Universiteit
Leiden
The Netherlands

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

Hooij, A. van

**Citation**
Hooij, A. van. (2021, November 17). *Development of immunodiagnostic tests for leprosy: from biomarker discovery to application in endemic areas*. Retrieved from https://hdl.handle.net/1887/3240171

| | |
|---|---|
| Version: | Publisher's Version |
| License: | Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden |
| Downloaded from: | https://hdl.handle.net/1887/3240171 |

**Note:** To cite this publication please use the final published version (if applicable).

# 9 Chapter

# General discussion

Anouk van Hooij[1]

[1]*Dept. of Infectious Diseases, Leiden University Medical Center, The Netherlands;*

## 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).**

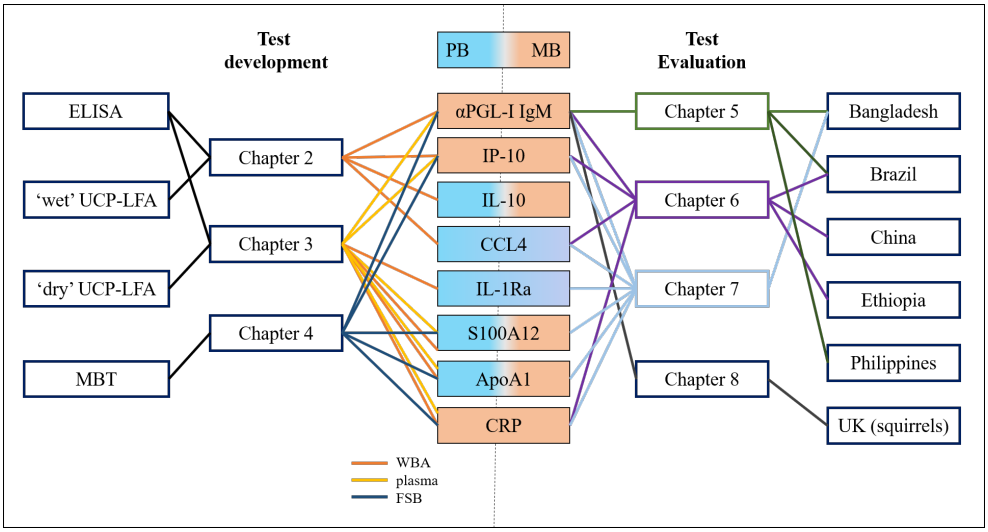


**Figure 1: Summarizing overview of the thesis' chapters.** The middle column indicates the biomarkers identified in this thesis and their diagnostic value for either multibacillary (MB; orange) or paucibacillary (PB; blue) leprosy patients. Chapters 2-4 describe the development of the up-converting 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).

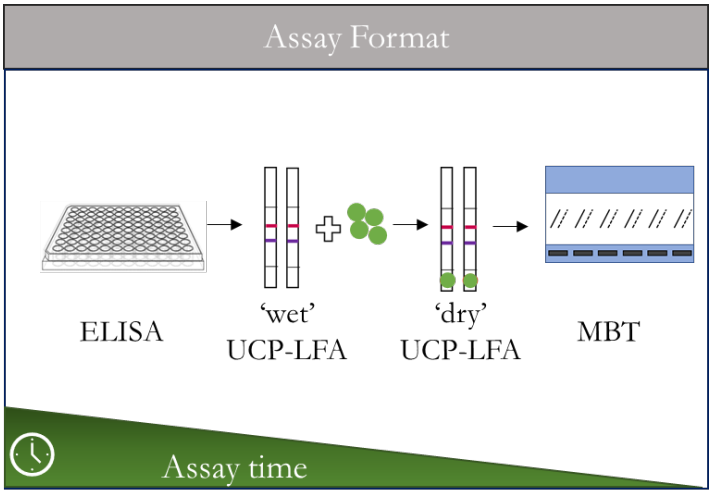


**Figure 2: Overview of the assays described in this thesis**

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.

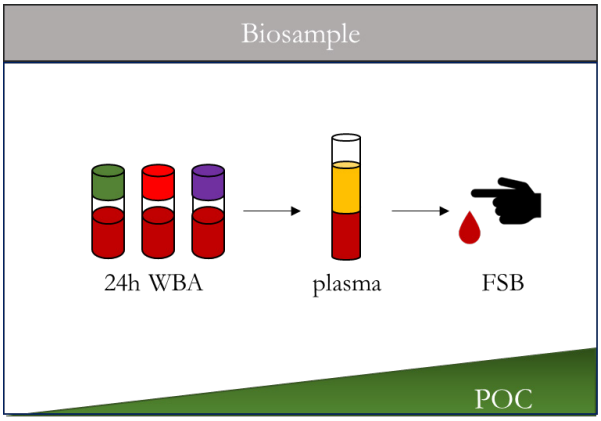


**Figure 3: Biosamples described in this thesis, with increasing point-of-care (POC) applicability.**

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.

## References


1. GPZL Gpfzl, Decatur, Georgia USA. GPZL reports on research priorities. Lepr Rev. 2019;90:237-89.
2. Blok DJ, de Vlas SJ, Geluk A, Richardus JH. Minimum requirements and optimal testing strategies of a diagnostic test for leprosy as a tool towards zero transmission: A modeling study. PLoS Negl Trop Dis. 2018;12(5):e0006529.
3. Montoya D, Modlin RL. Learning from leprosy: insight into the human innate immune response. Adv Immunol. 2010;105:1-24.
4. Fava VM, Dallmann-Sauer M, Schurr E. Genetics of leprosy: today and beyond. Hum Genet. 2019.
5. Joyce MP. Historic aspects of human susceptibility to leprosy and the risk of conjugal transmission. Mem Inst Oswaldo Cruz. 2012;107 Suppl 1:17-21.
6. Gurung P, Gomes CM, Vernal S, Leeflang MMG. Diagnostic accuracy of tests for leprosy: a systematic review and meta-analysis. Clin Microbiol Infect. 2019;25(11):1315-27.
7. Penna ML, Penna GO, Iglesias PC, Natal S, Rodrigues LC. Anti-PGL-1 Positivity as a Risk Marker for the Development of Leprosy among Contacts of Leprosy Cases: Systematic Review and Meta-analysis. PLoS Negl Trop Dis. 2016;10(5):e0004703.
8. Matteelli A, Sulis G, Capone S, D'Ambrosio L, Migliori GB, Getahun H. Tuberculosis elimination and the challenge of latent tuberculosis. Presse Med. 2017;46(2 Pt 2):e13-e21.
9. Kwiatkowska S. The IGRA tests: where are we now? Pneumonol Alergol Pol. 2015;83(2):95-7.
10. Spencer JS, Marques MA, Lima MC, Junqueira-Kipnis AP, Gregory BC, Truman RW, et al. Antigenic specificity of the Mycobacterium leprae homologue of ESAT-6. Infect Immun. 2002;70(2):1010-3.
11. Geluk A, van Meijgaarden KE, Franken KL, Subronto YW, Wieles B, Arend SM, et al. Identification and characterization of the ESAT-6 homologue of Mycobacterium leprae and T-cell cross-reactivity with Mycobacterium tuberculosis. Infect Immun. 2002;70(5):2544-8.
12. Geluk A, van Meijgaarden KE, Franken KL, Wieles B, Arend SM, Faber WR, et al. Immunological crossreactivity of the Mycobacterium leprae CFP-10 with its homologue in Mycobacterium tuberculosis. Scand J Immunol. 2004;59(1):66-70.
13. Spencer JS, Kim HJ, Marques AM, Gonzalez-Juarerro M, Lima MC, Vissa VD, et al. Comparative analysis of B- and T-cell epitopes of Mycobacterium leprae and Mycobacterium tuberculosis culture filtrate protein 10. Infect Immun. 2004;72(6):3161-70.
14. Malhotra S, Vedithi SC, Blundell TL. Decoding the similarities and differences among mycobacterial species. PLoS Negl Trop Dis. 2017;11(8):e0005883.
15. Geluk A, Bobosha K, van der Ploeg-van Schip JJ, Spencer JS, Banu S, Martins MV, et al. New biomarkers with relevance to leprosy diagnosis applicable in areas hyperendemic for leprosy. J Immunol. 2012;188(10):4782-91.
16. Georgila K, Vyrla D, Drakos E. Apolipoprotein A-I (ApoA-I), Immunity, Inflammation and Cancer. Cancers (Basel). 2019;11(8).
17. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. J Immunol. 2008;180(9):5771-7.
18. Sengupta MB, Saha S, Mohanty PK, Mukhopadhyay KK, Mukhopadhyay D. Increased expression of ApoA1 after neuronal injury may be beneficial for healing. Mol Cell Biochem. 2017;424(1-2):45-55.
19. Lemes RMR, Silva C, Marques MÂ M, Atella GC, Nery J, Nogueira MRS, et al. Altered composition and functional profile of high-density lipoprotein in leprosy patients. PLoS Negl Trop Dis. 2020;14(3):e0008138.
20. Bagheri V. S100A12: Friend or foe in pulmonary tuberculosis? Cytokine. 2017;92:80-2.

21. Lui G, Wong CK, Ip M, Chu YJ, Yung IM, Cheung CS, et al. HMGB1/RAGE Signaling and Pro-Inflammatory Cytokine Responses in Non-HIV Adults with Active Pulmonary Tuberculosis. PLoS One. 2016;11(7):e0159132.
22. van Zoelen MA, Wieland CW, van der Windt GJ, Florquin S, Nawroth PP, Bierhaus A, et al. Receptor for advanced glycation end products is protective during murine tuberculosis. Mol Immunol. 2012;52(3-4):183-9.
23. Realegeno S, Kelly-Scumpia KM, Dang AT, Lu J, Teles R, Liu PT, et al. S100A12 Is Part of the Antimicrobial Network against Mycobacterium leprae in Human Macrophages. PLoS Pathog. 2016;12(6):e1005705.
24. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med. 1999;340(6):448-54.
25. Oved K, Cohen A, Boico O, Navon R, Friedman T, Etshtein L, et al. A novel host-proteome signature for distinguishing between acute bacterial and viral infections. PLoS One. 2015;10(3):e0120012.
26. Hungria EM, Freitas AA, Pontes MA, Goncalves HS, Sousa AL, Costa MB, et al. Antigen-specific secretion of IFNgamma and CXCL10 in whole blood assay detects Mycobacterium leprae infection but does not discriminate asymptomatic infection from symptomatic leprosy. Diagn Microbiol Infect Dis. 2017;87(4):328-34.
27. Freitas AA, Hungria EM, Costa MB, Sousa AL, Castilho ML, Gonçalves HS, et al. Application of Mycobacterium Leprae-specific cellular and serological tests for the differential diagnosis of leprosy from confounding dermatoses. Diagn Microbiol Infect Dis. 2016;86(2):163-8.
28. Bezerra-Santos M, do Vale-Simon M, Barreto AS, Cazzaniga RA, de Oliveira DT, Barrios MR, et al. Mycobacterium leprae Recombinant Antigen Induces High Expression of Multifunction T Lymphocytes and Is Promising as a Specific Vaccine for Leprosy. Front Immunol. 2018;9:2920.
29. Sampaio LH, Stefani MM, Oliveira RM, Sousa AL, Ireton GC, Reed SG, et al. Immunologically reactive M. leprae antigens with relevance to diagnosis and vaccine development. BMC Infect Dis. 2011;11:26.
30. Oliveira RM, Hungria EM, de Araujo Freitas A, de Sousa AL, Costa MB, Reed SG, et al. Synergistic antigen combinations for the development of interferon gamma release assays for paucibacillary leprosy. Eur J Clin Microbiol Infect Dis. 2014;33(8):1415-24.
31. Chen X, You YG, Yuan YH, Yuan LC, Wen Y. Host immune responses induced by specific Mycobacterium leprae antigens in an overnight whole-blood assay correlate with the diagnosis of paucibacillary leprosy patients in China. PLoS Negl Trop Dis. 2019;13(4):e0007318.
32. Cassatella MA, Gasperini S, Calzetti F, Bertagnin A, Luster AD, McDonald PP. Regulated production of the interferon-gamma-inducible protein-10 (IP-10) chemokine by human neutrophils. Eur J Immunol. 1997;27(1):111-5.
33. Aabye MG, Ruhwald M, Praygod G, Jeremiah K, Faurholt-Jepsen M, Faurholt-Jepsen D, et al. Potential of interferon-γ-inducible protein 10 in improving tuberculosis diagnosis in HIV-infected patients. Eur Respir J. 2010;36(6):1488-90.
34. Fallows D, Peixoto B, Kaplan G, Manca C. Mycobacterium leprae alters classical activation of human monocytes in vitro. J Inflamm (Lond). 2016;13:8.
35. Pinheiro RO, Schmitz V, Silva BJA, Dias AA, de Souza BJ, de Mattos Barbosa MG, et al. Innate Immune Responses in Leprosy. Front Immunol. 2018;9:518.
36. van Hooij A, Geluk A. In search of biomarkers for leprosy by unraveling the host immune response to Mycobacterium leprae. Immunol Rev. 2021.
37. Pacheco FS, Prata R, Brandão SS, Ferreira H, Rodrigues TF, Brandão Dos Santos J, et al. Erythema Nodosum Leprosum Neutrophil Subset Expressing IL-10R1 Transmigrates into Skin Lesions and Responds to IL-10. Immunohorizons. 2020;4(2):47-56.
38. Guerreiro LT, Robottom-Ferreira AB, Ribeiro-Alves M, Toledo-Pinto TG, Rosa Brito T, Rosa PS, et al. Gene expression profiling specifies chemokine, mitochondrial and lipid metabolism signatures in leprosy. PLoS One. 2013;8(6):e64748.
39. Joosten SA, van Meijgaarden KE, Savage ND, de Boer T, Triebel F, van der Wal A, et al. Identification of a human CD8+ regulatory T cell subset that mediates suppression through the chemokine CC chemokine ligand 4. Proc Natl Acad Sci U S A. 2007;104(19):8029-34.
40. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. Front Immunol. 2014;5:491.
41. Richardus RA, Alam K, Pahan D, Feenstra SG, Geluk A, Richardus JH. The combined effect of chemoprophylaxis with single dose rifampicin and immunoprophylaxis with BCG to prevent leprosy in contacts of newly diagnosed leprosy cases: a cluster randomized controlled trial (MALTALEP study). BMC Infect Dis. 2013;13:456.
42. Tió-Coma M, Kiełbasa SM, van den Eeden SJF, Mei H, Roy JC, Wallinga J, et al. Blood RNA signature RISK4LEP predicts leprosy years before clinical onset. EBioMedicine. 2021;68:103379.

43. Corstjens P, Zuiderwijk M, Brink A, Li S, Feindt H, Niedbala RS, et al. Use of up-converting phosphor reporters in lateral-flow assays to detect specific nucleic acid sequences: a rapid, sensitive DNA test to identify human papillomavirus type 16 infection. Clin Chem. 2001;47(10):1885-93.
44. Corstjens PL, Chen Z, Zuiderwijk M, Bau HH, Abrams WR, Malamud D, et al. Rapid assay format for multiplex detection of humoral immune responses to infectious disease pathogens (HIV, HCV, and TB). Ann N Y Acad Sci. 2007;1098:437-45.
45. Corstjens PL, Li S, Zuiderwijk M, Kardos K, Abrams WR, Niedbala RS, et al. Infrared up-converting phosphors for bioassays. IEE Proc Nanobiotechnol. 2005;152(2):64-72.
46. Corstjens PL, Tjon Kon Fat EM, de Dood CJ, van der Ploeg-van Schip JJ, Franken KL, Chegou NN, et al. Multi-center evaluation of a user-friendly lateral flow assay to determine IP-10 and CCL4 levels in blood of TB and non-TB cases in Africa. Clin Biochem. 2016;49(1-2):22-31.
47. Hagge DA, Parajuli P, Kunwar CB, Rana D, Thapa R, Neupane KD, et al. Opening a Can of Worms: Leprosy Reactions and Complicit Soil-Transmitted Helminths. EBioMedicine. 2017;23:119-24.
48. Corstjens P, van Hooij A, Tjon Kon Fat EM, Alam K, Vrolijk LB, Dlamini S, et al. Fingerstick test quantifying humoral and cellular biomarkers indicative for M. leprae infection. Clin Biochem. 2019.
49. Espinosa OA, Benevides Ferreira SM, Longhi Palacio FG, Cortela D, Ignotti E. Accuracy of Enzyme-Linked Immunosorbent Assays (ELISAs) in Detecting Antibodies against Mycobacterium leprae in Leprosy Patients: A Systematic Review and Meta-Analysis. Can J Infect Dis Med Microbiol. 2018;2018:9828023.
50. Kundakci N, Erdem C. Leprosy: A great imitator. Clin Dermatol. 2019;37(3):200-12.
51. Scollard DM. Leprosy treatment: Can we replace opinions with research? PLoS Negl Trop Dis. 2020;14(10):e0008636.
52. Khadge S, Banu S, Bobosha K, van der Ploeg-van Schip JJ, Goulart IM, Thapa P, et al. Longitudinal immune profiles in type 1 leprosy reactions in Bangladesh, Brazil, Ethiopia and Nepal. BMC Infect Dis. 2015;15:477.
53. Tió-Coma M, van Hooij A, Bobosha K, van der Ploeg-van Schip JJ, Banu S, Khadge S, et al. Whole blood RNA signatures in leprosy patients identify reversal reactions before clinical onset: a prospective, multicenter study. Sci Rep. 2019;9(1):17931.
54. Geluk A, van Meijgaarden KE, Wilson L, Bobosha K, van der Ploeg-van Schip JJ, van den Eeden SJ, et al. Longitudinal immune responses and gene expression profiles in type 1 leprosy reactions. J Clin Immunol. 2014;34(2):245-55.
55. Memon RA, Hussain R, Raynes JG, Lateff A, Chiang TJ. Alterations in serum lipids in lepromatous leprosy patients with and without ENL reactions and their relationship to acute phase proteins. Int J Lepr Other Mycobact Dis. 1996;64(2):115-22.
56. Silva EA, Iyer A, Ura S, Lauris JR, Naafs B, Das PK, et al. Utility of measuring serum levels of anti-PGL-I antibody, neopterin and C-reactive protein in monitoring leprosy patients during multi-drug treatment and reactions. Trop Med Int Health. 2007;12(12):1450-8.
57. Sehgal VN, Bhattacharya SN, Shah Y, Sharma VK, Gupta CK. Reaction in leprosy: acute phase reactant response during and after remission. Int J Dermatol. 1992;31(9):632-4.
58. Hussain R, Lucas SB, Kifayet A, Jamil S, Raynes J, Uqaili Z, et al. Clinical and histological discrepancies in diagnosis of ENL reactions classified by assessment of acute phase proteins SAA and CRP. Int J Lepr Other Mycobact Dis. 1995;63(2):222-30.
59. Corstjens PL, van Hooij A, Tjon Kon Fat EM, van den Eeden SJ, Wilson L, Geluk A. Field-Friendly Test for Monitoring Multiple Immune Response Markers during Onset and Treatment of Exacerbated Immunity in Leprosy. Clin Vaccine Immunol. 2016;23(6):515-9.
60. van Beers S, Hatta M, Klatser PR. Seroprevalence rates of antibodies to phenolic glycolipid-I among school children as an indicator of leprosy endemicity. Int J Lepr Other Mycobact Dis. 1999;67(3):243-9.
61. Hambridge T, Nanjan Chandran SL, Geluk A, Saunderson P, Richardus JH. Mycobacterium leprae transmission characteristics during the declining stages of leprosy incidence: A systematic review. PLoS Negl Trop Dis. 2021;15(5):e0009436.
62. Tió-Coma M, Avanzi C, Verhard EM, Pierneef L, van Hooij A, Benjak A, et al. Genomic Characterization of Mycobacterium leprae to Explore Transmission Patterns Identifies New Subtype in Bangladesh. Front Microbiol. 2020;11:1220
63. Fischer EA, de Vlas SJ, Habbema JD, Richardus JH. The long-term effect of current and new interventions on the new case detection of leprosy: a modeling study. PLoS Negl Trop Dis. 2011;5(9):e1330.
64. Schoenmakers A, Mieras L, Budiawan T, van Brakel WH. The State of Affairs in Post-Exposure Leprosy Prevention: A Descriptive Meta-Analysis on Immuno- and Chemo-Prophylaxis. Res Rep Trop Med. 2020;11:97-117.

65. Richardus RA, Butlin CR, Alam K, Kundu K, Geluk A, Richardus JH. Clinical manifestations of leprosy after BCG vaccination: an observational study in Bangladesh. Vaccine. 2015;33(13):1562-7.
66. World Health Organization. Guidelines for the diagnosis, treatment and prevention of leprosy [Internet]. WHO. [cited 2019 Feb 19] 2018 [Available from: http://www.who.int/lep/resources/9789290226383/en/.
67. Moet FJ, Pahan D, Oskam L, Richardus JH. Effectiveness of single dose rifampicin in preventing leprosy in close contacts of patients with newly diagnosed leprosy: cluster randomised controlled trial. BMJ. 2008;336(7647):761-4.
68. Richardus JH, Tiwari A, Barth-Jaeggi T, Arif MA, Banstola NL, Baskota R, et al. Leprosy post-exposure prophylaxis with single-dose rifampicin (LPEP): an international feasibility programme. Lancet Glob Health. 2020.
69. Ortuno-Gutierrez N, Younoussa A, Randrianantoandro A, Braet S, Cauchoix B, Ramboarina S, et al. Protocol, rationale and design of PEOPLE (Post ExpOsure Prophylaxis for LEprosy in the Comoros and Madagascar): a cluster randomized trial on effectiveness of different modalities of implementation of post-exposure prophylaxis of leprosy contacts. BMC Infect Dis. 2019;19(1):1033.
70. Avanzi C, Del-Pozo J, Benjak A, Stevenson K, Simpson VR, Busso P, et al. Red squirrels in the British Isles are infected with leprosy bacilli. Science. 2016;354(6313):744-7.
71. Oliveira I, Deps PD, Antunes J. Armadillos and leprosy: from infection to biological model. Rev Inst Med Trop Sao Paulo. 2019;61:e44.
72. Truman RW, Singh P, Sharma R, Busso P, Rougemont J, Paniz-Mondolfi A, et al. Probable zoonotic leprosy in the southern United States. N Engl J Med. 2011;364(17):1626-33.
73. da Silva MB, Portela JM, Li W, Jackson M, Gonzalez-Juarrero M, Hidalgo AS, et al. Evidence of zoonotic leprosy in Para, Brazilian Amazon, and risks associated with human contact or consumption of armadillos. PLoS Negl Trop Dis. 2018;12(6):e0006532.