In search of biomarkers for leprosy diagnosis : in silico identification, screening & field application

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Chapter 1

General Introduction

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Leprosy is a chronic infectious disease that affects hundreds of thousands of people every year. Its prevalence has dramatically reduced from millions in the 1980s to hundreds of thousands in the last 30 years as a result of the introduction of multidrug treatment (MDT) which is a combination of dapsone, clofazimine and rifampicin [7;204]. However, since the last decade, the annual numbers of new leprosy cases have become consistent indicating the continuing disease transmission [183;203;205]. The numbers of new cases in children, and the numbers of patients with grade 2 disability (visible deformity in hands/feet and/or visual impairment) reported every year are good indicators of the ongoing transmission and the delayed detection of cases, respectively. This is mainly due to the lack of early diagnostic tools [83] as well as poor awareness and knowledge of the signs and symptoms of the disease among the public and health professionals.

Leprosy primarily affects the peripheral nerves and skin and its cardinal signs are skin lesions, loss of sensation and nerve thickenings. The disease manifests itself in different clinical forms. For treatment purposes, WHO classifies leprosy cases as multi-bacillary (MB) and pauci-bacillary (PB) where cases with more than 5 skin lesions and high bacterial load are considered as MB and those with less than 5 skin lesions and low bacterial load as PB [1]. The clinical forms are further classified in to five groups based on the host's immunological responses, bacterial load and histopathological features of the lesions into tuberculoid leprosy (TT), borderline tuberculoid leprosy (BT), borderline borderline leprosy (BB), borderline lepromatous leprosy (BL) and lepromatous leprosy (LL). The TT and LL are stable forms whereas the borderlines are immunologically unstable. The cell mediated immunity decreases from TT to LL whereas the bacterial load and antibody level increase [181].

The discovery of *Mycobacterium leprae* (*M. leprae*) as an etiologic agent of leprosy a century ago, and later the development of efficient treatment regimens have proven that leprosy is a curable infectious disease [157;158;247]. The recent whole *M. leprae* genome sequencing project [39] has further opened the way for new avenues in leprosy research. Hence, there are currently more opportunities than ever before to better understand *M. leprae* and leprosy.

Following the availability of the whole genome sequence of *M. leprae*, several unique *M. leprae* proteins have been identified [81;191;218]. These were tested in different endemic sites in Asia, Africa and South America for their immunogenicity and for their discriminating potential of those infected and at risk of becoming infected, such as household contacts [22;86]. The *M. leprae* unique proteins identified as well as the host biomarkers induced by stimulation of blood cells by these proteins (including cytokines, chemokines, growth factors and others) provide important new tools for leprosy disease control, and are currently being validated in large-scale studies for their potential application in the early detection of leprosy (before the onset of clinical signs and symptoms of the disease) [65;68;83].

1.1. The Global Leprosy burden

"*We can endure losing fingers and toes, eyes and nose, but what we cannot endure is to be rejected by those nearest and dearest*"; a leprosy victim from Nepal [174].
The disfiguring character of leprosy and the wrong perception on the cause and transmission of the disease are the main reasons for stigmatizing leprosy affected people [43;174;230]. Leprosy is a curable chronic infectious disease; however this scientific fact is not yet well perceived in minds of many people irrespective of their level of knowledge [15;92]. Some

studies have also shown the strong stigma in leprosy compared to other diseases and its devastating impact on the lives of patients and their close relatives [177].

The dramatic reduction in leprosy prevalence and number of new cases in the post MDT era is undeniable, which has been the result of the determined efforts to eliminate leprosy. However, in the last couple of years, more than 200,000 new leprosy cases including children have been registered each year. Among the 5 WHO regions, the South-East-Asia region has the largest number of new cases (166,445) per year, followed by the Americas (36,000) and the African countries (20,599). Multi-bacillary (MB) new cases dominated in most of the regions and percentages of children affected ranges from 0.6% in Argentina to 24.5% in Cameroon and disabilities from 0.7 % in Marshall Islands to 25.4% in Uganda [7]. Here the major concerns are the considerable number of children and the grade 2 disabilities among new leprosy cases.

Number of new cases per given period of time and death or disability adjusted life years (DALY) are among the many ways to express a disease burden [178]. The disability together with self and society driven psychological distresses leave leprosy affected persons with a life time of misery and pain [212]. Despite the fact that leprosy is the leading cause of disability, leprosy associated DALYs are not registered in many high burden countries except the recent assessment in 3 states in India which showed the loss of 13.4 productive working years due to disability (DAWLY) of the 42 years estimated productive years [178]. Unlike TB or malaria, mortality due to leprosy is not a major concern although some deaths may occur from indirect effects of leprosy [72;138] and there were also a few reports showing the higher risk of death in lepromatous leprosy (LL) patients compared with the general population in pre MDT era [156]. However, the chain of disability to poor quality of life, to inevitable economic and physical dependence on family and society, to noticeable stigma, to mental distress greatly affects the lives of leprosy patients [132;201;235].

Leprosy control activities have been integrated with other health services since 1997 with the intention of better management of leprosy and reducing stigma [5]. However, the emergence of TB in the 1990s [3] and its co-infection with HIV diverted the focus of TB-leprosy control programs in most countries for the last two decades heavily towards TB/HIV. As a result leprosy has been left as poorly managed disease due to lack of knowledge, interest and commitment. The integration of leprosy management in the general health services has had its own advantages [173] and disadvantages. The service is meant to be easily accessible as long as there are health facilities in the vicinity of the patients, but its use depends on their health seeking behavior and the attitude of the community, since active case detection has not been part of the control programs so far. The knowledge, attitude and practice (KAP) of the health professionals at each facility are also main factors determining the quality of the leprosy management. This includes the focus of the control program to keep health professionals updated and committed as there is evidence of below average KAP of health professionals in leprosy [16].

The WHO “leprosy elimination by 2000” goal has also had an impact on the leprosy control programs, leading to less commitment in dealing with the true burden of leprosy. Indeed, it brought budget limitations in most leprosy activities due to the pulling out of donors [129]. However, the WHO leprosy strategy for the years 2011 to 2015 has put renewed emphasis on major issues: early detection of leprosy through active case detection, contact tracing especially in hotspots, and reducing disability [6] which is a step forward towards controlling leprosy.

1.2. Leprosy manifestations

1.2.1. Signs and symptoms of leprosy

Leprosy is caused by *Mycobacterium leprae* (*M. leprae*). It is a unique and challenging pathogen that primarily affects peripheral nerves and the skin through invading and residing in Schwann cells (SC) and macrophages. It also affects the eyes, the testis, the extremities, the hands and feet, and the upper respiratory tract [181;205]. Being primarily a nerve and skin disease, the clinical examination of leprosy is mainly based on the assessment of skin lesions and nerve involvements. There are three basic signs and symptoms to identify leprosy. These are: loss of sensation, presence of skin lesions and nerve thickenings [181]. The degree of sensation loss, the number and features of lesions and the number and level of nerve involvement vary widely in patients depending on the bacillary load and the host immunological capability, which strongly correlate with the different clinical forms of leprosy. In most patients, all three signs of leprosy are present. However, there are also cases with only skin lesions and no or undetectable nerve involvement, while on the other hand there are some cases with only nerve involvement and no skin manifestations, referred to as neuritis. These are the commonly missed cases during diagnosis since they are not fully covered by standard WHO classification methods [169].

In addition, a new mycobacterium species, *Mycobacterium lepromatosis* which can cause a fatal diffuse lepromatous form of leprosy was discovered in 2008 [99]. Preliminary phylogenetic analysis of few genes including the 16S rRNA gene revealed a significant difference of *M. lepromatosis* and *M. leprae* [101;213]. This fatal type of leprosy has been reported as endemic in Mexico and Costa Rica for a century [101;102;237] and according to a recent report it is also found in Singapore [100], Brazil and Myanmar [97].

1.2.2. Clinical forms of leprosy and diagnosis

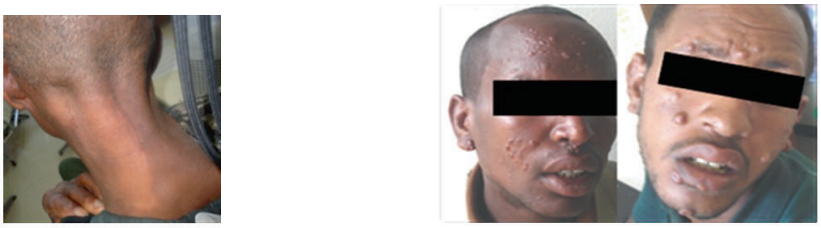
i. *Clinical forms of leprosy*

The diverse clinical manifestations of leprosy are primarily associated with the immunological and genetic variability of the host [181;216]. This renders leprosy an immunologically interesting and challenging human disease [146]. There are three main aspects that have led to the categorization of leprosy into five clinical forms. These are:

1. The level and type of the immunological response.
2. The bacillary load.
3. The histopathological features.

The five clinical forms of leprosy established as standard classification especially for research purposes by Ridley and Jopling [181] are tuberculoid leprosy (TT), borderline tuberculoid leprosy (BT), borderline borderline leprosy (BB), borderline lepromatous leprosy (BL) and lepromatous leprosy (LL). The TT and LL are the two stable forms of leprosy at the opposite extremes or poles. The BT, BB and BL forms are immunologically unstable groups which could possibly downgrade or upgrade, depending on e.g. initiation and efficiency of treatment. The TT and BT forms (Fig 1A) are characterized mainly by few skin lesions and high levels of cell mediated immunity (CMI). This involves the activation and proliferation of T helper 1 (Th1) cells; subsets of effector $CD4^+$ T cells important in activating macrophages via production of pro-inflammatory cytokines like IFN-γ. Along with this, a well characterized granuloma with undetectable or few bacilli are important features characterizing this group [153;181;205]. The "indeterminate" leprosy type is very similar to the TT group except that the lesions can be at an early stage and need to be confirmed histopathologically. Both the TT and indeterminate forms of the disease may spontaneously heal.

On the other side of the spectrum, the BL and LL forms (Fig 1B) are characterized by multiple skin lesions, poor CMI and strong humoral responses. Their granulomas are disorganized and filled with foamy macrophages and numerous bacilli which is a typical histopathological feature for these clinical forms. The BB group which is in the middle of all forms is difficult to clearly define with clinical or histopathological features as it shares characteristics from both BT and BL groups.



A B

Figure 1. A patient with borderline tuberculoid leprosy (BT) (1A) and patients with lepromatous leprosy (LL) (1B). Patients were enrolled in one of the studies in this thesis.
Photo by S/r Genet Amare; AHRI (Posted with permission)

1.3. Diagnostic tools

Leprosy diagnosis requires significant expertise because of the multi-facetted leprosy manifestations and its complications. In most leprosy burdened countries, diagnosis mainly relies on clinical examination, supplemented to some extent by the acid fast bacilli (AFB) staining of the skin slit smear (SSS) reported as BI (Bacterial Index). However, clinical diagnosis can only detect an already manifested and visible stage of leprosy, at which stage often irreversible tissue damage has already occurred. This illustrates the key importance of developing diagnostic tools that can detect leprosy earlier.

Clinical

As mentioned in the previous section, clinical examination includes 1) lesion characterization: number, demarcation, pigmentation, formation (raised, nodular), symmetry and loss of sensation; 2) voluntary muscle testing (VMT) and 3) examining of possible nerve enlargement. It is challenging to clearly diagnose leprosy and classify it into one of the 5 forms based on the clinical signs and symptoms unless it is supported with Acid Fast Bacilli staining (AFB) from skin slit smear (SSS) and with Hematoxylin and Eosin (H&E) staining of biopsy samples. The AFB staining and bacterial index (BI) reporting (Table 1) is within the capacity of many poor resource settings but the histopathological examination needs advanced lab facilities and a pathologist. Hence, classifying leprosy into the five classical forms remains important mainly for research purposes but not for routine diagnosis and treatment. Instead, World Health Organization (WHO) has recommended a more pragmatic, two arm classification of leprosy patients; paucibacillary (PB) patients with less than 5 lesions; and multibacillary (MB) with more than 5 lesions (WHO 1982) which simplifies leprosy management in resource poor settings.

Table 1
BI grading in leprosy diagnosis

BI grading	Definition
1+	At least 1 bacillus in every 100 fields
2+	At least 1 bacillus in every 10 fields
3+	At least 1 bacillus in every field
4+	At least 10 bacilli in every field
5+	At least 100 bacilli in every field
6+	At least 1000 bacilli in every field

Source: WHO (www.who.int/lep/microbiology/en.)

Other than clinical signs and symptoms, there are some serological tests which have been developed but none of these has been used for routine diagnosis; they are of limited value since they mainly detect MB but not PB patients. These tests include the following:

Anti PGL-I IgM antibody ELISA
Phenolic glycolipid I (PGL-I) is the dominant lipid component of the cell wall of *M. leprae* and is "specific" for *M. leprae* although there are some reports of its presence in *Leishmania* parasites [95]. The IgM antibodies level produced against PGL-I in patients is measured using ELISA and is usually high in MB (BL/LL) which have poor CMI and high humoral responses [181]. *M. leprae* has never been grown on artificial media and can only be cultured in armadillos or in the mouse foot-pad, which severely limits its availability and research into disease mechanisms. Therefore, synthetic PGL-I made of natural disaccharide octyl human serum albumin (ND-O-HSA) is used in these ELISAs. This technique clearly detects BL/LL patients and can also be used to monitor their treatment outcome as it is reduced during treatment [168]. TT/BT patients are usually seronegative and do not reproducibly produce detectable circulating antibodies to PGL-I [205]. Clinically, identifying BL/LL patients is not difficult for dermatologists and in that case the anti-PGL-I ELISA may not contribute substantially to the diagnosis as the main challenges are detecting TT/BT patients and sub-clinically infected individuals. Nevertheless, there is some evidence showing its utility in identifying HHC with higher risk of developing the disease [18;60].

Anti PGL-I IgM antibody ML-Flow test
This is an immunochromatographic test which is also based on PGL-I but detects IgM faster [27;28]. It does not require special skills to perform the test and does not need a reader as it can be graded by visual inspection [133] unlike the ELISA. However, it has the same limitations as the anti-PGL-I ELISAs in that it mainly detects MB patients and is not quantitative.

NDO-LID® rapid test
This is a recently developed rapid serological diagnostic test which measures IgM antibodies against PGL-I through the use of NDO-BSA and measures IgG antibodies against LID-1, a fusion protein made of ML0405 and ML2331 [62;65;191]. It is an immunochromatographic test which requires small amounts of serum or whole blood. Like the other serological tests, it detects most MB patients and relatively improved numbers of PB patients (32.3%) compared with NDO-BSA (6.5%) [62]. However, a large scale evaluation of this test in different endemic sites is necessary to evaluate its precise utility in leprosy.

Lepromin Skin Test

This is a skin test using a suspension of whole, killed *M. leprae,* called lepromin, injected intradermally. The reaction to this test is measured as mm induration 4 weeks after injection. Lepromin is not used for diagnosis of leprosy; rather it measures or provides information about the individual's immune response: a positive lepromin reaction indicates the ability of the individual to develop a granulomatous response to *M. leprae* while a negative reaction is commonly seen in lepromatous patients who are incapable to contain or clear the bacilli [103;205].

PCR based M. leprae viability assay

Several attempts have been made since the last 3 decades to establish clinically applicable PCR based leprosy diagnostic tools [192;193;245]. However, the major challenges had been identifying potential target genes for specific detection of *M. leprae* and determination of bacilli viability. In recent years, real time PCR based amplification of the repetitive element of *M. leprae* (RLEP) DNA had shown strong correlation with AFB count and was found reliable for specific quantification of *M. leprae* from mouse and armadillo tissues but with limitations of providing absolute data on viability [48]. In recent development, expressions of number of genes were assessed [135;232] among which *hsp18* (encoding 18kd heat shock protein) and *esxA* (encoding the ESAT 6 protein) were found reliable in detecting viable *M. leprae* [48]. This newly established *hsp18* and *esxA* based viability assay can be used as an assessment tool for early detection of *M. leprae* infection in close household contacts of leprosy patients, in monitoring treatment outcome and in detection of drug resistance by eliminating the tiresome bacterial isolation process. However, the cost and the demand for trained personnel remain limiting factors to implement the assay as point of care (POC) in resource poor settings.

1.4. Treatment and drug resistance

Despite being an ancient disease, treatment for leprosy was not available until the 1940s. The first modern treatment was dapson (diaminodimethyl sulfone) and was given for long-term to life time in case of MB cases. Poor compliance to the long term treatment contributed to the occurrence of dapsone-resistant *M. leprae* isolates in most countries and was a challenge for the leprosy control programs [77;108;167;241]. Several efforts were made to replace dapsone with other monotherapies such as clofazimine, ofloxacin or rifampin but resistance against these antimicrobial agents developed when given as monotherapy. To resolve this, a combined treatment consisting of dapsone, clofazimine and rifampicin which commonly referred as multi-drug treatment (MDT) was recommended by WHO in 1981[1]. Currently, the WHO recommended treatment period is 6 months for PB patients and one year for MB patients (reduced from two years since 1997) [4], however, there are countries which still treat MB cases for two or more years especially those countries with high resources and low leprosy burden). In both PB and MB 100 mg dapsone daily and 600 mg rifampicin monthly is given while for MB cases an additional 50 mg daily and 300 mg monthly dose of clofazimine is given. There is also a third regimen which is recommended to single-lesion PB patients; this is a single dose rifampicin (600 mg), ofloxacin (400 mg) and minocycline (100 mg), commonly referred as ROM. Currently, ROM is being tested for MB patients as 12 month regimen [79;126]. For patients who are unable to take medications because of allergy or suspected complications, fluoroquinolones, ofloxacin, moxifloxacin, or pefloxacin and minocyline, macrolide clarithromycine can be given as second line drugs. MDT is efficient as witnessed by the dramatic reduction of leprosy prevalence since its initiation.

Drug resistance to MDT is not considered as a major issue (ILEP report 2013), however, there are recent reports which identified either mono or multiple drug resistant *M. leprae* strains [244;246]. A study which evaluated samples from 230 new and 3 relapse cases from Venezuela and Brazil using sequencing and real-time PCR Taqman technologies showed drug (rifampicin and dapson) resistance-associated mutations in *fol*P1 and *rpo*B genes in the 3 relapse cases [214]. Similarly, SNPs in biopsy samples of 4 among a total of 92 (4.3%) relapse cases in Brazil were found where in 2 relapse cases multi drug resistance (SNPs in *fol*P1, *rpo*B and *gyr*A) was observed [46]. Although cases with SNPs were small in number, the reports in general indicate the importance of regular drug resistance monitoring especially in relapse cases.

1.5. Vaccines against leprosy

Mycobacterium bovis BCG (Bacillus Calmette–Guérin) is the only vaccine currently available for TB. It protects young infants from severe forms of TB, mainly the miliary and meningeal forms, but its protection wanes over time and varies across age, different countries and the type of vaccine strain used. The BCG vaccine was initially developed for both TB and leprosy but its protective role in leprosy became neglected because of MDT campaigns. There is evidence for higher BCG induced protection in young individuals that wanes overtime and for increased protection through several doses of BCG [41;141;182;208;248].

There were long (up to 8 years) follow up studies that assessed the protection of killed *M. leprae* combined with BCG in comparison with BCG alone. In a Venezuelan study, no better protection was observed after five years follow up [40]. In contrast, in an Indian study, the combined *M. leprae* killed/BCG vaccine showed 64% protection compared to BCG alone which was 34.1% [96].

Further assessment and use of killed *M. leprae* vaccines need large production of *M. leprae* which is very challenging. Therefore, other easily cultivable mycobacteria such as *M. w*, *M. vaccae* and *M. habanna* used in vaccine preparations were tested in household contacts of leprosy patients and showed more than 50% protection for at least 3 to 6 years [209;229;238]. Crude *M. leprae* antigens were also assessed in mice [80;152]. rBCG vaccines that express Ag85 and MMP- II also inhibited *M. leprae* multiplication [134;160]. From recent antigen screenings, those antigens recognized by PB patients were also further assessed for their potential as a vaccine in mice work [63]. So far no successful vaccine has been developed for use except BCG implicating the need for continuous effort to develop potential leprosy vaccines.

1.6. *Mycobacterium leprae* and its unique characteristics

Leprosy has been with humans since ancient times as evidenced by some records in India and China and by more reliable archaeological discoveries of the 2nd Century BC in Egypt [224]. However, *M. leprae,* the etiologic agent of leprosy was discovered only a century ago as the first human pathogen, notably by the Norwegian scientist Gerhard Henrik Armauer Hansen [104;105]. Other human pathogens discovered later have been more intensely investigated than *M. leprae* due to its challenging unique characteristics and the inability to culture *M. leprae* [38;69].

1.6.1. *M. leprae* classification and its cell wall composition

M. leprae is an intracellular obligate, acid-fast, capsulated, rod shaped bacillus which belongs to the order Actinomycetales, family Mycobacteriaceae and genus *Mycobacterium*. As any other mycobacteria it replicates by binary fission but unlike any other species which belong to either fast or slow growers in this genus, *M. leprae* divides in 12-14 days and it has never been grown in artificial media/*in vitro* [179].

The *M.leprae* capsule is an electron transparent zone composed of phthioceroldimycoserosate and phenolic glycolipid (PGL) composed of three sugar molecule linked to phthiocerol (fat) with a phenol component. The outer layer of the cell wall is similar to other mycobacteria containing lipopolysaccharides composed of branched chains of arabinogalactan and long chains of mycolic acids. The inner part of the cell wall is composed of a peptidoglycan layer which is formed from chains of alternating N-glucosamine and N-glycolyl muramyl linked by peptides cross bridges [61;205].

1.6.2. Genome and metabolism

The genome of *M. leprae* was first sequenced in 2001 [38]. The genome size, 3.3Mb, is smaller than that of *M. tuberculosis* (Mtb) and contains 1133 pseudogenes, leaving a total of 1614 genes encoding for functional proteins, and displaying a major reduction of G+C content. The reduced genome size with nearly 50% inactivated genes is the reason for the absence of several metabolic pathways, a feature which distinguishes *M. leprae* from other mycobacteria [38]. As a result of this, *M. leprae* utilizes glucose but no other carbon sources as an energy source, which makes it highly dependent on the host. The *M. leprae* genome project also revealed that the genes encoding enzymes degrading other carbon sources such as acetate and galactose are in fact pseudogenes while in *Mtb* these genes encode functional enzymes capable of degrading these carbon sources [38;69;243]. Being intracellular mycobacteria, *M. leprae* and *Mtb* are also known to utilize host-derived lipids as energy sources through lipolysis. However, *M. leprae* has very few lipase genes as compared to *Mtb*. *M. leprae* has also lost the anaerobic pathways and has less efficient aerobic pathways left due to its incapability to generate ATP by oxidizing NADH which is one possible reason why it is unable to grow in artificial media as it will be difficult to provide appropriate levels of oxygen in the media [243]. It also lost the *mbt* operon, leading to deficiency in iron acquisition from its environment as it is not able to produce mycobactin [113]. However, there are many other genes related to iron storage still active, indicating *M. leprae* is able to use host iron.

1.6.3. Host preference

Besides humans, the wild nine-banded armadillo (*Dasypus novemcinctus)* is a highly susceptible natural host of *M. leprae.* Primates such as Chimpanzee and Mangabey Monkey were reported as hosts as well but not to a major extent [2]. The wild nine-banded armadillos are common in the south central USA [226] and recently, a study in Texas showed genotype similarities of the isolates from US leprosy patients and infected armadillos, indicating the possible existence of zoonotic leprosy in that specific area [228]. Nine banded armadillos and nude mice are very important for leprosy related experiments. The mouse foot pads are convenient to grow *M. leprae* and the nine-banded armadillos also show fully disseminated, MB infection when inoculated with *M. leprae* [205]. These experimental animals are important for drug resistance monitoring, to assess bacilli viability and to culture bacilli for *in vitro* or *in vivo* immunological studies.

1.6.4. *M. leprae* in the environment

M. leprae is known for being an obligate pathogen. However, there are recent reports indicating the presence of viable *M. leprae* in soil mainly in areas with high prevalence of leprosy [121;231]. The genotypes of those isolated from soil were found similar with those isolated from patients in that specific area [231]. MB patients carry very high number of bacilli in their upper respiratory tract and the bacilli can easily be excreted from the nasal area through washing or sneezing [210]. Hence, finding live *M. leprae* in the surrounding soil and water might not be surprising. Previous studies also showed its survival outside the host for 9 days [53] and in an extended study it was found to survive 46 days in wet soil and 60 days in saline at room temperature [54]. *M. leprae* was also found viable and intact after being ingested by a free living pathogenic amoeba for at least 72 hours [119] and a recent study revealed a long term (up to 8 months) survival of *M. leprae* in free living amoebae cyst while retaining its virulence [242]. However, further investigations are required to further understand the capability of the *M. leprae* bacilli found in the surrounding environment including free living amoebae to infect the host and if so these environmental sources can be considered as potential reservoirs for *M. leprae*.

1.7. Host-pathogen Interactions in leprosy

The manifestation of the disease in its different forms, closely correlating with host genetic and immunological factors, has made leprosy an immunological disease model [144;146;147]. Besides its great potential to provide a research model for human immune mediated diseases, extensive knowledge about host-pathogen interactions in leprosy will also help to improve and innovate leprosy control activities.

1.7.1. The route of infection/ entrance of *M. leprae*/ transmission

Untreated lepromatous patients carrying high bacillary load reaching 10^{11} per gram of tissue, are the main sources of infection where the aerosol spreading of nasal droplets from these patients infects healthy individuals through nasal/respiratory routes [107]. Due to lack of active case detection strategy in high burden countries, there is high possibility for untreated MB patients to transmit the infection to their close contacts [175]. Sub-clinically infected individuals as source of infection cannot be ruled out although no clear evidence is available as yet. Transmission through skin contact [55], via nine-banded Armadillos [120;227] and via free living amoebae that contained *M. leprae* [242] also need due attention and further investigation.

1.7.2. Innate Immunity; uptake of *M. leprae* by host cells

M. leprae and Schwann cells

The presence of considerable number of bacilli in the endothelial cells lining the blood vessels and lymphatics revealed that endothelial cells could be sites for *M. leprae* replication and establishment of infection [42;137]. Moreover, they might further assist *M. leprae* to reach peripheral nerve tissues through the blood stream [17] and act as reservoirs to further infect Schwann cells [199;200;207]. A layer of basal lamina which covers the Schwann cell-axon complex is the area where *M. leprae* can interact with laminin-2 as an entrance to infect Schwann cells. In addition, the expression of C-type lectin (CD209) on the surface of Schwann cells also enhances the binding and uptake of *M. leprae* which is regulated by Th2 cytokines like IL-4 [223]. There are also evidences that infected Schwann cells are capable of processing and presenting *M. leprae* antigens to inflammatory type 1 T cells which results demyelination, lyse of infected Schwann cells and nerve function impairment especially in tuberculoid patients with high CMI [161;220;221].

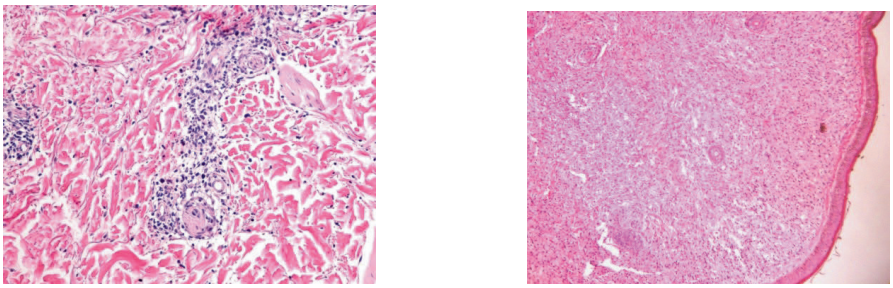
M. leprae and Macrophages

The various pattern recognition receptors (PRR) such as toll like receptors (TLR), C-type lectins, nucleotide-binding oligomerization domain (NOD2) and others recognize pathogen associated molecular patterns (PAMPs) and play a role in determining the function of macrophages during *M. leprae* infection [144]. In mycobacterial infections like TB and leprosy, microbial lipoproteins trigger TLR2, TLR2/1 or TLR2/6 heterodimers and this activation is enhanced by Th1 type cytokines and inhibited by Th2 cytokines.

Macrophages (Mϕ), being mediators of both the innate and adaptive immune systems, show distinct features in different forms of leprosy and determine the outcome of the pathogenesis. In tuberculoid leprosy, Mϕs are activated and their vitamin D dependent antimicrobial function (VDR) is dominant leading to killing of the bacteria. In lepromatous patients, the phagocyte function is more dominant and the Mϕs appear to be foamy, filled with lipid droplets derived from *M. leprae* and the host itself [9;44;146;147;222]. These features are visible in the types of granulomas formed in skin lesions of TT/BT and BL/LL patients (Fig 3 Granulomas). There is also evidence *in vitro* and in tissues that TLR2 and TLR1 are more expressed in TT/BT patients than BL/LL [114]. The mannose receptor (CD206) is mainly expressed on mature macrophages and facilitates phagocytosis through binding to the mannose capped liproarabinomanan.

The dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN); CD209 is also a major receptor on DCs, having similar ligand properties with CD206 for mannose capped liproarabinomanan and mycobacteria target DC-SIGN to suppress DC maturation through induction of IL-10 and inhibition of IL-12 [78;155]. There is also evidence for the expression of CD209 on macrophages of both TT and LL patients, and this is important in recognition of mannose-rich glycoconjugates like mycobacterial lipoglycan mannosylated lipoarabinomanan (ManLam), facilitating the binding and phagocytic process.

Along with this, innate cytokines in the lesions regulate the functions of macrophages in leprosy where IL-10 induces the phagocytic pathway and IL-15 induces the vitamin D dependent antimicrobial pathway [144;146;147]. The complement receptors (CR1, CR3 and CR4) also play a role in facilitating the phagocytosis process; CR3 especially facilitates the uptake of PGL-I by macrophages [197;198]. However, the activated phagocytic pathway in BL/LL patients will not culminate in the fusion of phagosome and lysosome because of the foamy nature of the Mϕs: this impairs lysosomal function and proper antigen presentation due to disrupted HLA-DR rafts [118;144;147]. Similarly, the higher frequency of regulatory T cells with higher production of anti-inflammatory cytokines in lepromatous compared with tuberculoid patients can also be considered as a potential factor modulating the formation of foamy macrophages which eventually form a diffused, unorganized granuloma in BL/LL patients unlike in TT/BT [165;188].



A. BT B. LL

Figure 3. H&E staining of tissues taken from a BT patient skin lesion (A) and LL patient skin lesion (B) showing the differences in granuloma formation.
Image A is taken from www.dermpedia.org and image B is photographed by Dr. Munir Hussien (Dermatovenorologist and Pathologist, AHRI)

Dendritic cells and Langerhans cells in leprosy

Dendritic cells (DCs) are professional APCs and are very efficient in priming and activating naïve T cells. In lesions of lepromatous patients, a deficit in DCs both in the dermis and epidermis was observed [211]. In addition, peripheral monocytes do not differentiate into $CD1^+$ DC following TLR activation in lepromatous patients [115]. These $CD1^+$ DCs ($CD83^+$) are capable of presenting non-peptide components; lipids and glycolipids to $CD1^+$ restricted T cells which in turn produce enormous levels of IFN-γ. Expression of the co-stimulatory molecule B7.1 is also decreased in LL patients [194]. This shows how *M. leprae* is capable of impairing the antigen presenting role of DCs. However, a recent study has indicated that activation of monocytes via NOD2 (Nucleotide-binding oligomerization domain containing protein 2) with its ligand muramyl dipeptide induces their differentiation into DC a process which was dependent on IL-32. Indeed, the expression of NOD2, IL-32 and $CD1b^+$ DC in leprosy lesion was found to correlate with bacterial control, being higher in TT/BT patients [196].

Langerhans cells are known to block dissemination of *M. leprae* at the infection sites and there is evidence showing reduced number of Langerhans cells in lepromatous patients compared to tuberculoid ones, which does not change after treatment [136;143].

1.7.3. Adaptive immunity in leprosy

Leprosy manifests itself in a wide-ranged spectrum where differences among the various forms are characterized by the type and level of immune responses. Cell mediated immunity (CMI) is important in controlling intracellular pathogens like *M. leprae* and *Mtb*. In tuberculoid leprosy patients, *M. leprae* infected macrophages eliminate the bacilli and present antigens in the context of MHC Class II molecules which induces IL-12 and stimulate $CD4^+$ Th1 cells [9]. These dominant Th1 cells, $CD4^+$ T helper and memory T cells in lesions of TT patients produce pro-inflammatory cytokines mainly IFN-γ, IL-2, TNF and other Th1 associated factors [117] and play an important role in activating macrophages to initiate their microbicidal activity and control bacillary multiplication [171]. Simultaneously, the numerous cytotoxic T cells, $CD4^+$ and $CD8^+$ T cells, which are restricted to MHC-Class II and I respectively produce effector molecules such as perforin, granzyme B and granulysin and lyse the infected macrophages.

In lepromatous leprosy patients (LL), suppressor type $CD8^+$ T cells are present, which are distributed in the lesions together with $CD4^+$ T cells. The suppressor $CD8^+$ T cells are

important in down regulating the macrophage activation and suppressing CMI. On the other hand, there is high production of antibodies which leads to accumulation of immune complexes activating the complement system[93]. The IgM level in the circulation facilitates the *M. leprae* evasion through activated C3 which are capable of co-stimulating naive T cells via complement regulation protein CD46, which leads to differentiation of IL-10 secreting regulatory T cells [29]. The Th2 type responses in the lepromatous patients are mainly characterized by the production of cytokines like IL-4, IL-5 and IL-13 and lack of IFN-γ and IL-2 to the extent of *M. leprae* specific anergy in LL patients. In these patients, a higher number of regulatory $CD4^+$ T cells characterized either by their expression of CD25, FoxP3, or production of cytokines such as IL-10 and TGF-β and also higher numbers of anti-inflammatory macrophages (Mϕ2) in the circulation and in skin lesions have been reported in several studies [24;116;165;188]. Along with this, Kumar *et al*., pointed out the importance of the molecular cross-talk of TGF-β, CTLA-4 and Cb1-b and the disruption of HLA-DR rafts leading to *M. leprae* persistence and T-cell hypo-responsiveness in lepromatous patients which they claim is a Th3 type response [117;118]. Other than the classical Th1 and Th2 type responses, a role for Th17 has been uncovered recently in a study in which higher level of IL-17 associated cytokines IL-1, IL-22 and RORC (Th17 transcription factor) were reported in the tuberculoid form of leprosy [187].

1.7.4. Host genetics and susceptibility to leprosy

It has been a century since leprosy was described as not inherited but rather caused by an infectious pathogenic bacterium. Nevertheless, the very different manifestations of leprosy with its distinct clinical presentations and immunological responses has led to investigations which collectively revealed the involvement of various human host genes involved in susceptibility to leprosy [11] and in developing specific forms of leprosy and leprosy reactions [8;11;75;91;236].

Human leukocyte antigens (HLA) are known for their association with several diseases. The association with leprosy was demonstrated three decades ago [49;50] although the presence of genetic susceptibility or predisposition was speculated since 1841 [74]. Later, the major role of the HLA Class II linked genes in determining the type/form of disease to be developed was demonstrated [51;162;236] as for instance shown in previous studies that HLA-DR3 and -DR2 are more associated with tuberculoid leprosy [49] where as HLA-DQ1 is associated with lepromatous leprosy [164]. The polymorphic nature of the peptide binding groove in HLA molecules is the main factor for HLA-peptide binding differences and this basic understanding has led to the development of *in silico* tools that predict immunogenic epitopes relevant in biomarker search for leprosy diagnosis [85].

Two genes highly associated to leprosy susceptibility more recently are PARK2 and PACRG [122;142]. The PARK2 gene is known for its association with Parkinson's disease. The PARK2 and LRRK2 genes have a role in cell apoptosis regulation. Another gene associated with susceptibility or resistance to leprosy and also linked with development of different forms of leprosy is NRAMP1 [8;30;94;139;215]. TLRs, NOD2 and MRC1 are also among the genes which are important in the early phase of host pathogen interaction with roles of bacterial recognition and uptake. The LTA4H gene regulates Lipoxin A4, one of the factors in the formation of macrophages filled with lipid droplets in LL patients. Genes such as TNF, LTA and IFN-γ and other related genes are involved in the formation of granulomas and in the maintenance of adaptive immunity [31]. Since TLR and VDR are important in recognition and killing of the bacilli respectively, polymorphisms in these genes are also important factors in determining the outcome of the leprosy infection. Other than the

previously reported susceptible loci, a recent genome-wide association study of leprosy in a Chinese population revealed six new susceptible loci where BATF3, CCDC88B and CIITA-SOCS1 were reported as new susceptible genes [123].

Genetic studies are powerful tools to decipher host pathogen interactions. Obtaining more detailed insights into the genetic control of infection and disease susceptibility of leprosy affected people, their household contacts and endemic controls may further help in designing strategies for early detection of individuals who are at risk of developing leprosy and contribute towards reducing leprosy transmission.

1.8. Reactions in Leprosy

Leprosy reactions are acute immunologic hypersensitivity episodes which can occur before, during or after MDT in about 30% to 50% of the leprosy patients. These complications are the main reasons of nerve impairment and disabilities in leprosy which increase leprosy related morbidity [26;111;130;131;240].

There are two commonly known leprosy reactions referred to as reversal reaction (RR) or type 1 reaction (T1R) and erythema nodosum leprosum (ENL) or type 2 reaction (T2R) [190;239]. There is also a third category of reactions, called the Lucio phenomenon, but this is less common and is reported mainly in central and south America in non-nodular lepromatous patients[145] as well as in patients with lepromatosis [98].

1.8.1. Reversal Reaction (T1R)

T1R mainly occurs in borderline patients (BT, BB and BL). It is characterized by inflammations in the skin and/or nerves with edema of the hands, feet and face [34;234] (Fig 4A). The diagnosis is mainly clinical and sometimes typical T1R histologic features can help the diagnosis. As T1R are frequently recurrent, close follow up of patients is necessary to avoid additional nerve damage [234]. Although not yet exhaustive, various host and pathogen factors are associated with T1R such as enhanced CMI with expression of IFN-γ, IL-6, IL-8, IL-13, TNF-α and other pro-inflammatory cytokines and chemokines like IP-10 which are typical characteristics of T1R [14;154;166;206]. Recent analysis of T1R has also revealed an increased production of CXCL9, IL-17A and VEGF in addition to IFN-γ and IP-10, and a reduction in IL-10. G-CSF and cytotoxicity associated genes such as granulysins, granzymes, perforins were shown to be up regulated during T1R compared with their levels before T1R [90]. An increased serum level of IL-17F during T1R was also reported which might indicate a role in inflammation [33]. Infiltration of $CD4^+$ T cells and presence of pro-inflammatory cytokines observed in the periphery were reported in skin lesions in patients with T1R. Upregulation of human beta-defensin 3 was also reported during T1R, which later subsided during corticosteroid treatment [37]. The association of certain genes or proteins either being upregulated or downregulated during T1R or after treatment with corticosteroids is an important clue towards a better understanding and management of reactions through developing tools that can possibly predict those at risk of developing reactions.

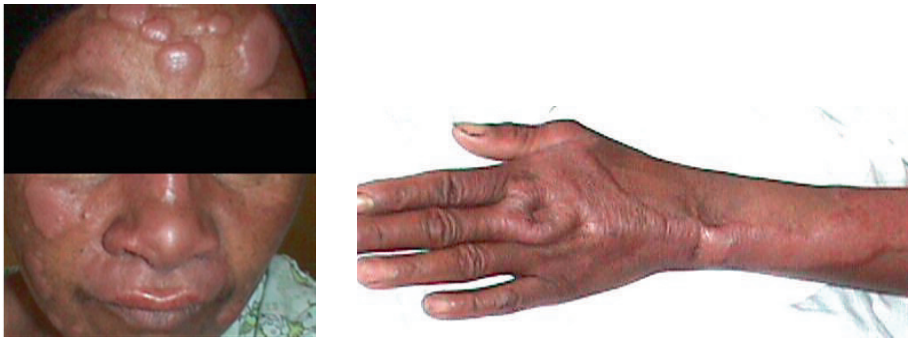


Figure 4 A. Type 1 reaction (T1R) or Reversal reaction (RR)
Photo by Dr. Elizabeth Bizuneh, ALERT (posted with permission)

1.8.2. Erythema nodosum leprosum (ENL)

ENL or T2R is an immune complex mediated episode which is more common in LL patients and some BL because of high level of *M. leprae* antigens and anti-*M. leprae* antibodies in the circulation of these patients [12;111]. Unlike T1R, ENL is characterized by infiltration of neutrophils into the lesions [45]. ENL is commonly diagnosed clinically as patients have tender red papules and nodules associated with fever (Fig 4B). As this type of reaction is a systemic disorder, it may affect organ systems and confer systemic complications. ENL starts as acute but can progress to a chronic phase and can also be recurrent [239]. High levels of serum C-reactive protein, amyloid A protein and alpha-1-antitrypsin are ENL markers [110] but are not commonly tested for.

Figure 4B. Type 2 Reaction (T2R) or Erythema Nodosum Leprosum (ENL)
Photo by S/r Genet Amare; AHRI (Posted with permission)

1.8.3. Risk factors for developing leprosy reactions

Leprosy reactions can occur at any time before, during or after MDT. However, the proportions of patients that develop reactions during and after treatment are higher compared to those before treatment. This is mainly because of the bactericidal effect of rifampicin which kills high numbers of bacilli leading to the release of *M. leprae* antigens in the circulation that trigger inflammatory reactions. Clinically, high BI (or being an MB patient), anti-PGL-I antibody level and being on MDT are among the potential risk factors. A genetic

study revealed that polymorphisms in genes such as TLR1 and TLR2, VDR, NRAMP-1, C4B and IL-6 have associations with developing leprosy reactions [73].
In addition, co-infections, especially oral infections, hepatitis C and hepatitis B are among reported possible risk factors for developing leprosy reactions [149-151]. The majority of leprosy patients co-infected with HIV show T1R [52] but further investigation is required to conclude this with more certainty.

1.8.4. Treatment of T1R and ENL

For both reaction types, corticosteroids (commonly prednisolone) are given to control the inflammation and reverse the nerve impairment. The regimen starts with high dose according to the severity of the reaction and is tailored based on clinical assessments made every 2 weeks. To avoid the long term side effects of corticosteroids, cyclosporine, methotrexate and tacrolimus are also considered as options to manage T1R.

Unavailability of treatment options is a big challenge in management of leprosy reactions but lack of knowledge present among health professionals in identifying reactions properly and prescribing the right dose of corticosteroids and tailoring down or up as to the degree of severity of the reaction is a major knowledge gap as well.

Thus, in addition to the currently available diagnostics tools and treatment, there is a demand to investigate leprosy-specific host or pathogen biomarkers for early detection of leprosy and prediction of leprosy reactions.

1.9. Search for leprosy-specific Biomarkers

1.9.1. Major challenges in leprosy and current opportunities

There have been major achievements in the control of leprosy, especially after the introduction of MDT since the 1980s [205]. However, the presence of pocket areas in different endemic countries contributes to the consistent number of new cases every year, the percentage of patients reporting with grade 2 disabilities and considerable numbers of new pediatrics cases [125;131;202]. This indicates how much effort is needed to "eliminate" leprosy and prevent leprosy associated disabilities. MB patients with high bacillary load and visible deformities stay in the population from months to years due to lack of awareness or being threatened by stigma. Hence, these patients serve as potential sources of infection. On the other hand, preclinical patients without any visible signs and symptoms of leprosy but harboring *M. leprae* in their body also live in the population until the disease is manifest because *M. leprae* has a long incubation period. Although it needs solid evidence, these "sub-clinically" infected individuals might be other sources of infection and there are no clinical or laboratory diagnostic tools to detect them [83].

Leprosy research has provided several key findings in the pre-genomics era. However, the area has benefitted and developed more in the postgenomic era mainly from the whole genome sequencing and bioinformatics which avails all necessary information about *M. leprae,* related *Mycobacterial* species and other species including the human genome. This has shortened the search for unique *M. leprae* antigens and the production of recombinant proteins and synthetic peptides enabling the evaluation of these antigens in a larger endemic population [20;23;83;86] to further use the promising ones in the biomarker search and development of novel tools for early detection of leprosy and prediction of leprosy reactions.

1.9.2. The search for unique and immunogenic M. leprae antigens in post genomic era

In the search for potential and promising antigens, both T and B cell based approaches have been used. As recently reviewed, about 200 *M. leprae* proteins and more than 10 fusion proteins have been tested for CMI and HMI in endemic countries in several studies in Brazil, Bangladesh, Ethiopia, India, Nepal, Pakistan, Philippines, Venezuela, China and in countries with relatively low number of leprosy cases like Japan and South Korea [81] .

T cell-based approaches

Previously, several *M. leprae* antigens like 18kDa, 35kDa, 45kDa or undefined mixtures of *M. leprae* components were tested for their immunogenicity in endemic and non-endemic populations [25;58;59;106;225]. Later our group at LUMC and others, using bio-informatics tools and whole genome sequences of *M. leprae* and other related species, identified *M. leprae* unique regions, with either known or unknown functions in infection biology. Genes with unique sequences were selected and their recombinant proteins were produced by cloning [76]. The recombinant *M. leprae* proteins and/or their synthetic peptides with 9-15 unique sequential amino acids fitting into MHC-I and II binding grooves were first tested for their specificity in non-infected and non-exposed individuals living in non-endemic countries, and next tested in endemic countries for their immunogenicity in whole blood assays (WBA) or in lymphocyte stimulation tests (LST) through analysis of IFN-γ by $CD4^+$ or $CD8^+$ T cells stimulated with these specific antigens in different groups: leprosy patients (TT/BT, BL/LL, patients with reactions), household contacts (HHC), healthy endemic controls (EC) and TB patients.

The main purpose of this evaluation process was to select potential T cell epitopes for diagnostics that could enable to differentiate *M. leprae*-infected from non-infected or exposed individuals, or detecting those who are at risk of developing the disease [84;88;218] or to accelerate leprosy vaccine development [66;67;87;176;191]. For instance, *M. leprae* proteins; ML0049 and ML0050, homologues of *Mtb* ESAT-6 and CFP-10 respectively were tested with the intention to develop similar diagnostic test for leprosy. However, cells of TB patients in endemic countries also recognized these *M. leprae* proteins as measured by IFN-γ response although their sequences are not similar (only 36% and 40% similarity) probably indicating cross-reactivity by the highly similar peptides, although co-infection of the TB patients with *M. leprae* could not be excluded [89]. Other *M. leprae* specific proteins which were initially tested in Brazil [84;218] were further tested in different populations in Africa and Asia [23;86]. Proteins ML1989 and ML1990 were almost fully recognized by all groups which included leprosy patients, household contacts and endemic controls questioning their potential as diagnostic tools. On the other hand, proteins such as ML0126 and ML1601 were found to be immunogenic and showed specific responses in leprosy patients although also nearly half of the healthy endemic controls responded to these antigens [23;86]. Further analysis of these proteins and their peptides including ML1601 and ML2478 in healthy endemic controls with different levels of exposure confirmed the diagnostic potential of these antigens, since endemic controls with relatively higher levels of exposure to *M. leprae* showed increased level of IFN-γ, IP-10, IL-1β, IL-6, TNF-α and CCL2 [22]. Similarly based on the cellular responses to these antigens, biomarkers other than IFN-γ, MCP-1, MIP-1β and IL-1β were shown to differentiate TT/BT patients from HHC [83].

Serological approaches

Antibody responses against *M. leprae*-unique proteins and other components of *M. leprae* have been analysed in several studies for potential use as diagnostic tools to monitor treatment, or disease progression [64;172;191;219]. Assessment of antibody responses

against 12 *M. leprae* specific antigens in leprosy patients and household contacts showed that antibodies to ML2028, ML0286, ML2038, LID-1, ML0405 and ML2055 proteins were detectable in MB patients and antibodies to ML2028, ML0286, ML2038 proteins were detectable in PB patients. The antibody titers against LID-1 and ML2028 were found increasing in two household contacts of MB index for about 15 months and one of these HHC showed progressively higher antibody titers to MLSA (*M. leprae* soluble antigen), LAM and PGL-I and developed borderline leprosy after two years of enrollment [219]. One of these proteins, the fusion protein LID-1 that induces strong IgG responses mainly in MB cases [170;172;180] is also used in the development of NDO-LID rapid test [62] (see page 17).
The fundamental reason for identifying and evaluating *M. leprae* specific antigens based on T cell or humoral approaches is to use these antigens in the development of rapid and field-friendly diagnostic tools to detect *M. leprae* infection earlier and for rapidly monitoring treatment outcome.

1.9.3. Cytokines/chemokines as potential biomarkers in leprosy

The search for biomarkers induced by these antigens, however, has many challenges in itself [82;163]. Several immunological biomarkers for T-cell based assays have now been identified and are discussed below:

Interferon-gamma

IFN-γ is a stable cytokine which has been and will remain a good indicator of pro-inflammatory/Th1 host responses against *M. leprae* and other mycobacteria. If the *M. leprae* protein used to activate its production is specific enough, unexposed individuals will not produce IFN-γ in *in vitro* assays. This would confirm the absence of cross reactivity with other mycobacteria which is important in selecting specific and immunogenic proteins [13;84;86]. As shown in TB diagnostics, two commercially available IFN-γ release assays (IGRAs), QuantiFERON©-TB Gold assay and T-SPOT TB, are developed based on the IFN-γ response to *Mtb* specific peptides of ESAT-6, CFP-10 and TB7.7, and has been successful in detecting latent *Mtb* infection in non-endemic areas and BCG vaccination does not interfere as these peptides are from the RD1 region unique to *Mtb* [35;36]. There is a continuing effort to develop relatively similar diagnostic tests for leprosy and it was shown that IFN-γ can be used to differentiate levels of *M. leprae* exposure as a response to *M. leprae* specific antigens [22;83]. However, prospective longitudinal studies are required to clearly define the different levels of exposure as risk or protective signals [82].

IFN-γ induced protein 10 (IP-10)

IP-10 is a small chemokine produced by many cell types but mainly by antigen presenting cells (APCs). There are several signals that induce IP-10 secretion such as T-cell derived IFN-γ, IL-2, IL-17, IL-23 and others. This makes IP-10 a reliable downstream marker as readout of cytokines like IL-2 and IFN-γ in CMI assays. Its expression is much higher which makes it more preferable for application in diagnostic tests [184]. In TB diagnostics, IP-10 has become a potential alternative to IFN-γ as it was found comparable to QuantiFERON© In-Tube test (QFT-IT) with higher level of expression [186] and also in combination with IFN-γ for detection of *Mtb* infection [185]. In leprosy, it has been decades since Kaplan *et al*. showed the potential of IP-10 in differentiating the different forms of leprosy by measuring its expression in skin lesions of TT/BT and LL patients where more IP-10 level was observed in TT/BT lesions [112]. Recent reports also revealed the potential of IP-10 in leprosy diagnosis by correlating with the level of exposure [22] and its potential in predicting T1R

[90;206]. It has now also been optimized for use in the field friendly UCP-LF assay [21] see chapter 5.

Monocyte chemo attractant protein 1 (MCP-1) (CCL2)
MCP-1 is a CC chemokine which is produced by various types of cells but mainly by monocytes/macrophages. It promotes macrophage infiltration in various inflammatory diseases and there is evidence for its importance in granuloma formation [47] which is an important phenomenon in containment of mycobacterial infections like leprosy and TB. There is evidence in TB studies that plasma levels of MCP-1 associate with TB disease and treatment responses [109]. Its potential to differentiate TT/BT patients from endemic controls was reported [83]. Its association with the PARK2 gene where level of MCP-1 increases along with increased expression PARK2 gene further confirms its involvement in the host defense against *M. leprae* infection [142].

Interleukin 1 beta (IL-1β)
IL-1β is a prominent member of the IL-1 family and is mainly produced by activated DCs and macrophages. Its expression regulates the Th1/Th2 balance such that higher expression of IL-1β leads to Th1 dominated response like in TT/BT patients while lower expression levels of IL-1β were found in LL lesions [124]. It was recently reported by our own group as a potential biomarker in detecting *M. leprae* exposure [22] as well as infection [83]

Interleukin-6 (IL-6)
IL-6 is a pro-inflammatory cytokine associated with inflammation. In leprosy, from previous assessments of plasma cytokines and chemokines in plasma of leprosy patients with type 1 and 2 reactions, IL-6 was reported as being elevated in both episodes as compared with non-reactional patients [19;148;159]. It was also significantly higher in endemic controls living in high leprosy endemic area than in those living in low endemic areas [22]. Recent studies also showed associations of single nucleotide polymorphism (SNP) in the IL-6 encoding gene with susceptibility to T2R [217], indicating the potential of IL-6 as genetic predictive risk marker. Similarly, increased IL6 expression in lesions of leprosy patients with reactions was reported [148] which decreased after prednisolone treatment[14].

1.10. Leprosy and co-infections

Being a mycobacterial infection, leprosy like TB, was predicted to manifest immune reconstitution disease (IRD) in HIV co-infected patients initiating treatment with anti retroviral therapy (ART). IRD or immune reconstitution inflammatory syndrome (IRIS) is an immune restoration disease in immunocompromised individuals such as HIV infected patients usually after the initiation of ART or a change to more active ART. As HIV mainly affects the host Th1 immune arm by affecting $CD4^+$ T cells, failure in containment of the disease in tuberculoid patients, an increase in lepromatous leprosy cases and increased transmission of leprosy was expected [128;233]. However, this has not been the case thus far: lepromatous forms have not increased although some patients on ART are being diagnosed as new leprosy cases, which requires attention [127;233]. Previous and recent reports on cellular and immunological parameters in leprosy and HIV co-infected patients demonstrated low numbers of $CD4^+$ T cells in the periphery but extensive $CD4^+$ T cells infiltration in the lesions of BT [189] with higher $CD8^+$ T cells [32]. In addition, no differences in histopathological features of leprosy were observed in HIV co-infected versus non-HIV patients [52].

In some studies, an association of type 1 leprosy reactions with ART was reported [140;195], suggesting ART and immune reconstitution could be risk factors for the development of leprosy reactions; however this requires further investigation.

In addition, intestinal parasites, mainly helminthic infections (non-protozoan intestinal parasites) are known to immune modulate the host by upregulating Th2 responses [10]. In mycobacterial infections like TB, studies have shown the importance of helminth infestation in weakening the Th1 immunity. For instance, poor immunogenicity of BCG in a helminth infested group compared to a de-wormed group was observed in an Ethiopian cohort [70;71]. The presence of intestinal helminthes may also facilitate the establishment of *M. leprae* infection or the progression to more severe forms of leprosy. A direct correlation between mycobacterial index and the frequency of intestinal helminthes in leprosy patients was also previously observed [56;57]. Therefore, further information needs to be collected in the area of helminth-leprosy co-infections, since this may have impact on clinical diagnosis and management.

Aims and outline of the Thesis

This thesis describes advances in the search for biomarkers relevant to leprosy diagnosis focusing in particular on early detection of the disease. It includes the screening of immunogenic and specific *M. leprae* antigens in endemic countries, identification of potential host biomarkers and development of field applicable diagnostic tools. It discusses the achievements and the unresolved challenges.

The approach for searching potential pathogen-derived biomarkers has changed dramatically after the year 2000 with the availability of the *M. leprae* whole genome sequence. The studies in this thesis selected antigens based on *in silico* algorithms, tested in leprosy patients, household contacts and endemic controls and endemic TB patients for their immunogenicity and specificity. Those unique and immunogenic proteins and peptides were investigated further as potential biomarkers in patients and healthy control groups, and eventually taken further to develop a field friendly diagnostic test based on both T cell and antibody responses. **Chapter 1** provides a detailed introduction into all areas studied in this thesis.

In **Chapter 2,** the screening of recombinant *M. leprae* proteins and synthetic peptides for immunogenicity and specificity in populations with different genetic background is described. T cell responses of stimulated PBMC from MB and PB patients, household contacts of MB patients, healthy endemic controls and TB patients were analysed using IFN-γ as a read-out for antigen-specific T cell activation. The positive responses found in 50% of healthy endemic controls for most of the antigens raised the issue of what level of *M. leprae* exposure was present in that subgroup.

In **Chapter 3** detailed analysis of host biomarker profiles in responses to the selected *M. leprae*-specific and immunogenic recombinant proteins and synthetic peptides is reported. The discriminating potential of these biomarkers among groups based on level of *M. leprae* exposure is an important result, clarifying why 50% of the healthy endemic controls responded to specific antigens. Other than the commonly known pro-inflammatory cytokine IFN-γ, several cytokines, chemokines and growth factors induced by adaptive and innate immunity were studied as well among which several were identified with discriminating potential. **Chapter 4** describes potential biomarker profiles in patients, in a longitudinal assessment, in an effort to predict leprosy reactions. Increased IFN-γ, IP-10, IL-17 and VEGF

were demonstrated whereas IL-10 was decreased in PBMCs simulated with *M. leprae* antigens during the onset of a reaction. This implicates the importance of combined assessments of Th1/Th2 responses in development of potential diagnostic assays. **In Chapter 5**, detection of identified biomarkers using lateral flow tests utilizing Up-converting Phosphor Technology (UPT) is demonstrated. The highly correlating results of the Up-converting Phosphor-lateral flow assay (UCP-LF) and ELISA demonstrated reliability of the assay. The potential of the UCP-LF assay to measure more than one biomarker (cellular and humoral responses) at a time has also revealed its appropriateness for field applicability.
Chapter 6 deals with assessment of the role of Tregs in view of the *M. leprae*-specific T cell non responsiveness in lepromatous patients. Using depletion assays, we demonstrated the recovery of T cell responses in one third of lepromatous patients after depletion of $CD25^+$ T cells. The increased presence of FoxP3 expressing T cells in the vicinity of Mϕ2 in LL lesions further revealed Tregs as one of the key factors responsible for poor CMI in lepromatous patients.

Chapter 7 describes co-infections in leprosy patients. Although the number of leprosy-HIV co-infected patients is small, understanding the influence of one disease on the other is important for proper patient management and for implementing proper control mechanisms. In recent studies including ours, most of the leprosy HIV co-infected patients on ART developed T1R, which requires further investigation to detect the underlying risk factors. In addition, we have also analysed and compared IFN-γ responses in helminth free and helminth-leprosy co-infected patients.

Finally, in **Chapter 8**, the main themes of the thesis are discussed in the broad context of leprosy diagnosis.

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