Exploring host and pathogen biomarkers for leprosy

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General Introduction

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Highlights & Hallmarks of leprosy

Leprosy, a chronic disease affecting the skin and nerves, is caused by *Mycobacterium leprae* or *Mycobacterium lepromatosis* (1). Leprosy has been reported in humans for millennia with ancient reports in Roman, Egyptian and Greek manuscripts. In Europe it was endemic during the Middle Ages until the sixteenth century when it dissipated. In 1873, during his work in a leprosarium in Bergen (Norway), Gerhard Armauer Hansen discovered *M. leprae* in unstained material. *M. leprae* is unculturable in present media and this has hindered the study of the disease, bacterial transmission and pathogenesis. Nevertheless, leprosy is a curable disease currently treated with multi-drug therapy (MDT) (2). The first antimicrobial treatment for leprosy was introduced in 1943 and was based on sulphones. In 1950s dapsone monotherapy was established, in 1982 MDT was recommended to multibacillary (MB) patients and in 2002 this was extended to all patients (3).

Leprosy classification

Leprosy can be distributed according to the Ridley Jopling (4) or the World Health Organization (WHO) classifications (5) based on clinical features and the immunological response against the leprosy bacilli. The Ridley Jopling classification consist of five forms, at one end of the spectrum tuberculoid leprosy (TT) patients display a low bacterial load and a strong cell-mediated immune response, characterized by Th1 and Th17 cells (6, 7), granuloma formation and elimination of bacteria. At the other end, lepromatous leprosy (LL) is characterized by a high bacterial load and humoral immunity with Th2 cells but almost no protective cell mediated immunity, allowing accumulation of high numbers of bacilli around foamy macrophages (8-12). However, the majority of leprosy patients present unstable borderline forms, between LL and TT and classified as borderline lepromatous (BL), borderline borderline (BB) and borderline tuberculoid (BT) (9).

The WHO classifies leprosy in two groups, paucibacillary (PB) and multibacillary (MB) leprosy and is based on the number of skin lesions and presence of acid fast bacilli in slit skin smears (SSS) (5). Leprosy patients with up to five skin lesions and negative SSS are considered PB whereas patients with six or more skin lesions and a positive SSS are classified as MB.

Leprosy reactions

Leprosy reactions present a major challenge in the prevention of (permanent) disabilities (13) because reactions can cause peripheral nerve damage and are the major cause of irreversible neuropathy (14). Leprosy reactions are increased inflammation episode which may occur at any time before, during or after MDT (15, 16). Up to 30-50% of leprosy patients may experience leprosy reactions, however, these are most common in unstable BL patients presenting a high number of bacilli (17). There are two types of reactions: type

1 reactions (T1R) or reversal reactions (RR) and type 2 reactions (T2R) or erythema nodosum leprosum (ENL). While T1R are characterized by inflammation of nerves and/or skin lesions, T2R, which can be very painful, are characterized by erythematous skin lesions which may present in combination with fever and/or malaise (18). T1R are delayed hypersensitivity reactions caused by an increased cellular immune response against the bacteria mediated via Th1 cells resulting in a switch from borderline leprosy to TT leprosy (19, 20). Several cytokines and chemokines such as IFNγ, IL-12, CXCL10 and IL-6 show higher levels in lesions or plasma during T1R (18). Moreover, anti-helminth treatment (21-24), extensive anti-TNF-α therapy (16, 20), HIV highly active antiretroviral therapy (HAART) or BCG vaccination (25) produce a shift from Th2 to Th1 cells that may trigger T1R.

ENL are likely immune complex-mediated reactions, however, the mechanism of action is still unclear (18). Nevertheless, lower expression of Treg cells and higher CD4/CD8 ratios are observed (26) in patients presenting ENL.

Leprosy epidemiology

In 2000 leprosy elimination as a public health problem, defined as a prevalence of less than 1 case per 10,000 in the world population, was achieved (2). However, the number of new cases has been steady during the last decade, with 208,619 new leprosy cases worldwide in 2018 (27). The majority of new cases occur in low- and middle-income countries where leprosy is still endemic. India with 120,334 new cases in 2018 is the country with the highest number of new cases, followed by Brazil with 28,660 new cases and Indonesia with 17,017 new cases. Numerous studies of this thesis were performed in Bangladesh, a leprosy endemic country reporting up to 3,729 new leprosy cases in 2018 (27).

The incubation period of leprosy is significantly long being 5-10 years for MB cases and 2-5 for PB cases (28). Moreover, only 5% of people exposed to *M. leprae* become infected, and from those, barely 20% eventually develop the disease (29). The long incubation period and the low amount of leprosy progressors within individuals exposed to *M. leprae* in addition to the limited awareness of leprosy by the public and healthcare providers as well as the social stigma hinders the identification of new cases, particularly amongst household contacts (HC) of leprosy patients who are at highest risk of developing disease (30).

The incidence of leprosy in females is lower than in males. Women represent around 35-37% of the new cases, however, this lower rate may be influenced by an under-diagnosis due to limitations women face in endemic countries such as restricted access to health services, illiteracy and low status (28). Moreover, MB cases are more common in men than in women. From all new cases children represent 9% and since these are recent infections, it indicates that *M. leprae* transmission is still ongoing.

Leprosy: the pathogen side

Transmission and One Health

M. leprae transmission is still not completely understood, however, aerosol transmission via the respiratory route and skin-to-skin contact are assumed to be the most probable ways of bacterial dissemination (31, 32). Besides bacterial exposure, other risk factors have been shown to be associated with leprosy development, including host genetic polymorphisms (33-37), close contact with untreated, MB patients (38), infection with soil transmitted helminths (21), immunosuppression (38), nutritional factors (39), food shortage (40), living conditions (41, 42) and individual characteristics (43, 44). HC of leprosy patients present the highest risk to develop leprosy (45), due to the continuous contact with a person infected with the leprosy bacilli. Besides, they might not develop leprosy, but bear *M. leprae* serving as asymptomatic carriers who contribute to transmission.

M. leprae and *M. lepromatosis* have been identified in several animals as well as environmental samples representing a reservoir that could potentially become a source of infection (46, 47). Moreover, a leprosy-like disease caused by *Mycobacterium lepraemurium*, *Mycobacterium tarwinense* or *Mycobacterium lepraefelis* has been reported in cats (48).

In the British Isles, Scotland and Ireland *M. leprae* and *M. lepromatosis* were detected in red squirrels causing a leprosy-like disease (46, 49-52). However, molecular testing by PCR showed absence of the leprosy bacilli in squirrels from France, Germany, Switzerland, Italy and Mexico (53), indicating that leprosy diseases and reservoirs of *M. leprae* and *M. lepromatosis* in squirrels are only present in the British Isles.

M. leprae was first found in nine-banded armadillos from southern United States in 1977 (54) and was thereafter extensively studied (55-57). Probable zoonotic transmission of *M. leprae* from armadillos was also identified in the southeastern United States where wild armadillos and patients were infected with the same genotype (3I-2-v1) (58). This genotype was also identified both in armadillos and humans from Florida (59) and is closely related to *M. leprae* strains circulating in medieval Europe, suggesting that leprosy arrived in the United States from Europe before it disappeared in the sixteenth century. Armadillos bearing *M. leprae* have also been found in Brazil, Mexico, and Colombia (60-63). A study in Pará (Brazilian Amazon), Brazil found a higher rate of leprosy amongst armadillo hunters and people who eat armadillo meat more than 12 times per year (60). However, other studies from Brazil found no association with armadillo meat consumption and leprosy in Curitiba (Paraná) (64) and absence of *M. leprae* in armadillos from Coari (Amazonas state) (65).

Furthermore, *M. leprae* infections have been reported in wild and captive nonhuman primates (66-68). The *M. leprae* strain identified in a captive nonhuman primate (branch 0, 3K genotype) in The Philippines was phylogenetically close to the human strains, suggest-

ing possible transmission between humans and nonhuman primates (66). Whereas wild chimpanzees in West Africa were found to be infected with *M. leprae* strains belonging to different and rare genotypes: 2F and 4N/O. Genotype 4N/O was also identified in a captive nonhuman primate in West Africa (67).

In addition to animals, *M. leprae* DNA has also been detected in the environment, namely in soil (47, 69-74) and water (74-76) from India and Brazil. It has been described that viable bacilli could survive in the environment in free living amoebic cysts up to 8 months (77). Thus, these environmental reservoirs should be taken into consideration to investigate transmission chains.

Pathogen genomics

M. leprae genome was first sequenced in 2001 (78) leading to the identification of several repetitive elements such as RLEP which is currently used as the PCR target for *M. leprae* DNA detection (79-82). *M. leprae* underwent a massive gene reduction resulting in a genome of 3.27 Mb, whilst the closely related *Mycobacterium tuberculosis* possess a genome of 4.41 Mb (78). The majority of *M. leprae* genome is composed of pseudogenes which correspond to active genes in *M. tuberculosis*. Several of the genes absent in *M. leprae* are involved in vital metabolic activity, thus converting *M. leprae* into an obligate intracellular bacterium. *M. leprae* phylogeny is composed of four genotypes (1-4) (83) and 16 subtypes (A-P) (58, 84). This phylogenetic information has been recovered from *M. leprae* genomes obtained from contemporary leprosy patients (83-85), but also from ancient skeletons (86-90), red squirrels (46, 53), armadillos (58, 59) and non-human primates (66, 67). Extensive whole genome sequencing (WGS) from different sources has proven to be a relevant tool to study *M. leprae* transmission.

The genome of *M. leprae* is highly conserved, presenting a mutation rate of 18 ± 30 mutations per 1000 years (87), which facilitates reconstruction of historic *M. leprae* transmission and human migration patterns (91). The most ancestral linages of *M. leprae* are 3K-0 or branch 0 followed by 3K-1 or branch 5, which are predominant in modern East Asia, particularly in China, Japan and Korea (85, 87). In Medieval Europe different *M. leprae* genotypes were present reflecting ancient human migrations (91). Genotypes 2F and 3I were common in north Europe, whilst in Hungary, Byzantine Turkey and the Czech Republic subtypes 3M and the ancestral lineage 3K were present, likely introduced trough the Silk Road from central Asia (86, 92). The origin of *M. leprae* is still ambiguous and two possible locations have been suggested: Western Eurasia or East Asia and the Middle East. Genotype 3K-0 is found in modern East Asia whilst this ancestral lineage was also present in medieval East Europe indicating that either of these regions could be the origin of leprosy (87). Genotype 3K-0 possibly spread through Europe, the Middle East and East Asia giving rise to the different genotypes 3 whilst genotype 1 is predominant in south Asia.

Genotype 4 is thought to have evolved from European genotype 3 into West Africa and later arrived to America through the slave trade. In addition to genotype 4, subtype 3I has also been identified in America, suggesting a likely introduction of leprosy by European immigrants (83, 84).

In 2008 *M. lepromatosis* was identified in two patients presenting diffuse lepromatous leprosy and a set of genes also present in *M. leprae* were sequenced (1). Although *M. lepromatosis* is closely related to *M. leprae*, 2.1% of divergence can be observed in the highly conserved bacterial marker 16S rRNA (1). In addition, protein coding genes show 93% nucleotide sequence identity between the two species and pseudogenes 82% (93). It was suggested that both *M. leprae* and *M. lepromatosis* diverged from the most recent common ancestor around 13.9-20 Mya (93, 94). Since *M. lepromatosis* has retained all functions required to infect Schwann cells of the peripheral nervous system it can also cause leprosy.

The host side

Host transcriptomic biomarkers for leprosy diagnosis

Leprosy, particularly PB leprosy, is difficult to diagnose and current diagnostic tests are not sensitive and/or specific enough, thus diagnosis still strongly relies on clinical symptoms. Early detection of leprosy together with identification of *M. leprae* asymptomatic carriers is crucial for an effective intervention aimed at stopping *M. leprae* transmission. Besides, an early diagnosis can prevent the development of disabilities. Late diagnosis or misdiagnosis of leprosy, particularly in non-endemic areas, is common due to the infrequent encounters of health personnel with this disease. Leprosy diagnosis is usually assisted by detection of acid-fast bacilli in tissue smears, lymph or histological sections using a Ziehl-Neelsen staining (95). This technique is not sensitive enough for the diagnosis of PB cases and also during the early stages of leprosy. Currently, molecular techniques based on (quantitative) PCRs to detect *M. leprae* DNA are also in use to support the diagnosis (79, 96, 97). While the sensitivity of such molecular techniques is higher than microscopy it is still challenging to detect *M. leprae* DNA in PB cases. In addition, it has been observed that HC, the group with the highest risk to develop leprosy, may be asymptomatic carriers of *M. leprae* (31, 70, 98-104). Therefore, presence of *M. leprae* DNA without clinical symptoms in HC is not a useful predictive marker of leprosy (105). Nevertheless, detection of *M. leprae* carriage or infection can be applied for targeted prophylactic treatment to reduce transmission.

Several host markers have been proposed to diagnose leprosy based on the immunological response to *M. leprae* (106-111). The most commonly used is detection of serum levels of IgM anti-*M. leprae* phenolic glycolipid I (PGL-I) which has been implemented as a point-of-care (POC) test (97, 109, 112). However, the majority of the available host markers fail to diagnose PB leprosy cases (106, 113) and some markers, such as PGL-I, could also be

present in HC who have been exposed to *M. leprae* but do not develop leprosy.

Transcriptomic host profiles have been proven to be effective to identify correlates of risk for tuberculosis (114, 115). In leprosy, some studies employed transcriptomics to investigate the immunological response to *M. leprae* but others also aimed at identifying potential biomarkers for leprosy or leprosy reactions (116-125). However, the studies that focused on biomarkers to identify leprosy were employed after clinical symptoms, thus are not useful for early or predictive diagnosis. In addition, the biosamples used for these transcriptional studies were skin biopsies, nerve biopsies or cell culture, and although these are particularly useful to study the pathogenesis of the disease they are not practical for POC diagnostic tests. Instead, blood or urine are less invasive and easy-to collect samples that would be favored for diagnosis. For this reason, blood samples were employed in a study to identify longitudinal differential gene expression (DGE) during T1R (126). Using dual-color Reverse Transcriptase Multiplex Ligation-dependent Probe Amplification (dcRT-MLPA) an increased expression during T1R was observed in genes associated with cytotoxic T-cell response, IFN-induced genes and *VEGF* whilst a decrease was found in T-cell regulation genes. In line with this, a blood signature including type I IFN components, autophagy, parkins and Toll like receptors was also identified during T1R (127). In a different study, applying RNA-Seq a 44-gene signature in blood was established that could differentiate between leprosy patients suffering a T1R and no leprosy reaction (121). The signature was formed by pro-inflammatory regulators, arachidonic acid metabolism mediators and regulators of antiinflammation. However, to date none of these studies has resulted in application of host transcriptomics to diagnostic (POC) tests.

Influence of host genetics on leprosy

Leprosy is an infectious disease, however, host genetics strongly influences the outcome of the disease. This is observed from the low rate of disease development in people exposed to *M. leprae* (29) and the wide spectrum of leprosy disease whilst bacterial genome variation is very limited, suggesting that host genetics play a crucial role in this variation. In the last 20 years the genetics of leprosy has been extensively investigated through genome-wide association studies (GWASs) and candidate gene association studies (CGASs) (128, 129). In 2009 the first GWAS in leprosy was performed in a Chinese population (33) and since then many studies have used GWAS to characterize the association of genetics with leprosy (130-133).

Most of the genes that have been associated with leprosy or leprosy reactions are immune related and involved in the innate or adaptive immunity. Numerous studies have explored the association of genetics with leprosy per se, leprosy type and leprosy reactions.

Leprosy per se, referring to the presence or absence of disease, was the first subject to be researched by genetic studies. Several genes have been associated with leprosy per se,

such as *TLR1*, *SLC11A1*, *VDR*, *NOD2, LACC1* and *TYK* or variants in the promoter regions of *PRKN*, *IL10* and *LTA* (Figure 1) (129). The major histocompatibility complex (MHC) has been strongly linked to leprosy, however, the large variation of HLA alleles has hampered its study (129). Most of the variants associated with leprosy per se identified in GWASs are non-coding, such as eQTLs for *NOD*2 or *IL18RAP* (134).

The association of genetic variation to leprosy polarization has been less studied than leprosy per se. Besides, classification into a leprosy subtype, particularly using the Ridley Jopling classification, depends on the physician's expertise and definition used, affecting the resulting classification. Most of the genes related to leprosy type have been found in CGASs whereas GWASs have focused mostly on leprosy per se (129). Genes such as *IL10*, *MBL2*, *MRC1*, *TGFB1*, *TLR2*, *TNF*, *CUBN* and *NEBL* have been associated to the subtype of leprosy (Figure 1) (135-140).

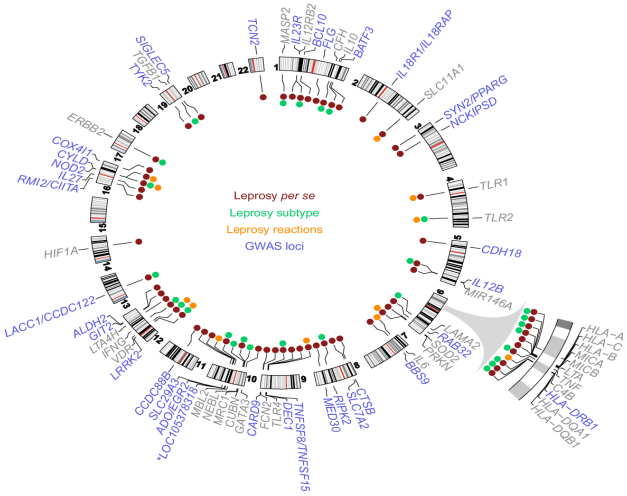

Figure 1. Genes and genome-wide association studies (GWAS) loci associated with leprosy per se (red), leprosy types (green) and leprosy reactions (orange). The human chromosomes 1–22 are presented in the circular plot. In blue, genes identified by GWAS and in gray genes identified using other approaches. Figure retrieved from Fava et al. (129).

CGASs have also been employed to study the relationship between host genetics and leprosy reactions. Since T1R are more common than ENL, association of genetics with T1R has been more widely investigated than ENL. Nevertheless, a study identified that allelic variance or absence of MHC class III protein C4B was associated with ENL occurrence (141).

Several *LRRK2* and *PRKN* variants as well as eQTLS for *LRRK2* were suggested to be linked with occurrence of T1R (142, 143). The mutations identified in leprosy patients presenting T1R have also been found in patients with Parkinson's disease, showing an overlapping inflammatory profile. In addition, eQTLs for lncRNA ENSG00000235140 (*LOC105378318*)

represent a risk for development of T1R (144). A selection of genes found to be associated with leprosy per se have also been linked to the occurrence of T1R: *PPARG*, *TNFSF8*, *TNFSF15*, *NOD2*, *LRRK2*, *TLR1*, and *TLR2* (Figure 1) (36, 135, 142, 144-148).

Outline of this thesis

The aim of the research described in this thesis was to combine the study on the pathogen *M. leprae* with the identification of transcriptomic and genetic host biomarkers associated with leprosy to aid the development of diagnostic tests as well as reduce transmission.

First, pathogen transmission was investigated through a One Health approach which incorporated analyses of *M. leprae* DNA derived from human, environmental and animal samples. In **chapter 2**, we identified leprosy patients and their asymptomatic HC carrying or infected with *M. leprae* in Bangladesh. We explored *M. leprae* genetic variation between individuals and intra-individually by WGS to identify transmission patterns. In **chapter 3**, we assessed whether *M. leprae* or *M. lepromatosis* were present in the environment. For this, we analyzed by PCR soil samples from the homes of leprosy patients in Bangladesh, the area where squirrels infected with *M. leprae* and *M. lepromatosis* were found in the British Isles and around holes of armadillos in Suriname. Since leprosy was previously observed in squirrels from the British Isles, in **chapter 4** we investigated whether Dutch and Belgian squirrels infected with *M. leprae* or *M. lepromatosis* could be identified.

Next, transcriptomic and genetic host biomarkers were identified to predict leprosy, leprosy reactions and genetic markers associated with susceptibility for leprosy. In **chapter 5**, we aimed to develop a transcriptomic signature that could predict leprosy development in HC of leprosy patients, 4 to 61 months before clinical symptoms. For this purpose, we analyzed gene expression variation in leprosy progressors using RNA-Seq: gene expression of leprosy progressors before clinical symptoms was compared with HC who remained without leprosy symptoms (cross-sectional analysis) and with the timepoint of clinical diagnosis, when symptoms were present (longitudinal analysis). In **chapter 6**, we studied gene expression differences in leprosy patients from Bangladesh, Brazil, Ethiopia and Nepal who developed leprosy reactions and identified a signature that predicted reversal reactions in leprosy patients before onset. In **chapter 7**, we investigated the association of 11 host genetic markers with leprosy in Bangladesh through a family-based study consisting of leprosy patients and both progenitors.

Finally, in **chapter 8** all findings and conclusions of this thesis are summarized and discussed.

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