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

Hooij, A. van

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).

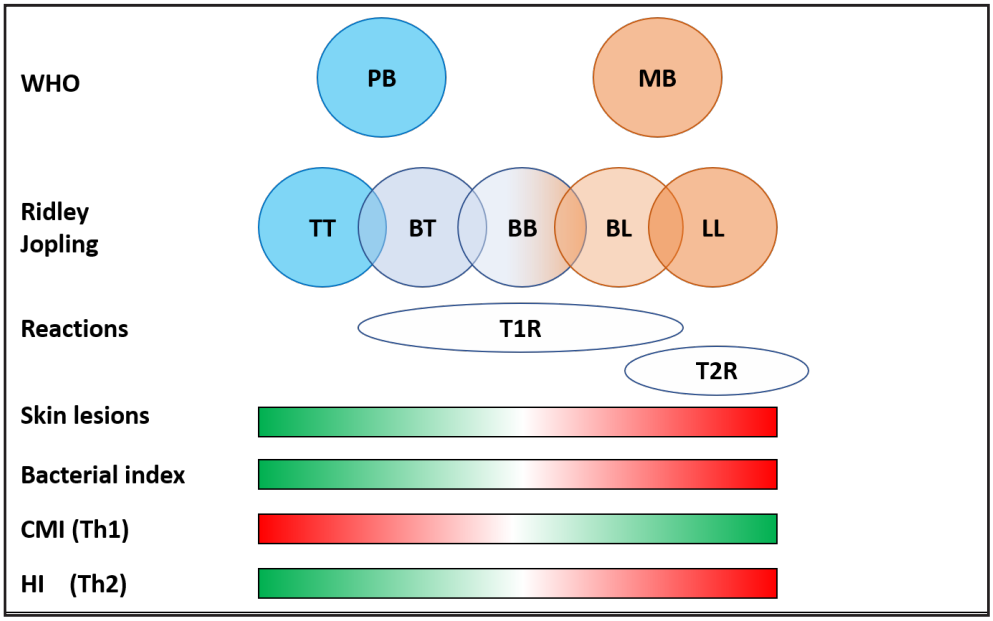

Figure 1: The leprosy spectrum. Leprosy is a spectral disease ranging from patients with few lesions, low bacterial loads and strong Th1-cell-mediated responses (Cell-mediated immunity (CMI)) to patients with multiple lesions, high bacterial loads and predominantly Th2 antibody responses (Humoral immunity (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.

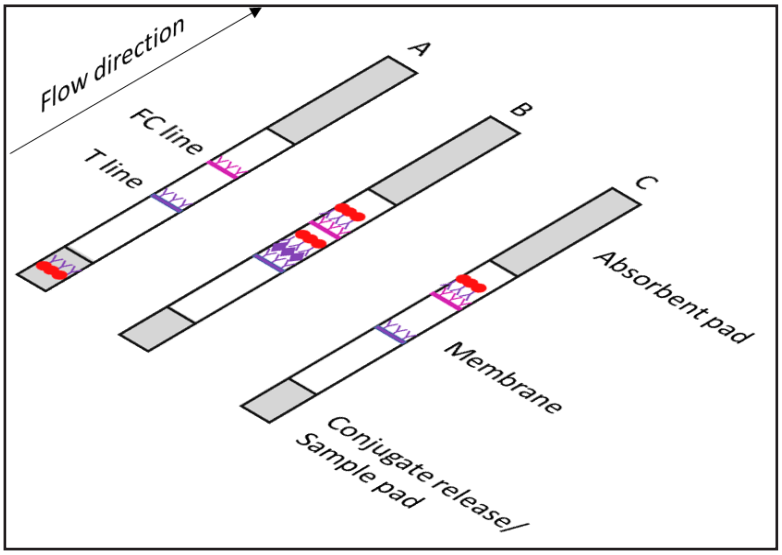

Figure 2: Schematic representation of lateral flow assays. Lateral flow assays (LFAs) generally consist of a sample pad, conjugate release pad, membrane and absorbent pad. On the sample pad/conjugate release pad colored or fluorescent particles conjugated (red dot) to an antibody (Y) specific for the 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.

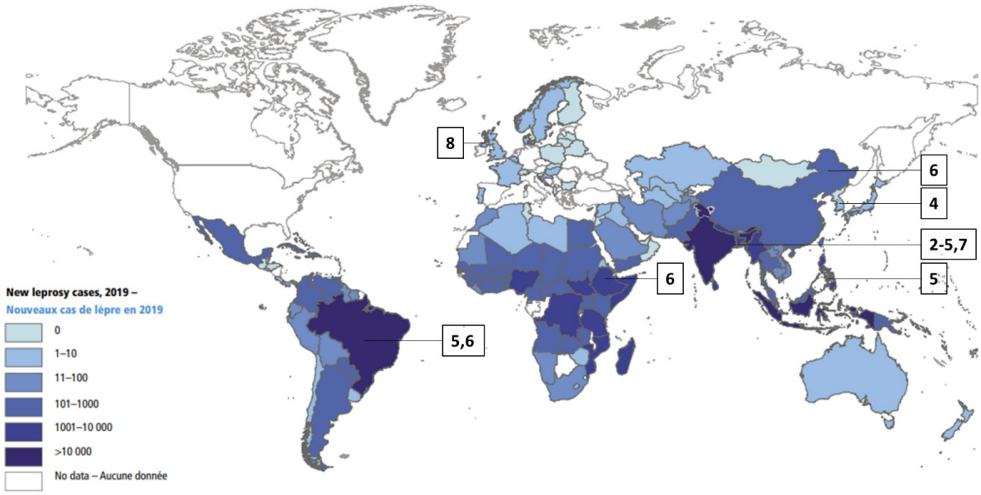

Figure 3: Geographical distribution of cohorts tested in this thesis and the corresponding number of new leprosy cases in 2019. Indication per chapter from which country the samples originated and the corresponding number of new cases reported per country. Bangladesh (chapter 2-5,7), Brazil (chapter 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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