.aspx).

UCP Conjugates

Lateral flow assays were developed and performed using luminescent up-converting reporter particles (UCP) allowing quantitative detection of the targeted biomarker (28-

30). Sodium yttrium fluoride upconverting nano materials (85 nm, $NaYF_4$:Yb^{3+},Er^{3+}) functionalized with polyacrylic acid were obtained from Intelligent Material Solutions Inc. UCP conjugates were prepared with goat anti-human IgM (I0759, Sigma-Aldrich, St.Louis, Missouri, USA), mouse-anti-IP-10 (BC-50; Diaclone Research, Besancon, France), goat-anti-CCL4 (AF-271-NA; R&D systems, Minneapolis, USA) or mouse-anti-CRP (CRP135; Labned.com, Amstelveen, Netherlands) at a concentration of 125 µg (αIP-10, αCRP) or 25 µg (αCCL4) antibody per mg UCP according to the method described previously (15).

LF strips

Lateral flow strips (LF strips) were assembled by mounting 10 mm glass fiber sample/conjugate pad (Ahlstrom 8964), 25 mm laminated nitrocellulose membrane (Sartorius UniSart CN95) and 20 mm absorbent pad (Whatman Cellulose 470) on a plastic backing. Sample pad and absorbent pad each overlap 2 mm with the nitrocellulose, respectively at the beginning and the end. All LF strip components were obtained via Kenosha (Amstelveen, the Netherlands). The nitrocellulose was provided with an assay-specific test (T) line and an upstream Flow Control (FC) line. Ready to use LF strips were stored at ambient temperature in plastic containers with silica dry pad.

For PGL-I strips the nitrocellulose was provided with a test (T) line comprised of synthetic PGL-I (ND-O-HSA, see above) and a flow-control (FC) line comprised of rabbit anti-goat IgG (RαG; G4018, Sigma-Aldrich) at a concentration of 100 and 50 ng per 4 mm width, respectively. UCP reporter conjugate was applied to the sample/conjugate-release pad at a density of 200 ng per 4 mm in a buffer containing 5% (w/v) sucrose, 50 mM HEPES pH 7.5, 135 mM NaCl, 0.5 % (w/v) BSA, and 0.25% Tween-20. The pads were dried 1 hour at 37 ˚C. For IP-10, CCL4 and CRP LF strips the T line comprised mouse-anti-IP-10 mAb (Clone BC-55; Diaclone Research), mouse-anti-CCL4 mAb (MAB271; Clone # 24006; R&D systems) or mouse-anti-CRP mAb (Clone C5; Labned.com, Amstelveen, Netherlands) respectively, at a concentration of 200 ng per 4 mm width. The FC line comprised goat-anti-mouse IgG antibody (M8642; Sigma-Aldrich) for IP-10 and CRP LF strips and 100 ng rabbit anti-goat IgG (RαG; G4018, Sigma-Aldrich) for CCL4.

LFA protocol

10 µl, 1 µl and 0.1 µl WBA supernatant was diluted in high salt lateral flow (HSLF) buffer (100 mM HEPES pH 7.5, 270 mM NaCl, 1% (w/v) BSA, 0.5% (v/v) Tween-20). 50 ul of diluted sample was added to microtiter plate wells and mixed with 250 ng of target-specific UCP conjugate (IP-10, CCL4 and CRP) before target-specific LF strips were placed in the corresponding wells. Immunochromatography was allowed to continue for at least 30

min until dry.

LF strip analysis

LF strips were scanned locally using portable LF strip readers adapted for measurement of the UCP label (ESEQuant *LFR* reader with 980 nm excitation and 550 nm emission; QIAGEN Lake Constance GmbH, Stockach Germany). LF strips were shipped to the LUMC and re-analysed using a UCP dedicated benchtop reader (UPCON; Labrox, Finland). Results are displayed as the ratio value between Test and Flow-Control signal based on relative fluorescence units (RFUs) measured at the respective lines.

Ethics

This study was performed according to the Helsinki Declaration as described previously (26). The national and institutional Research Ethics Committee, IRB or Beijing Tropical Medicine Research Institute, Beijing Friendship Hospital-affiliate of Capital University of Medical Sciences have approved the study protocol (Colorado State University IRB human protocol 15-6340H; Ethical Appreciation Certificate Nº 26765414.0.0000.0018 (Brazil), ethical approval number 3-10/014/2015 (Ethiopia), ethical approval number BJFH-EC/2014-053 (China). Participants were informed about the study-objectives, the samples and their right to refuse to take part or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. Informed consent was provided by parents/guardians on behalf of all child participants. All patients who were diagnosed with leprosy received free multidrug treatment (MDT) according to national guidelines.

Data analysis

Graphpad Prism version 7.02 for Windows (GraphPad Software, San Diego CA, USA) was used to perform Mann-Whitney U tests, Kruskal-Wallis with Dunn's correction for multiple testing, plot receiver operating characteristic (ROC) curves and calculate the area under curve (AUC). The cut-off with the optimal sensitivity and specificity was determined using the Youden's index (31).

Results

Patient cohorts

The extent of humoral and cellular immune responses against *M. leprae* differs within the leprosy spectrum (10), ranging from predominantly humoral in MB patients to largely cellular immune responses in PB patients. The reported ratio of MB/PB patients as well

as the level of endemicity differ between the three regions where the study cohorts were recruited (Table 1). LL/BL, on the MB side of the leprosy spectrum, was the major form of leprosy observed in China and Ethiopia (respectively 76% and 71%), whereas in the Brazilian cohort the different forms of leprosy were more equally divided (Table 1). In China and Ethiopia the majority of the patients were male, while in Brazil a more even distribution of males and females was observed (Table 1).

Table 1: Patient characteristics

	Brazil	China	Ethiopia
LL/BL	30 (31%)	47 (76%)	17 (71%)
BT/TT	41 (42%)	10 (16%)	4 (17%)
Others*	26 (27%)	5 (8%)	3 (12.5%)
age (median (min-max)) years	39.5 (8-78)	35 (13-72)	29 (6-75)
male/ female	52/45	40/18	17/7
Prevalence (per 10,000)	NA	0.085 (Qianxinan) 0.011 (Guiyang)	0.32
New case detection rate (per 100,000)	30.4	NA	NA

Patient characteristics of the Brazilian, Chinese and Ethiopian test cohorts. Patients were stratified by clinical form based on Ridley-Jopling classification. The number of lepromatous leprosy (LL)/ borderline lepromatous (BL) and borderline tuberculoid (BT)/ tuberculoid (TT) patients are indicated for each group. *Patients that were not classified in one of these two groups (borderline, indeterminate, neural leprosy or not assessed) are referred to as others. The prevalence or new case detection rate is region specific.

Diagnostic value of single UCP-LFAs

Supernatants of WBAs (both *M. leprae* antigen-stimulated, WCS and Mlep, and non-stimulated (Nil)) were used to assess the significance of 4 single UCP-LFAs. The diagnostic potential of each marker (anti-PGL-I IgM, CRP_{Nil}, $IP\text{-}10_{Nil}$, $IP\text{-}10_{Mlep}$, $IP\text{-}10_{WCS}$, $CCL4_{Nil}$, $CCL4_{Mlep}$, $CCL4_{WCS}$) was evaluated based on Ridley-Jopling classifications by assessing the ability of the markers to discriminate LL/BL and BT/TT patients from HHC and EC (Supplementary Table S1). The diagnostic value of each marker was assessed by computing ROC curves, and the associated AUCs (Figure 1).

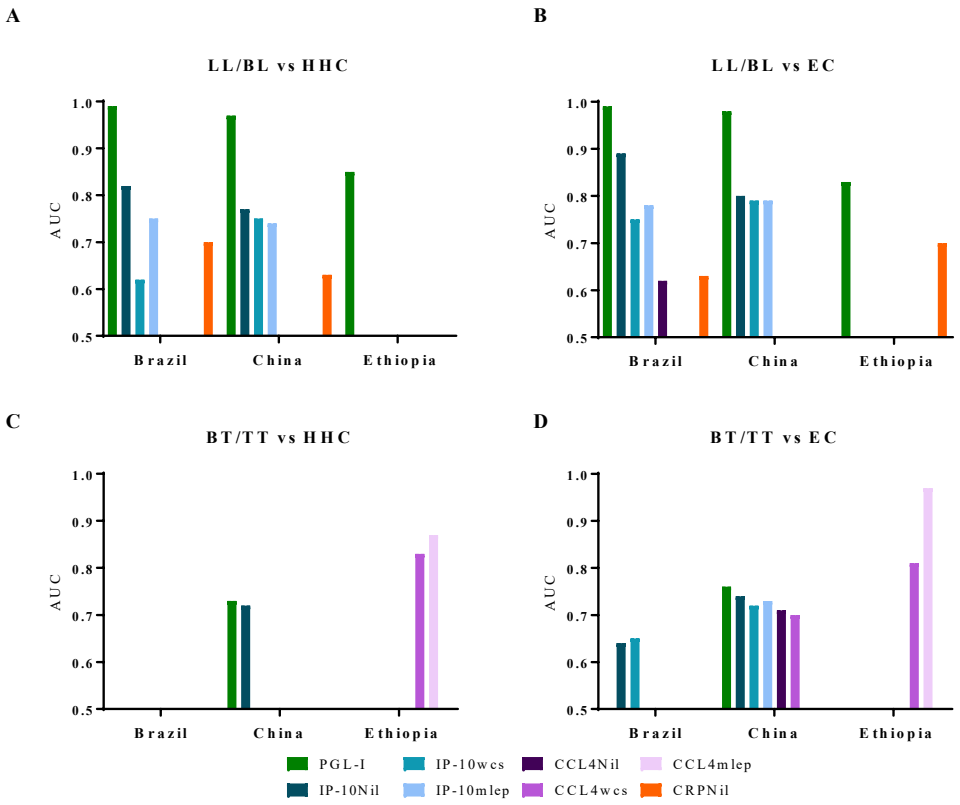

Figure 1: Performance of up-converting phosphor (UCP) lateral flow assays (LFAs). UCP-LFA for detection of anti-PGL-I IgM levels, IP-10, CCL4 and CRP in whole blood (Nil). IP-10 and CCL4 levels were also assessed in whole blood stimulated with *Mycobacterium leprae (M. leprae)* whole cell sonicate (WCS) and two *M. leprae* specific proteins (Mlep). The area under the curve (AUC) was calculated for each individual marker and the significant AUCs are shown per cohort (Brazil, China, Ethiopia). (A) Significant AUCs discriminating lepromatous leprosy (LL)/ borderline lepromatous (BL) patients from healthy household contacts (HHC). (B) Significant AUCs discriminating LL/BL patients from endemic controls (EC). (C) Significant AUCs discriminating borderline tuberculoid (BT)/ tuberculoid (TT) patients from HHC. (D) Significant AUCs discriminating BT/TT patients from EC.China: 47 LL/BL patients, 10 BT/TT patients, 87 HHC and 56 EC. Brazil: 30 LL/BL patients, 41 BT/TT patients, 103 HHC and 237 EC. Ethiopia: 17 LL/BL patients, 4 BT/TT patients, 24 HHC and 25 EC.

In the hyperendemic Brazilian cohort (Supplementary Figure S1), anti-PGL-I IgM, CRP and IP-10 (Nil, WCS and Mlep) levels differed significantly between LL/BL patients versus HHC and EC as indicated by the significant AUC values (Figure 1). Similarly, $CCL4_{Nil}$ levels for LL/BL patients and EC in Brazil were significantly different (Figure 1), while a good distinction between BT/TT patients and EC was provided by $IP\text{-}10_{Nil}$ and $IP\text{-}10_{WCS.}$

In the Chinese cohort (supplementary Figure S2), anti-PGL-I IgM and IP-10 (Nil, WCS and Mlep) levels significantly differed in LL/BL patients from HHC and EC (Figure 1). Additionally,

CRP levels distinguished LL/BL patients from HHC. BT/TT patients significantly differed from HHC and EC in anti-PGL-I IgM levels and IP-10_{Nil}. Moreover, IP-10_{WCS}, IP-$10_{Mlep,}$ $CCL4_{Nil}$ and $CCL4_{WCS}$ differentiated BT/TT patients from EC.

In the Ethiopian cohort (Supplementary Figure S3), anti-PGL-I IgM levels distinguished LL/BL patients from HHC and EC, whereas CRP levels reached significance between these patients and EC. $CCL4_{WCS}$ and $CCL4_{Mlep}$ discriminated BT/TT patients from HHC and EC.

Overall, the data show that IP-10 was the most significant cellular marker to identify both LL/BL and BT/TT leprosy patients in low- and high endemic populations, anti-PGL-I IgM and CRP are relevant for diagnosis of LL/BL patients and CCL4 contributes to the detection of BT/TT patients.

Identification of multi-biomarker signatures

To identify a biomarker signature specific for leprosy disease in general, we included in this signature besides anti-PGL-I IgM also cellular markers based on the AUCs (Figure 1).

In the Chinese cohort both IP-10 and CCL4 enabled the distinction between BT/TT patients and EC, with the highest AUC for a single analyte for IP-10_{Nil} and $CCL4_{Nil}$ (Figure 1). In Brazil, IP-10_{Nil} and IP-10_{WCS} discriminated BT/TT patients from EC, whereas in Ethiopia $CCL4_{WCS}$ and $CCL4_{Mlep}$ showed diagnostic value for these patients. Optimal cut-offs of the selected biomarkers were determined based on the Youden's index (31). All individuals positive for both selected cellular markers were designated positive (Supplementary Figure S4).

The use of multi-biomarker signatures consisting of cellular markers and humoral anti-PGL-I IgM seropositivity resulted in four possible outcomes depicted in Figure 2. With a sensitivity for LL/BL patients of 91%, 97% and 75% in China, Brazil and Ethiopia respectively, the majority of LL/BL patients was identified by the PGL-I UCP-LFA with little added value of the cellular markers identifying 2%, 3% or 5% additional patients respectively in line with the immune responses within the leprosy spectrum.

On the other hand, for BT/TT patients the combination of cellular and humoral markers increased the test sensitivity with 50% to 54%, resulting in an overall sensitivity for BT/TT leprosy of 80% (China), 71% (Brazil) and 75% (Ethiopia). Similar analysis was applied to previously described data from a cohort in Bangladesh (7), additionally detecting 39% of BT/TT patients resulting in an overall sensitivity of 63%. Importantly, specificity was not relevantly affected by the inclusion of cellular biomarkers in China and Ethiopia and was only moderately decreased in Brazil and Bangladesh. Of the HHCs, 10%, 13%, 15% and 49% were positive for cellular markers in the Chinese, Ethiopian, Bangladeshi and Brazilian

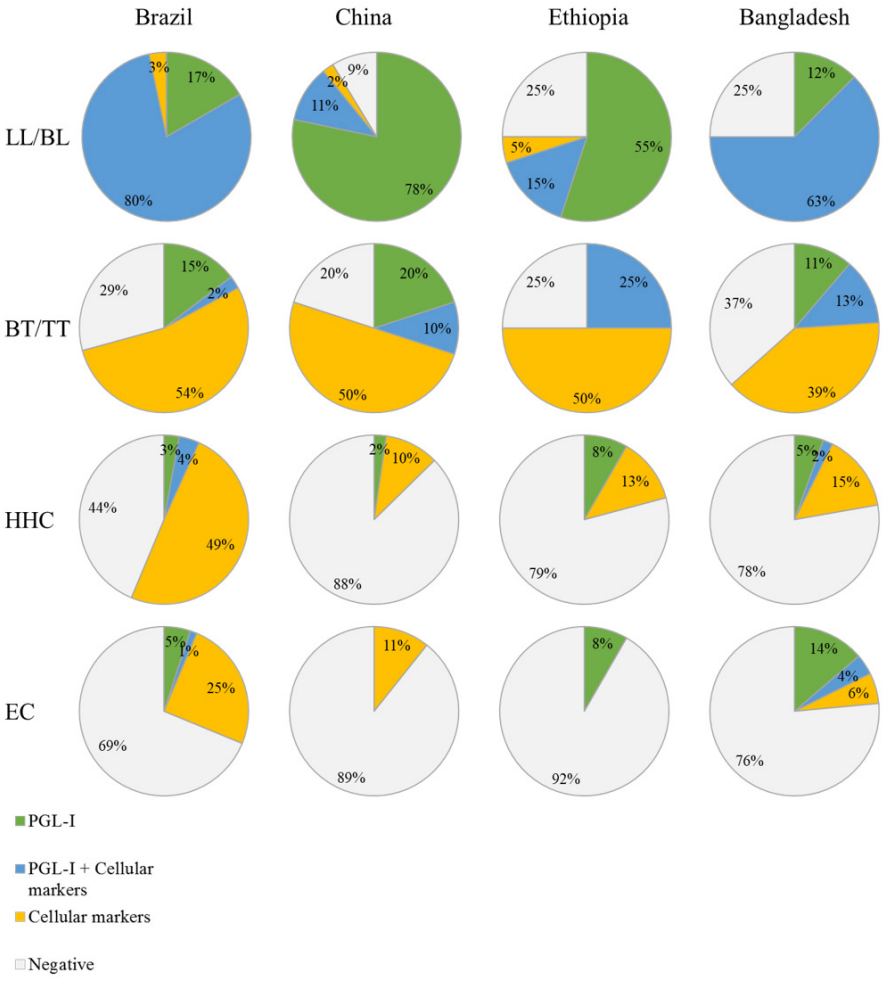

Figure 2: Combination of cellular and humoral markers improves the detection of leprosy patients. Pie charts showing the percentage of individuals with a positive test result for anti-PGL-I IgM (light green), cellular markers (yellow), both anti-PGL-I IgM and cellular markers (blue) or without positive test results (light grey; Supplementary Figure S4) per test group (lepromatous leprosy/ borderline lepromatous (LL/BL), borderline tuberculoid / tuberculoid (BT/TT) patients, healthy household contacts (HHC) and endemic controls (EC)). PGL-I IgM was included to identify LL/BL patients. The threshold for positivity was determined based on the Youden's index, resulting in a cut-off of >0.205, >0.61 and >1.195 for Brazil, China and Ethiopia respectively for PGL-I IgM. The threshold for positivity was determined as well for two cellular markers that were selected per cohort based on the areas under the curve (AUC) depicted in figure 1: $CCL4_{Nil}$ and $IP\text{-}10_{Nil}$ (China; cut-off >0.355 and >0.105 respectively), $IP\text{-}10_{Nil}$ and $IP\text{-}10_{WCS}$ (Brazil; cut-off >0.395 and >0.855 respectively); $CCL4_{WCS}$ and $CCL4_{MIep}$ (Ethiopia; cut-off <1.03 and <1.13 respectively) China: 47 LL/BL patients, 10 BT/TT patients, 87 HHC and 56 EC. Brazil: 30 LL/BL patients, 41 BT/TT patients, 103 HHC and 237 EC. Ethiopia: 17 LL/BL patients, 4 BT/TT patients, 24 HHC and 25 EC. For comparison data obtained from a previous study performed in Bangladesh, using $IP\text{-}10_{WCS}$ and $CCL4_{WCS}$ as cellular markers, was shown (8 LL/BL, 71 BT/TT, 54 HHC and 51 EC).

cohorts, respectively. These data show that biomarker profiles based on humoral and cellular markers can identify patients at both ends of the leprosy spectrum, irrespective of geographical region.

Decision tree as a field-tool to assess leprosy risk profiles

For detection of leprosy patients and/or individuals at risk of developing leprosy in field situations, a decision tree based on low-complexity diagnostic tests may facilitate decision making by local health workers. As biomarker based diagnostic tests should be globally applicable, we constructed a decision tree irrespective of cohort applying anti-PGL-I IgM and IP-10 as general biomarkers for leprosy patients. As anti-PGL-I IgM levels measured by UCP-LFA were highly specific for *M. leprae* infection and can be considered a correlate of risk for developing leprosy, this was designated the first step of the decision tree. This step identified 132 individuals (based on the cut-offs described in Figure 2), among whom 104 leprosy patients (57% of total patients in the 3 cohorts) and 28 individuals without leprosy (5%) (Figure 3).

In the next step, IP-10_{Nil} levels were used for anti-PGL-I IgM seronegative individuals, which detected an additional 59 patients thereby identifying 89% of all leprosy cases. Of the individuals without clinical leprosy 230 (43%) were IP-10_{Nil} positive. Subsequent assessment of the IP-10 levels in response to *M. leprae* WCS in the IP-10_{Nil} positive individuals increased specificity; while 81% of the patients were identified this step reduced the number of individuals without leprosy that were identified as being at risk to 137 (26%; Supplementary Figure S5A). Stratifying these data by cohort showed that based on this three-step decision model of the individuals without leprosy 38% in Brazil (n=131; 46 HHC; 74 EC), 22% (n=11; 7 HHC; 4 EC) in Ethiopia and 16% (n=23; 16 HHC; 7 EC) in China are considered at risk of developing leprosy or transmitting *M. leprae* (Supplementary Figure S5B). Thus, use of a decision tree based on multiple, different types of markers measured in low-complexity assays can therefore guide decision making on who needs (prophylactic) treatment in large-scale screening efforts in field settings.

Discussion

Diagnostic tests for *M. leprae* infection will provide a useful asset in large scale screening efforts to identify individuals who need prophylactic treatment. In a previous study we demonstrated that the combination of field-friendly UCP-LFAs for leprosy detecting cellular and humoral biomarkers as compared to humoral markers alone, increased the sensitivity for detection of patients in Bangladesh by 32% for MB and 36% for PB cases (7). The current study investigates the use of similar UCP-LFAs in three other cohorts

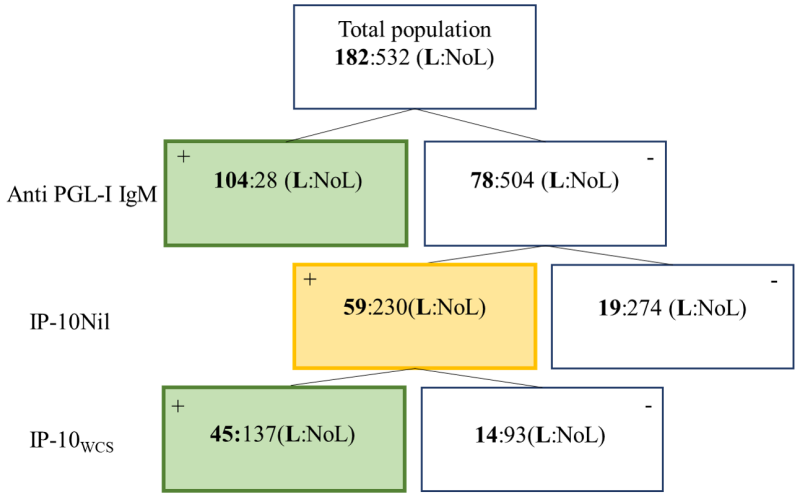

Figure 3: Decision tree as a tool to assess leprosy risk profiles. Decision tree to identify individuals with *M. leprae* infection or at risk of developing leprosy based on the data obtained from all three cohorts. The total population is first stratified by anti-PGL-I IgM levels indicating the total number of individuals positive (left box) and negative (right box) with the number of leprosy patients indicated in bold (L=leprosy; NoL=no leprosy). In the second step, the anti-PGL-I IgM seronegative individuals are stratified by IP-10_{Nil} levels indicating the total number of individuals positive (left box) and negative with the number of leprosy patients (L) indicated in bold. In the third step all individuals positive for IP-10_{Nil} (yellow box) are stratified by IP-10_{WCS} levels indicating the total number of individuals positive (left box) and negative with the number of leprosy patients (L) indicated in bold. The green boxes indicate the individuals that are identified as *M. leprae* infected or at risk of developing leprosy.

with different leprosy endemicity in order to evaluate their worldwide applicability as field-friendly, point-of-care tests for leprosy based on combined multiplex detection of biomarker profiles.

In line with previous studies (4, 6-8), anti-PGL-I IgM levels identified the majority of LL/BL patients with high bacillary loads. The detection of cellular markers (IP-10 and CCL4) further improved the sensitivity of BT/TT leprosy patients up to 54%. Elevated levels of CRP in LL/BL (MB) leprosy were in agreement with other reported studies (17).

Cellular markers thus increased sensitivity of leprosy diagnosis and in particular showed added value for BT/TT patients in the three cohorts compared to assessment of anti-PGL-I IgM seropositivity alone. BT/TT patients at the PB side of the leprosy spectrum worldwide comprise 41% of the leprosy patients, signifying the relevance of cellular markers in leprosy diagnostics (11). Especially with respect to detection of *M. leprae* infection in the absence of clinical symptoms, in particular relevant for the HHC group, cellular markers also diagnosed *M. leprae* infection more often than anti-PGL-I IgM testing.

The low rate (6%) of anti-PGL-I IgM positivity in the Brazilian HHC contrasted earlier observations from the same region (22) that indicated a remarkably high 77.6% seropositivity in a comparable student population. This difference is thought to be a consequence of improved assay-specificity of the UCP-LFA used in the current study compared to the earlier applied ELISA (6): the UCP-LFA format is a virtually background-free reporter technology (29) thereby detecting *M. leprae* infection with higher specificity.

The rate of positivity for cellular markers in HHC correlated with the level of endemicity ranging from 10% in China (low endemic) to 53% in Brazil (high endemic), which is in agreement with previous findings (25). In Brazil intense transmission continues in the area of this study as revealed by particularly high rates of leprosy cases amongst children (32). Moreover, it has been reported that in the majority (19 out of 27) of Brazilian states 50% of the individuals is exposed to high or hyperendemic rates of infection (33). Thus, the Brazilian EC group tested is therefore not unaffected by *M. leprae*, as these school children are likely to have been in contact with *M. leprae* infected individuals (22). However, using quantitative signals as measured by UCP-LFA in field-settings, cut-off values are adjustable. This facilitates a stepwise approach that can be accommodated for various diagnostic questions (postexposure prophylaxis, monitoring, classification) each with different sensitivity/specificity requirements.

Factors to be considered for the appropriate applicability of UCP-LFAs based on combined biomarker profiles thus are regional differences in the MB/PB ratio and the level of endemicity. Cellular markers clearly represent valuable diagnostic tools in countries with high percentages of PB patients (i.e. Bangladesh) (11). The level of cellular markers is more frequently elevated in HHC in regions with high leprosy endemicity as the rate of *M. leprae* infection corresponds with high new case detection (25). Consequently, HHC resemble BT/TT patients with respect to positivity for certain cellular biomarkers. In this regard, the use of a multi-step decision approach, using initial categorization based on anti-PGL-I IgM seropositivity, followed by additional steps based on a cellular biomarkers can identify more sensitively and specifically those at risk of developing leprosy or transmitting bacteria.

The UCP-LFA format applied in this study facilitates rapid testing based on the presence of selected biomarkers as it is compatible with the use of finger stick blood (FSB). UCP-LFAs could thus serve as a rapid FSB screening test for *M. leprae* infection applicable as triage in large scale screening of HHCs aiming to provide PEP to infected individuals, to reduce transmission by infected but non-symptomatic individuals (12, 13). For leprosy diagnosis, on the other hand, a subsequent test using overnight stimulation with *M. leprae* antigens similar to the Quantiferon TB test(34) can be applied to increase test specificity for leprosy

avoiding unnecessary use of antibiotics.

Longitudinal studies sampling contacts of leprosy patients during yearly follow-up are ongoing. This approach will include intra-individual biomarker comparison of individuals before and at diagnosis of clinical leprosy aimed at identification of biomarker signatures specific for early disease in individuals yet lacking symptoms (7, 26). The biomarker profile investigated in the current study indicates a high level of similarity of the immunological response of BT/TT and HHC based on 4 biomarkers. Longitudinal studies will provide biomarker signatures that can be used as correlates of disease in infected individuals before clinical manifestation of leprosy.

In summary, despite minor differences in biomarker specificity due to levels of leprosy endemicity, this study demonstrates that UCP-LFA rapid tests are well suited for diagnosis of leprosy patients and *M. leprae* infected individuals irrespective of geographical region. Multiplex UCP-LFAs will enable the assessment of biomarker signatures in leprosy endemic areas, which can facilitate guidance of prophylactic treatment within large-scale screening efforts to reduce transmission and disease while limiting administration of antibiotics (13).

Acknowledgements

The authors gratefully acknowledge all patients and blood donors.

Funding Statement

This study was supported with grants from the Order of Malta-Grants-for-Leprosy-Research (MALTALEP), the Netherlands Leprosy Relief Foundation (NLR; ILEP#: 7.01.02.48, 7.02.02.73 and 7.03.15.07) together with the Turing Foundation, the German Leprosy and TB Relief Association (GLRA) the Leprosy Research Initiative (LRI), The Science and Technology Fund project of Guizhou Provincial Health and Planning Commission (# gzwjkj2014-1-078), 2. National Natural Science Foundation of China –#81260436), the Heiser Program for Research in Leprosy of The New York Community Trust (grants P15-000827 and P16-000796), the J. William Fulbright Scholar to Brazil award 2015-2016 (to JSS), CNPq (308980/2015-8 and 428964/2016-8), CAPES PROAMAZONIA (3288/2013) (to CS) and the Q.M. Gastmann-Wichers Foundation (to AG).

Conflict of interest

The authors declare to have no financial/commercial conflicts of interests.

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Supplementary material

Tables

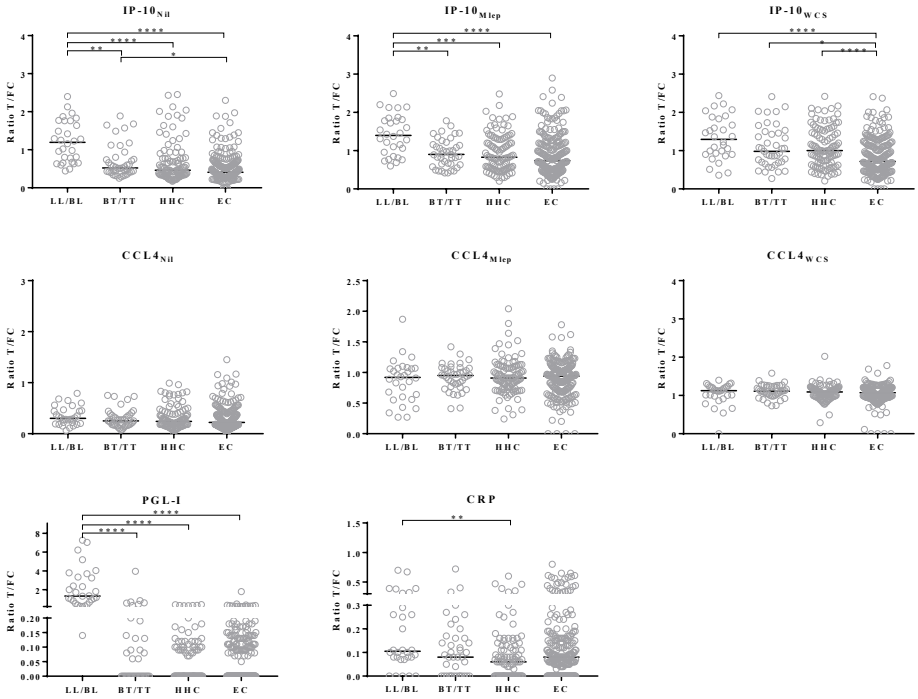
Supplementary Table S1: Biomarker potential of anti-PGL-I IgM, IP-10, CCL4 and CRP

		PGL-I	IP-10$_{Nil}$	IP-10$_{Mlep}$	IP-10$_{WCS}$	CCL4$_{Nil}$	CCL4$_{Mlep}$	CCL4$_{WCS}$	CRP$_{Nil}$
Brazil	LL/BL vs HHC								
China									
Ethiopia									
Brazil	LL/BL vs EC								
China									
Ethiopia									
Brazil	BT/TT vs HHC								
China									
Ethiopia									
Brazil	BT/TT vs EC								
China									
Ethiopia									
Brazil	LL/BL vs BT/TT								
China									
Ethiopia									
Brazil	HHC vs EC								
China									
Ethiopia									

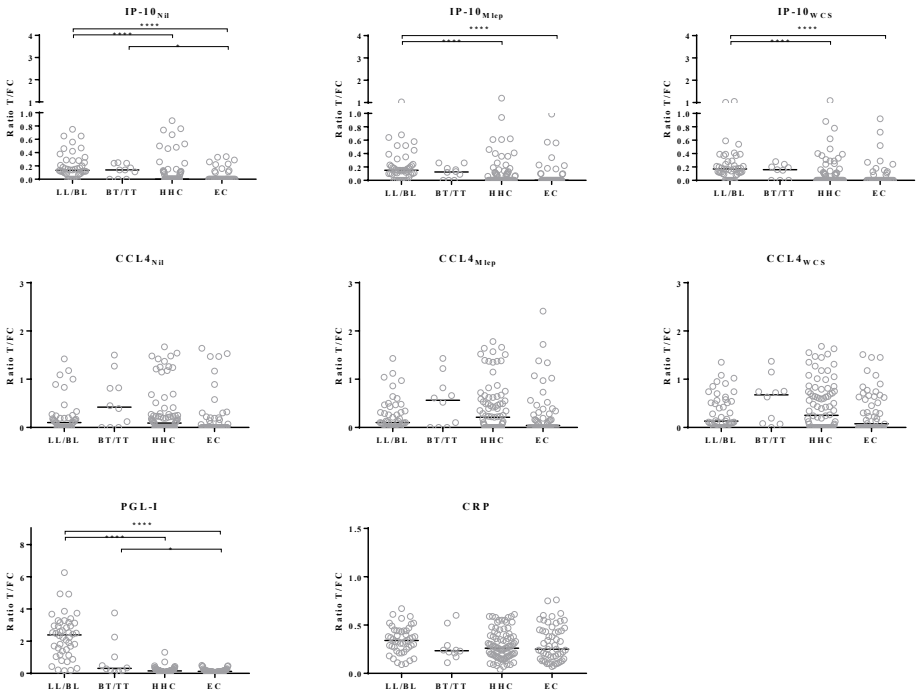
P-values	
	<0.05-0.01
	<0.01-0.001
	<0.001-0.0001
	<0.0001

Significantly different markers per cohort (Brazil, China, Ethiopia) between two groups as determined by Mann-Whitney U test for lepromatous leprosy (LL)/ borderline lepromatous (BL) patients compared to healthy household contacts (HHC) and endemic controls (EC), borderline tuberculoid (BT)/ tuberculoid (TT) patients compared to HHC and EC, LL/BL compared to BT/TT patients and HHC compared to EC. P-values are indicated by colour coding.

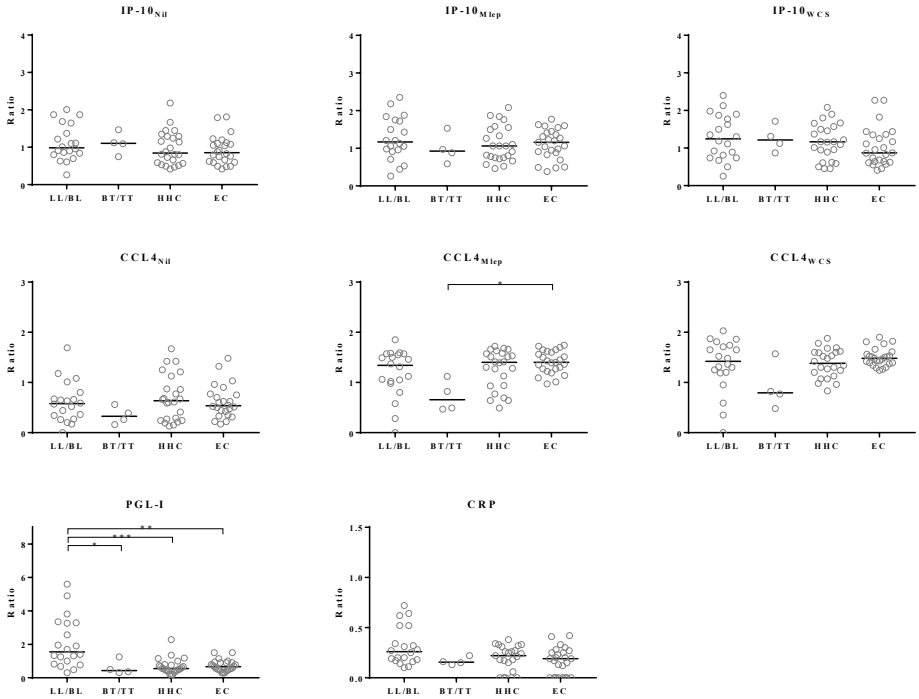
Figures



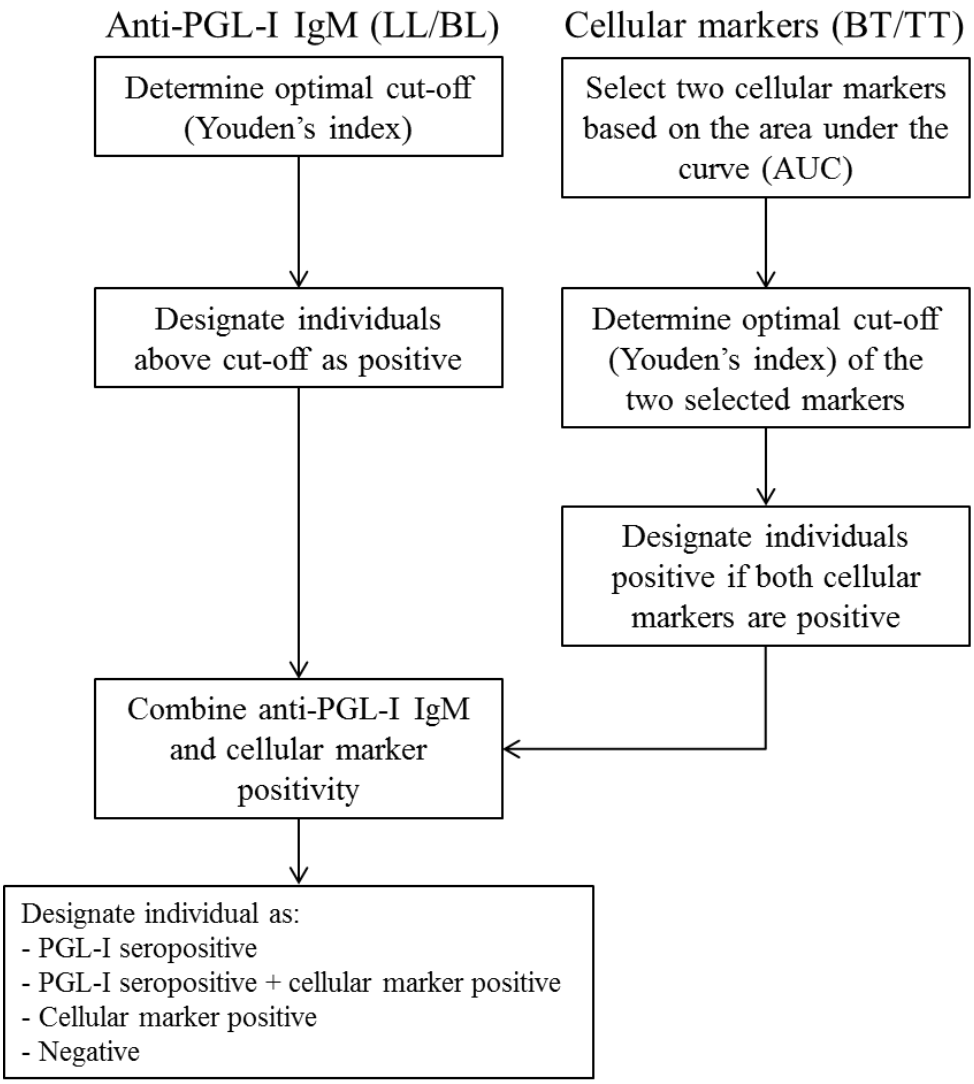
Supplementary Figure S1: Up-converting phosphor (UCP) lateral flow tests (LFAs) performed for the Brazilian cohort. Ratio values of the peak areas of the Test line (T) and flow control (FC) on UCP-LFA strips are shown for IP-10, CCL4, CRP and anti-PGL-I IgM measured in both unstimulated and stimulated whole blood (*M. leprae* whole cell sonicate (WCS) or 2 *M. leprae*-specific recombinant proteins (Mlep)) of lepromatous leprosy/borderline lepromatous patients (LL/BL; n=30), borderline tuberculoid/tuberculoid patients (BT/TT; n=41), healthy household contacts (HHC; n=103) and endemic controls (EC; n=237). P-values were determined by the Kruskal-Wallis test with Dunn's correction for multiple testing. P-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.



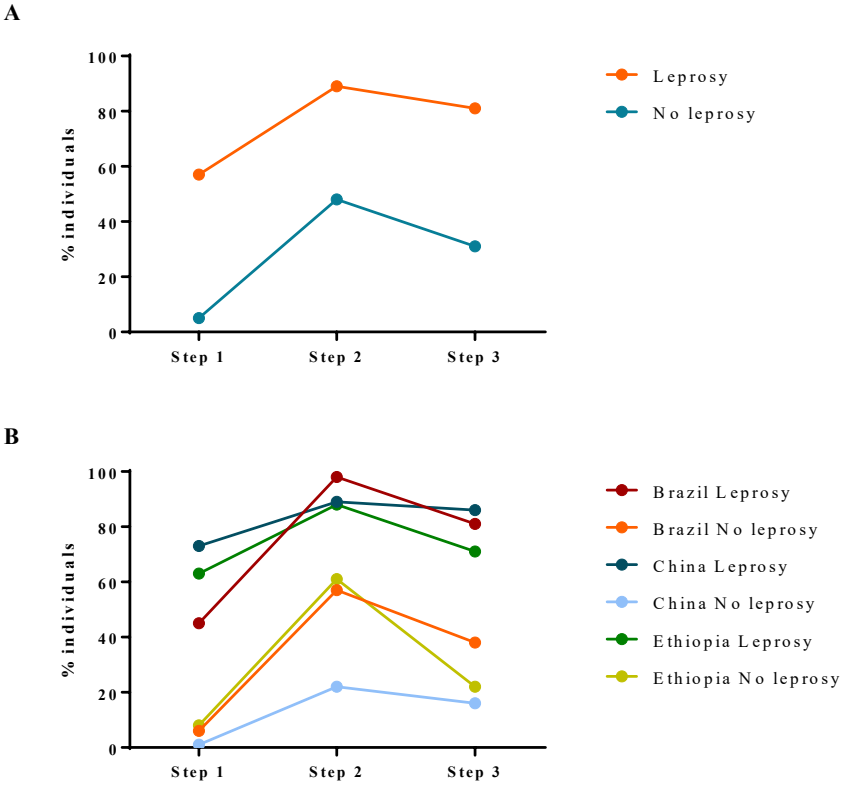
Supplementary Figure S2: Up-converting phosphor (UCP) lateral flow tests (LFAs) performed for the Chinese cohort Ratio values of the peak areas of the Test line (T) and flow control (FC) on UCP-LFA strips are shown for IP-10, CCL4, CRP and anti-PGL-I IgM measured in both unstimulated and stimulated whole blood (*M. leprae* whole cell sonicate (WCS) or 2 *M. leprae*-specific recombinant proteins (Mlep)) of lepromatous leprosy/borderline lepromatous patients (LL/BL; n=47), borderline tuberculoid/tuberculoid patients (BT/TT; n=10), healthy household contacts (HHC; n=87) and endemic controls (EC; n=56). P-values were determined by the Kruskal-Wallis test with Dunn's correction for multiple testing. P-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.



Supplementary Figure S3: Up-converting phosphor (UCP) lateral flow tests (LFAs) performed for the Ethiopian cohort. Ratio values of the peak areas of the Test line (T) and flow control (FC) on UCP-LFA strips are shown for IP-10, CCL4, CRP and anti-PGL-I IgM measured in both unstimulated and stimulated whole blood (*M. leprae* whole cell sonicate (WCS) or 2 *M. leprae*-specific recombinant proteins (Mlep)) of lepromatous leprosy/borderline lepromatous patients (LL/BL; n=17), borderline tuberculoid/tuberculoid patients (BT/TT; n=4), healthy household contacts (HHC; n=24) and endemic controls (EC; n=25). P-values were determined by the Kruskal-Wallis test with Dunn's correction for multiple testing. P-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.



Supplementary Figure S4: Selection procedure of markers. Description of the selection procedures and the combination of cellular and humoral markers (anti-PGL-I IgM) per cohort, resulting in the pie charts in Figure 2



Supplementary Figure S5: Stepwise identification of individuals by a three-step decision tree
Percentage of individuals identified stepwise as positive based on anti-PGL-I IgM (step 1), IP-10_{Nil} (step 2) and IP-10 in response to *M. leprae* whole cell sonicate (step 3) according to the decision tree in Figure 3. (A) Percentage of individuals with (orange) and without (blue) leprosy identified by each step of the decision tree. (B) Percentage of individuals with and without leprosy identified by each step of the decision tree stratified by cohort. Brazil: leprosy (red)/no leprosy (orange), China: leprosy (dark blue)/no leprosy (light blue), Ethiopia: leprosy (green)/no leprosy (light green).

Chapter 7

Household contacts of leprosy patients in endemic areas display a specific innate immunity profile

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Front Immunol. 2020 Aug 11;11:1811.

Abstract

Leprosy is a chronic infectious disease, caused by *Mycobacterium leprae*, that can lead to severe life-long disabilities. The transmission of *M. leprae* is continuously ongoing as witnessed by the stable new case detection rate. The majority of exposed individuals does, however, not develop leprosy and is protected from infection by innate immune mechanisms.

In this study the relation between innate immune markers and *M. leprae* infection as well as the occurrence of leprosy was studied in household contacts (HCs) of leprosy patients with high bacillary loads. Serum proteins associated with innate immunity (ApoA1, CCL4, CRP, IL-1Ra, IL-6, IP-10 and S100A12) were determined by lateral flow assays (LFAs) in conjunction with the presence of *M. leprae* DNA in nasal swabs (NS) and/or slit-skin smears (SSS).

The HCs displayed ApoA1 and S100A12 levels similar to paucibacillary patients and could be differentiated from endemic controls based on the levels of these markers. In the 31 households included the number (percentage) of HCs that were concomitantly diagnosed with leprosy, or tested positive for *M. leprae* DNA in NS and SSS, was not equally divided. Specifically, households where *M. leprae* infection and leprosy disease was not observed amongst members of the household were characterized by higher S100A12 and lower CCL4 levels in whole blood assays of HCs in response to *M. leprae*.

Lateral flow assays provide a convenient diagnostic tool to quantitively measure markers of the innate immune response and thereby detect individuals which are likely infected with *M. leprae* and at risk of developing disease or transmitting bacteria. Low complexity diagnostic tests measuring innate immunity markers can therefore be applied to help identify who should be targeted for prophylactic treatment.

Introduction

Leprosy is a debilitating disease that is one of the leading causes of long-term nerve damage worldwide (1). Multidrug therapy (MDT) effectively kills *Mycobacterium leprae,* the causative agent of leprosy, providing an effective cure when treatment is initiated timely (2, 3). To achieve elimination of leprosy, however, it is vital to not only treat adequately and timely but also to prevent transmission (4). The stable new case detection rates in many leprosy endemic countries (5) indicate that MDT insufficiently reduces transmission of *M. leprae*. Recognition of the often subtle cardinal clinical signs is of major importance for leprosy diagnosis (6). The declaration of the WHO in 2000 that leprosy had been eliminated as a public health problem (7), however, caused a reduction of leprosy control activities. The reduced intensity in case detection activities and training in the diagnosis and treatment of leprosy results in many cases that remain undetected for several years (8), allowing the transmission of *M. leprae* to continue.

Contacts close to leprosy patients have a higher risk of acquiring the infection, especially when the patients carry high bacillary loads (9-11). Fortunately, the majority of exposed individuals is naturally immune to *M. leprae* infection (12). Host immunity also determines the clinical phenotype of leprosy, ranging from paucibacillary (PB) patients with a strong proinflammatory response (Th1/Th17) leading to bacterial control to multibacillary (MB) patients with an anti-inflammatory immune response (Th2) producing large quantities of antibodies but unable to control the bacteria (13, 14). In the innate immune response macrophages are critical mediators that define the course of *M. leprae* infection and clinical outcome. In PB patients IL-15 induces antimicrobial activity and the vitamin D-dependent antimicrobial program in macrophages restricting bacterial dissemination (proinflammatory M1 macrophages) (15). In contrast, in MB patients a scavenger receptor program is induced by IL-10, leading to foam cell formation by increased phagocytosis of mycobacteria and oxidized lipids, and persistence of *M. leprae* (anti-inflammatory M2 macrophages) (16, 17).

Markers of the innate immune response can thus be helpful to identify *M. leprae* infected individuals who are prone to develop leprosy disease and thereby, since they are unable to kill and remove *M. leprae*, contribute to the ongoing transmission. No practical tools are yet available to identify individuals that should be prioritized for prophylactic treatment. Recently, biomarkers for leprosy and *M. leprae* infection were identified (18, 19), including serum proteins that play a role in innate immunity. For example, S100A12 is required to decrease *M. leprae* viability in infected macrophages (20). CCL4 and IP-10 attract innate immune cells such as natural killer (NK) cells and monocytes, whereas IL-1Ra-stimulated monocytes turn into M2 macrophages that produce high levels of the anti-inflammatory

cytokine IL-10 (21).

Two other identified biomarkers (18) that play a role in the innate immune system were contrasting acute phase proteins: anti-inflammatory ApoA1 and pro-inflammatory CRP. ApoA1 inhibits the recruitment of monocytes and macrophage chemotaxis (22), whereas CRP can recognize pathogens and activate the classical complement pathway (23). Together with αPGL-I IgM, the well-established biomarker for MB leprosy (24), the identified biomarkers were implemented in quantitative up-converting phosphor lateral flow assays (UCP-LFAs) (18). These user-friendly tests are applicable in resource-limited settings, essential for diagnostic tools in large-scale contact screening of leprosy contacts, and provide quantitative results. The latter allows monitoring of drug treatment as well as discriminating high from low responders.

Previously, we analysed nasal swabs (NS) and slit-skin smears (SSS) of household contacts (HCs) of MB leprosy patients with high bacillary loads for the presence of *M. leprae* DNA (25). Here we analysed the same individuals to examine the correlation of the presence of *M. leprae* DNA with the levels of innate immune markers. *M. leprae* DNA in NS indicates colonisation of the HC with the bacterium, but not invasion of the tissue. Detection of *M. leprae* DNA in SSS does indicate that a HC is infected. In this study, levels of ApoA1, CCL4, CRP, IL-1Ra, IL-6, IP-10, αPGL-I IgM and S100A12 were determined by UCP-LFAs in supernatants of 24 hour *M. leprae* antigen-stimulated whole blood assays (WBA) addressing newly diagnosed MB patients with a high bacteriological index (BI) and their HCs in Bangladesh.

Materials and methods

Study participants

The cohort used in this study originates from four districts in Bangladesh (Nilphamari, Rangpur, Panchagar and Thakurgaon) and has been extensively described previously (25). The prevalence of leprosy in these districts was 0.9 per 10,000 and the new case detection rate 1.18 per 10,000 (Rural health program, the leprosy mission Bangladesh, yearly district activity report 2018). Between July 2017 and May 2018, newly diagnosed leprosy patients (index case; n=31) with BI ≥ 2 and between 3 to 15 HCs per index case (n=279) were recruited (25). Leprosy was diagnosed based on clinical and bacteriological observations and classified as MB or PB as described by the WHO (5) and the BI was determined. HCs were examined as well for signs and symptoms of leprosy upon recruitment and followed up yearly for surveillance of new case occurrence for ≥ 24 months after sample collection. Control individuals without known contact to leprosy or TB patients and without clinical

disease symptoms from the same leprosy endemic area (EC) were included and assessed for the absence of clinical signs and symptoms of leprosy and TB. Staff of leprosy or TB clinics were excluded as EC.

Household contacts

The coding system used to describe physical and genetic distance of contacts from the patient has been extensively described previously (26). In short, 4 categories of physical distance are relevant for this study:

- KR: contacts living under the same roof and the same kitchen
- K: contacts living under a separate roof but using the same kitchen
- R: contacts living under the same roof, not using the same kitchen
- N1: next-door neighbors

In this study the KR and R group were considered as one group. For genetic distance 7 categories were defined: spouse (M), child (C), parent (P), sibling (B), other relative (O), relative in-law (CL, PL, BL, or OL), and not family related (N). CL, PL and OL were considered as one group in this study, referred to by OL.

Ethics

This study was performed according to the Helsinki Declaration (version Fortaleza, Brazil, October 2013). The studies involving human participants were reviewed and approved by the Bangladesh Medical Research Council/National Research Ethics Committee (BMRC/NREC/2010-2013/1534). Participants were informed about the study-objectives, the samples and their right to refuse to take part or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. All patients received treatment according to national guidelines.

Sample collection

SSS from the earlobe and NS were collected for detection of *M. leprae* DNA as described previously (25). For the WBA, 4 ml venous blood was drawn and 1 ml was applied directly to a microtube precoated with 10 µg *M. leprae* whole cell sonicate (WCS) or without stimulus (Med). After 24 h incubation at 37 °C the microtube was frozen at –20 °C, shipped to the LUMC and stored at –80 °C until further analysis.

DNA isolation and RLEP PCR/qPCR

DNA isolated from the NS and SSS was used to perform RLEP PCR and qPCR as described

previously (25). Presence of *M. leprae* DNA was considered if a sample was positive for RLEP qPCR with a Ct lower than 37.5 or was positive for RLEP PCR at least in two out of three independently performed PCRs to avoid false positives.

UCP-LFAs

Levels of αPGL-I IgM, CRP, IP-10, S100A12, ApoA1, IL-6, IL-1Ra and CCL4 in WBA supernatant were analyzed using UCP-LFAs. αPGL-I IgM, CRP, IP-10, S100A12 and ApoA1 UCP-LFAs have been described previously (18, 19). IL-6, IL-1Ra and CCL4 UCP-LFAs were produced similarly, with a Test line of 200 ng MQ2-39C3 (IL-6; BioLegend, San Diego, USA), AF280 (IL-1Ra) and clone 24006 (CCL4) (R&D systems, Minneapolis, USA) and a Flow Control line with 100 ng Goat-anti-Rat (IL-6; R5130, Sigma-Aldrich), Goat-anti-Mouse (IL-1Ra; M8642; Sigma-Aldrich) and Rabbit-anti-Goat (CCL4; G4018, Sigma-Aldrich). Complementary antibodies were conjugated to the UCP particles, MQ2-13A5 (BioLegend, San Diego, USA), clone 10309 (IL-1Ra) and AF-271-NA (CCL4)(R&D systems, Minneapolis, USA). Yttrium fluoride upconverting nano materials (200 nm, NaYF4:Yb3+,Er 3+) functionalized with polyacrylic acid were obtained from Intelligent Material Solutions Inc. (Princeton, New Jersey, USA).

To perform the UCP-LFAs WBA supernatant was diluted 5-fold (IP-10, IL-1Ra and CCL4), 50-fold (IL-6, αPGL-I IgM and S100A12), 500-fold (CRP) and 5000-fold (ApoA1) in high salt buffer (100mM Tris pH 8, 270mM NaCl, 1% (w/v) BSA, 1% (v/v) Triton X-100). As WCS stimulation does not affect the levels of ApoA1, CRP and αPGL-I IgM these three markers were only determined in medium. Strips were analyzed using a UCP dedicated benchtop reader (UPCON; Labrox, Finland). Results are displayed as the ratio value between Test and Flow-Control signal based on relative fluorescence units (RFUs; excitation at 980nm and emission at 550 nm) measured at the respective lines.

Statistical analysis

GraphPad Prism version 8.1.1 for Windows (GraphPad Software, San Diego CA, USA) was used to perform Mann-Whitney U tests, Kruskal-Wallis with Dunn's correction for multiple testing, Wilcoxon matched-pairs signed rank test, plot receiver operating characteristic (ROC) curves and calculate the area under curve (AUC). The Pearson correlation coefficient and the corresponding p-values and heatmap were also determined using GraphPad Prism.

Results

M. leprae DNA in nasal swabs/slit-skin smears and the occurrence of leprosy in HCs

The presence of *M. leprae* DNA in NS and SSS of HCs was assessed in 31 households of MB index cases with BI ≥ 2 (25) (Figure 1). Out of 279 HCs, 29 were diagnosed with leprosy upon first physical investigation at intake, and four were diagnosed with PB leprosy during follow-up. Of the patients diagnosed at intake the majority (93%) had a low bacillary load: 22 were PB and seven were MB, of whom five with BI 0 (MB/BT) and two with BI ≥ 4 (Supplementary Figure S1). The HCs diagnosed with leprosy at intake (DevLep) were not evenly distributed over the different households: in 14 households none of the HCs had developed leprosy, whereas in the other 17 households, 9-42% suffered from leprosy (Figure 1). Applying previous results on the presence of *M. leprae* DNA (25), indicated that in 10 households *M. leprae* DNA was not detected in any of the HCs in NS and in 13 households all HCs were negative in the SSS. Of the households where *M. leprae* DNA was detected, percentages of colonization varied from 7 to 100% (NS) and for infection from 10 to 66% (SSS; Figure 1). The proportion of *M. leprae* DNA presence in NS or SSS and identified leprosy in HCs upon first physical screening thus varies between households even if the index cases have similarly high bacillary loads.

ApoA1 and S100A12 levels differentiate HCs from EC

Levels of αPGL-I IgM, CRP, IP-10, S100A12, ApoA1, IL-6, IL-1Ra and CCL4 were determined by UCP-LFA in WBA supernatant. Levels of these eight markers in patients (n=62; 38 MB and 24 PB), HCs (n=244) and EC (n=20) without known contact to leprosy patients were compared. Stimulation with *M. leprae* WCS had a significant impact on the CCL4, IL-1Ra and IL-6 levels (Supplementary Figure S2). Significant differences between the groups were observed for αPGL-I IgM, $S100A12_{Med}$, $S100A12_{WCS,}$ ApoA1 and CRP (Figure 2A). Compared to EC, the AUC values for αPGL-I IgM and CRP were significant only for MB patients, whereas ApoA1 and S100A12 levels significantly differed in both MB and PB patients. In HCs, however, the levels of S100A12 were comparable to those in (MB and PB) patients with similar AUCs (ranging from 0.85 to 0.91; Figure 2B). Interestingly, the difference in ApoA1 levels between EC was more profound for HC (AUC:0,81; $p<0.0001$) than for PB (AUC:0.76; $p=0.0039$) or MB patients (AUC: 0.7; $p=0.0126$). As described for other cohorts previously (19), MB patients can be discriminated from HCs based on αPGL-I IgM ($p<0.0001$) and CRP ($p=0.0024$), but these markers cannot differentiate PB patients from HCs with similar rates of *M. leprae* DNA presence in NS and SSS (25). These data thus indicate that PB patients and HCs respond similarly to *M. leprae*.

(A)

Household					Index case					
HH ID	Contacts (n)	% DevLep	%NS+	%SSS+	Gender	Age	BI	RLEP+	SSS (Ct)	NS (Ct)
HH1	5	20	20	0	Male	19	6	SSS+NS	16,25	20,06
HH2	11	0	55	36	Female	46	6	SSS+NS	16,81	21,24
HH3	8	13	25	63	Male	26	6	SSS+NS	17,98	21,21
HH4	10	0	10	10	Male	45	6	SSS+NS	18,40	22,73
HH5	0	NA	NA	NA	Male	48	6	SSS+NS	18,94	24,20
HH6	9	11	0	22	Male	45	6	SSS+NS	19,04	24,95
HH7	8	0	0	0	Male	30	6	SSS+NS	19,17	24,65
HH8	10	10	40	50	Male	33	5	SSS+NS	20,73	23,86
HH9	8	0	50	13	Male	25	6	SSS+NS	21,21	23,31
HH10	12	25	25	0	Male	34	6	SSS+NS	21,65	30,79
HH11	7	0	0	14	Male	31	5	SSS+NS	21,76	33,18
HH12	10	20	20	20	Male	38	6	SSS+NS	22,40	28,58
HH13	13	0	0	0	Male	48	5	SSS+NS	22,55	29,89
HH14	4	0	25	25	Male	36	5	SSS+NS	22,67	31,57
HH15	12	0	33	0	Male	67	5	SSS+NS	22,96	27,78
HH16	3	33	33	66	Female	12	6	SSS+NS	23,05	35,83
HH17	6	0	0	33	Male	27	6	SSS+NS	23,13	28,19
HH18	15	0	7	0	Male	35	6	SSS+NS	23,19	24,82
HH19	5	40	100	0	Male	45	6	SSS+NS	23,25	21,15
HH20	12	42	42	33	Female	17	6	SSS+NS	23,71	26,27
HH21	10	10	30	0	Female	33	5	SSS+NS	24,33	23,27
HH22	11	9	0	0	Male	32	6	SSS+NS	25,35	29,21
HH23	7	14	57	14	Male	36	5	SSS+NS	25,59	26,70
HH24	9	0	0	0	Male	50	6	SSS+NS	25,78	31,75
HH25	14	21	0	0	Male	50	5	SSS+NS	26,21	30,46
HH26	10	0	10	0	Male	30	6	SSS+NS	26,27	20,70
HH27	9	0	22	11	Female	36	4	SSS+NS	27,84	32,31
HH28	11	18	0	18	Male	48	2	SSS	32,58	Undetermined
HH29	12	0	17	25	Male	26	6	NS	NA	33,66
HH30	10	40	30	10	Male	56	4	None	NA	Undetermined
HH31	6	17	0	0	Female	30	4	NS	NA	29,25

(B)

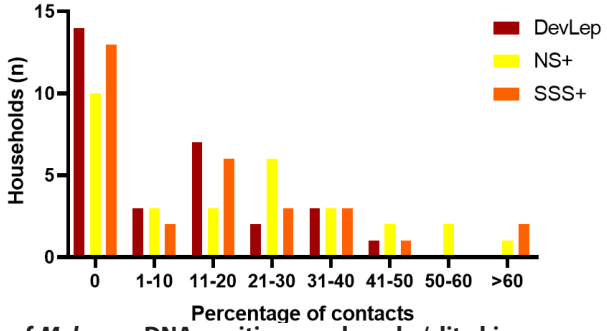

Figure 1: Percentage of *M. leprae* DNA positive nasal swabs/slit-skin smears and occurrence of leprosy in contacts per household. (A) Table indicates the number of household contacts per index case, the percentage of contacts that were diagnosed with leprosy during contact screening (%DevLep) and the percentage of contacts with *M. leprae* DNA detected in nasal swabs (%NS+) and skin-slit smears (%SSS+). The characteristics of the index case of each household (HH) are also indicated in this table. RLEP+ indicates whether *M. leprae* DNA was detected in the NS or SSS of the index case, the corresponding Ct values are indicative of the amount of *M. leprae* bacilli in NS and SSS. A low Ct value corresponds to high amounts of bacteria. BI = bacteriological index; NA=Not applicable.(B) On the *x*-axis the percentage range of household contacts (HCs) diagnosed with leprosy during contact screening (DevLep; dark red bars), that were *M. leprae* DNA positive in nasal swabs (NS+; yellow bars) or slit-skin smears (SSS+; orange bars) is indicated. The *y*-axis depicts the number of households for the percentage range indicated on the *x*-axis. The number of households within each percentage range was determined using the data table from (A).

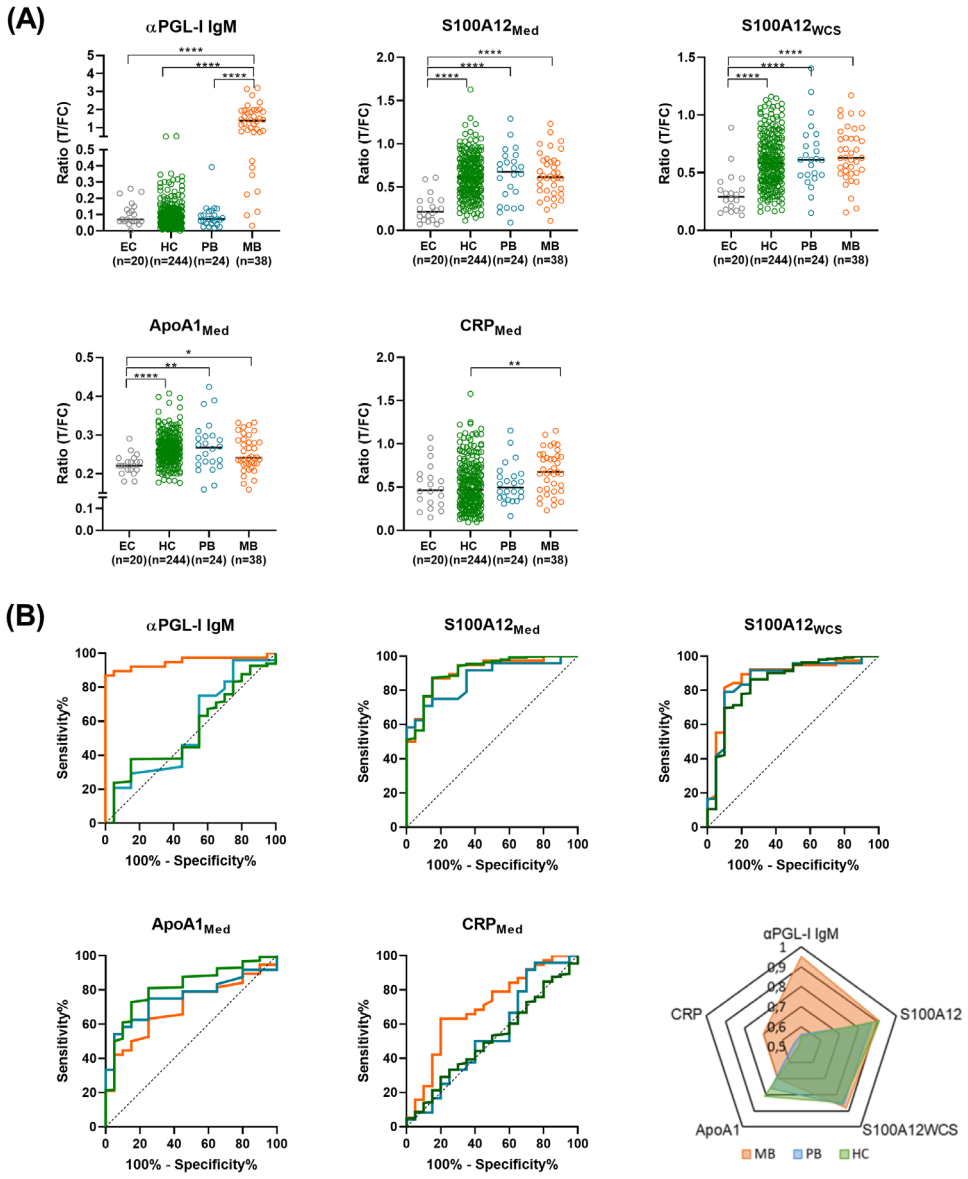

Figure 2: Differentiation of leprosy patients and household contacts (HC) from endemic controls (EC) by immune markers. Whole blood without stimulus (Med) or stimulated with *M. leprae* whole cell sonicate (WCS) was frozen after 24 hours. Levels of 8 proteins (αPGL-I IgM, S100A12, ApoA1, CCL4, IP-10, IL-6, IL-1Ra and CRP) were assessed by up-converting phosphor lateral flow assays (UCP-LFAs) in these whole blood assay supernatants for 31 households of index cases with multibacillary (MB) leprosy (bacteriological index ≥ 2).(A) UCP-LFA ratio values were calculated by dividing the peak area of the test line (T) by the peak area of the flow control line (FC; *y*-axis). As ratio values are marker dependent the *y*-axis scale differs per marker. The levels of MB (orange circles) and paucibacillary (PB; blue circles) patients, household contacts (HC; green circles) and endemic controls (EC; grey circles) were compared using the Kruskal-Wallis test with (*legend continues on the next page)*

(*Continuation of legend Figure 2)* Dunn's correction for multiple testing. The data of CCL4, IP-10, IL-6 and IL-1Ra were not shown as no significant differences were observed in the levels of these proteins between groups. P-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.(B) Receiver operating characteristic (ROC) curves were computed comparing the levels of αPGL-I IgM, CRP, S100A12, ApoA1 in multibacillary (MB)/ paucibacillary (PB) patients and HC to EC. These levels were determined by up-converting phosphor lateral flow assays in supernatant of 24 hour *M. leprae* antigen-stimulated whole blood assays (WBA; medium = Med, *M. leprae* whole cell sonicate = WCS). A summary of the areas under the curve (AUC) for MB (orange), PB (blue) and HC (green) is depicted in the spider plot showing the markers in which significant differences were observed (lower right panel).

S100A12 and CCL4 response is associated with the occurrence of leprosy in households

The relationship between disease and infection/colonisation status in households was examined into more detail by determining the correlation between the immune markers and the percentage of HCs with detectable *M. leprae* DNA in NS (%NS) and SSS (%SSS) or diagnosed with leprosy (%DevLep) (Figure 3A). A highly significant ($p<0.0001$) positive correlation was identified for the %DevLep with $CCL4_{WCS}$ and a negative correlation for %SSS with $S100A12_{Med}$ and $S100A12_{WCS}$ (Supplementary Table S1). For a subset of individuals qPCR Ct values were available indicative of the quantity of *M. leprae* DNA in NS (n=105) or SSS (n=71). These Ct values showed an inverse correlation with αPGL-I IgM antibodies in this cohort, indicating a strong positive correlation between the amount of *M. leprae* and the PGL-I antibody titer (25). For $IL\text{-}1Ra_{Med}$/$IL\text{-}1Ra_{WCS}$ and inversely for CRP, a significant correlation was observed with the Ct values for both NS and SSS as well (Supplementary Table S1).

A cross-sectional analysis was performed to compare households in which HCs developed leprosy to households where this was not observed. The same analysis was performed for households where *M. leprae* DNA was present in NS or SSS of HCs. In households where *M. leprae* DNA was detected in NS significantly lower levels of $S100A12_{Med}$ ($p <0.0001$) and $S100A12_{WCS}$ ($p=0.0005$) and higher levels of $IL\text{-}1Ra_{WCS}$ were observed (Figure 3B). S100A12 levels were also significantly lower in households where *M. leprae* DNA was detected in SSS (Figure 3C; $p<0.0001$). CCL4 levels were higher in these households, especially in response to *M. leprae* WCS ($p<0.0001$). Higher levels of $CCL4_{WCS}$ were also observed in the households where HCs of the primary index case were diagnosed with leprosy upon first physical investigation at intake ($p=0.0002$) as well as increased levels of CRP ($p=0.025$; Figure 3D).

The levels of CCL4 and S100A12 showed a significant result in both the correlation and cross-sectional analysis, indicating an association of these markers with leprosy and/or *M. leprae* infection among HCs.

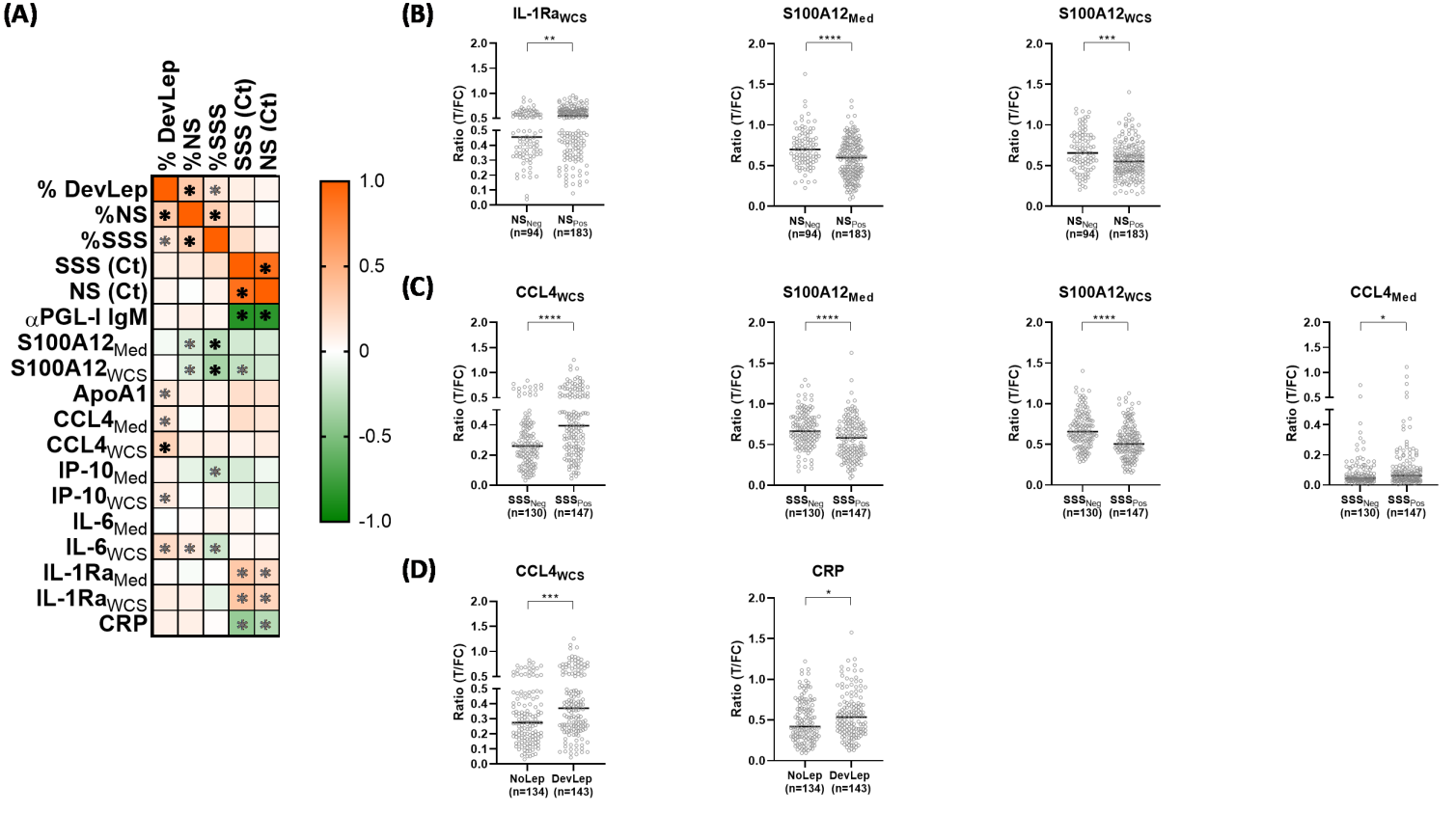

Figure 3: Correlation of leprosy disease and M. leprae infection/colonization status in households with innate immune markers. (legend on next page)

Legend Figure 3: (A) Whole blood without stimulus (Med) or stimulated with *M. leprae* whole cell sonicate (WCS) was frozen after 24 hours. Levels of 8 proteins (αPGL-I IgM, S100A12, ApoA1, CCL4, IP-10, IL-6, IL-1Ra and CRP) were assessed by up-converting phosphor lateral flow assays (UCP-LFAs) in supernatants of WBA for 31 households of index cases with multibacillary (MB) leprosy (bacterial index ≥ 2). The proportion of household contacts (HCs) diagnosed with leprosy upon first clinical examination (%DevLep) or with *M. leprae* DNA presence in nasal swabs (%NS) or slit-skin smears (%SSS) was calculated per household. These percentages and the RLEP Ct values determined by qPCR in NS and SSS were correlated with the levels of the assessed immune markers. The heatmap indicates the correlation coefficient (R), ranging from -1 (green) to 1 (orange) as determined using GraphPad Prism. Significant correlations ($p < 0.05$) are indicated with an asterisk (*), highly significant ($p<0.0001$) are indicated with a black asterisk (*). (B) Significantly different ($p<0.05$) levels of immune markers observed in HCs of *M. leprae* DNA positive (NS_{Pos}) and negative (NS_{Neg}) households. Ratio values (*y*-axis) represent the level of the assessed marker and were determined by dividing the signal of the test line (T) by the signal of the flow control (FC) line of the up-converting phosphor lateral flow assays. (C) Significantly different ($p<0,05$) levels of immune markers observed in HCs of *M. leprae* DNA positive (SSS_{Pos}) and negative (SSS_{Neg}) households. (D) Significantly different ($p<0.05$) levels of immune markers between HCs living in households where leprosy was diagnosed among contacts (DevLep) and in households where leprosy was not observed (NoLep).

M. leprae colonisation in HCs correlates with physical distance to the index case

To examine the influence of the characteristics of the index case (all MB patients with high bacillary loads) on the development of leprosy and *M. leprae* colonisation (NS) or infection (SSS) in HCs, a correlation and cross-sectional analysis was performed (Supplementary Figure S3). Cross-sectionally, higher $S100A12_{Med}$ levels were observed in index cases without detectable *M. leprae* DNA in NS of their HCs ($p=0.035$). No other significant differences were observed in index cases for the other markers nor in the amount of bacteria in SSS or NS. Thus, characteristics of the index case in this cohort have little influence on the observed differences between the households (Figure 1).

The influence of genetic relationship and physical distance of HCs to the index case was also examined. HCs were stratified by genetic distance against the percentage of leprosy and *M. leprae* DNA presence in NS and SSS in these groups (Figure 4). Development of leprosy was most frequently observed in spouses (37%), followed by siblings (23%) and siblings in law (17%) (Figure 4A). Spouses also showed the highest frequency of *M. leprae* presence in NS and/or SSS (58%), followed by children (42%) and parents (41%) (Figure 4B). Spouses, children and parents live in the closest proximity of patients (Figure 4C; living under the same roof or sharing a kitchen) and thus have the highest level of exposure. Physical distance indeed correlated significantly ($p=0.003$; $R^2=0.8$) with the $\%NS_{Pos}$ (colonization), though this was not observed for the development of leprosy in HCs ($p=0.07$; $R^2=0.44$).

The levels of the innate immune markers were also stratified by genetic distance. Based on the median levels of the assessed markers, the HC groups that were diagnosed with

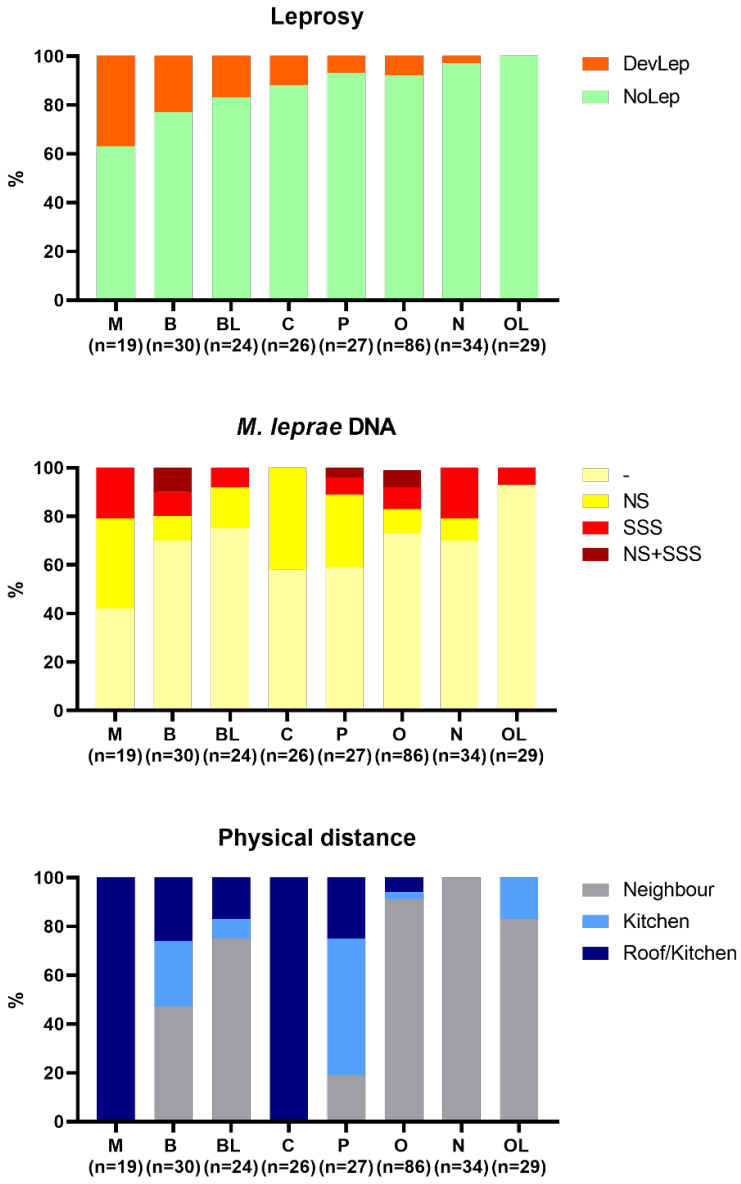

Figure 4: Stratification of household contacts by genetic distance to the index case. Eight different groups were classified for genetic distance: spouse (M), child (C), parent (P), sibling (B), other relative (O), brother/sister in law (BL), other relatives in law (OL) and not family related (N). (A) Percentage of individuals diagnosed with leprosy upon first clinical examination (DevLep; orange) stratified by genetic distance and ranked by percentage. (B) Percentage of *M. leprae* DNA presence in nasal swabs (NS; yellow), slit-skin smears (SSS; red) or both (NS + SSS; dark red) stratified by genetic distance. (C) Distribution of physical distance (Roof/kitchen = dark blue, kitchen = blue, Neighbor = grey) to the index case stratified by genetic distance.

leprosy clustered apart from the HC groups that did not show symptoms of disease (Figure 5). Across the groups with different genetic distance to the index case, similar innate immune mechanisms seem to play a role in the development of leprosy in HCs. Additionally, the index case group clustered apart from all HC groups rendering the assessed markers useful for leprosy diagnostics.

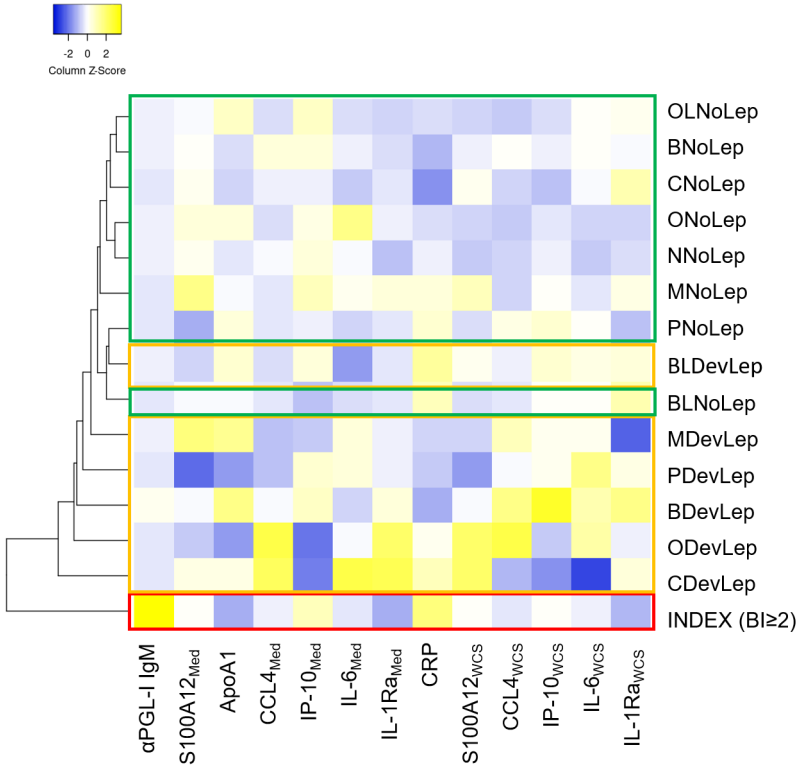

Figure 5: Contacts diagnosed with leprosy upon first clinical screening cluster together based on their immune response, irrespective of genetic distance. Whole blood without stimulus (=Med) or stimulated with *M. leprae* whole cell sonicate (=WCS) was frozen after 24 hours. Levels of 8 proteins (αPGL-I IgM, S100A12, ApoA1, CCL4, IP-10, IL-6, IL-1Ra and CRP) were assessed by up-converting phosphor lateral flow assays in supernatants of whole blood assays (WBA) for 31 households of index cases with multibacillary (MB) leprosy (bacteriological index ≥ 2). The heatmap shows clustering based on average linkage performed by heatmapper (47) of the median level of eight serum protein markers in contacts diagnosed with leprosy upon first clinical screening of the HCs (DevLep) and without leprosy (NoLep) stratified by genetic distance; spouse (M), child (C); parent (P); sibling (B); other relative (O); brother/sister in law (BL); other relatives in law (OL) and not family related (N). The z-score indicates the deviation from the average level of the marker across groups, higher Z-scores are indicated in yellow and lower Z-scores in blue. Red = index case, yellow = contacts diagnosed with leprosy; green = household contacts without leprosy.

Discussion

To examine the link between innate immunity and *M. leprae* colonisation/infection in HCs, immune markers were assessed in 24 hour *M. leprae* antigen-stimulated WBAs by UCP-LFAs. Even though all HCs were exposed to comparable levels of *M. leprae*, as all 31 index cases were MB patients with BI ≥ 2, there was a difference in the percentage of *M. leprae* DNA presence in NS/SSS and the occurrence of leprosy cases between households. Characteristics of the index case, such as the amount of *M. leprae* bacilli in NS or the αPGL-I antibody titer, had little influence on the development of leprosy nor on *M. leprae* colonization/infection in other household members. Physical distance of HCs to the index case was, however, significantly correlated with *M. leprae* colonization, though not with *M. leprae* infection or development of leprosy demonstrating the role of innate immune responses to remove bacteria.

In this study, S100A12 was associated with a protective response to *M. leprae* colonization/infection in HCs. As previously demonstrated (18), S100A12 also remained a useful marker to discriminate leprosy patients from EC. S100A12 has a dual role inducing both proinflammatory and antimicrobial effects by interacting with different receptors, such as RAGE and TLR4 (27). RAGE expression is associated with disease severity and levels of proinflammatory cytokines in active tuberculosis (TB) (28). Contrary, RAGE is protective against the development of pulmonary TB in mouse models (29) in line with reduction of antimicrobial activity in human macrophages upon TLR2/1 ligand activation by S100A12 knockdown (20). S100A12 thus seems to protect exposed individuals from *M. leprae* colonization and infection, but once infected, S100A12 can contribute to maintain a detrimental, pro-inflammatory state in leprosy patients.

ApoA1 levels in HCs were similar to those in PB patients, suggesting that ApoA1 plays a role in limiting bacterial growth. This is in line with the finding that PB patients showed a similar low rate of *M. leprae* DNA presence in NS and SSS as HCs (25). Increased levels of ApoA1 have been observed in cells exposed to activated complement, where ApoA1 inhibits the formation of the membrane attack complex thereby contributing to complement clearance (30). Decreased levels are associated with destructive chronic inflammation, as ApoA1 exerts anti-inflammatory effects (31). The effects of ApoA1 do, however, not only rely on the protein level but also on the functionality, oxidative modification can for instance transform ApoA1 to an inflammatory agent (32). The role and functionality of ApoA1 in leprosy thus remains to be further elucidated. The influence of ApoA1 on lipid metabolism is of interest as dysfunctional high-density lipoprotein (involved in cholesterol transport to the liver of which the main protein is ApoA1) related to altered ApoA1 levels has been observed in MB patients (33). Moreover, it was suggested that

M. leprae can directly affect ApoA1 biosynthesis.

Other markers in this study were associated with *M. leprae* colonization (IL-1Ra), whereas CCL4 was associated with infection and disease. These responses were most profound upon stimulation with *M. leprae* WCS, reflecting the innate immune response of these individuals to mycobacterial antigens. Interestingly, in whole blood of BCG-vaccinated infants the production of IL-1Ra and CCL4 was decreased upon stimulation of several TLRs (34). This observed response can be a result of BCG-induced trained innate immunity, which is immunological memory of the innate immune response that leads to an enhanced response to a subsequent trigger (35). Moreover, in Systemic Lupus Erythematosus (SLE) a pathogenic three-marker signature, including high levels of IL-1Ra and CCL4, was identified in monocytes (36). The signature was associated with the immune dysregulation in this autoimmune disease, in which flares occur similar to leprosy reactions (37). High levels of IL-1Ra and CCL4 thus seem indicative of pathogenic innate immune responses, corroborating earlier results on the identification of IL-1Ra and CCL4 as biomarkers associated with a pathogenic immune response to *M. leprae* (18, 19, 38).

One of the challenges of application of host immune markers for diagnostics is the influence of co-morbidities or co-infections on biomarker levels. Helminth infections dampen the Th1 response and increase the risk for MB leprosy (39, 40). A biomarker study to examine the influence of helminth co-infection in leprosy patients is currently ongoing. Moreover, the influence on biomarker levels of co-morbidities, such as diabetes mellitus which is known to increase the risk of active TB (41), on the disease outcome should be further studied. Another issue impeding straightforward implementation of biomarkers is that inflammatory markers are not disease-specific. For example, S100A12 has been described as biomarker for rheumatoid arthritis (42), TB (43) as well as inflammatory bowel disease (44). As the UCP-LFA allows quantitative measurement of biomarkers it would be interesting to compare disease-specific S100A12 levels for these conditions. Taking into account the multiple factors that influence host immune responses, a biomarker signature that combines several innate immune markers is required to identify individuals at risk of developing leprosy. This signature should also be evaluated in other inflammatory conditions.

In conclusion: Frequent exposure of HCs to *M. leprae* results in a continuously active innate immune response. This allows differentiation of HCs from EC by user-friendly diagnostic tests measuring specific serum protein levels. If the innate immune response is sufficient, pathogens and pathogen-infected cells are being successfully removed. However, prolonged (intense) activation can lead to an immune response directed against the host (45). The resemblance of the innate immune response of PB patients and HCs observed in

this and previous studies (18, 38) indicates that PB leprosy can be a result of an imbalance in innate immunity. HCs that do not develop disease seem to effectively clear the bacteria without overactivation of the innate immune response. Elucidation of this delicate balance in innate immune responses by quantitation of appropriate biomarker signatures (46) can contribute to the identification of individuals at risk of developing leprosy upon *M. leprae* exposure. To gain more insight in this balance longitudinal analysis is required, which is currently ongoing. Diagnostic user-friendly rapid tests, as applied in this study, that allow quantitative measurement of combinations of innate immune markers represent useful tools to identify individuals that could benefit from prophylactic treatment.

Acknowledgements

We thank all patients and control individuals for their voluntary participation.

Funding statement

This study was supported by an R2STOP Research grant from Effect hope/ The Leprosy Mission Canada, the Order of Malta-Grants-for-Leprosy-Research (MALTALEP), the Q.M. Gastmann-Wichers Foundation (to AG), and the Leprosy Research Initiative (LRI) together with the Turing Foundation (ILEP#: 703.15.07).The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing financial interests

The authors declare to have no financial/commercial conflicts of interests.

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Supplementary material

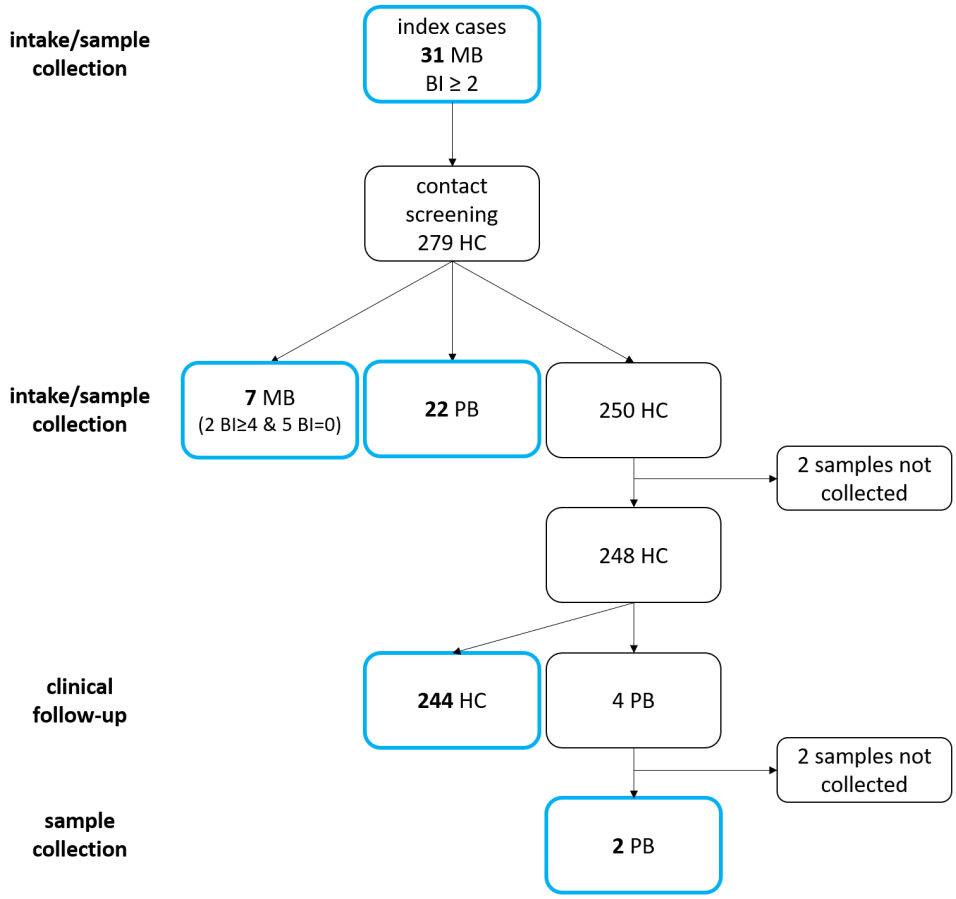
Tables

Supplementary Table S1: Correlation of leprosy disease and *M. leprae* infection/colonization status in households with innate immune markers

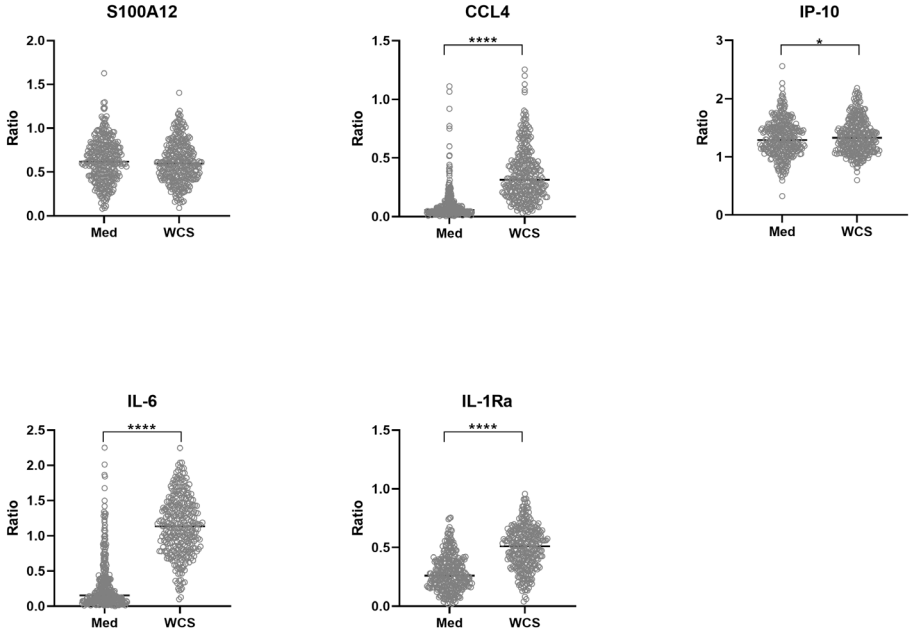
	P-value					Correlation Coefficient (R)				
	% DevLep	%NS	%SSS	SSS (Ct)	NS (Ct)	% DevLep	%NS	%SSS	SSS (Ct)	NS (Ct)
% DevLep		1,83E-09	0,01	0,48	0,62	1,000	0,333	0,146	0,085	0,050
%NS	1,83E-09		4,56E-08	0,30	0,93	0,333	1,000	0,304	0,125	-0,009
%SSS	0,01	4,56E-08		0,10	0,44	0,146	0,304	1,000	0,201	0,076
SSS (Ct)	0,48	0,30	0,10		2,87E-17	0,085	0,125	0,201	1,000	0,890
NS (Ct)	0,62	0,93	0,44	2,87E-17		0,050	-0,009	0,076	0,890	1,000
αPGL-I IgM	0,33	0,12	0,26	2,32E-21	2,64E-28	0,055	0,089	0,064	-0,852	-0,831
$S100A12_{Med}$	0,46	0,005	2,53E-06	0,14	0,12	-0,042	-0,159	-0,264	-0,178	-0,154
$S100A12_{WCS}$	0,83	0,01	5,49E-10	0,04	0,08	0,012	-0,146	-0,343	-0,242	-0,171
ApoA1	0,01	0,10	0,13	0,10	0,08	0,144	0,093	0,085	0,197	0,171
$CCL4_{Med}$	0,01	0,81	0,41	0,09	0,11	0,148	-0,014	0,047	0,205	0,156
$CCL4_{WCS}$	1,35E-06	0,08	0,09	0,52	0,23	0,270	0,098	0,096	0,078	0,118
$IP\text{-}10_{Med}$	0,16	0,08	0,001	0,19	0,53	0,081	-0,100	-0,189	-0,158	-0,063
$IP\text{-}10_{WCS}$	0,04	0,92	0,34	0,34	0,12	0,119	-0,006	0,054	-0,115	-0,153
$IL\text{-}6_{Med}$	0,91	0,81	0,30	0,67	0,99	-0,007	0,014	0,060	0,051	0,001
$IL\text{-}6_{WCS}$	0,0001	0,02	0,001	0,73	0,65	0,215	0,128	-0,193	0,042	0,045
$IL\text{-}1Ra_{Med}$	0,66	0,62	0,94	0,004	0,03	0,025	-0,029	0,004	0,336	0,215
$IL\text{-}1Ra_{WCS}$	0,07	0,12	0,10	0,003	0,01	0,103	0,089	-0,094	0,351	0,252
CRP	0,12	0,11	0,81	0,0004	0,003	0,089	0,092	0,014	-0,409	-0,287

Whole blood without stimulus (Med) or stimulated with *M. leprae* whole cell sonicate (WCS) was frozen after 24 hours. For 31 households of index cases with multibacillary leprosy (bacteriolgocial index ≥ 2), levels of 8 proteins (αPGL-I IgM, S100A12, ApoA1, CCL4, IP-10, IL-6, IL-1Ra and CRP) were assessed by up-converting phosphor lateral flow assays (UCP-LFAs) in whole blood assay supernatants. Per household the percentage of household contacts (HCs) diagnosed with leprosy upon first clinical screening (%DevLep) or positive for *M. leprae* DNA in nasal swabs (%NS) or skin slit smears (%SSS) at that same time, was calculated. Correlation between these percentages and the RLEP Ct values, determined by qPCR in NS and SSS (25), with the levels of the assessed immune markers was determined. The p-value and the corresponding correlation coefficient (R) are shown. Significant p-values (green) indicate which innate immune markers are correlated with the amount of bacteria in NS and SSS assessed by qPCR or are correlated with the %DevLep, %NS or %SSS.

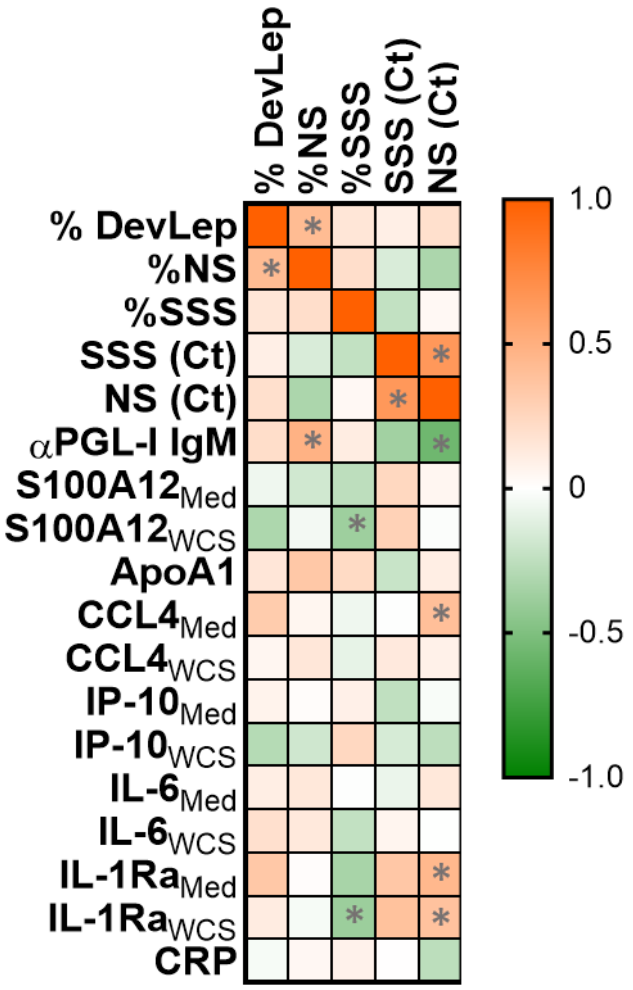
Figures



Supplementary Figure S1: Schematic representation of sample collection. First, index cases diagnosed with multibacillary (MB) leprosy and a bacteriological index (BI) ≥ 2 were recruited. 279 household contacts (HC) of these index cases were screened for the signs and symptoms of leprosy, of which 7 were diagnosed with MB leprosy and 22 with paucibacillary (PB) leprosy at intake. Subjects included in the study were followed up for surveillance of new case occurrence for ≥ 24 months after sample collection (clinical follow-up), identifying 4 additional PB patients. At clinical follow-up samples of HC that developed leprosy were collected. The blue rectangles indicate the amount of MB (n=38), PB (n=24) and HC (n=244) samples used in this study.



Supplementary Figure S2: Influence of whole blood stimulation with *Mycobacterium leprae* whole cell sonicate (WCS) on biomarker levels. S100A12, CCL4, IP-10, IL-6 and IL-1Ra levels were detected in both unstimulated (Med) and M. leprae WCS-stimulated (WCS) whole blood assays (WBA) using up-converting phosphor lateral flow assays. Paired comparison between Med and WCS ratio values (y-axis; signal detected at the test line divided by the signal at the flow control line) of all study subjects was performed using the Wilcoxon matched-pairs signed rank test. P-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.



Supplementary Figure S3: Correlation of index case characteristics with the development of leprosy and *M. leprae* colonisation or infection in the same household. Levels of eight markers in 24 hour *M. leprae* antigen-stimulated whole blood assays (medium = Med, *M. leprae* whole cell sonicate = WCS) were determined for 31 index cases with multibacillary (MB) leprosy (bacteriological index ≥ 2). Per household, the percentage of contacts (HCs) diagnosed with leprosy upon first clinical screening (%DevLep) or with *M. leprae* DNA positivity in nasal swabs (%NS) or skin slit smears (%SSS) was calculated. These percentages and the RLEP Ct values determined by qPCR in NS and SSS were correlated with the levels of innate immunity markers. The heatmap indicates the correlation coefficient (R), ranging from -1 (green) to 1 (orange). Significant correlations ($p < 0.05$) are indicated with a grey asterisk (*).

8 Chapter

The anti-PGL-I antibody response in naturally *M. leprae* infected squirrels, a free-roaming animal model

Adapted from:

Detection of humoral immunity to mycobacteria causing leprosy in Eurasian red squirrels (Sciurus vulgaris) using a quantitative rapid test[§]

&

Clinical Progression of leprosy in Eurasian red squirrels (Sciurus vulgaris) in A Naturally infected wild population¶

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[§]Eur J Wildl Res 65, 49 (2019)
.J Zoo Wildl Med. *In press*

Abstract

Eurasian red squirrels (Sciurus vulgaris, ERS) in the British Isles are a recently discovered natural host for *Mycobacterium leprae* and *Mycobacterium lepromatosis*. Infected squirrels can develop skin lesions or carry the bacteria without showing clinical signs. Until now the clinical diagnosis of leprosy could only be confirmed in squirrels by isolating DNA of leprosy bacilli from carcasses or by establishing the presence of acid-fast bacilli in skin sections of carcasses with clinical signs. In this study, we assessed the performance of a field-friendly diagnostic test for detection of *M. leprae*/*M. lepromatosis* infection in ERS. This up-converting phosphor lateral flow assay (UCP-LFA) is well established for detection of *M. leprae* specific anti-phenolic glycolipid-I antibodies (αPGL-I) IgM antibodies in humans and associated with bacterial load. Assessment was performed on serum and blood drops from live squirrels. Clinically diseased squirrels showed significantly higher αPGL-I antibody levels than healthy animals or subclinically infected animals ($p < 0.0001$), both in serum and whole blood drop samples. Subclinically infected animals were identified using molecular methods to detect the presence of leprosy bacilli DNA in punch biopsy tissue samples. This study shows that the αPGL-I UCP-LFAs presented here allows a field-friendly serological confirmation of *M. leprae* infection in clinically diseased live ERS. For surveillance purposes, the combination of clinical assessment, αPGL-I UCP-LFAs, and molecular methods allow the identification of both diseased animals and subclinically infected animals.

Introduction

Leprosy was first described in Eurasian red squirrels (*Sciurus vulgaris*; ERS) in 2014 (1). Since then, DNA of the causative mycobacteria (*Mycobacterium leprae* and *Mycobacterium lepromatosis*) has been detected in ERS populations throughout the British Isles (2-5), but not on the European continent (6, 7). Individual squirrels may carry leprosy bacteria without showing clinical signs (2) or present pathognomonic clinical lesions with individual variation (1). The most clinically similar disease in ERS is atypical histiocytosis, which has been described only in a few animals in Scotland (8). Differential diagnosis using histological and molecular methods is possible using carcasses but for live ERS accurate diagnosis is more challenging. Minimally invasive rapid tests would provide a field-friendly and humane method to confirm clinical diagnosis of leprosy.

In humans levels of antibodies against the *M. leprae*-specific phenolic glycolipid I (αPGL-I) closely correlate with bacterial load and higher risk of developing leprosy. αPGL-I serology is used to detect infections with leprosy bacilli in humans (9) and nine-banded armadillos (Dasypus novemcinctus) (10). Previously, a qualitative lateral flow test was used to detect αPGL-I in body fluid samples of ERS (2). However, this test lacked a quantified read-out, was subject to operator bias, and test results could not be correlated with disease severity. In humans, lateral flow assays (LFAs) combined with up-converting phosphor (UCP) reporter particles as a quantitative label to assess αPGL-I antibody levels (αPGL-I UCP-LFA) are highly sensitive, field friendly, low-complexity diagnostic tools in leprosy endemic areas (11). We investigated the applicability of an αPGL-I UCP-LFA in ERS for diagnostic purposes using different sample types; serum and blood drops from live squirrels.

Materials and methods

Sampling and ethical approval

Samples were obtained from two squirrel populations (Isle of Arran, Ayrshire; Brownsea Island, Dorset) in which leprosy had been confirmed previously (2). They were collected between 2016 and 2018 from 90 different ERS (87 adult, 3 sub-adult). Field-based clinical assessment and sampling took place under general isoflurane anesthesia, following a previously published protocol (12). Once fully recovered from anesthesia, ERS were released at the trapping site. A microchip was placed subcutaneously between the shoulder blades as permanent identification to document repeated assessments of individual ERS. Since the population was free-living, return and re-assessment of a previously trapped individual could not be guaranteed. Scanning for the presence of a microchip was always performed at the end of an assessment, to avoid subconscious bias in lesion assessment

and categorization. To be able to compare information from individual ERS seen for the first time at different sessions, results were noted as a timeline of 0 to a maximum of 24 months (up to 5 time points).All procedures took place under Home Office license authority (Project license 70/9023), Natural England License 2016–24517-SCI-SCI and with ethical approval from the University of Edinburgh's Animal Welfare and Ethical Review Body.

Diagnostic methods to establish the leprosy status of ERS

At the time of sampling, all animals were clinically assessed by a veterinarian and grouped according to the absence or presence of clinical pathognomonic leprosy lesions (areas of alopecia, shininess, and firm rubbery swelling of the skin). Those with lesions were classified into four severity categories (1 = mild, 2 = mild-moderate, 3 = moderate, 4 = severe) derived from an additive numerical (2–96) score assigned after assessing the number of affected body areas, size, shape, and ulceration of lesions (Supplementary Table S1). For 64 live ERS without clinical lesions, a small tissue punch sample was taken from the left ear under general anesthesia. Tissue punch samples from clinically diseased ERS were not collected to avoid altering the progression of lesions in the first four sampling sessions (autumn 2016 to spring 2018), but were collected from all ERS assessed in the final session (autumn 2018). The presence of leprosy bacilli DNA in these tissues was assessed via PCR as described by Avanzi et al. (2016).

Blood samples were taken from the femoral vein under general anesthesia. Serum samples (n = 132) were prepared at room temperature by centrifugation (10 min/2000g) and were either used immediately or stored at − 20 °C until required. Blood drop samples (n = 65) were obtained either using remaining blood in the syringe after ejection of the whole blood sample (n = 26) or via a skin prick using disposable 20-µL Minivette® collection tubes (heparin coated; Sarstedt) (n = 39). Where the blood flow from the prick site was insufficient to fill the minivette, filling was completed from the whole blood sample. Full details of samples are provided in Online Resource 1 (https://link.springer.com/article/10.1007/s10344-019-1287-1#Sec5). The information obtained from clinical assessment, serological and molecular diagnostics were combined to establish each ERS' leprosy status (Supplementary Table S2).

αPGL-I UCP-LFA

The αPGL-I UCP-LFA were produced as described previously (13). Briefly, the nitrocellulose of the LF strips was provided with a test line (T) of 100 ng synthetic PGL-I and a 50-ng rabbit anti-goat IgG (G4018, Sigma-Aldrich) flow control line (FC). The reporter, 85-nm-

sized NaYF4:Yb3+, Er3+ up-converting reporter particles (UCP; Intelligent Material Solutions Inc., Princeton, NJ, US) was covalently coated with 125 µg goat anti-human IgM (I0759, Sigma-Aldrich) per mg UCP (14) and 200 ng dried on the conjugate-release pad of the LF strip. LF strips were stored at ambient temperature in plastic containers with silica dry pad. Irrespective of type (serum, blood drop) samples were diluted 50-fold in LF assay buffer (100 mM Tris pH 7.2, 270 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) BSA). The diluted sample (50 µl) was applied to the PGL-I strips and immunochromatography continued until strips were dry.

Data analysis

LF strips were scanned in a Packard FluoroCount microtiter plate reader adapted for measurement of the UCP label (980 nm IR excitation, 550 nm emission) (15). Test results were displayed as ratio (R) between T and FC signals (550 nm emission) measured upon IR excitation.

Graphpad Prism version 7.00 for Windows (GraphPad Software, San Diego CA, USA) was used to perform Mann-Whitney U tests, one-way ANOVA for non-parametric distribution (Kruskall-Wallis), and Dunn's correction for multiple testing and to plot ROC curves and calculate the area under curve (AUC). Cut-offs were calculated using Youden's index (16).

Results

αPGL-I antibody levels indicate clinical leprosy and correlate to disease severity

Only adult animals showed clinical signs of leprosy or tested positive for the presence of *M. leprae* DNA. *M. lepromatosis* DNA was not detected in this study. Detailed information on all ERS including clinical category and lesion score is presented in Online Resource 1 (https://link.springer.com/article/10.1007/s10344-019-1287-1#Sec5).

Of the serum samples analyzed, 25 were from ERS with pathognomonic leprosy lesions, 11 from individual ERS with no clinical signs but detectable *M. leprae* DNA, 53 from ERS with no lesions or detectable *M. leprae* DNA, and 43 samples from ERS with no lesions and from which no tissue sample was available. Elevated αPGL-I antibody levels clearly discriminated clinically positive ERS from clinically negative/PCR positive ($p < 0.0001$; AUC 0.94) and clinically negative/PCR negative ERS ($p < 0.0001$; AUC 0.96; Figure 1a). However, they did not significantly differ between clinically negative/PCR positive ERS and clinically negative/PCR negative ERS ($p > 0.9999$). The UCP-LFA has a sensitivity of 88% and a specificity of 96% in sera for detection of *M. leprae* infection in clinically diseased animals (cut-off ratio > 0.1).

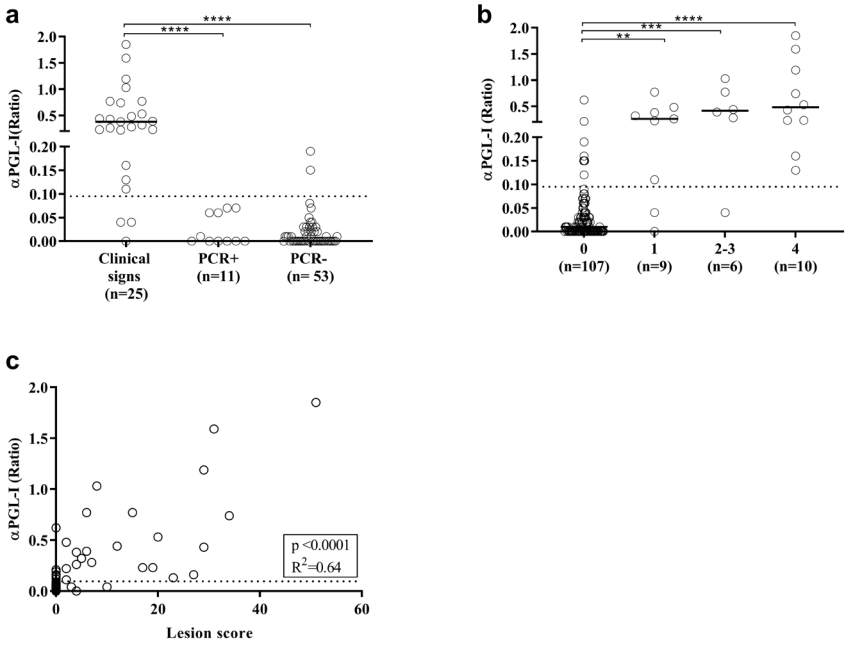

Figure 1: αPGL-I antibody levels correlate with clinical presentation of leprosy in squirrels. αPGL-I antibody levels were determined by UCP-LFA and ratio values are displayed on the y-axis. The cut-off for positivity (R = 0.1) is indicated by the dotted line. Comparisons were made using Kruskal-Wallis tests with multiple Dunn's correction. **a** Comparison of serum αPGL-I antibody levels from ERS with clinical lesions with ERS PCR+ without lesions or ERS negative for both PCR and lesions (only animals with clinical disease or tissue sampled for PCR included). **b** Comparison of serum αPGL-I antibody levels from ERS classified into different lesion categories ranging from negative (0) to severe (4). **c** Comparison of serum αPGL-I antibody levels from ERS classified according to severity of lesions expressed by a continuous numerical score (Pearson's correlation). **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

The αPGL-I antibody levels showed to correspond with disease severity; for ERS in category 1 elevated levels of αPGL-I antibodies could be detected (p = 0.0012; AUC 0.88; Figure 1b) compared with ERS lacking lesions, but this difference became more significant for animals with lesions of a higher category (2–3: p = 0.0005; AUC 0.96; 4: p < 0.0001; AUC: 0.99. Figure 1b). By representing the clinical signs as a continuous numerical score a significant correlation (p < 0.0001; $R^2 = 0.64$) between αPGL-I antibody levels and severity of the lesions confirmed this observation (Figure 1c).

Longitudinal αPGL-I antibody levels correlate to disease progression

A total of 31 ERS were trapped in multiple assessment sessions. Two ERS were assessed the maximum of five times (i.e. at 0, 6, 12, 18 and 24 months), two were assessed at four time points, 12 at three time points and 15 at two time points. Eight (25.8%) ERS were

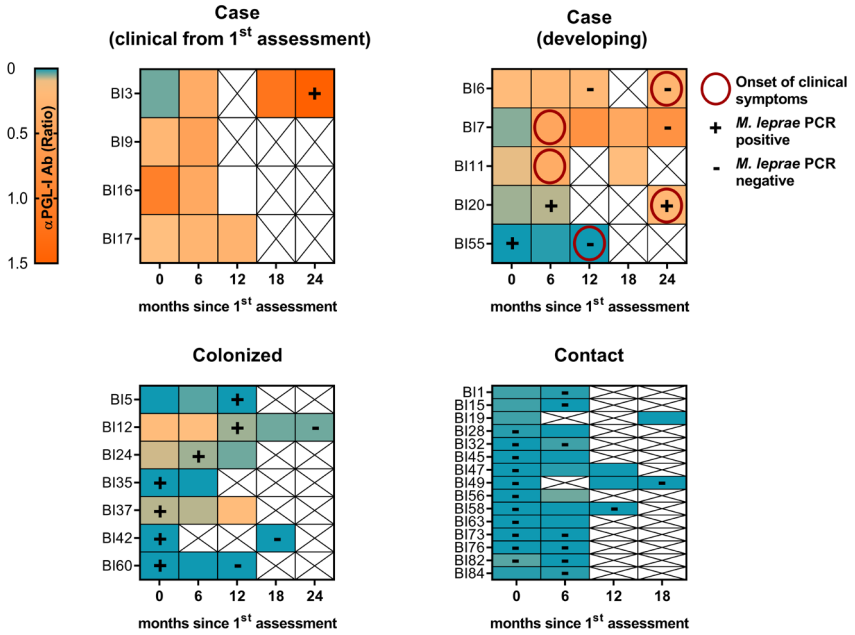

Figure 2 Longitudinal anti-phenolic glycolipid-I (PGL-I) antibody levels and presence of *M. leprae* DNA in 31 free-roaming Eurasian red squirrels (Sciurus vulgaris). Squirrels were assessed for αPGL-I antibody levels at all time points of assessment, with a maximum of 5 timepoints (Supplementary Figure S1). The color indication at each timepoint corresponds to the Ratio value observed in the αPGL-I antibody lateral flow assay. Blue indicates Ratio values below the cut-off for positivity determined for this study (0.1) and orange represents Ratio values above this cut-off. PCR was performed to determine the presence of *M. leprae* DNA for the time points where either a plus (+, PCR positive) or a minus (-, PCR negative) is indicated. Squirrels were divided in 4 groups; Clinical leprosy, squirrels that showed clinical signs at initial assessment (top left panel). Developing leprosy, squirrels that developed leprosy during the study, timepoint of leprosy diagnosis is indicated with a red circle (top right panel). Colonized squirrels, colonized with *M. leprae* at a certain time point during the study showing no signs of leprosy (bottom left panel). Contact ERS, squirrels without symptoms of leprosy and no presence of M. leprae DNA (bottom right panel).

identified at some point during the study as leprosy cases, one as leprosy suspect (3.2%, included with leprosy cases in figures, BI55), seven as colonized (22.6%) and 15 (48.4%) as contacts (Figure 2). Three out of four squirrels that already presented with symptoms at the first clinical assessment showed αPGL-I antibody levels above the cut-off for positivity during the entire study and one from the six month timepoint until the end of the study. This animal (BI3) could be followed for two years, displaying an increase in the αPGL-I antibody levels over time (Supplementary Figure S1). Longitudinal αPGL-I antibody levels in the squirrels that developed leprosy symptoms during the study showed a clear association with the appearance of clinical symptoms. All subjects with symptoms except the suspect (BI55) showed positive αPGL-I antibody titers in the 2-year study period. Only

in one animal (Bl6), the antibodies were detected before the onset of clinical symptoms, in the other 3 animals the antibodies were detected at the appearance of clinical symptoms

In the colonized animals αPGL-I antibodies were hardly detected, two out of seven had αPGL-I antibody levels above the cut-off for positivity (Figure 2). However, the R-values in these two ERS were not as high as observed in the confirmed clinical cases (Supplementary Figure S1). Of the contacts, in which no *M. leprae* DNA was detected, the αPGL-I antibody levels remained below the cut-off for positivity at all timepoints. It is apparent from these data that αPGL-I antibody levels correspond to the appearance of clinical symptoms, rather than *M. leprae* infection.

αPGL-I antibody levels in blood drops

We assessed the performance of αPGL-I UCP-LFA on blood drop samples by comparing results for 65 sample pairs for which both serum and blood drop samples were collected from the same ERS at the same time point. Eight of these sample pairs were from ERS with lesions and 57 from ERS without lesions, in five of the latter, *M. leprae* DNA was detected. αPGL-I levels showed a significant correlation ($p < 0.0001$; $R^2 = 0.9$), indicating the compatibility of αPGL-I UCP-LFA with blood drops (whole blood) as well as serum (Figure 3).

This offers the potential to reduce the impact of sampling on the animal. However, we found it difficult to reliably get sufficient blood drop formation in the prick sites that were evaluated (ear, front and hind foot, tail; selected on the basis of accessibility without risk of injury to ERS and handler in a handling cone). Prick sites either did not bleed enough or the blood drop dispersed along the fur, even if it was clipped very short. Limited success was achieved on the underside of the last third of the tail by clipping the fur very short, disinfecting the site with ethanol and warming the tail on a heat pad prior to pricking.

Discussion

The ability to study squirrels developing leprosy is an unique opportunity to gain insight in the antibody response to *M. leprae* before the onset of clinical symptoms. Using the αPGL-I UCP-LFA previously developed for humans (11, 17) antibody levels could be easily monitored in both serum and blood drops. These levels showed to correlate to the severity of disease and corresponded with the appearance of clinical symptoms. *M. leprae* infection without clinical symptoms is not detected using the UCP-LFA, requiring PCR. On the other hand, the PCR result was not positive for all squirrels that showed clinical symptoms and the unavailability of PCR data for all time-points does not enable a direct comparison of PCR and αPGL-I data in all samples. The αPGL-I UCP-LFA thus offers a useful

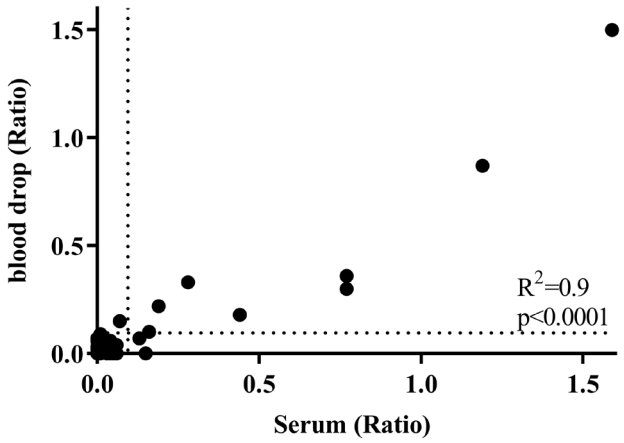

Figure 3: Significant correlation between αPGL-I levels in serum and blood drop samples. αPGL-I levels were detected by UCP-LFA in serum and blood drops of the same squirrels and Pearson's correlation indicated a significant correlation between both sample types.

rapid test to confirm clinical leprosy in ERS in the field.

For the identification of subclinical carriers, tissue sampling and molecular assessment for the presence of leprosy bacilli DNA was more sensitive. For three squirrels, *M. leprae* DNA was no longer detected at a later timepoint, suggesting that these animals were able to efficiently clear the mycobacterium without developing disease symptoms. In humans, αPGL-I antibodies are predominantly detected in leprosy patients with high bacterial loads and are difficult to detect in paucibacillary patients (11, 17). The observation that the highest αPGL-I antibody levels were observed in the animals with the most severe disease symptoms suggests that this correlation is also present in squirrels. qPCR enables the quantification of the *M. leprae* bacilli, which was not performed in this study, but would be of interest to confirm that the high αPGL-I antibody levels indeed correlate to a high bacterial load (18).

In line with observations in humans (19), αPGL-I antibody levels are poor predictors of the development of leprosy. Only in one squirrel αPGL-I antibody levels preceded the development of symptoms, but in general corresponded with the appearance of clinical symptoms. In the squirrels that did not develop clinical symptoms, both colonized and contacts, high αPGL-I antibody levels were not observed, again confirming the correlation with clinical disease.

Since none of the samples included in this study were derived from animals infected with *M. lepromatosis*, we cannot confirm that the UCP-LFA could be used to detect

infection with this bacterium in ERS. However, for humans, it is shown that αPGL-I-based immunodiagnostics are able to detect infections with *M. lepromatosis* as well (20). It will be important to verify this assumption particularly for surveillance efforts in ERS populations in which *M. lepromatosis* infections have been described to occur in more locations than *M. leprae* infections (2). Future efforts should investigate whether additional cellular immune markers can augment the identification of subclinically infected squirrels, something that has been done successfully in humans (17).

In summary, we present a field- and animal-friendly serological test to detect specific αPGL-I antibodies and confirm clinical leprosy in ERS. While it will be necessary to add other tools and/or additional biomarkers in animals that do not show visible signs of disease to estimate the prevalence of leprosy bacilli in this species, the αPGL-I UCP-LFA is a valuable tool to exclude or confirm clinical leprosy or severe infection in a captured squirrel with lesions.

Acknowledgements

We thank the local staff of the National Trust(Brownsea Island) and Dorset Wildlife Trust, especially Angela Cott, Timothy Hartley, John Lamming and Adam Poole, Luke Johns, Nicki Tutton, and Chris Thain (Brownsea Island), the National Trust for Scotland and Forestry Commission Scotland, especially Kate Sampson, Corinna Goeckeritz, Jackie Kemp, Amy Bennett, Andy Walker, Robert Logan, and Robin (Isle of Arran) for their invaluable help in preparing and executing our field research. We also thank Mizuho Suzuki, Katy Barrett, Anna George, Rebecca Amos, Julia Coats, and Amy Fish for their assistance in the field.

Funding information

This study was supported by the Q.M. Gastmann- Wichers Foundation (to AG), the Principal's Career Development PhD Scholarship by the University of Edinburgh (to AKS), and the Scottish Government Rural and Environment Science and Analytical Services Division.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary material

Tables

Supplementary Table S1: Scoring of lesions

Points per body section*					
Points	**0**	**1**	**2**	**3**	**4**
Lesion size (mm)	None	< 2	< 5	< 10	> 10
Lesion description	None	A	B	C	D
Ulceration	None/N	T	–	–	Y
Ulcus description	None	–	Dry	Bleeding	Purulent

*To calculate the score four characteristics of lesion's present in each of the 6 body sections are assessed:

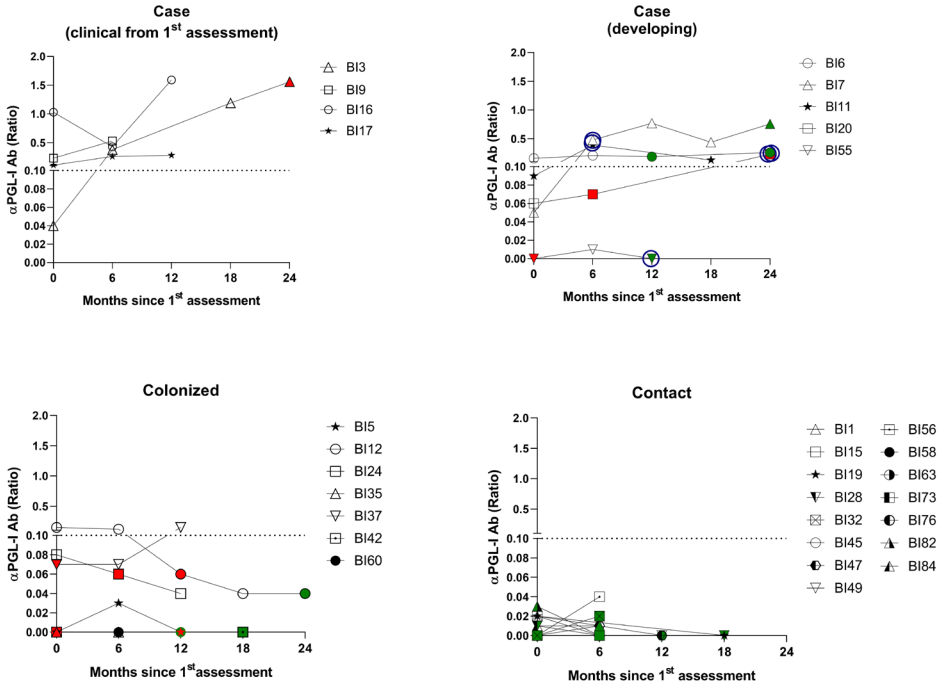
1. Lesion size (< 2, < 5, <10, > 10 mm)
2. Lesion description (*A*, one lesion with a clearly defined rim, or just alopecia; *B*, several lesions, separate with clearly defined rim; *C*, several lesions, rim not always clear/merging; *D*, cauliflower appearance due to excessive merging of several lesions)
3. Ulceration (traumatic injury or ulceration of the lesion are present or absent)
4. Ulcus description (dry, bleeding, or purulent)

The sum of the scores of all six body areas is used to calculate the total score per squirrel

Supplementary Table S2. Leprosy status definitions for Eurasian red squirrels based on clinical assessment and two diagnostic tests (anti-phenolic glycolipid-I antibody detection and PCR detection of *M.leprae* DNA)

Leprosy status	1. Clinical signs of leprosy	2. αPGL-I levels above positivity cut-off	3. *M. leprae* DNA detected in tissue
Leprosy case	Yes	Yes	Yes
	Yes	No	Yes
	Yes	Yes	No
Colonized squirrel	No	No	Yes
Leprosy contact (endemic area)	No	No/Yes	No
Leprosy suspect, further tests necessary	Yes	No	No

Figures



Supplementary Figure S1: Longitudinal anti-phenolic glycolipid-I (αPGL-I) antibody levels and presence of *M. leprae* DNA in 31 free-roaming Eurasian red squirrels (Sciurus vulgaris). Squirrels were assessed for αPGL-I levels at all time points of assessment, with a maximum of 5 timepoints. PCR was performed to determine the presence of *M. leprae* DNA for the time points indicated in red (PCR positive) or green (PCR negative) is indicated. Squirrels were divided in 4 groups; Clinical leprosy, squirrels that showed clinical signs at initial assessment (top left panel). Developing leprosy, squirrels that developed leprosy during the study, timepoint of leprosy diagnosis is indicated with a blue circle (top right panel). Colonized squirrels, colonized with M. leprae at a certain time point during the study showing no signs of leprosy (bottom left panel). Contact ERS, squirrels without symptoms of leprosy and no presence of M. leprae DNA (bottom right panel).

9 Chapter

General discussion

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Introduction

To decrease the yearly new case detection rate of leprosy patients it is vital to reduce the transmission of its causative agent, *Mycobacterium leprae*. Although the exact mode of transmission of *M. leprae* still needs to be elucidated, undiagnosed leprosy patients are believed to form a major bacterial reservoir. Unfortunately, misdiagnosis of leprosy symptoms occurs frequently, causing a delay in treatment initiation. Prompt treatment is essential as it will reduce the potential period of *M. leprae* transmission and may prevent the irreversible disabilities as a result of permanent nerve damage. Tests to facilitate diagnosis and treatment of leprosy patients are thus high on the leprosy research agenda (1) as an important tool towards zero transmission (2). This thesis focused on the development of user-friendly diagnostic tests based on the host immune response.

The studies in this thesis aimed to:

- Characterize host immune biomarkers that accurately identified both the disseminated (high bacterial load; multibacillary, MB) and self-limiting (low bacterial load; paucibacillary, PB) form of leprosy disease.
- Develop field-friendly lateral flow assays (LFAs) for application of biomarker-based diagnostic tests in various remote and resource limited, leprosy-endemic settings.
- Extend development to point-of-care (POC) application of the LFAs, using fingerstick blood as biosample.

Finally, the prospect of future implementation of this type of diagnostic tests in leprosy control activities will be discussed.

Host immune biomarkers for leprosy

Previous examinations of the host response to *M. leprae* has provided general insight into host defense and immunopathology in human infectious diseases (3). However, diagnostic application of this host response to *M. leprae* infection is still quite limited in clinical practice. This is a missed opportunity in our opinion, as the clinical outcome upon *M. leprae* infection is determined by the host response (4). It is estimated that upon exposure to *M. leprae* approximately 10% of individuals are susceptible to infection (5). Infection can result in either: I) disseminated infection (MB leprosy); II) a self-limiting form of the disease (PB leprosy) or III) asymptomatic infection. Diagnosis of leprosy patients is difficult, especially of the self-limiting form in which symptoms are less apparent. PB patients generally lack the relatively easy to detect humoral antibody response (Th2) to the *M. leprae* specific cell-wall glycolipid PGL-I as seen in MB patients (6, 7). Instead,

PB patients predominantly induce a cell-mediated immune response characterized by amongst others Th1 cytokines (3).

For tuberculosis (TB), caused by a different *Mycobacterium (M. tuberculosis),* the hallmark cytokine for Th1 responses (IFN-γ) has been implemented in a diagnostic test (i.e. Quantiferon). This interferon gamma release assay (IGRA) measures the IFN-γ production in whole blood samples after 24 hours of stimulation with specific *M. tuberculosis* antigens ESAT-6 and CFP-10. IGRA enables the identification of infected individuals, but does not distinguish latent from active infection (8, 9). Upon identification of the ESAT-6 and CFP-10 homologues in *M. leprae*, a similar test has been evaluated in leprosy patients, but with disappointing results due to cross-reactivity observed with other mycobacterial infections (10-13). Besides the mycobacteria that cause TB or leprosy, there are several other atypical mycobacterial infections (nontuberculous mycobacteria) that can cause opportunistic infections placing individuals with immune related diseases (e.g. AIDS) at increased risk. The conservation of pathways and functions across different species and the associated proteins (14) can cause a cross-reactive response in TB or leprosy diagnostic tests in individuals infected with these environmental mycobacteria. Continued search for new diagnostic antigens identified two proteins specific for the *M. leprae* genome, ML0840 and ML2478. The *in vitro* response to these antigens in leprosy patients and exposed individuals resulted in alternatives to IFN-γ i.e. IP-10 and CCL4 (15). That timepoint and status of research represented the starting point of this thesis.

IL-10, IL-1Ra, S100A12, ApoA1 and CRP were identified in this thesis as biomarkers for leprosy in addition to αPGL-I IgM, IP-10 and CCL4 (Figure 1). IL-10, S100A12 and ApoA1 identified leprosy patients irrespective of their classification, whereas αPGL-I IgM, IP-10 and CRP were useful biomarkers for MB leprosy and CCL4 and IL-1Ra for PB leprosy. These findings challenge the traditional Th1/Th2 paradigm for leprosy, as IL-10 responses (Th2) were observed in PB patients and IP-10 responses (cell-mediated immunity) in MB patients. The host immune response to *M. leprae* at the poles of the leprosy spectrum seems thus not mutually exclusive and consists of innate, adaptive cellular and humoral mediated immunity as reflected by the identified biomarkers.

Biomarkers for M. leprae infection

The identification of ApoA1, IL-10 and S100A12 as new biomarkers for both MB and PB leprosy suggest that these proteins contribute to a general response to *M. leprae* infection. Especially since levels of these biomarkers also differed for household contacts who are continuously exposed to *M. leprae,* compared to endemic controls **(Chapters 2-4 and 7).**

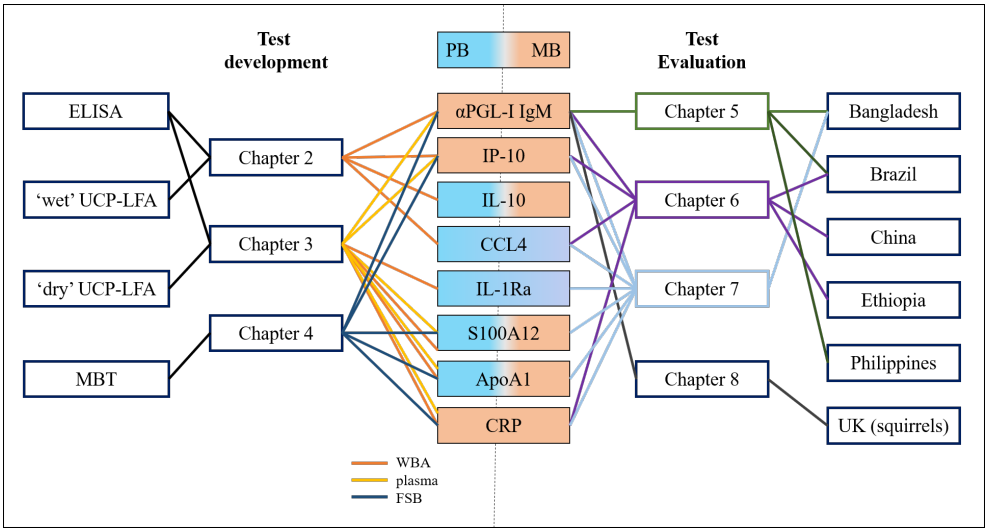

Figure 1: Summarizing overview of the thesis' chapters. The middle column indicates the biomarkers identified in this thesis and their diagnostic value for either multibacillary (MB; orange) or paucibacillary (PB; blue) leprosy patients. Chapters 2-4 describe the development of the up-converting phosphor (UCP) lateral flow assays (LFA) for leprosy diagnostics. On the left side the techniques used per chapter are indicated by the connecting lines (MBT=multi-biomarker test). The coloured lines indicate which biomarkers where first identified in whole blood assay samples (WBA; orange), plasma (yellow) or fingerstick blood (FSB; blue) in which chapter. Chapters 5-8 describe evaluation of the LFAs developed for each biomarker, the connecting lines indicated which biomarkers were tested in each chapter and in which (endemic)area.

ApoA1 and IL-10 both exert anti-inflammatory effects and decreased levels are associated with destructive chronic inflammation (16, 17). IL-10 is well-known to inhibit Th1-mediated immunity, hence the association with MB leprosy as a Th1 response is important for optimal pathogen clearance. However, both ApoA1 and IL-10 levels were similar in MB and PB leprosy patients, as well as exposed/potentially infected household contacts, suggesting that an anti-inflammatory response is initiated to dampen the immune response to *M. leprae*. Increased levels of ApoA1 have been described in relation to neuronal injury as a self-protecting mechanism to dampen the inflammatory response after injury contributing to the healing process (18). ApoA1 also affects lipid metabolism as the main protein of high density lipoprotein (HDL; involved in cholesterol transport to the liver). Dysfunctional HDL, in combination with altered ApoA1 levels has been observed in MB patients (19). Altered ApoA1 levels thus are not only a result of the altered immune response, but indicate a change in lipid metabolism as well.

The third identified biomarker for *M. leprae* infection, S100A12, has a dual role inducing both proinflammatory and antimicrobial effects by interacting with receptors such as RAGE and TLR4 (20). These pattern recognition receptors both activate the innate immune

response. RAGE expression is positively associated with disease severity and levels of proinflammatory cytokines in active TB (21). In contrast, RAGE expression is also described to be protective against the development of pulmonary TB in mouse models (22), in line with reduction of antimicrobial activity to *M. leprae* in human S100A12 knock-down-macrophages (23). S100A12 thus seems to protect exposed individuals from *M. leprae* colonization and infection. Once infected, however, S100A12 can contribute to maintain a detrimental, pro-inflammatory state in leprosy patients.

Biomarkers for MB leprosy

Biomarkers specific for either one pole of the leprosy spectrum were also identified. For MB leprosy, in addition to the extensively studied αPGL-I IgM antibody response (6, 7), IP-10 and CRP were identified and assessed in different leprosy endemic areas (Figure 1). CRP levels were predominantly increased in MB leprosy patients with high bacillary loads. This acute phase protein is produced by the liver and its level in blood can increase up to 1000-fold during inflammatory disorders (24). As a well-established biomarker for systemic disease, e.g. upon bacterial infection, CRP has many pathophysiologic roles by induction of an inflammatory response and activation of the complement system (25). The higher bacterial burden in MB patients could cause the upregulation of CRP, which is less apparent in PB patients presenting with few bacilli.

As mentioned earlier, the identification of IP-10 as a biomarker for MB leprosy is contradictory to the established Th1/Th2 paradigm, as IP-10 is induced by IFN-γ (Th1). Most studies that examined the IFN-γ response to various *M. leprae* specific antigens showed an IFN-γ response in PB patients and contacts, which was absent in MB patients (26-30). Only few studies describe the assessment of IP-10 for potential leprosy diagnostics. The IP-10 response to ML2044 (31) and ML0276 + LID-1 (26) indicated diagnostic potential for PB patients. In this thesis, IP-10 levels were shown to be elevated in MB patients, even without *M. leprae* antigen stimulation. Monocytes, keratinocytes, endothelial cells and neutrophils produce IP-10 (32) and even HIV-patients with very low CD4 T cell counts can still produce IP-10 in perhaps IFN-γ independent pathways (33). These data indicate that IP-10 can be induced without the help of Th1 cells. Increased production of IP-10 was observed in pro-inflammatory macrophages exposed to *M. leprae* before polarization (34), suggesting that *M. leprae* might predispose macrophages to increased IP-10 production. The IP-10 response in MB patients is thus most likely a result of the innate immune response to *M. leprae*.

Biomarkers for PB leprosy

The focus of explaining the immune response to *M. leprae* has been primarily on adaptive immune responses (B and T cells) in the past, but the contribution of innate immune responses to the spectral pathology of leprosy has been extensively reviewed recently (35, 36). The production of CCL4 by neutrophils (37) and monocytes (38) in PB leprosy patients and its upregulation after only 24 hours of *in vitro* stimulation with *M. leprae* whole-cell sonicate (WCS) confirm a role for innate immune responses in PB leprosy. Stratification of contacts also showed an elevated CCL4 response in households where *M. leprae* DNA was detected in slit-skin smears (a marker for infection) or where leprosy developed among household contacts (**Chapter 7**). These data indicate a role for CCL4 in the (early) immune response to *M. leprae*. CCL4 is a chemoattractant for amongst others natural killer cells and monocytes, but is also described to mediate suppression of T cells (39). The second biomarker identified specifically for PB leprosy is IL-1Ra, which can induce the production of high levels of IL-10 in macrophages (40) supporting the association of anti-inflammatory responses with PB leprosy.

The new biomarkers significantly improved the identification of PB leprosy patients and were applied successfully to LFAs targeting test applicability in remote and resource limited settings. However, though PB patients and endemic controls could be easily discriminated based on the combination of these markers, few differences in the immune response between the self-limiting form of leprosy and household contacts of leprosy patients were observed (**Chapters 2,3,6 and 7**). This indicates that a disbalance in the immune response probably causes pathogenic immunity to *M. leprae*. The interplay between innate and adaptive immunity, as well as lipid metabolism (ApoA1), in leprosy patients requires further mechanistic studies to gain more insight in the factors that contribute to the development of disease (36). Longitudinal studies are ongoing, examining in an unbiased manner both gene expression and proteomic markers in household contacts who develop leprosy during follow-up (41, 42).

Test development, from ELISA to MBT

The described biomarkers were identified and validated using multiplex bead assays and ELISAs **(Chapter 2-3)**. To enable biomarker-based diagnostics in leprosy endemic areas, often remote and with limited resources, LFAs were developed aiming at a more field-friendly test format. The lateral flow strips are composed of a sample pad, nitrocellulose membrane with immobilized antibodies and an absorbent pad. The antibodies on the nitrocellulose membrane capture the target of interest (Test line) or antibodies coupled to the reporter up-converting phosphor (UCP) nanoparticles that did not bind target

antigen (Flow Control) (43-45). At first, LFAs were developed requiring pre-incubation of UCP particles with the sample diluted in buffer in a 96-well plate before insertion of the lateral flow strip ('wet' UCP-LFA; **Chapter 2**). To decrease the assay time, in **Chapter 3** the particles were incorporated in the sample pad ('dry' UCP-LFA), thereby removing the pre-incubation step of one hour. The final stage of development was to incorporate multiple biomarkers on a single lateral flow strip, resulting in the multi-biomarker test (MBT). Compared to ELISAs, which require at least one work day to complete, the LFAs greatly reduced the assay time and eventually could be completed in less than one hour from sample addition to test result (Figure 2).

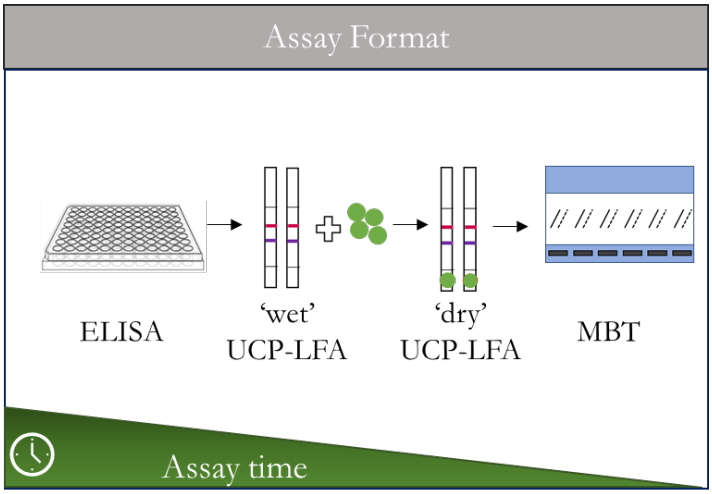

Figure 2: Overview of the assays described in this thesis

A major advantage of UCP as reporter particle is the quantitative readout, measured as the Ratio value of the fluorescence signal observed at the Test line divided by the signal at the Flow Control. This signal is determined by an operator independent reader, the up-converting reporter particle is excited with infrared light to generate a visible light. As the signal does not fade over time, strips can be stored as a permanent record allowing re-analysis at a reference lab. The Ratio values determined by the reader can be converted to concentrations if standard curves are generated, as described in **Chapter 2**, or used as a stand-alone value as described in the remainder of the chapters. Both concentrations and Ratio values determined by UCP-LFA corresponded to the data observed in ELISAs (**Chapter 2-3**), proving the quantitative ability of this assay.

The UCP-LFA format allows quick implementation of newly identified biomarkers. However, before implementation new biomarkers have to be assessed for feasibility with the UCP-LFA format. Differences between the median concentration in the patient and

control group should be large enough to in order to observe this difference in the UCP-LFA readout, ideally more than threefold (46). The quantitative aspect additionally requires the use of a measured amount of sample. Sample volume should be optimized per biomarker to match the appropriate dynamic range. Available antibody pairs may not always allow reaching the required lower limit of detection. Generally, levels below 100 pg/ml are difficult to detect reproducibly in rapid POC tests.

The incorporation of multiple biomarkers in an adapted lateral flow strip provides an ideal format regarding the spectral nature of leprosy disease. A five-biomarker signature of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 was assessed in MBT strips, showing 97% sensitivity and 75% specificity. Furthermore, application of the MBT strip allowed discrimination of MB and PB leprosy patients from control individuals in both high and low leprosy endemic areas (**Chapter 4**).

Numerous factors can influence the host response to *M. leprae*, such as the level of endemicity (15) or helminth co-infection (47), which differ per endemic area. Therefore, the developed UCP-LFAs have been evaluated in multiple endemic regions for the identified biomarkers as depicted in figure 1, confirming the diagnostic potential across studies (**Chapters 5-7**) . Moreover, in Brazil and China the tests have been performed and analyzed locally with a portable reader, indicating feasibility of the protocol in leprosy endemic areas. The UCP-LFA format thus provides an efficient format for immunodiagnostic tests for leprosy in remote and resource limited settings.

Towards point-of-care application

In order to apply the developed UCP-LFAs at the point-of-care, fingerstick blood (FSB) is the preferred biosample as it does not require the presence of a phlebotomist and is less-invasive. Therefore, the studies in this thesis worked towards the application of the UCP-LFA format with FSB, after evaluation of the developed tests in 24-hour stimulated whole-blood assays (WBA) and plasma (Figure 3).

Figure 1 shows an overview of the biosample in which the biomarkers showed diagnostic value **(Chapters 2 – 4).** CCL4, IL-10 and IL-1Ra were identified as specifically useful biomarkers in WBA. This 24-hour *in vitro* stimulation of whole blood with either *M. leprae* whole-cell sonicate (WCS) or the two earlier mentioned *M. leprae*-specific proteins ML0840 and ML2478 was performed to assess the host response to these stimuli. In addition, one vial without stimulus was included to assess the baseline production of the biomarkers in each individual, resulting in three vials per person. To add 1 ml of blood per vial and subsequently incubate the sample for 24 hours at 37 ^{0}C, laboratory equipment

is required rendering this biosample not useful for point-of-care application. However, *in vitro* stimulation showed added value in identifying PB patients (CCL4 and IL-1Ra; **Chapter 3**) and contacts prone to establish *M. leprae* infection (CCL4; **Chapter 7**). These results indicate that *in vitro* stimulation can reveal immune responses in *M. leprae*-infected individuals that improve their identification, rendering WBAs useful diagnostic tools for leprosy in settings where rapid result is not required, such as in-patient care.

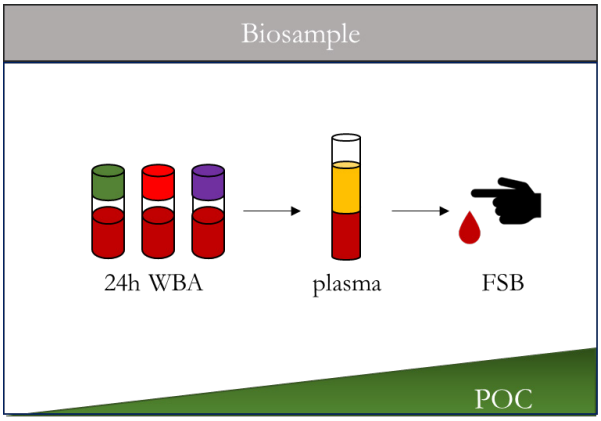

Figure 3: Biosamples described in this thesis, with increasing point-of-care (POC) applicability.

The five biomarkers that enabled identification of leprosy patients in plasma samples **(Chapter 3)** were also applicable with FSB **(Chapter 4).** For αPGL-I IgM, IP-10 and CRP a good correlation between plasma and FSB in the UCP-LFA was already observed previously (48). ApoA1 and S100A12 were additionally confirmed as biomarkers suitable for FSB applications. For accurate quantitation it is important that a measured amount of FSB is collected. Heparin-coated minivettes were used for this purpose, designed as collection devices for capillary blood. The heparin coating prevents blood clotting, enabling easy and precise application of the exact FSB volume to a specific buffer, resulting in the lysis of red blood cells. The reporter technology applied in the UCP-LFA format is not hampered by lysis of the erythrocytes (the red colour of heme groups of hemoglobin) and as a result this test format does not require a blood filter to be incorporated for FSB use. After mixing the diluted FSB was applied to the LF strip. Removing this mixing step will increase the ease of use, direct application of FSB to the LF strip in combination with chasing the sample with buffer is being examined currently. An advantage of the lysed FSB in buffer is, however, that this sample can be easily stored for later use. Currently, incorporation of the UCP-LFA in cassettes is ongoing and has been evaluated in India (in collaboration the Banaras Hindu University, Varanasi, India). This format will further improve the POC applicability, allowing near-patient testing and on site availability of test results.

Implementation of diagnostic tests in leprosy control strategies

1. Leprosy diagnosis & patient monitoring

The signs and symptoms of leprosy are often not recognized which results in delayed diagnosis, increasing the risk of severe disability if treatment is not initiated timely. Clinical diagnosis is especially difficult for those patients where *M. leprae* bacteria cannot be detected in routine slit-skin smears. The multi-biomarker signature described in this thesis (**Chapter 3**) identified leprosy patients across the leprosy spectrum and outperforms diagnosis with the well-established biomarker for leprosy (αPGL-I IgM (6, 49)). The five-biomarker signature discriminated leprosy patients from patients with other dermatological diseases (**Chapter 4**). This shows the potential of our test to aid in the (differential) diagnosis, as leprosy has clinical manifestations similar to several dermatological diseases (i.e. psoriasis, sarcoidosis, dermal leishmaniasis) (50).
In conjunction with the initial application as an adjunct diagnostic to confirm leprosy diagnosis made clinically, a diagnostic test such as developed here, can also be used to monitor treatment efficacy. Today, the duration of multidrug therapy is based on the diagnosis as MB (1 year) or PB (6 months) leprosy. Biomarker-based monitoring will allow an improved personalized treatment regimen. An optimized biomarker signature that accurately reflects the response to treatment needs to be determined and could also provide insights in the optimal treatment regimen for leprosy, which is still under debate (51).

2. Monitoring leprosy reactions

Another aspect of leprosy pathogenesis that requires adequate monitoring is the onset of acute inflammatory episodes, so-called leprosy reactions. These reactions are the main cause of permanent nerve damage, which can be prevented if treatment of these episodes is initiated timely. The potential of immune profiles and biomarker signatures to monitor (52) or even predict (53) leprosy reactions has been described previously. Of the biomarkers for which an UCP-LFA was developed in this thesis, IP-10 (52-54) and CRP (55-58) have shown to be increased during these reactional episodes. For the anti-inflammatory IL-10 an opposite pattern was observed, during reactional episodes IL-10 levels decreased (52, 54). This shift in immune response, from anti- to pro-inflammatory, indicates that longitudinal monitoring of biomarker levels in leprosy patients can aid in recognizing the onset of reactional episodes, thereby guiding the initiation of treatment to reduce the nerve-damage causing hyperinflammation. Application of additional biomarkers reflecting this shift in the host response, such as VEGF and IL-17 (52), to the

UCP-LFA should be explored further.

The IP-10 UCP-LFA has been applied to longitudinal samples of patients suffering from a leprosy reaction, clearly showing a peak response during the reactional episode (59). Currently, studies in India (in collaboration with the Molecular Biology Institute of Health-Research and Leprosy Center (SIH-R&LC), Karigiri, Vellore, Tamil Nadu, India) and Nepal (in collaboration with the Mycobacterial Research Laboratories, Anandaban Hospital, Kathmandu, Nepal) are ongoing to monitor leprosy patients longitudinally to evaluate the kinetics of the additional biomarkers identified in this thesis in patients that develop reactions. In both studies the UCP-LFA is performed locally to assess the feasibility to perform these tests in the field and POC.

3. Indication of M. leprae transmission rate

Zero transmission of *M. leprae* is necessary to achieve the elimination of leprosy, requiring population screenings to ensure that transmission in a certain area has stopped. To gain insight in the transmission rate in an endemic area, screening of children for the presence of αPGL-I antibodies provides a good proxy (60). Children are specifically targeted as *M. leprae* has a long incubation time and symptoms can take up to 20 years to appear; the presence of antibodies in children indicates per definition recent infection.

The αPGL-I IgM UCP-LFA has been used for each of the studies described in this thesis (Figure 1) and sensitively identifies MB patients. In populations where the transmission and leprosy incidence is declining, the proportion of MB leprosy cases has been shown to increase (61). These patients can be readily detected, particularly if we include the here identified markers such as IP-10, CRP and S100A12 in combination with αPGL-I IgM. The good correlation with the quantity of *M. leprae* DNA in slit-skin smears of patients, determined by qPCR (**Chapter 7**(62)), indicates that the readout of the αPGL-I IgM UCP-LFA can provide information on the bacterial burden in an individual. Antibody screening is a useful and important tool to identify those infected with sufficient bacteria, as they are more likely to transmit *M. leprae* to others. Infection with few bacilli, however, requires additional biomarkers as only 20-30% of the PB patients in the cohorts described in this thesis elicited an detectable antibody response.

4. POC test in targeted PEP strategies

Modelling studies indicate that a diagnostic test for subclinical leprosy with a sensitivity of at least 50% could already substantially reduce the *M. leprae* transmission, identifying

individuals eligible for chemo- or immunoprophylaxis after exposure to *M. leprae* (2, 63). Post-exposure prophylaxis (PEP) with BCG provides protection against leprosy in household contacts of patients and (re)vaccination of contacts is recommended in Brazil, although reports on the efficacy are conflicting (64). In the first months after BCG vaccination a relative increase in the number of PB leprosy cases among contacts was observed, suggesting that boosting the cell-mediated immune response can induce pathology in subclinically infected individuals (65). Single-dose rifampicin as PEP (SDR-PEP) has reported less adverse events and is now recommended by the WHO for leprosy prevention (66). A reduction in the incidence of leprosy of 57% among contacts was observed in the first two years after SDR (67). In a large international multi-center feasibility study implementation of SDR-PEP showed to be safe and was well accepted by the eligible individuals (68).

Identification of *M. leprae*-infected individuals eligible for SDR-PEP is therefore a useful application for a leprosy diagnostic test. Administration of SDR-PEP to neighborhood contacts based on the outcome of the αPGL-I IgM UCP-LFA as a measure for infection is currently evaluated in the PEOPLE study. This is a large-scale trial comparing different SDR-PEP modalities in Madagascar and the Comoros (69). The UCP-LFA test indicates individuals at the highest risk of transmitting the bacteria. Other biomarkers described in this thesis are being evaluated longitudinally in FSB of contacts receiving SDR-PEP to gain more insight on the direct, immunological effect of SDR-PEP in contacts.

5. Animal models and reservoirs

The applicability of the αPGL-I IgM UCP-LFA in squirrel samples (**Chapter 8**) opened the possibility to use the developed UCP-LFAs in animal models as well. The longitudinal samples of squirrels developing leprosy allowed the evaluation of αPGL-I antibody levels in a non-experimental animal model, which were recently identified as natural hosts for *M. leprae and M. lepromatosis* (70). Increasing αPGL-I antibody levels mostly coincided with the development of leprosy and correlated with disease severity in squirrels. These animal data further support the evidence that the presence of αPGL-I antibodies is a good proxy for the infection status, with high levels corresponding to severe disease (and concomitantly a higher bacterial load).

Natural infection has also been described in armadillos, which have become the primary experimental animal model for leprosy (71). The αPGL-I IgM UCP-LFA proved feasible with armadillo samples (Zhou *et al.*, in press), allowing monitoring of antibody levels during experimental studies in these animals. Currently, application of UCP-LFAs for additional

biomarkers in these animals is being examined. In non-human primates experimentally infected with *M. tuberculosis* UCP-LFAs monitoring pro-inflammatory responses, including IP-10 and CRP, have already been successfully applied showing the potential of this assay for quantitative biomarker detection in animal studies (Zhou *et al*. submitted).

Evidence for zoonotic leprosy has also been reported, humans with no contact to leprosy patients were shown to be infected with the same *M. leprae* strain as the armadillos in the area (72, 73). Besides the application in experimental models, UCP-LFAs can thus also be applied to screen animals population which can be naturally infected with *M. leprae* and live in close proximity to humans, in order to gain insight in this potential reservoir for transmission.

In summary, diagnostic tests can be implemented in various leprosy control strategies to stop *M. leprae* transmission, either supporting the early diagnosis of leprosy patients to initiate prompt treatment, identifying infected individuals for prophylactic treatment, screen populations to gain insight in the transmission rate or identify potential animal reservoirs for *M. leprae*.

Concluding remarks

In this thesis, it was demonstrated that the combination of multiple biomarkers, reflecting the diverse host response to *M. leprae*, is required to sensitively detect MB and PB leprosy patients and discriminate these from infected individuals. Quantitative UCP-LFAs were developed based on the identified biomarkers and evaluated in several leprosy endemic countries, confirming the added value of biomarker signatures. As a result, a MBT strip was developed, representing a step forward in the development of the urgently needed immunodiagnostic test for detection of *M. leprae* infection and early stage leprosy. The feasibility of this platform with FSB enables POC application, facilitating implementation in leprosy control strategies. Longitudinal proteomic and transcriptomic profiling of household contacts that develop leprosy is currently ongoing to identify biomarkers that can even predict those at risk of developing leprosy. Through the simultaneous process of identification of biomarkers and test development, quick implementation of newly identified biomarkers into the POC test format is feasible allowing custom design per purpose.

Implementation of a diagnostic tool in current leprosy control strategies might be the game-changer to break the chain of the decade-long stable *M. leprae* transmission. UCP-LFAs provide such a diagnostic tool, using sophisticated technology in a low-complexity format. These LFAs enable the assessment of biomarker signatures in low-resource

settings, both at reference centers or at the point-of-care. Applications of this multi-purpose format range from adjunct leprosy diagnostic to population screening for *M. leprae* infection. Developed LFAs in this thesis can thus contribute to early diagnosis of patients and accurate insight into the ongoing *M. leprae* transmission, which is key to reduce the number of new leprosy cases, prevent leprosy-associated disabilities and finally establish worldwide leprosy elimination.

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AIDS	acquired immunodeficiency syndrome
αPGL-I	anti-PGL-I
ApoA1	Apolipoprotein A1
BB	Mid-borderline leprosy
BCG	Bacillus Calmette-Guerin
BI	Bacteriological index
BL	Borderline lepromatous leprosy
BT	Borderline tuberculoid leprosy
CMI	Cell-mediated immunity
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
FC	Flow control
FSB	Fingerstick blood
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IgM	Immunoglobulin M
IGRA	Interferon gamma release assay
IL-1β	Interleukin 1 Beta
IL-1Ra	Interleukin 1 receptor antagonist
IL-10	Interleukin 10
IL-15	Interleukin 15
IL-17	Interleukin 17
IP-10	Interferon gamma-induced protein 10 a.k.a. C-X-C motif chemokine ligand 10 (CXCL10)
LFA	Lateral flow assay
LID-1	Leprosy IDRI diagnostic-1
LL	Lepromatous leprosy
MB	Multibacillary
MBT	Multi-biomarker test
MCP-1	Monocyte chemoattractant protein 1 a.k.a. C-C Motif Chemokine Ligand 2 (CCL2)
MDT	Multidrug therapy
MIP-1β	Macrophage inflammatory protein 1 beta a.k.a. Chemokine (C-C motif) ligands 4 (CCL4)
M. leprae	*Mycobacterium leprae*
M. lepromatosis	*Mycobacterium lepromatosis*
M. tuberculosis	*Mycobacterium tuberculosis*

NDO-LID	Conjugate of synthetic PGL-I and LID-1
NS	Nasal swabs
PB	Paucibacillary
PCR	Polymerase chain reaction
PEP	Post-exposure prophylaxis
PGL-I	Phenolic glycolipid-I
POC	Point-of-care
RAGE	Receptor for advanced glycation endproducts
RT-PCR	Reverse transcriptase PCR
SDR	Single dose rifampicin
SNP	Single nucleotide polymorphism
SSS	Slit-skin smears
S100A12	S100 calcium-binding protein A12
T-line	Test line
TB	Tuberculosis
Th1	T-helper 1
Th2	T-helper 2
Th17	T-helper 17
TLR4	Toll-like receptor 4
TST	Tuberculin skin test
TT	Tuberculoid leprosy
T1R	Type 1 reaction
T2R	Type 2 reaction
UCP	Up-converting phosphor
VEGF	Vascular endothelial growth factor
VNTR	Variable number tandem repeat
WBA	Whole blood assay
WCS	Whole cell sonicate
WHO	World Health Organization

Nederlandse samenvatting

Lepra is een infectieziekte die de perifere zenuwen aantast en kan leiden tot ernstige blijvende of irreversibele handicaps. De ziekte beïnvloedt de sociaaleconomische status van individuen, onder andere door het stigma rondom lepra. De beschikbaarheid van een effectieve remedie sinds de jaren tachtig gaf hoop voor de eliminatie van lepra. Deze multi-drug therapie (MDT), bestaande uit een combinatie van drie antibiotica, wordt in landen waar lepra voorkomt gratis aangeboden sinds ruim 20 jaar aan patiënten door de Wereldgezondheidsorganisatie, waardoor grootschalige toepassing mogelijk is. Als gevolg daarvan daalde de prevalentie van lepra enorm. Sinds 2010 wordt echter een redelijk stabiel aantal nieuwe lepragevallen van ongeveer 200.000 per jaar gerapporteerd. Dit toont aan dat het verstrekken van MDT alleen, niet voldoende is om lepra te elimineren.

Het stabiele aantal nieuwe gevallen geeft aan dat de transmissie van *Mycobacterium leprae* en *Mycobacterium lepromatosis*, de mycobacteriën die lepra veroorzaken, nog steeds plaats vindt. Nog niet alles is bekend over de overdracht van deze mycobacteriën, maar onbehandelde patiënten worden als een belangrijke besmettingsbron beschouwd. Tijdige diagnose van deze patiënten is daarom van vitaal belang, zodat het tijdsbestek waarin een persoon besmettelijk is wordt verkort. Bovendien kan behandeling in een vroeg stadium van lepra onomkeerbare zenuwbeschadiging en lepra-geassocieerde handicaps voorkomen. Hulpmiddelen die de diagnose lepra bevestigen en/of *M. leprae* geïnfecteerde personen in een preklinisch stadium identificeren kunnen, vooral indien deze goedkoop en makkelijk te gebruiken zijn, een bijdrage leveren aan het (tijdig) starten van een therapeutische of profylactische behandeling. Echter zijn deze nog niet beschikbaar. Dit proefschrift richt zich daarom op de ontwikkeling van gebruiksvriendelijke diagnostische testen voor lepra.

De hoofdstukken 2-4 richten zich op het identificeren van biomarkers en de ontwikkeling van diagnostische testen om de detectie van leprapatiënten te verbeteren. De klinische symptomen van lepra hebben een spectraal karakter en worden beïnvloed door de immuunrespons van de gastheer. Multibacillaire (MB) patiënten induceren voornamelijk een antilichaam-gemedieerde respons (humoraal), terwijl paucibacillaire (PB) patiënten een T-helper 1-gemedieerde immuunrespons (cellulair) induceren, waardoor de bacteriële groei beter kan worden beperkt. De diversiteit aan immuun responsen binnen het lepra spectrum vereist de detectie van zowel cellulaire als humorale biomarkers in een diagnostische test. Om biomarkers te identificeren zijn zowel ELISAs als multiplex-bead-assays uitgevoerd voor 71 verschillende cytokines, chemokines en groeifactoren (hoofdstuk 2-3). Acht biomarkers (ApoA1, CCL4, CRP, IL-1Ra, IL-10, IP-10, αPGL-I IgM,

S100A12) lieten in meerdere onafhankelijke cohorten uit Bangladesh een goed onderscheid zien tussen lepra patiënten en gezonde controles uit dezelfde regio.

De geïdentificeerde biomarkers zijn vervolgens geïmplementeerd in up-converting phoshor (UCP) lateral flow assays (LFA) (hoofdstuk 2-3). Deze kwantitatieve testen vereisen weinig apparatuur en kunnen resultaat geven binnen een uur. Dit maakt deze testen uitermate geschikt voor lepra endemische gebieden die vaak afgelegen zijn en beschikken over beperkte middelen. De LFAs waren net zo goed in staat om patiënten van gezonde controles te onderscheiden als de ELISA, met het voordeel dat de test point-of-care kan worden uitgevoerd. In hoofdstuk 4 is vervolgens een multi-biomarker test (MBT) strip ontwikkeld, waarbij tot zes verschillende biomarkers op een enkele strip tegelijk kunnen worden gemeten. Een biomarker signature bestaande uit ApoA1, CRP, IP-10, αPGL-I IgM en S100A12 geïmplementeerd op de MBT strip identificeerde zowel de MB als PB patiënten en biedt daarmee de mogelijkheid om met een enkele test patiënten aan beide kanten van het spectrum te identificeren. Daarnaast is de MBT strip ook te gebruiken met vingerprik bloed als biologisch sample, wat de point-of-care toepasbaarheid nog verder vergroot.

In de hoofdstukken 5-7 zijn de ontwikkelde UCP-LFAs verder geëvalueerd. In hoofdstuk 5 zijn twee verschillende testen die anti-PGL-I-antilichamen detecteren met elkaar vergeleken: de kwantitatieve αPGL-I IgM UCP-LFA geproduceerd in het LUMC en een kwalitatieve test waarbij NDO-LID als doelwitantigeen werd gebruikt (een conjugaat van zowel het PGL-I als het LID-1 antigen). PGL-I is een glycolipid dat specifiek voorkomt in de celwand van *M. leprae*, antilichaam titers zijn daarom vooral hoog in MB patiënten. Beide testen zijn geëvalueerd in samples uit drie endemische lepragebieden (Bangladesh, Brazilië en de Filippijnen) en lieten inderdaad zien dat voornamelijk patiënten met een hoge bacteriële index goed geïdentificeerd konden worden, waarbij de αPGL-I IgM UCP-LFA de hoogste sensitiviteit liet zien.

In hoofdstuk 6 is de αPGL-I IgM UCP-LFA in combinatie met de LFAs voor IP-10, CRP en CCL4 verder geëvalueerd om te bepalen of deze biomarkers ook diagnostische waarde hadden voor lepra patiënten in Brazilië, China en Ethiopië. Ongeacht de mate van endemiciteit verbeterde de combinatie van meerdere LFAs de identificatie (sensitiviteit) van PB patiënten met 50% of meer. Dit laat zien dat de eerder geïdentificeerde biomarker signatures ook toepasbaar zijn in andere endemische gebieden dan Bangladesh.

De isolatie van *M. leprae* DNA uit nasal swabs en slit-skin smears van zowel lepra patiënten met een hoge bacteriële index en hun contacten gaf de mogelijkheid om de relatie tussen de geïdentificeerde biomarkers in hoofdstuk 2-3 en *M. leprae* kolonisatie (*M. leprae* DNA

aanwezig in nasal swabs), *M. leprae* infectie (*M. leprae* DNA aanwezig in slit-skin smears) en lepra diagnose in contacten te onderzoeken (hoofdstuk 7). De contacten van de MB patiënten hadden significant hogere ApoA1- en S100A12-niveaus dan endemische controles, vergelijkbaar met PB patiënten. Huishoudens waar *M. leprae* infectie en lepra niet voorkwamen werden gekenmerkt door hogere S100A12- en lagere CCL4-waarden. Behalve het aantonen van PB lepra, zijn de biomarkers geïdentificeerd in deze thesis dus ook nuttig voor de screening van contacten op *M. leprae* infectie.

Tot slot is in hoofdstuk 8 de toepasbaarheid van de anti-PGL-I IgM UCP-LFA voor diagnostiek bij rode eekhoorns onderzocht. Recent is ontdekt dat sommige rode eekhoorns op de Britse eilanden geïnfecteerd waren met *M. leprae* of *M. lepromatosis*, terwijl lepra al eeuwenlang niet meer voorkomt in dat gebied. Eekhoorns van deze populatie werden gevolgd in de tijd en gescreend op tekenen en symptomen van lepra en de aanwezigheid van *M. leprae* DNA. Eekhoorns met klinische symptomen vertoonden significant meer anti-PGL-I-antilichamen dan gezonde dieren of subklinisch geïnfecteerde dieren. De anti-PGL-I IgM UCP-LFA kan bijdragen aan de diagnose bij eekhoorns door deze serologisch te bevestigen en heeft dus potentie om ook bij andere potentiele dierlijke reservoirs, zoals armadillo's, *M. leprae* infectie op te sporen.

Concluderend, de implementatie van een diagnostische test als hulpmiddel in de huidige strategieën voor het elimineren van lepra zou de gamechanger kunnen zijn om de keten van stabiele *M. leprae*-transmissie te doorbreken. UCP-LFAs bieden de mogelijkheid om biomarker signatures te bepalen in omgevingen met weinig middelen, zowel in referentiecentra als point-of-care. Toepassingen van dit multifunctionele format variëren van aanvullende lepradiagnostiek tot populatiescreening op *M. leprae*-infectie. De in dit proefschrift ontwikkelde LFAs kunnen bijdragen aan een tijdige diagnose van patiënten en geven een nauwkeurig inzicht in de aanhoudende transmissie van *M. leprae*. Dit is essentieel om het aantal nieuwe lepragevallen te verminderen, lepra-geassocieerde handicaps te voorkomen en uiteindelijk wereldwijde eliminatie van lepra tot stand te brengen.

Dankwoord

De vraag wanneer ga je nu eigenlijk promoveren is mij de afgelopen jaren meerdere malen gesteld, maar na zes jaar is het eindelijk zo ver. Bij deze wil ik graag iedereen bedanken die eraan heeft bijgedragen dat dit proefschrift nu voor jullie ligt.

Allereerst Annemieke, bedankt dat je mij de kans hebt gegeven om promotieonderzoek in jouw groep te gaan doen aansluitend aan mijn afstudeerstage. Van jou kreeg ik de mogelijkheid om een aantal buitenlandse labs te bezoeken, waardoor ik verschillende culturen heb leren kennen. Tijdens de lange vliegreizen hadden we altijd voldoende tijd om bij te praten over alle lopende projecten. Bedankt voor alle feedback en begeleiding de afgelopen jaren.

Susan en Mariateresa, als mijn begeleiders op het lab tijdens mijn stage hebben jullie mij mede enthousiast gemaakt voor dit onderzoek. Naast professionele begeleiding was het ook altijd mogelijk om jullie om advies te vragen en kon ik altijd bij jullie terecht. Daarnaast zorgden jullie voor een gezellige sfeer op kantoor door het organiseren van activiteiten met collega's.

Maria, naast een fijne mede-Phd waarmee ik alles kon bespreken is ook een vriendschap ontstaan, waardoor je zelfs een keer in Roosendaal carnaval met me bent komen vieren. Jouw kijk op dingen heeft mij vaak aan het denken gezet en jouw werk aan de database heeft ook veel voor mijn onderzoek opgeleverd.

Krista, al zes jaar zitten we naast elkaar op kantoor en daarin hebben we veel besproken. Bedankt voor het luisteren en de goede adviezen, maar bovenal de gezelligheid. Samen met Annelies heb je daarnaast tijdens de coronapandemie ook heel veel werk eraan gehad om ervoor te zorgen dat er genoeg voorraad was om experimenten te blijven doen, mijn waardering voor jullie allebei daarvoor.

Paul, bedankt dat ik de kans had om met jouw groep samen te werken en zelfs een plek bij jullie op kantoor kreeg, zodat ik niet iedere keer tussen gebouw 1 en 2 heen en weer hoefde te lopen. Elisa, bedankt dat je me alle praktische vaardigheden hebt geleerd voor het maken van de lateral flow strips. Ik heb niet durven tellen hoeveel strips, MBTs en buffer we in al die jaren samen hebben gemaakt. Bedankt dat jij, Danielle en Claudia me altijd welkom hebben laten voelen bij jullie op het lab.

Louis, jij straalde zoveel rust uit dat ik vanzelf wat relaxter werd. Na je pensioen nam Els het stokje over voor de lepradiagnostiek, allebei bedankt voor de fijne samenwerking.

Matthias, Frank, Teresa, Emile, bedankt voor het onvergetelijke PhD retreat en de vele PhD dinners. Met veel plezier heb ik ook een aantal jaar met de FECO de labuitjes en borrels georganiseerd, samen met Kimberley, Anne, Michella, Jonathan, Arthur en Miriam. Cassandra, Calinda, Suzanne, Paula, Eleonora en alle andere collega's die in de loop der jaren op K5, maar ook E5 hebben gewerkt, bedankt voor het bijdragen aan de fijne werksfeer.

Els, Jolijn, Lea, Lonneke, Maaike, Randall en Rosita, bedankt dat jullie je tijdens je stage hebben ingezet voor het onderzoek in onze groep. Het begeleiden van jullie heeft ook mij veel geleerd. Louise, als student was het al fijn samen werken met jou en dat is nu je PhD student bent nog steeds zo.

Liesbeth en Ingrid, bedankt voor de secretariële ondersteuning en het plaatsen van de vele bestellingen. Kees, bedankt voor het produceren van de *M. leprae* specifieke eiwitten.

Belangrijk voor de totstandkoming van mijn proefschrift zijn de vele internationale samenwerkingen. In het bijzonder wil ik iedereen in Bangladesh, Brazilië, China en Nepal bedanken voor hun gastvrijheid. Vooral de collega's in Bangladesh, Khorshed, Marufa, Santosh, dr. Sufian, Johan en de gehele field-staff, ben ik erg dankbaar. Door de grote hoeveelheid samples die jullie verzamelen heb ik een groot deel van mijn onderzoek kunnen uitvoeren. Jan Hendrik Richardus en Renate Verbiest-Richardus, wil ik ook bedanken voor hun bijdrage aan de projecten in Bangladesh.

Het allerbelangrijkst zijn natuurlijk mijn ouders, opa's en oma's, zus en man. Zij zijn de stabiele basis waar ik altijd op terug kan vallen. Erwin en Nicole, dankjewel dat jullie mijn keuzes altijd hebben ondersteund. Al van jongs af aan zijn de nodige peptalks en adviezen gegeven, waardoor ik dit uiteindelijk heb kunnen bereiken. Mijn opa's en oma's wil ik bedanken voor hun onvoorwaardelijke trots, opa Piet in het bijzonder. Evi, jij hebt een drive waar ik niet aan kan tippen en een sportmentaliteit waar ik nog steeds van leer.

Roel, tijdens mijn studie en promotietraject heb jij alle ups en downs meegekregen en hebben we de nodige discussies gevoerd. Bedankt dat je er altijd voor me was, menigmaal op de rem hebt getrapt en ervoor hebt gezorgd dat ik voldoende tijd nam voor de nodige ontspanning met vrienden en familie. We zijn al die tijd een goed team geweest en ik hoop dat we dat altijd zullen blijven.

Curriculum Vitae

Anouk van Hooij was born on September 29, 1992 in Roosendaal. In 2004, she started high school at the Norbertus college in Roosendaal and obtained her gymnasium diploma in 2010. In the same year she started the bachelor biomedical sciences at the University of Amsterdam. During her bachelor internship at the departments of Immunopathology and Haematopoiesis at Sanquin, Amsterdam, she developed her interest in immunological research. Continuing with the master biomedical sciences in 2013, she took a small detour from this research topic by performing her first internship at the department of experimental cardiology at the Academic Medical Center in Amsterdam. For her second internship at the department of Infectious Diseases at the Leiden University Medical Center (LUMC) she returned to her favourite topic. In the group of prof. dr. Annemieke Geluk she worked on different projects studying host immune responses to *Mycobacterium leprae* and *Mycobacterium tuberculosis*, the causative agents of leprosy and tuberculosis. After graduating in 2015, she had the opportunity to continue and expand this research as a PhD student at the same department. Her PhD project comprised an immunological approach to identify tools for early detection of leprosy with a focus on the development of immunodiagnostic tests for leprosy and tuberculosis. This included research in collaboration with the group of dr. ir. Paul Corstjens at the department of Cell and Chemical Biology. The results of the studies on leprosy are described in this thesis. After finalizing her thesis, Anouk will continue her work in the Geluk group at the LUMC as post-doctoral researcher in leprosy immunology and diagnostic test development.

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