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Hansen's Disease

The Forgotten and Neglected Disease

Edited by Wellman Ribòn

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Meet the editor

Wellman Ribón is a bacteriologist, clinical laboratory professional and specialist in environmental chemistry from the Universidad Industrial de Santander. He has a master's degree in Biochemistry from the Universidad Pontificia Javeriana and is a doctoral student in public health at the National Institute of Public Health of Mexico. He is also recognized as a senior researcher by COLCIENCIAS. Wellman Ribón has over 20 years of experience as a public health advisor and researcher in scientific and technological development in health. He has worked at the National Institute of Health of Colombia as coordinator of the Mycobacteria Group, has been a manager of scientific research projects, and is a member of the Colombian Center for Excellence in Research in Tuberculosis, EurolabTB Consortium and SLAMTB. He is currently a titular professor at the School of Medicine of the Faculty of Health of the Universidad Industrial de Santander where he works as a professor and researcher. He created the Mycobacterium Research Laboratory, in which he makes his research proposals, and contributes to the training of undergraduate and postgraduate students.

Mr. Ribón has published articles on diseases such as tuberculosis, leprosy and mycobacteriosis, and has written seven book chapters. He carried out several research projects on tuberculosis, leprosy and mycobacteriosis, and is currently developing a line of scientific research in the evaluation of health processes, plans, policies and programs. He is the editor of the books *Tuberculosis: Expanding Knowledge*, *Mycobacterium: Research and Development* and *Hansen's Disease - The Forgotten and Neglected Disease*.

The area of interest of Mr. Ribón's professional work has always been public health focused on diseases such as tuberculosis, leprosy, mycobacteriosis and their comorbidities and relationships with the social determinants. These are areas that are being linked to the processes of scientific evaluation due to the great negative impact of these diseases on public health.

For his contributions Mr. Ribón received the distinction "Orden Lorencita Villegas de Santos" on behalf of the Colombian Antituberculosis League and Respiratory Diseases - LAC.

Contents

Preface

The purpose of this book is to expand the knowledge and investigations of a disease feared by humanity since biblical times. Leprosy was once related to sin and thought to be caused by heredity or curses. Unfortunately, these stigmatizations still persist. This book about leprosy encompasses its nature, etiology, epidemiology, clinical studies, diagnosis, molecular techniques and treatment, which are still challenging research worldwide.

This book's goal is to contribute to science: the immunological, molecular and genetic advances that have been made in the management of leprosy. It contains important information in a clear, concise, and precise way about the history, epidemiology, pathology, diagnostic methods and genetic implications described for the disease, with the intention of emphasizing that leprosy remains a complex disease and of interest to public health. For this reason, the study of leprosy continues to represent a great challenge to humanity, because despite maintaining its control, it has not yet been possible to interrupt its chain of transmission. Public health agencies need to join forces to achieve eradication.

Here you will find important information about leprosy, dealing with major issues such as the characteristics of the etiological agent, the host's immune response and its updating in epidemiology, diagnosis and treatment. In addition the book studies the difficulty of early detection of the disease that has led to the search for existing techniques for its use in diagnosis, as well as the need to give importance to molecular methodologies, since the genetic study of *Mycobacterium leprae* has allowed the detection of genes or specific sequences for drug identification and resistance.

The studies in this book will also give insight into the investigations of the participation of genetic factors that are involved in the susceptibility and immunopathogenesis of the disease, since the microorganism expresses a series of components involved in the pathogen–host interaction and its association of different genetic variants in the recognition of the clinical manifestations of leprosy.

With the rapidity of advances in knowledge about leprosy, this book incorporates contributions from professionals and experts in the study of *M. leprae* to ensure a broad review and update of each of the chapters presented here, showing perspectives that open new possibilities to address the study of leprosy and its impact on clinical, immunological, molecular and epidemiological research in the coming years. We hope that this book will be of great interest and expand information on and contribute to relevant aspects of the disease.

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Chapter 1

Introductory Chapter: Hansen´s Disease – The Forgotten and Neglected Disease

Lina Fernández and Wellman Ribón

Additional information is available at the end of the chapter

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1. Introduction

Leprosy has been one of the diseases most feared by mankind and has managed to stigmatize societies for its lamentable symptoms and consequences. It is known since the remote epochs of the Biblical Times, the Middle Ages, the modern era, the Renaissance, and its lyrical poems, dedicated to those patients with leprosy in which their suffering was interpreted as a curse, a divine punishment, or a hereditary disease. The condemnation of those patients diagnosed with Hansen's disease was despair, physical, integral, and social death, promoted by rejection, persecution, and exile, because they had to be expelled and live hiding their shame and physical decomposition, trying to adapt to the spiritual change and its real beauty as the disease spread through their bodies; being recruited forcibly in those "cemeteries for the living" known as leprosaria, leprocomios, or lazaretos were dragging their lives and awaited death face to face; being neglected by a society that considered that the disease was the worst abomination that could exist, and that at the same time it did not know that it was through no fault of their own that those Hansen's disease patients contracted the disease [1, 2].

Leprosy is a disease believed to be incurable and characterized by marked changes in physical appearance and evolutionary skin lesions affecting the skin, peripheral nerves, and nasal mucosa. It was discovered in 1873 by Gerhard Hansen, who demonstrated that it was an infectious disease and not a curse, helping to establish the fundamental principles of immunology, bacteriology, and also public health and thus putting an end to the idea of leprosy being un-curable. Even so, because of the social stigma and fear, humanity continued to consider those with the disease as cursed pariahs, and the patients had to take refuge in places where those suffering from the same ailment were housed, the lazaretos; hidden there, they were isolated from the horror and repugnance of an uneducated society [3].

Today, the scientific community recognizes *Mycobacterium leprae* as the causative agent of the disease, although the postulates of the scholar Koch do not apply to this bacillus. This is a microorganism that prefers the colder zones of the body, and the most documented way of transmission is the one which expresses that the disease is transmitted from person to person by prolonged contact with a baciliferous patient through the respiratory tract reaching the nerves, skin, and eyes where its incubation period is long and years may pass from the infection to the emergence of evident clinical manifestations in a leprosy patient. Belonging to the *Mycobacteriaceae* family, this slightly curved bacillus measuring from 1 to 8 microns in length and 0.3 microns in diameter is intracellular, with tropism toward the macrophages and Schwann cells of the peripheral nerves. It is an acid alcohol-resistant bacillus as its wall consists of polysaccharides and mycolic acids that make it hydrophobic and resistant to discoloration and is not cultivable, a factor that makes it difficult to identify the microorganism by conventional methods [3, 4].

M. leprae contains in its genome around 3,200,000 base pairs and has 57.5% of guanine-cytokine; its sequencing identifies 1614 genes that encode proteins, 1116 considered as pseudogenes and 50 genes that encode stable RNA (Cole, Eiglmeier et al. 2001). The recombination between repetitive sequences and chromosomal rearrangements, translocations, and genetic deletions probably caused the genome reduction and mutation of the metabolic areas of *M. leprae* (Eiglemeier, Parkhill et al., 2001), making it conserve only the genes necessary for its transmission, fixation, and survival in the host and making it a "really astute microorganism" [5, 6].

Symptomatic skin (SP) patients have a skin lesion, anesthetic, hypopigmented, or reddish with limited borders or diffuse, non-congenital, unlike a scar, and symptomatic peripheral nervous system (SSNP) are defined as people with anesthetic body areas with distal problems of the feet or eyelids. People affected by leprosy have historically been diagnosed according to medical criteria using conventional methodologies, such as sputum smear that identifies acid- and alcohol-resistant bacilli, as well as samples of mucus and lymph, and skin biopsy that allow to observe the acid- and alcohol-resistant bacilli or the destruction of the peripheral nerve, achieving the classification of the disease as paucibacillary or multibacillary and thus establishing the treatment determined by the World Health Organization (WHO). Once *M. leprae* infects the skin and nervous tissue, it replicates slowly for years, and several mechanisms of skin lesions are triggered, reflecting those clinical manifestations of the disease that depend on the immune status. Based on the above, the WHO established two categories of the disease: paucibacillary, characterized by sporadic lesions on the skin with low presence of bacilli and immunological reactions Th1 type with high production of cytokines that favor the formation of granulomas that include tuberculoid leprosy (LT) and borderline tuberculoid leprosy (TB), or multibacillary, characterized by numerous lesions on the skin and a high bacillary load, Th2-type immune response with the absence of granuloma, including lepromatous leprosy (LL), borderline lepromatous leprosy (LB), and borderline-borderline (BB) leprosy [7–9]. They are the unstable forms of leprosy, and if they are not treated on time, these can evolve into lepromatous or tuberculoid forms gradually causing the disfigurement of the extremities and the development of physical disabilities, which are defined by WHO in three degrees of disability: grade 0 indicates the absence of disability; grade 1 indicates the loss of sensitivity in eyes, hands, and feet; and grade 2 indicates severe visual impairment and deformity of hands and feet [10].

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The treatment of the disease occurred in three stages: firstly, the incurability when there were no accurate studies of the disease and the patients had to be isolated and removed from the society. Secondly, the monotherapy that began in 1941 when Guy Farget found a derivative of dapsone as a cure for leprosy and was the only medicine available until then in the world, but in the middle of the 1960's *M. leprae* began to show resistance to the drug, causing the health authorities to begin the fight to maintain control over the disease and the search for strategies to eliminate it since it was a public health problem. Finally, rifampicin and clofazimine were discovered and added to the treatment of the disease known as polychemotherapy. In 1981, the WHO recommended multidrug therapy (MMT) consisting of the administration of dapsone, rifampicin, and clofazimine in multibacillary patients, with treatment for prolonged periods to completely eliminate the causative agent of the disease [11].

Worldwide, the diagnosis of Hansen's disease is based on criteria established by the WHO, and it is determined by a careful and thorough clinical examination in the search for characteristic lesions of the disease, such as looking for hypopigmented spots accompanied by loss of sensitivity, temperature, and pain or thickening of the peripheral nerves. However, there are disadvantages in the effectiveness of the diagnosis due to the nonexistent standard method that differentiates the infection from the disease. Laboratory tests are still established as a diagnostic support through the visualization of the bacillus by smear microscopy, the histopathology, or the intradermal reaction to lepromin. Since the advent of molecular biology techniques, a great impact has been made in different fields of science. They are currently used not only in the diagnosis of diseases but also in the study of pathologies, finding and understanding a wide variety of infectious diseases (immunological and genetic) [12].

Following the arrival of a highly sensitive and specific technique such as the polymerase chain reaction (PCR), used in the detection and quantification of DNA to differentiate species and aid the rapid identification of drug resistance, this molecular methodology has been fundamental in the investigation of infectious diseases, thus developing methods based on PCR. It is certainly necessary to adopt it to detect and identify *M. Leprae* in the shortest possible time and as a diagnostic support for the amplification of nucleic acids with high purity of different molecular targets, thus interrupting the chain of transmission and the sequelae of disability, since leprosy is an unheeded disease despite WHO's efforts to improve leprosy control programs [13].

For Hansen's disease, the use of PCR is based on the knowledge of gene sequences that code proteins and repeated sequences, allowing the analysis of different sequences on the genome of *M. leprae*, preparing specific complementary primers of the opposite strand of DNA, and achieving in vitro dissociation and reassociation by heating and cooling. The primers are incubated with the DNA to amplify it, and a DNA polymerase synthesizes the complementary chain through a series of specific temperatures that seeks its denaturation, binding and synthesizing the nucleotides corresponding to the *Mycobacterium* [14]. Through the cyclical application of these processes, exponential copies of the nucleic acid fragment of the microorganism are achieved.

2. Conclusion

Although the control of leprosy in the world was achieved, it has not yet been eradicated, and the lack of an effective diagnostic method is one of the limitations in the control of the disease, since the long period of incubation of the disease and the dissemination of *M. leprae* mean that the conventional methodologies used are not conclusive and are only useful in symptomatic patients or in those with physical changes, and infected cohabitants or patients without symptoms or injuries are not diagnosed in a timely manner. Therefore, molecular methodologies are an alternative of causality and are needed for the diagnosis of the disease. The evolution of the disease and the continuous use of basic methodologies for its diagnosis highlight the importance of implementing molecular methods to achieve early diagnosis of the disease and thus diminishing the emergence of disabling forms, since methods based on PCR are capable of generating large amounts of DNA, analysis of genetic variability, typing of strains, either through the use of genetic markers, repeated sequences, genetic polymorphisms, microsatellites, and white sequences, among others, demonstrating that PCR is the method of the future for the diagnosis of leprosy, its sensitivity, specificity, diversity, and simplicity allows identifying sources of infection, patterns of transmission, monitoring treatment, and detecting resistance to drugs of the disease, which would be of great support for follow-up and timely treatment sought by health programs, and thus maintaining the control of a disease considered as unattended.

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Chapter 2

The Distribution and Origins of Ancient Leprosy

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Abstract

Human leprosy is primarily caused by *Mycobacterium leprae*, but also by the related '*M. lepromatosis*'. Ancient leprosy can be recognised in archaeological materials by the paleopathology associated with multi-bacillary or lepromatous forms of the disease. Whole *M. leprae* genomes have been obtained from human skeletons, and diagnostic aDNA fragments have been recovered. The derived *M. leprae* phylogenies, based on single nucleotide polymorphisms, mirror past human migrations, as *M. leprae* is usually an obligate pathogen. The detection of *M. leprae* in historical leprosy cases is assisted by the hydrophobic *M. leprae* cell envelope, which is composed of unusual lipids that can be used as specific biomarkers. Lipid biomarkers are more stable than aDNA and can be detected directly without amplification. Indigenous human leprosy is extinct in Western Europe, but recently, both *M. leprae* and '*M. lepromatosis*' were found in British red squirrels. Leprosy may also be found in nine-banded armadillos (*Dasypus novemcinctus*) where it can cause a zoonotic human infection. Certain leprosy-like diseases, caused by uncultivable species in cats, for example, may be related to *M. leprae*. The closest extant relatives of leprosy bacilli are probably members of the *M. haemophilum* taxon, emerging pathogens with genomic and lipid biomarker similarities.

Keywords: ancient DNA, lipid biomarkers, genotyping, leprosy, paleopathology, evolution

1. Introduction

Leprosy (Hansen's disease) is a chronic infectious disease that has been recognised over millennia. In the majority of human cases, it is caused by *Mycobacterium leprae*, but recently a

related organism, '*M. lepromatosis*', has also been implicated [1] and appears to cause diffuse lepromatous leprosy (DLL). Both organisms are obligate pathogens that are uncultivable in cell-free growth media. Although '*M. lepromatosis*' has been the subject of many recent publications [2–5], there is still discussion about whether it is a distinct species [6]; currently, it is a name without standing in nomenclature (http://www.bacterio.net/-nonvalid.html). Leprosy is primarily a disease of peripheral nerves and skin, but it also affects bones. The genomes of *M. leprae* and '*M. lepromatosis*' have been sequenced, and it is clear that they diverged from a common ancestor many millennia ago [7, 8]. The genome of '*M. lepromatosis*' confirms a close but distinct relationship with *M. leprae*, and both organisms can also cause disease in animals, such as armadillos and squirrels [9–12]. The closest ancestors of these leprosy bacilli are probably relatives of *M. haemophilum* that has genomic and lipid biomarker similarities [13–16].

Initially, ancient leprosy was recognised by the paleopathology associated with multi-bacillary or lepromatous forms of the disease [17, 18]. Leprosy causes skeletal changes in the rhinomaxillary area, including pitting and perforation in the palate, resorption of the nasal spine and the maxilla leading to loss of the upper teeth. The tubular bones of the hands and feet are frequently involved. In the tibia and fibula, inflammatory periostitis can be recognised; the metatarsals and metacarpals are often resorbed so these small bones develop a pencil shape. In sub-adult individuals afflicted with multibacillary leprosy, the development of the secondary dentition can be affected, leading to a rare condition, *leprogenic odontodysplasia* (LO), where the incisor teeth exhibit a characteristic root constriction [19]. Intriguingly, this has been seen only in archaeological cases and not in a clinical setting. Cases have been described from medieval Denmark [20] and in four individual medieval inhumations from the St. Mary Magdalen, Winchester leprosarium [21]. Subtle skeletal changes like grooving on the volar surfaces of the proximal phalanges may also accompany paucibacillary forms of leprosy that cause digital contracture or loss of pain sensation [22].

Suspected leprosy cases can be confirmed by the detection of *M. leprae* ancient DNA (aDNA) [23, 24] and further characterised by repetitive DNA sequences and genotyping [25, 26]. The aDNA detection of *M. leprae* in historical cases is probably assisted by the protective presence of unusual lipids in the *M. leprae* cell envelope. These lipids can be used as specific biomarkers; they are more stable than aDNA and can be directly detected without amplification (*vide infra*). Lipid biomarkers have been used to confirm aDNA findings [21, 27–29]. However, due to their stability, lipid biomarkers can also confirm a diagnosis of leprosy initially based on paleopathology, even in the absence of aDNA [30].

2. Causes and distribution of modern leprosy

2.1. *Mycobacterium leprae*

M. leprae, the main cause of leprosy in humans, is a slow-growing intracellular *Mycobacterium* and the average incubation period of the disease is about 5 years, although symptoms may occur within 1 year or up to 20 years after infection [31]. Leprosy mainly affects the skin, peripheral nerves, the mucosa of the upper respiratory tract and the eyes, as *M. leprae* has a tropism for

Schwann cells in nerves and macrophages in the skin [32]. The infection is transmitted by direct contact with untreated cases or healthy carriers or via infectious aerosols [33]. The clinical presentation of leprosy depends upon the cell-mediated immune (CMI) response to infection. If the host has an effective CMI response, few lesions develop, and there are only scanty bacilli in the tissues. However, some patients are anergic to *M. leprae*, so develop lepromatous leprosy with ineffective antibodies, a high bacterial load and multiple lesions. The clinical presentation of leprosy in a patient can vary over time, so there are borderline leprosy types where the immune response is unstable. It can show a wide range of clinical presentations from tuberculoid leprosy (TT) through borderline forms: borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL) to lepromatous leprosy (LL) [34]. A recent World Health Organization classification scheme recognises a simplified two-category system of either paucibacillary or multibacillary forms of leprosy [35]. The histopathology of skin lesions varies from compact granulomas to diffuse infiltration of dermis, which largely depend upon the immune status of the patient and may not be in agreement with the clinical diagnosis [36, 37]. The mycobacterial antigens can activate a chronic inflammatory response that is exacerbated by pro-inflammatory cytokines. Therefore, in late stages of leprosy, there may be no *M. leprae* bacilli in the tissues, but residual mycobacterial antigens can drive an inflammatory response that causes neurological damage [38].

2.2. '*Mycobacterium lepromatosis*'

'*M. lepromatosis*' appears to have a tropism for endothelial cells and can give rise to vasculitis and necrotic erythema. It seems to be less common than *M. leprae* and was initially believed to be geographically restricted to patients from Mexico and the Caribbean, where it was identified in patients suffering from diffuse lepromatous leprosy (DLL) [1, 39–41]. It was subsequently recognised in Brazil, Myanmar, Canada and Singapore and in mixed infections with *M. leprae* [3, 4]. Symptoms, characteristic of 'Lucio's phenomenon', have been associated with '*M. lepromatosis*' [1, 40, 42]. A case of two Mexican siblings infected with '*M. lepromatosis*' indicates facile transmission [5, 6]. However, '*M. lepromatosis*' has recently been found in the wild Eurasian red squirrel, *Sciurus vulgaris*, in the British Isles, from England, Scotland and Wales [11, 12]. In addition, *M. leprae* was found in red squirrels on the Isle of Wight and Brownsea Island, close to the south coast of England [43, 44]. This was very surprising, as although indigenous leprosy was prevalent in the human population of the British Isles in the first millennium (CE), it is now believed to be extinct. In these modern squirrels, the macroscopic signs and histopathology were characteristic of lepromatous leprosy, but no pathological differences were noted between infections caused by '*M. lepromatosis*' or *M. leprae* [12, 45]. The strain of '*M. lepromatosis*' in British wild squirrels is genetically distinguishable from Mexican strains found in modern day humans, and it appears that these strains diverged from a common ancestor about 26,000 years ago [12]. However, the *M. leprae* strain found in British red squirrels is similar to a strain found in human remains from a mediaeval leprosy hospital in Winchester [46], only 70 km from the Isle of Wight and Brownsea Island. One suggestion is that, in the past, humans may have been infected through direct contact with red squirrels as these were prized for their meat and fur [12]. They were also kept as pets, as is evident from various illustrated medieval manuscripts and art, for example 'A Lady with a Squirrel and a Starling' by Holbein the Younger (painted ca. 1526–1528, National Portrait Gallery, London).

2.3. Nature and distribution of *M. leprae* genotypes

Major collaborative studies based on single nucleotide polymorphism (SNP) typing have established that modern *M. leprae* consists of four distinct genotypes that are associated with different human populations [47]. It is believed that the ancestral precursor of *M. leprae* experienced an evolutionary bottleneck and thereafter developed independently in different human populations [26, 48]. In Europe, indigenous leprosy is now largely extinct, so a further study also looked at *M. leprae* from archaeological cases using aDNA methods [26]. This identified SNP type 3 cases from various European countries for the first time, including Denmark, Hungary, Croatia, Turkey and Britain. Some cases provided subtypes I, M or K. Genotype 3 strains were also found from Roman Egypt and by others in medieval Central Europe [30, 49]. Later studies also reported SNP type 2 strains for the first time in medieval cases from Winchester, UK [21] and from Sweden [50, 51]. Archaeological remains from Japan yielded a SNP type 1 from that country [52]. Several of the robust cases were subsequently amplified by whole genome sequencing (WGS) [46, 53].

Monot et al. [26] also recognised sub-genotypes from extant cases, thereby enabling more precise associations between *M. leprae*, geographical location and present human populations ranging from China [54] to South America [55]. In a detailed study of modern *M. leprae* that included SNP typing, variable-number-tandem-repeat (VNTR) analysis and WGS, Truman et al. [9] examined 50 patients with leprosy and 33 wild armadillos (*Dasypus novemcinctus*) in the United States, together with reference strains from other parts of the world. Seven *M. leprae* SNP types were detected. The SNP type for some patients with possible exposure by foreign residence was typical of *M. leprae* from foreign locations. The most abundant SNP type was 3I that is generally associated with historical northwest European or American populations. The SNP sub-type 3I-1 strains, with one copy of an 11-bp indel (indel_17915) had ancestral bases, but all other *M. leprae* strains have two copies. Type 3I-2 strains, a development of the ancestral 3I-1 strains, similarly have only one copy of indel ML_17915 and can be identified by base C at position 1527056 instead of base G present in type 3I-1 isolates [9]. These 3I-2 strains were found in all armadillos and most of the indigenous patients so the authors concluded that armadillos act as a reservoir for *M. leprae* and that there is zoonotic spread of leprosy in the Southern United States. As the disease was not present in the New World before European contact, it is assumed that the spread of the disease was linked to human migrations and that armadillos acquired leprosy from human cases [45, 56].

2.4. Transmission of leprosy

Recently it was realised that the enhanced hydrophobicity of tubercle bacilli is a key factor in aerosol transmission [57, 58]. Since it is becoming established that aerosol transmission is a prime mode for the spread of leprosy bacilli [33, 59], the transmissibility of the different manifestations of *M. leprae* should be considered. In a detailed study [33], it was demonstrated that MB/LL cases provided more transmissible bacilli than PB/TT patients. It would be of great interest to compare the relative cell envelope surface lipid composition of LL and TT leprosy bacilli to explore the possibility that the hydrophobicity of LL forms is enhanced or otherwise. It may also be possible to determine directly the relative hydrophobicity of *M. leprae* in biopsy

material, using micro fluorescence methods [60]. The evasion of airways epithelial clearance [33, 59] may be encouraged by enhanced hydrophobicity of infective agents.

3. Recognition, diagnosis and spread of ancient leprosy

3.1. Pathology and recognition of ancient leprosy

Leprosy is primarily a disease of the peripheral nervous system. In the past, the disease would run its natural course, resulting in both specific and nonspecific bony changes plus paleopathology due to secondary infections following nerve damage [17, 18, 61]. Ancient leprosy is typically recognised by the presentation known as *facies leprosa* or rhinomaxillary syndrome, in which the nasopharynx is remodelled, the nasal spine and palate are resorbed, and eventually also the maxilla, leading to loss of the upper teeth. There are changes to the tubular bones of the hands and feet including osteoporosis caused by disuse, pitting and perforation. The long bones of the lower leg also show paleopathology associated with inflammatory periostitis [30, 62–64].

M. leprae ancient DNA (aDNA) was first detected in skeletal remains with typical leprosy paleopathology soon after the introduction of PCR [23]. Subsequently, many further paleopathological cases of leprosy were confirmed by *M. leprae* aDNA from across Europe and the Middle East [24–27, 30, 49–51, 64–69]. Specific *M. leprae* short DNA sequences were targeted as ancient DNA (aDNA) becomes highly fragmented over time [70]. *M. leprae* aDNA amplification has confirmed leprosy and enabled genotyping of isolates from Europe, Byzantine Turkey and Roman Egypt (**Table 1**). As additional methodologies were developed, different *M. leprae* strains were distinguished by microsatellite analysis based on aDNA repetitive sequences [27, 71] and now whole *M. leprae* genomes have been obtained from historical human skeletons [46, 53]. The results of aDNA amplification studies, WGS and lipid biomarker detection are summarised in **Table 1**.

3.2. The potential of lipid biomarkers

The detection of *M. leprae* in historical leprosy cases is assisted by the *M. leprae* cell envelope, which is composed of unusual lipids some of which can be used as specific biomarkers (**Figures 1–3**). The mycolic acids of *M. leprae* are restricted to homologous α- and ketomycolates [79, 80], whose major components are shown in **Figure 1**.

Characteristic mycocerosic acids are components of both phthiocerol dimycocerosate waxes (PDIMs) (**Figure 2**) [81–83] and so-called phenolic glycolipids (PGLs) (**Figure 3**) [82–85]. *M. leprae* mycocerosates unusually include major amounts of a C_{34} component, accompanied by small proportions of a C_{33} acid (**Figure 2**). *M. haemophilum* produces a PGL with the same two internal sugars (3-*O*-Me-rhamnose and 2,3-di-*O*-Me-rhamnose), but in reversed order and with different linkages (**Figure 3**). Besra et al. [13] concluded that this mycocerosate profile was essentially the same, thereby revealing a close phylogenetic link between *M. leprae* and *M. haemophilum* for the first time.

Century (CE); location: cases	*M. leprae*		*M. leprae* genotype	Notes	Publications
	DNA	Lipids			
1st; Israel, Akeldema, Himmon valley: SC1	+				Matheson et al. [69]
1st–4th; Uzbekistan Devkesken 6: 5b	+	+	3L		Taylor et al. [27]
4th; Egypt, Dakhleh Oasis, Kellis 2: B116 and 7 other samples	+		3K/L/M (B116)		Donoghue et al. [72]; Monot et al. [26]
4th–7th; Israel, Jerusalem: HZ	+				Spigelman and Donoghue [67]
5th–6th; United Kingdom, Great Chesterfield: GC96	+	+	3I-1 (variant)	MTB –	Inskip et al. [73]
6th–7th; Israel, monastery on River Jordan: AR	+				Rafi et al. [23]
6th–8th; Italy, Morrione: T68, T108	1/2 + (T108)				Donoghue et al. [30]
7th; Hungary, Szeged-Kiskundorozsma-Daruhalom dűlő II: KD271, KD517, KD518	3/3+	KD517+	3K (KD271)	KD517 lipids+ and MTB+	Minnikin et al. [29]; Lee et al. [28]; Donoghue et al. [30]
7th; Italy, Vicenne: T18, T31, T144	1/3+ (T18)	2/2+ (T18, T144)		DNA-lipids+ (T144)	Donoghue et al. [30]
7th–8th; Hungary, Szentes-Kistőke: SK11	+				Donoghue et al. [30]
7th–9th; Hungary, Bélmegyer-Csömöki domb: 22	+	+		MTB lipid+	Donoghue et al. [30]; Molnár et al. [74]
7th–9th; Hungary, Szarvas Grexa, Téglagyár: SG-38	+	+			Minnikin et al. [29]; Donoghue et al. [30]
8th–9th; Turkey, Kovuklukaya: 9/1, 11/2, 20/1, 24/1	3/4+ (11/2−)	1/3+ (24/1+)	3K (20/1)		Minnikin et al. [29]; Donoghue et al. [30]
8th–9th; Croatia, Radasinovci: 2A, 3A	+				Watson et al. [49]
8th–9th; Austria, Zwölfaxing: 70, 88	2/2+			MTB DNA+ (88)	Donoghue et al. [30]
9th–10th; Czech Republic, Prušánky: 188	+		3M		Donoghue et al. [30]

Century (CE); location: cases	*M. leprae*		*M. leprae* genotype	Notes	Publications
	DNA	Lipids			
10th; Hungary, Hajdúdorog-Gyúlás: HG-56	+	+			Minnikin et al. [29]; Donoghue et al. [30]
10th; Hungary, Sárrétudvari-Hízóföld: S237	+			Palate+ Toe−	Haas et al. [65]
10th–11th; UK, Norwich: 11287, 11503, 11784	+		3		Watson et al. [49]
10th–11th; Hungary, Püspökladány-Eperjesvölgy 11, 222, 429, 503	+	+ (503)	3K (222) 3M (503)	222 and 503 MTB+	Donoghue et al. [30]
10th–12th; UK, Wharram Percy: G708	+		3		Taylor and Donoghue [71]
11th; Sweden, Björned: A4	+	+		MTB+	Donoghue et al. [72]; Minnikin et al. [29]
10th–12th; Sweden, Sigtuna: 10, 32H, 3077, 3092V, 3093F, 3159Hsin, 3320V, 3401H, F13320, S10V3	7/10+		2F (3092 and 3077) 3I (3093)	WGS	Economou et al. [50, 51]; Schuenemann et al. [46]
11th; Hungary, Felgyő, Kettőshalmi-dűlő: 2467, 3658	1/2+			3658+	Donoghue et al. [30]
11th; Hungary, Lászlófalva-Szentkirály: 79	+			MTB+	Donoghue et al. [30]
11th–12th; UK, Orkney: CC4	+				Taylor et al. [66]
9th–13th; UK, Winchester: Sk2, Sk7, Sk19 Sk8, Sk14, Sk27 Sk18	+	+	3I-1 2F	WGS Sk18 (weak)	Schuenemann et al. [46]; Taylor et al. [21] Roffey *et al*. 2017 [75]
11th–14th; Denmark, Refshale:2, 16, 26, 32, 36	1/5+	+	2F (Refshale16)	Refshale 16+	Schuenemann et al. [46]
12th; Spain, Seville: A43, A120	+				Montiel et al. [76]
12th; Czech Republic, Žatec: AO9611, AO9731	+				Likovsky et al. [77]
12th–14th; Poland, Suraz: A1	+				Donoghue et al. [70]; Witas et al. [78]

Century (CE); location: cases	*M. leprae*		*M. leprae* genotype	Notes	Publications
	DNA	Lipids			
13th–14th; Denmark, Odense: Jorgen 625, 1020	1/2+	+	3I (Jorgen 625)	Jorgen 625+	Schuenemann et al. [46]
13th–16th; UK, Ipswich, Blackfriars: 1914	+		3I* (variant)		Taylor et al. [25, 27]; Taylor and Donoghue [71]
13th–16th; Denmark, Odense: G483	+		3I/J		Watson et al. [49]
15th; Hungary, Szombathely: 10	+	+			Donoghue et al. [72]; Minnikin et al. [29]
15th–18th; Germany, Rain/Lech: R1788, R2208	2/2+				Haas et al. [65]
18th–20th; Japan, Aomori: SK26	+		1		Suzuki et al. [52]

Cases are listed in a chronological order.

Table 1. Detection of ancient leprosy using aDNA and lipid biomarkers.

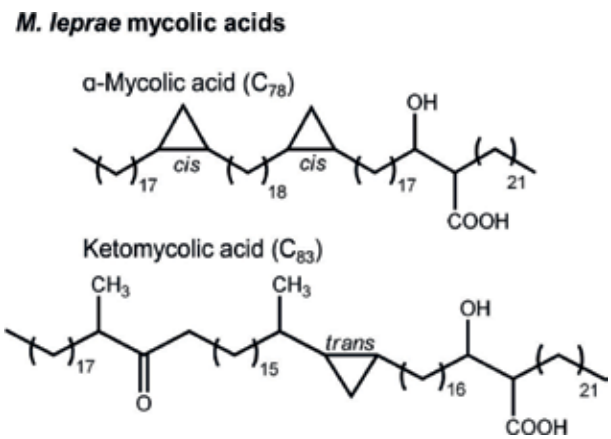

Figure 1. Mycolic acids of *M. leprae*. The main C_{78} α-mycolate and C_{83} ketomycolate are shown; additional homologous components are also present.

The lipid composition of '*M. lepromatosis*' remains to be determined, but limited information is available for *M. haemophilum*. In addition to α- and ketomycolates, *M. haemophilum* appears to have methoxymycolates, on thin-layer chromatography of extracts [86], but in a previous study, the patterns were unclear with material being degraded by acid methanolysis [87]. A gas chromatographic profile of *M. haemophilum* fatty acids [86] displayed an essentially typical mycobacterial profile, including tuberculostearic acid. The analysis was not extended to search for the unusual mycocerosic acids found previously in *M. haemophilum* (**Figure 2**) [13]. The only novel component was an incompletely characterised monounsaturated 2-methyl-branched C_{25} fatty acid and an enhanced proportion of C_{22} docosanoic acid was noted as being

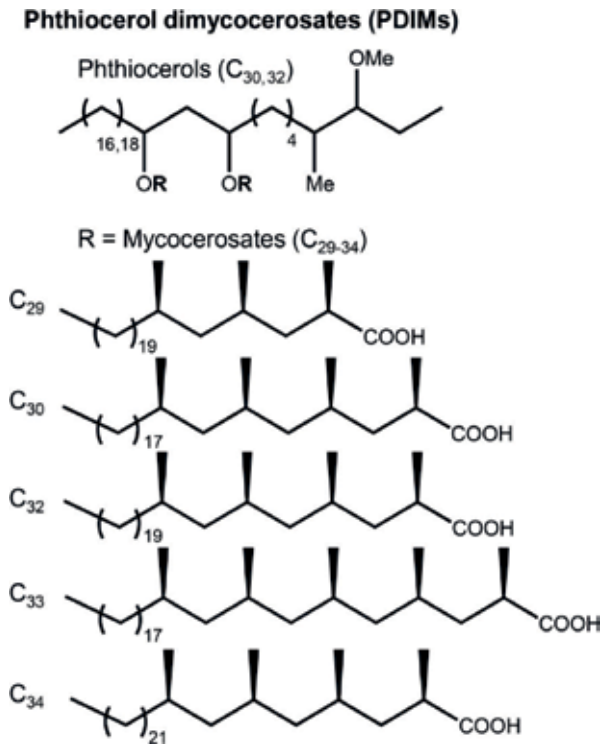

Figure 2. Phthiocerol dimycocerosates (PDIMs) of *M. leprae*. The C_{33} and C_{34} mycocerosates are diagnostic components for *M. leprae* and *M. haemophilum*, but C_{29}, C_{30} and C_{32} acids are shared with members of the *M. tuberculosis* complex [13, 81, 82].

similar to that found in *M. leprae* in a previous study [88]. However, an analysis of three *M. leprae* isolates did not record unusually enhanced proportions of docosanoic acid [80], nor did an additional analysis of *M. haemophilum* fatty acids [89]. It is interesting to compare the profile of uncharacterised fatty acids from *M. haemophilum* in an older study [87] with the more recent study [86]. An unusual large peak, labelled 19A, in the first analysis [87] could possibly correspond to the minor branched C_{25} acid in the later analysis [86]. This unusual C_{25} acid is a potentially valuable biomarker for *M. haemophilum* so its structure and cellular location should be investigated.

The biomarker potential of *M. leprae* lipids has been harnessed by fluorescence high performance liquid chromatography (HPLC) of pyrenebutyric acid (PBA) esters of mycolic acid pentafluorobenzyl (PFB) esters [90] and negative-ion chemical-ionisation gas-chromatography mass-spectrometry (NI-CI GC-MS) of mycocerosate PFB esters [91, 92]. Mycolate HPLC is exemplified in **Figure 4** for standard *M. leprae* and an extract of a skeleton (Sk2) from a mediaeval leprosy hospital near Winchester, UK [21]. Fluorescent mycolate derivatives are recognised by reverse-phase HPLC (**Figure 4A**), collected and analysed by normal phase HPLC to separate the α- and ketomycolate classes (**Figure 4B**). Reverse-phase HPLC provides the size and overall composition of the α-mycolates (**Figure 4C**) and ketomycolates (**Figure 4D**) for comparison with standard *M. leprae*.

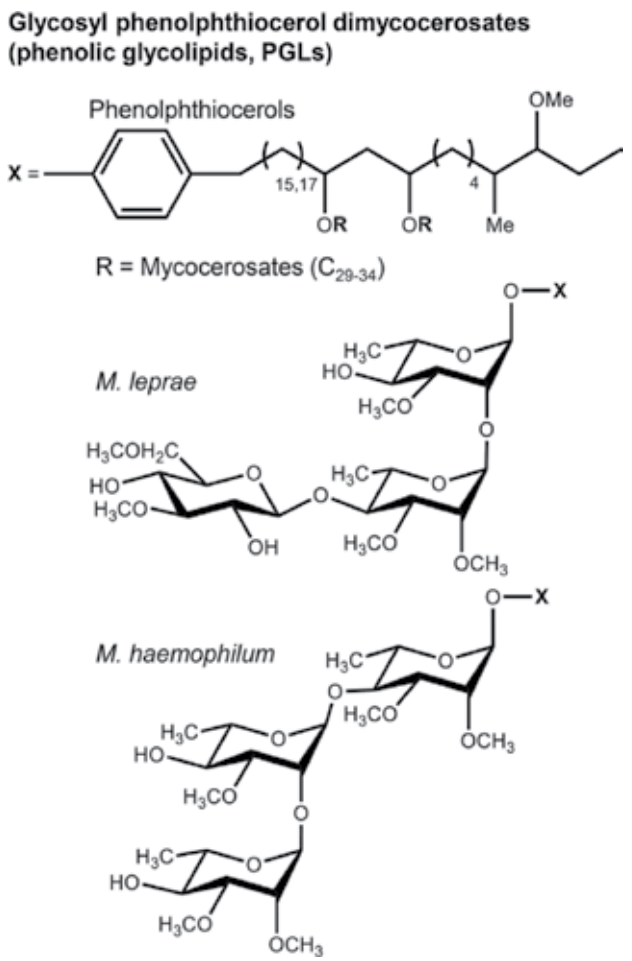

Figure 3. Phenolic glycolipids of *M. leprae* and *M. haemophilum*. The common phenolphthiocerol unit is attached to distinctive trisaccharides that share particular diagnostic sugars, 3-*O*-Me-rhamnose and 2,3-di-*O*-Me-rhamnose [13].

Selected ion monitoring NI-CI GC-MS analyses of mycocerosate PFB esters from Winchester skeleton Sk2 [21] and standard *M. leprae* are shown in **Figure 5**. There is good correspondence between the Sk2 extract and the standard; the Sk2 profile is unpublished work (O.Y-C. Lee, H.H.T. Wu, G.M. Taylor, K. Tucker, R. Butler, S. Roffey, P. Marter, D.E. Minnikin, G.S. Besra, G.R. Stewart, manuscript in preparation). In summary (**Table 1**), aDNA analysis with occasional lipid biomarker support has been successful in characterising ancient leprosy [21, 27, 29].

3.3. Distribution and phylogeny of ancient leprosy

Further, aDNA studies based on *M. leprae* sub-genotypes have given valuable information about the distribution of the disease in different human populations in the past [26]. The earliest known case of leprosy recognised by both skeletal paleopathology and aDNA, was from the early first millennium CE from the Ustyurt Plateau, Uzbekistan [93], with radiocarbon dating that suggests a date between the first and third centuries CE [94]. The *M. leprae*

http://dx.doi.org/10.5772/intechopen.75260

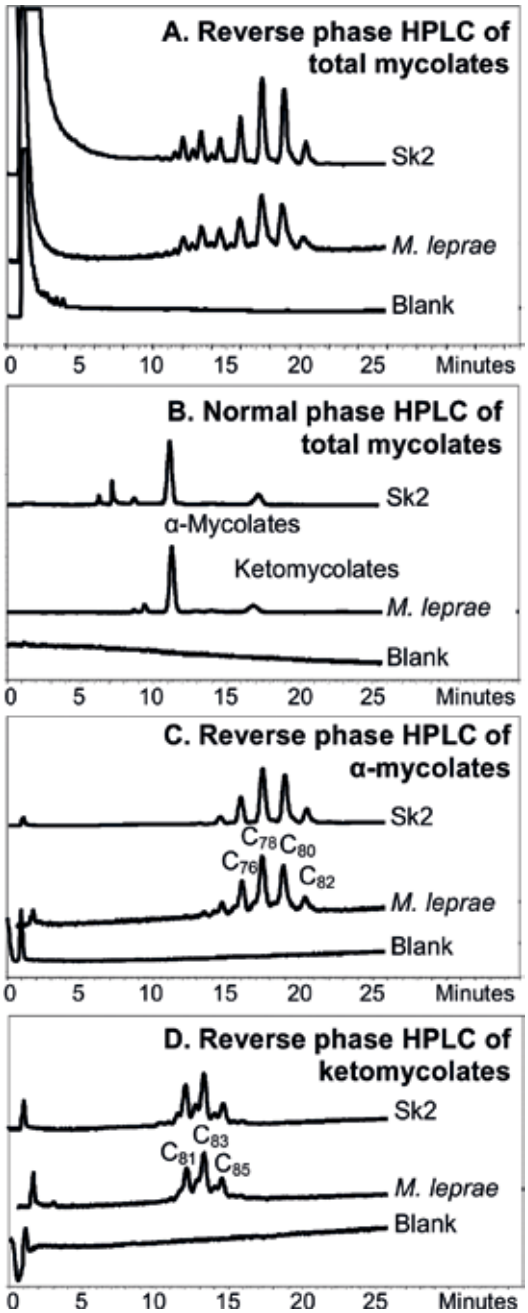

Figure 4. Mycolic acid profiles of Winchester skeleton Sk2. (A) Total mycolates, reverse phase HPLC; (B) collected total mycolates (MAs), normal phase; (C) collected α-mycolates, reverse phase; (D) Collected ketomycolates, reverse phase [21].

aDNA from this location was found to be of sub-genotype 3L [27] and the variable number tandem repeat analysis identified a unique aDNA profile [71]. Sub-genotyping has revealed that in historical Europe, there are clear differences between the leprosy found in human

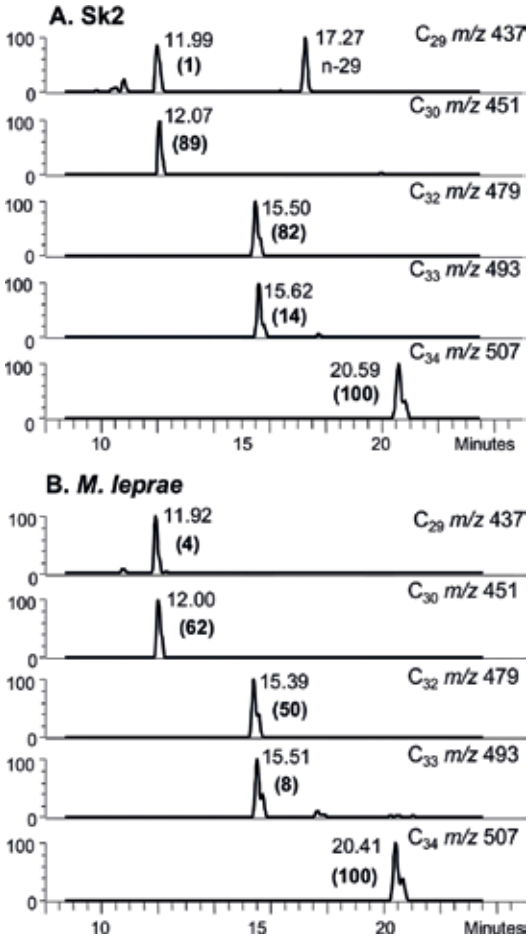

Figure 5. Mycocerosic acid profiles of Winchester skeleton Sk2. Selected ion monitoring NI-CI GC-MS of mycocerosic acid pentafluorobenzyl ester from A, Sk2 and B, *M. leprae* standard.

populations from central and southern Europe, compared with northwest Europe (**Table 1** and **Figure 6**). In Scandinavia and the British Isles, there are examples of *M. leprae* genotypes 2F and 3I [21, 46, 53, 75]. In historical northwest Europe, 3I-1 sub-genotypes were common, but in Hungary, Byzantine Turkey and the Czech Republic, sub-genotypes 3K and 3M were found [30]. It is believed that these differences reflect past human population movements. In northwest Europe, people travelled from Siberia and the Arctic, whereas central Europe was colonised by successive migrations from central Asia *via* ancient routes, such as the so-called Silk Road. WGS of the 3K subtype shows that it belongs to the earliest lineage of extant *M. leprae*, now termed branch 0 [46], and therefore carries characteristics of the most recent common ancestor (MRCA), not found in other groups. The distribution of the various European sub-genotypes is summarised in **Figure 6** and their phylogenetic relationship in **Figure 7**. It would be informative to have more data points for the Mediterranean basin and major countries, such as Spain, France and Germany.

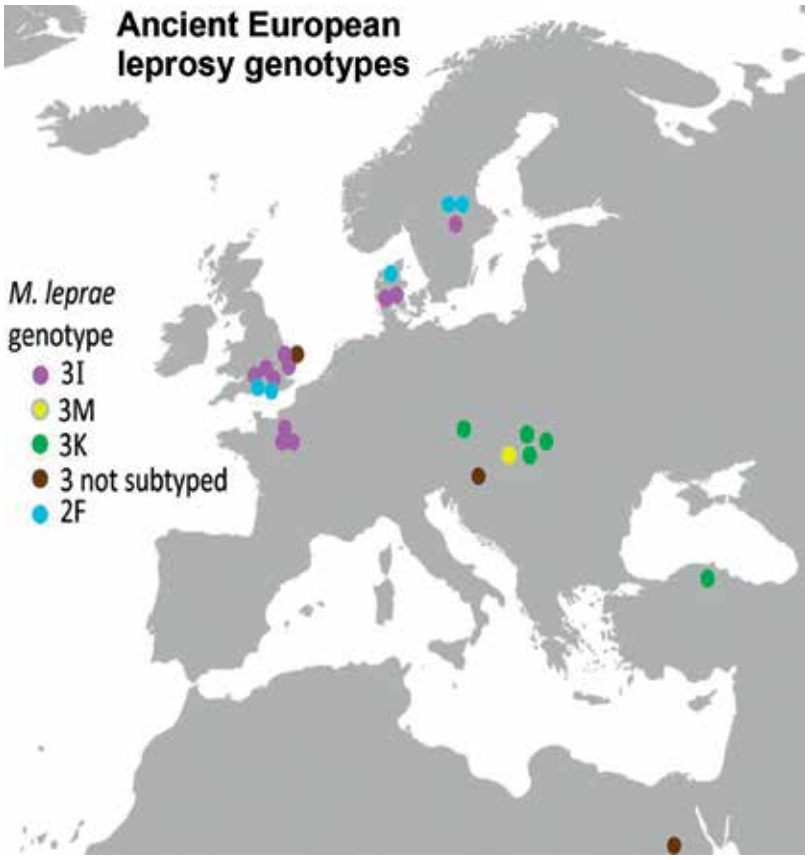

Figure 6. Geographical distribution of ancient leprosy sub-genotypes in the European area. Three type 3 strains are included where sub-typing was not determined.

3.4. Co-infection of leprosy and tuberculosis

Leprosy was a significant problem in Scandinavia until a century ago, leading to the identification of the leprosy bacillus by Hansen in 1871 [95], although publication was delayed due to the inevitable unsuccessful attempts at culture. In Central Europe, however, leprosy was prevalent in the first millennium CE, but a subsequent decline appeared to coincide with the upsurge of tuberculosis. Support for a period of overlap between leprosy and tuberculosis has been provided by a number of clear archaeological examples of dual infection, from first century AD Israel, fourth to fifth century Roman Egypt, seventh to eleventh century Hungary, eighth to ninth century Austria to tenth to thirteenth century Sweden [30, 72]. In one particular case, it was possible to use quantitative lipid biomarker analysis to estimate the relative amount of leprosy and tuberculosis infection [28–30]. Mathematical modelling to explore the epidemiological consequences of dual infection concluded that the disappearance of leprosy could indeed be explained by *M. leprae*/*M. tuberculosis* co-infections [96]. This may explain the present absence of indigenous human leprosy in Europe. Currently characterised *M. leprae*/*M. tuberculosis* co-infections are summarised in **Table 2**.

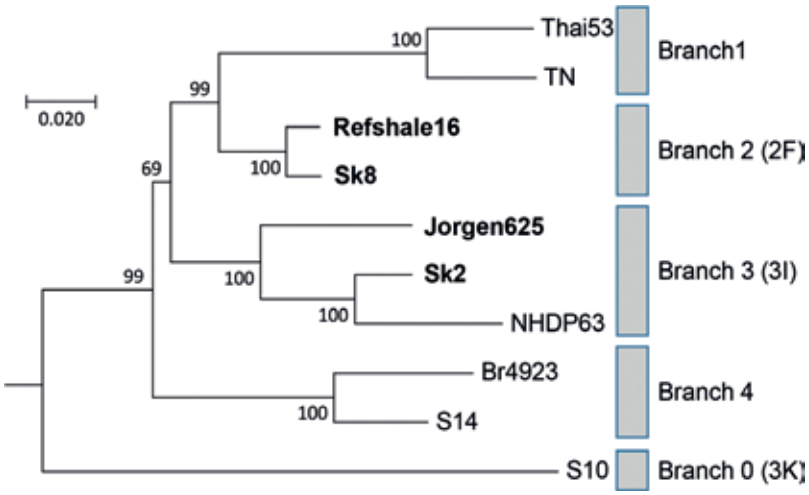

Figure 7. A phylogeny of selected *M. leprae* strains. The phylogeny was derived from an alignment of genomic SNPs [46]; ancient strains are denoted in bold. Phylogenies were generated in MEGA7 [105], using Maximum Likelihood methods. Phylogenies based on Neighbour Joining methods generated similar dendrograms. The scale represents the number of substitutions per site. Bootstrap values were determined from 500 replicates. '*M. lepromatosis*' was used as an out-group (not shown). Subtypes are indicated in brackets.

Authors	Year	Region	Century (CE)	Methods and comments
Nuorala et al. [97]	2004	Sweden	10th–13th	**PCR**: ML RLEP 129/99 bp; MTB 123 bp/92 bp. Nested products sequenced
Donoghue et al. [72]	2005	Egypt	4th–5th	**PCR**: ML RLEP 129/99 bp; MTB 123 bp/92 bp
		Hungary	10th–16th	
		Israel	1st	
		Sweden	10th–13th	
Matheson et al. [69]	2009	Israel	1st	**PCR**: ML RLEP 129/99 bp; MTB IS6110 123/92 bp
Minnikin et al. [29]	2011	Hungary	7th	**PCR**: Not re-tested; **Lipids**: mycolates and mycocerosates indicate relative disease load for Kiskundorozsma-Daruhalom dűlő II Grave KD517
Minnikin et al. [29]	2011	Hungary	15th	**PCR**: Not re-tested; **Lipids**: MTB methoxymycolates and ML ketomycolates for Szombathely Grave 6
Molnár et al. [74]	2015	Hungary	7th–9th	**PCR**: MTB IS6110 123/92 bp; IS1081 113 bp; ML not tested; **Lipids**: mycolates, mycolipenate and mycocerosates for Bélmegyer-Csömöki domb Grave 22
Donoghue et al. [30]	2015	Central and Eastern Europe	Various 6th–11th	**PCR**: ML RLEP 129/99 bp; 111 bp; 80 bp and probe; RepLep 66 bp and probe; SNP typing indicates migratory patterns into Europe. Coinfections suggest role of MTB in decline of European leprosy

Cases are listed according to year of study.

Table 2. aDNA and lipid biomarker detection of ancient ***M. leprae*** and ***M. tuberculosis*** complex co-infections.

4. Origins and evolution of leprosy

4.1. Genomics of modern leprosy

Whole genome sequencing has revealed large numbers of pseudogenes in both *M. leprae* and '*M. lepromatosis*' [7, 8, 98–100]. These genomic studies revealed that both *M. leprae* and '*M. lepromatosis*' have undergone a reductive evolution in which extensive recombination events have occurred between dispersed repetitive sequences, leading to less than half of their genomes containing functional genes. In a preliminary study [7], it was indicated that the genome of '*M. lepromatosis*' (~3.22 Mb) was 1.6% smaller than that (~3.27 Mb) of *M. leprae* [98, 99]. A comprehensive parallel study gave a similar genome size of ~3.21 for '*M. lepromatosis*' [8]. Functional comparisons revealed that whereas *M. leprae* has a defective *heme* pathway, '*M. lepromatosis*' lacked several genes needed for amino acid synthesis [8]. It is apparent that '*M. lepromatosis*' is the closest known mycobacterial taxon to the established species of *M. leprae*. Phylogenetic analysis indicates that '*M. lepromatosis*' and *M. leprae* diverged from a most recent common ancestor (MRCA) about 13.9 million years ago [8].

4.2. Evolutionary origins of leprosy bacilli

The deep origins of mycobacterial disease remain to be clearly defined [3, 47, 98, 99]. In contrast to tuberculosis, which appears to stretch back hundreds of thousands of years [57, 58], the earliest manifestations of human leprosy are found in skeletal remains only about 4000 years old [101]. However, the older participation of animal hosts cannot be ruled out, as it is increasingly evident that Pleistocene megafauna may have had a major involvement in tuberculosis evolution [58]. A possible ancestral organism to the organisms that cause leprosy may have been more like modern *M. haemophilum*, an emerging pathogen with a variety of possible natural reservoirs. The first significant link identified between *M. leprae* and *M. haemophilum* was established a quarter of a century ago in a study of the so-called 'phenolic glycolipids' (PGLs) [13]. As shown in **Figure 3**, the similarity in the oligosaccharide composition of the PGLs was striking and the mycocerosate profile (**Figure 2**) almost identical. This early key observation was subsequently reinforced by taxonomic studies that showed a close association of *M. leprae* and *M. haemophilum* [14, 15, 102]. Again, in studies comparing *M. leprae* and '*M. lepromatosis*', *M. haemophilum* was the nearest neighbour [8, 39], as illustrated in **Figure 8**. The recent determination of a full genome (~4.23 Mb) for *M. haemophilum* confirmed the close link [16], as shown in **Figure 8**. *M. haemophilum* is consistently placed outside of the *M. leprae*/*M. lepromatosis* group but between *M. leprae* and other mycobacteria such as the *M. tuberculosis* complex. It was suggested that the reductive evolution of *M. leprae* and '*M. lepromatosis*' was not shared with the most recent common ancestor but started after the divergence of *M. haemophilum* from both taxa [16]. The relatedness of *M. haemophilum*, *M. leprae*, '*M. lepromatosis*' and related taxa is shown in **Figure 8**.

4.3. Animal and environmental sources of leprosy ancestors

To assess the involvement of ancient relatives of *M. haemophilum* in the evolution of leprosy bacilli, it is necessary to consider the ecological, environmental and animal host preferences of

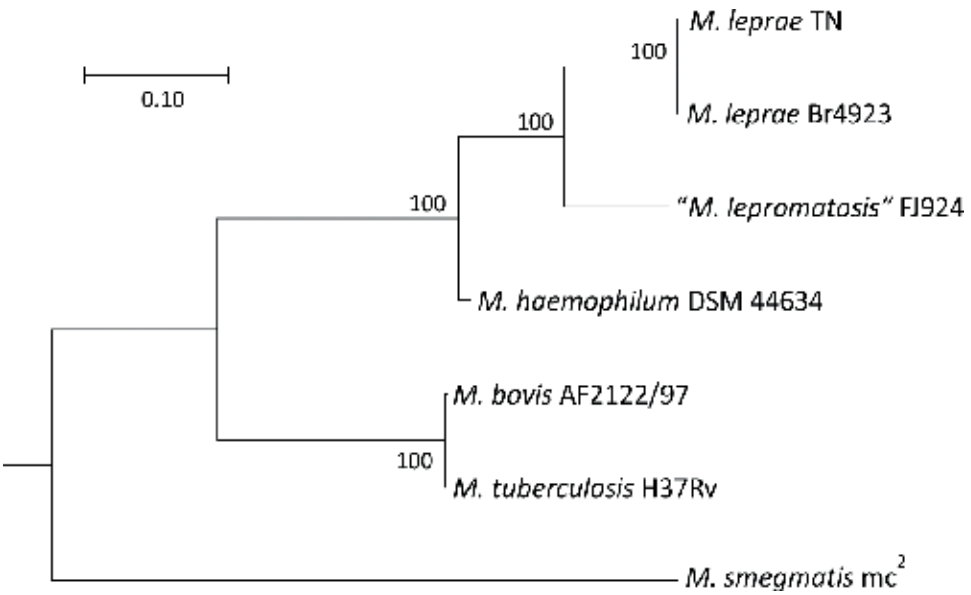

Figure 8. A phylogeny of *M. leprae* strains and other mycobacterial species. Genomic sequence coding for DnaN [103] from illustrative mycobacterial species was aligned with Clustal Omega [104] and their phylogeny inferred with MEGA7 [105] using the Maximum Likelihood methods and the Hasegawa-Kishino-Yano model with possible invariant sites. Phylogenies consistent with this interpretation were obtained with Neighbour-joining methods and when concatenated amino acid sequence of conserved proteins was used in the alignment. Bootstrap values are derived from 500 replicates.

this taxon. *M. haemophilum* is slow growing, requires iron supplementation and prefers a low growth temperature of 30°C. The first description of *M. haemophilum* was as a pathogen causing skin infections, particularly not only in immunocompromised patients [106, 107], but also in healthy children [108]. In a range of children, a variety of other clinical manifestations were encountered [15]. In two instances, *M. haemophilum* infections mimicked the appearance of leprosy [109, 110] and a co-infection of *M. leprae* and *M. haemophilum* has been reported [111]. Also, animal infections are common, with zebra fish (*Danio rerio*) being particularly susceptible [15]. More recently, a heavily infected leatherback sea turtle (*Dermochelys coriacea*) was found [112]. Infection of a haemophiliac with *M. haemophilum* was linked to contact with raw shrimp [113]. This suggests that *M. haemophilum* can move freely in a variety of environments, but it does not give a clear indication whether there is a particular zoonotic host in which the evolution of *M. haemophilum* may have occurred.

As noted previously, both *M. leprae* and '*M. lepromatosis*' can cause disease in squirrels [11, 12, 43, 44]. The presence of leprosy in armadillos is long established [9, 10, 114, 115] and, indeed, the armadillo was a prime source of material for early studies of the leprosy bacillus [79–81, 83, 84]. It is apparent that infected armadillos can spread leprosy to the human population [9, 10]. However, the leprosy introduced into the Americas by human migration was passed on to indigenous armadillos [46] so they can be eliminated as an environmental evolutionary source. The involvement of squirrels in the UK is more intriguing as it is difficult to envisage how

the diseases can have been contracted from human sources. A direct evolutionary pathway from ancient squirrel-like animals to humans is unlikely, but it is possible that squirrels are representative of other animals that may have acted as environmental reservoirs. In the case of '*M. lepromatosis*', a geographical association between patients and Mexican field rats (*Rattus rattus*) suggests a possible environmental reservoir [8].

4.4. Animal diseases resembling leprosy

Cases of tuberculoid nodular thelitis in both cattle [116] and goats [117] appear to be caused by uncultivable acid-fast species related to *M. leprae* and '*M. lepromatosis*'. However, the interrelationships between these agents, infecting cattle and goats, need to be defined more precisely before the disease can be considered as a true variety of leprosy. A complex scenario is emerging regarding the status of infections categorised as 'feline leprosy' [118–121]. After many early reports of diverse manifestations of cat leprosy, a definitive study clarified the scene [122]. It was apparent that the rat leprosy bacillus, *M. lepraemurium*, made a contribution to disease, but the influence of a novel uncultivable *Mycobacterium*, whose closest relative was *M. malmoense*, was noted. In a follow-up study [123], it was observed that younger cats were susceptible to *M. lepraemurium*, but more mature felines typically harboured the novel uncultivable agent. In an interesting development, PCR amplification of 16S rRNA sequences, from the uncultured feline agent AJ294740-6, showed that the greatest nucleotide identity was shared with *M. leprae* and *M. haemophilum*, as well as *M. malmoense*; indeed a specific additional nucleotide correlated with only with *M. leprae* [124]. This particular taxon, expressed in cases from eastern Australia, New Zealand and possibly Canada, has been provisionally labelled '*M. lepraefelis*' [121]. Three North American feline infections appeared to be caused by another uncultivable agent with close 16S rRNA relatedness to *M. leprae* and more distant affinity to *M. haemophilum*, among other species [125]. Initially labelled '*M. visibilis*', but more properly '*M. visibile*', this taxon remains uncharacterised and unfortunately unavailable for further study [120]. In a limited area of southeast Australia, studies of feline leprosy have revealed the presence of *M. lepraemurium* and an uncultivable novel agent, labelled '*M. tarwinense*'. This agent was indicated to be a fastidious member of the *M. simiae* complex [120, 126] so it does not appear to have a direct relationship with *M. leprae* or '*M. lepromatosis*'.

4.5. Overall interrelationships of leprosy affiliates

The precise interrelationships between all the bacterial taxa causing leprosy-like diseases require further study. It is clear that *M. leprae* or '*M. lepromatosis*' cause human leprosy and the same agents can routinely infect armadillos and squirrels. The apparent affinities of the feline leprosy taxon, labelled '*M. lepraefelis*', with *M. leprae* and *M. haemophilum* must be explored. The agents causing tuberculoid nodular thelitis in cattle and goats appear to have an affinity with established leprosy bacilli and this should be thoroughly investigated. In view of present uncertainties, it is premature to consider any concept of an *M. leprae* complex, as has been discussed [6, 8, 118, 127].

The possible origins and interrelationships of all agents causing leprosy-like disease are summarised in **Figure 9**. The phylogeny of *M. haemophilum* with *M. leprae* and '*M. lepromatosis*' indicates a deep common ancestor for all three taxa [16]; this ancestor is provisionally labelled

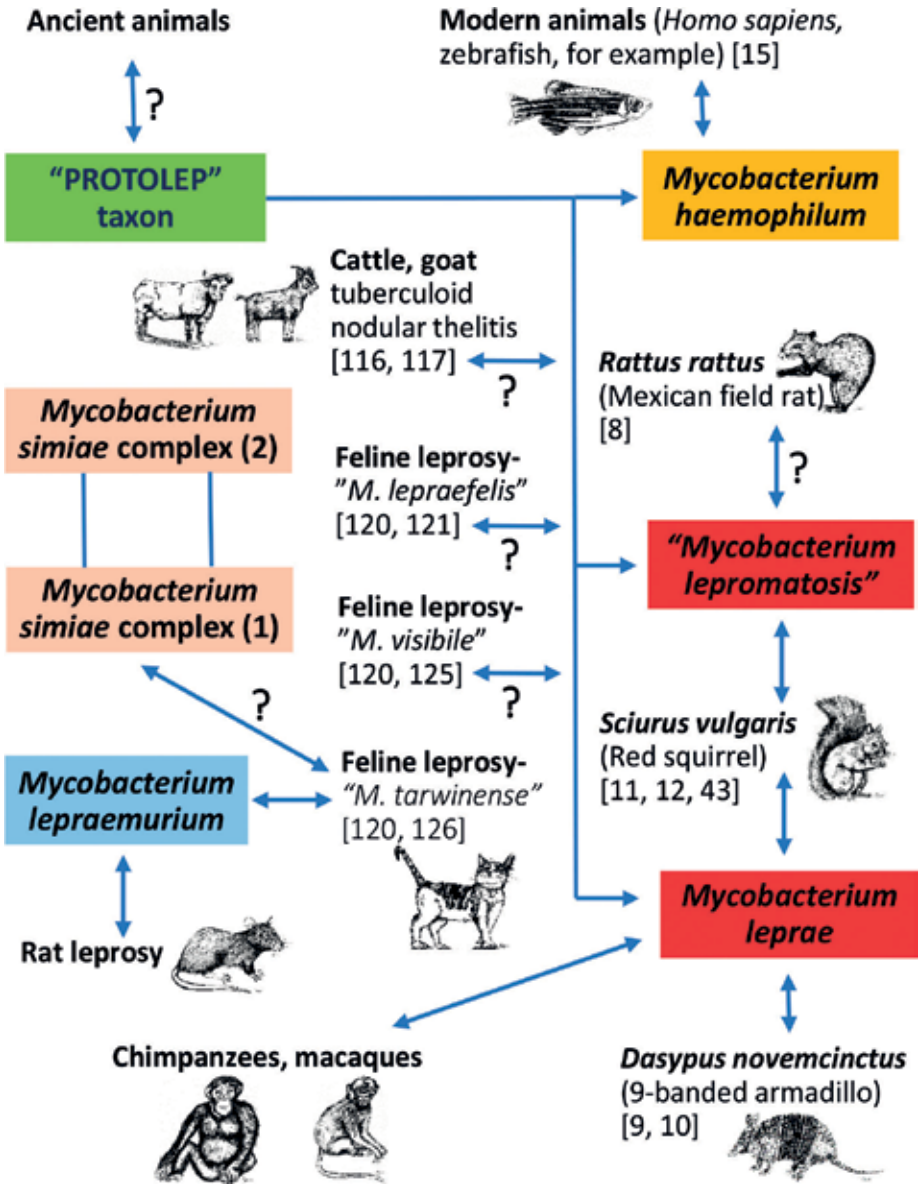

Figure 9. Origins and interrelationships of agents causing leprosy-like disease. Proposed relationships requiring further study are indicated (?). 'PROTOLEP' represents a prototype taxon with the specific type of outer membrane lipids expressed in *M. haemophilum*, *M. leprae* and possibly '*M. lepromatosis*'. *M. simiae* complex (**1**) represents species (*M. florentinum*, *M. interjectum*, *M. sherrissii*, *M. triplex*) apparently expressing genes for PDIM synthesis; *M. simiae* complex (**2**) includes the remaining species [128].

'PROTOLEP' in **Figure 9**. This hypothetical taxon is considered to incorporate characteristic cell envelope lipids, such as the C_{34} mycocerosates found in *M. leprae* and *M. haemophilum* (**Figure 2**). Sensitive lipid biomarker analysis has the potential to help identify the uncultivable agents causing feline leprosy ('*M. lepraefelis*', '*M. visibile*') and tuberculoid nodular thelitis in cattle and goats (**Figure 9**). It is an open question whether these agents have any affinity with *M. leprae*, '*M. lepromatosis*' or *M. haemophilum*, but it seems likely that the feline cases that are associated with both *M. lepraemurium* and '*M. tarwinense*' [119, 120, 126] (**Figure 9**) are distinct. '*M. tarwinense*' appears to be an affiliate of the *M. simiae* complex, which appeared to have little phylogeny with *M. leprae* and related taxa until detailed genomic characterisation of nontuberculous mycobacteria indicated that particular *M. simiae* complex members (*M. florentinum*, *M. interjectum*, *M. sherrissii*, *M. triplex*) apparently have genes for PDIM synthesis (**Figure 9**) [128]. It would be of interest to discover if there is any similarity between the proven PDIMs of *M. leprae* and those suggested to be expressed by these members of the *M. simiae* complex.

5. Conclusions

An understanding of the origins and spread of leprosy depends on establishing detailed knowledge of the ancient genotypes and their correlation with modern disease. The overall scenario has been expanded by the recent characterisation of the distinct modern clade, currently labelled '*M. lepromatosis*'. The availability of a full genome for '*M. lepromatosis*' is allowing specific probes to be developed to search for ancient expression of this biotype. Ongoing research is demonstrating that subtle lipid biomarker differences may be of value in distinguishing '*M. lepromatosis*' from *M. leprae*. The overall picture for the global development of leprosy suggests that the ancient disease evolved into a number of recognisable clades in Africa/Eurasia. It is clear that leprosy was introduced into the Americas by human migration, and the disease was passed on to indigenous armadillos. The deeper origins of leprosy appear to be inextricably linked to relatives of the environmental taxon *M. haemophilum*. Diseases in cats, cattle and goats, with affiliations and resemblances to leprosy, require detailed investigation.

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Chapter 3

An Update on the Epidemiology, Diagnosis and Treatment of Leprosy

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Additional information is available at the end of the chapter

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Abstract

Leprosy is a granulomatous, chronic infection caused by *Mycobacterium leprae* that has been reported for than 2000 years. The infection primarily affects the skin and peripheral nerves. *M. leprae* is bacterium that cannot be cultured in vitro and transmission and pathophysiological data is still uncertain and limited. Today the prevalence of this ancient disease is declining in most around the world. This decline is a direct effect of widespread administration by public health workers of multidrug therapy. However, emerging despite the use of multidrug therapy, identifying and monitoring resistance are still necessary.

Keywords: leprosy, Hansen's disease, epidemiology, clinical findings, multidrug treatment

1. Introduction

Leprosy is an ancient, granulomatous and chronic infection that caused by *Mycobacterium leprae*. It primarily affects the skin and peripheral nerves and late diagnosis of the leprosy related with the various complications and disabilities. Clinical findings of leprosy based on the cellular immune response of the patient and the duration of the disease.

Diagnosis is made by clinical examination; however, it must be supported with laboratory to determine the classification and the treatment of the disease.

In leprosy, Multi Drug Therapy (MDT) has been recommended since 1982 as the standard treatment. With MDT, relapse and the number of the new leprosy cases decreased. However, it is still important and necessary to closely monitorize the patients to prevent and eliminate the leprosy.

2. Epidemiology

According to data from the World Health Organization (WHO), there are 5.35 million leprosy patients in 1985, and this figure has declined to 210,758 in 2015 [1, 2]. The most probable cause of this decline in prevalence is the increasing public awareness, trainings made regarding diagnosis and treatment after the worldwide determination of leprosy as a public health problem [3]. When the prevalence of leprosy is determined, the data of registered patients receiving MDT every year are used. While the state of the disease on the world is determined, aside from prevalence, new patient detection rates and grade-2 disabilities (G2D) definitions and new cases with visible deformities are also used by WHO [2].

In reducing of the prevalence of leprosy over a period of several years, in particular, reducing the treatment with dapsone for many years (4–10) to up to 1 year with MDT was effective [4, 5]. In contrast with the decline in prevalence, the new patient detection rate continued to increase until 2001. After 2001, the number of new diagnoses in parallel with the decrease in prevalence due to the early diagnosis and the success of MDT has decreased [5]. The healthcare infrastructure, which plays a very important role in detecting and controlling the disease, and the accessibility of health services directly affect these rates [6].

At the end of 2015, the prevalence was calculated to be 0.29 (174,608 cases) per 100,000 population, and the rate of new cases was calculated to be 3.2 (210,758 cases) per 100,000 population, according to the number of patients receiving MDT in collected data by WHO from 138 countries [2]. Although the programs against leprosy are being prepared worldwide, currently, 14 countries, each reporting more than 1000 new patients per year, are generating 95% of newly diagnosed patients worldwide. In 2015, India alone accounts for 60% (127,326) of newly diagnosed patients, 13% of Brazil (26,395) and 8% of Indonesia. The Democratic Republic of the Congo, Ethiopia, Madagascar, Mozambique, Nigeria, the United Republic of Tanzania, Bangladesh, Nepal, Sri Lanka, Myanmar and Philippines totally have a new patient detection rate of 14%. Although the rates vary in different countries, on average, 38.8% of the patients reported worldwide are woman and 8.9% of them are children. It is rare to have leprosy in infancy due to long incubation period [7].

The rate of G2D, which indicates identification of early signs and symptoms of leprosy and the response to treatment, is around 6.7% worldwide (1409 cases), indicating a delay in the detection of cases where this rate is still high [2]. The aim of the Global leprosy 2016–2020 is to reduce G2D ratio to less than 1 per 1 billion worldwide; and to withdraw the G2D ratio to zero in children [8].

2.1. Contamination

Despite the fact that leprosy is a very old disease, we still have a limited knowledge of contamination routes and reservoirs. Contamination usually occurs after prolonged contact with the nasal and oral secretions of lepromatous leprosy (LL) patients infected and untreated with *M. leprae* [9]. However, many cases have been reported supporting the possibility of transmission by different ways, and discussions on different ways of transmission are continuing. There are reports that leprosy cases reported to develop by tattooing and accidental needle penetration support that they can be transmitted through damaged skin, there are also reports supporting undamaged

skin contamination [10–13]. In addition, leprosy cases seen in the infant period also suggest a possible infection from mother via blood or with breast milk [7, 14, 15]. Numerous cases of leprosy following direct or indirect contact, especially with nine-band armadillo, have been reported [16, 17]. In addition, a leprosy case developed after blood transfusion has been reported [18].

Bacillus *M. leprae* is showed by methods, e.g., skin biopsy, direct skin smear and electron microscopy; in different ratios in saliva, oral mucosa, hair follicle, hair shaft, sweat gland and canal, in the sebaceous gland orifice, in the mother's milk, and very little in the placenta [13, 15, 19–24]. It should be kept in mind that leprosy, which still continues to be a public health problem, may have different modes of transmission.

2.2. Reservoir

Although *M. leprae* is thought to be a largely human-depended parasite for a very long time, there is so much evidence supporting a reservoir other than human [25]. According to recent data, there is no significant decrease in new case detection rates between 2010 and 2015, suggesting that there may be reservoirs other than human [2]. The development of leprosy cases after direct or indirect contact with the nine-banded armodillo in the United States of America (USA) supports this idea [16, 17, 26]. In female chimpanzees brought from Africa to Japan for clinical trial purposes, development of leprosy after 30 years is important for the evidence regarding that leprosy may have both an incubation period and a non-human reservoir [27]. Findings also show that *M. leprae* is also present in environments such as insects, amoebae, soil, and water [25, 28–31]. Considering that bacillus can survive for 46 days in moist environment and 60 days in water; even backwater may become the medium in which they can survive for a long time [32].

2.3. Incubation

Since there is no serological or biological method that may detect *M. leprae* in the latent phase, which is subclinic, it is not yet possible to identify exactly the duration of the incubation; but observational estimates can be made. The estimated average incubation period for multibacillary leprosy (MB) is 5–10 years and sometimes more; for paucibacillary leprosy (PB), the average incubation period is 2–5 years [5].

2.4. Risk factors

2.4.1. Contact

In a case control study, when compared to the control group, those who had social contact and when compared to living in the same aquifer, those who are core households have a higher risk [33, 34].

2.4.2. Age

According to many specialist age is a risk factor and children below 14 years old who is in contact with MB patients as a householder are found to be at greater risk than adults [35–37].

In addition, a study showing that bimodal distribution of risk for age has been shown that the risk increase ages 5–15 years and over 30 years.

2.4.3. Gender

Although there are a number of studies showing no significant difference between men and women, there is also a study reported that men are at greater risk [34–36].

2.4.4. Leprosy type and physical distance to the patient

Compared to the general population, sharing the same house with a leprosy patient increases the risk. Contact with MB leprosy patients is more risky when compared to contact with patients with PB single lesion leprosy, while the risk of contact with MB patients is similar to contact with PB leprosy patients with 2–5 lesions [34, 36, 38]. If there are two or more patients in the same house, the risk of contamination doubles [11].

2.4.5. BCG vaccine

For individuals in contact with the general population and leprosy patients, BCG vaccination administration at repeated doses provides protection against the leprosy [39–41]. Those who live in the same household and do not vaccinated with BCG vaccine are at greater risk [42].

2.4.6. Genetic distance

Studies have been carried out for years to clarify whether there is a relationship between leprosy and genetics. Although not fully adequate, there are conclusions that support this idea. Moet et al. reported that genetic association is a risk factor predisposing to leprosy, regardless of physical distance [34]. Mire et al. found that the chromosome 6q25 locus was associated with leprosy susceptibility; Siddiqui et al. showed that the 10p13 locus was associated with PB leprosy [43]. There are also studies showing that HLA DR2 and non-HLA (SLC11A1, formerly NRAMP1 and TNF alpha) genes are also associated with leprosy [44, 45]. Genomic studies are important in combating leprosy in terms of having potential for improvement in treatment and vaccination. On the other hand, the presence of IL-17F (7488 t > C) single nucleotide polymorphism and the presence of IL-4 gene 4-590 T/C polymorphism are associated with decreased predisposition to leprosy [46, 47].

3. Microbiology and genetic

M. leprae is a compulsory intracellular organism, which is a fast-staining, very slow-growing (doubling time 14 days), which can reproduce at lower temperatures than body temperature [48]. Unlike other bacteria, it is thought that the reproduction pattern is not algorithmic. While the regions where it can reproduce in human body are at 25–33°C, whereas no *M. leprae* involvement is observed at 35–36°C regions [49].

Reproduction of *M. leprae* in vitro conditions has not been fully successful until now and the reason for this is still unclear. Amako et al. reached results showing that *M. leprae* Thai-53 strain grew in vitro in different media by digital droplet PCR method [50]. Most of the studies on the *M. leprae* have been carried out on armadillos. Low body temperatures (33–35°) of armadillos (*Dasypus novemcinctus*), long life cycles, and adequate body sizes have made them suitable hosts in experimental areas [26].

In 2001, by dissolving the genome sequence of *M. leprae*, new information about the disease has begun to be obtained [51]. *M. leprae* has the smallest genome among the mycobacteria species. *M. leprae* bacillus has undergone severe genomic disruption and diminution with reductive evolution [52]. Reductive evolution, especially in catabolism, has removed metabolic pathways along with control pathways [53]. More than half of the genome consists of pseudogenes, inactive reading frames or regulating sequences. In addition, dominance of the bacillus genome has led to advances in topics such as molecular epidemiology, drug-susceptibility testing, and understanding of the spread of the bacterium over the world [52].

4. Classification

Leprosy exhibits a broad spectrum of clinical and histopathological findings based on the cellular immune response of the host. In 1966, Ridley and Jopling classified leprosy according to clinical and histopathologic features [54, 55]. According to this classification system there is a tuberculous form (TT) consisting of a strong immune response and a small number of microorganisms at one end and a weak immunologic response and a lepromatous form (LL) overloading of microorganisms at the other end and three types of borderline leprosy; borderline tuberculoid leprosy (BT), mid-borderline (BB), borderline lepromatous leprosy (BL) between these two end. Conceptually, tuberculoid leprosy (TT) and lepromatous leprosy (LL) are clinically stable, while borderline forms may shift to stronger or weaker immunity.

In 1997, the WHO created a classification to provide leprosy treatment based on the number of lesions present, regardless of the size, localization, and histopathological features of the lesions, without laboratory support in endemic areas [56]. According to these, leprosy is divided to 3 subgroup: single lesion leprosy, PB, 2–5 lesions and MB, more than 5 lesions. According to the WHO classification, BT may be considered in the PB, BB and BL may be considered in the MB spectrum.

5. Clinics

Clinical findings of leprosy are primarily due to skin and nervous system involvement. There are five common types of peripheral nerve changes:

1. Enlargement of peripheral nerves: Peripheral nerves are more frequently affected by superficial placement. Unique findings such as anesthesia or hypoesthesia may develop as well as sensitivity and enlargement [55, 57]. Ulnar nerve in the elbow, median and superficial

radial cutaneous nerve in the wrist, large common auricular nerve in the neck, and common peroneal nerve enlargement in the popliteal fossa can be detected by palpation [57].

2. Presence of sensory defects such as anesthesia and hypoesthesia in skin lesions.
3. Sensory and motor function losses may occur depending on the location of nerves that are involved. General neurological examination was performed and neurological changes such as drop foot, flexion contracture 4–5 of the fingers, muscular atrophy, facial paralysis, and lagophthalmia may be detected [57].
4. Depending on the influence of thin, unmyelinated Type C fibers responsible for the transmission of senses such as light touch, pain, hot, and cold; the sensory loss in the glove-stocking pattern may be observed first in the hot-cold discrimination.
5. Anaphylaxis of palmoplantar area may be observed by the effected sympathetic nerve fibers.

Clinical forms of leprosy are determined according to clinical, bacteriological, immunological and histopathological criteria. According to that, leprosy has five clinical types:

5.1. Tuberculoid leprosy

It is a form of strong immune response that can be followed by spontaneous healing. Primary skin lesions are hairless, faintly elevated and endure, erythematous, squamous, annular plaques, which can be accompanied by neural involvements such as sharp anesthesia and hyperesthesia. The number of lesions is often solitary and does not exceed 10 cm in size. It can be seen as hypopigmented lesions in which partial pigment loss is observed, especially in dark-skinned individuals [54, 58]. Lesions should be examined thoroughly in terms of alopecia. Even if the enlargement and tenderness of the peripheral nerves near the cutaneous lesion are not detected, the lesion itself is typically hyperesthesia and anhidrotic [58].

5.2. Borderline tuberculoid leprosy

Although the immune response is sufficient to limit the disease, it is insufficient for spontaneous recovery [59]. Patients in this form may have a TT upgrade or borderline leprosy downgrade according to the change in the immunological response. Primary skin lesions are sharply defined, multiple, asymmetric, annular plaques and papules [54]. Lesions are less indurated and eleve, less erythematous, scarless, or slightly squamous than TT. The lesions can be seen in size to cover the entire limb. The lesions can be seen in size to cover the entire extremity. Loss of sense is observed in all lesions and nerve involvement (enlargement and paralysis) is usually asymmetry.

5.3. Mid-borderline leprosy

Immunologically, the two extremities are the midpoint of the spectrum [59]. The severity of cutaneous findings and neurological changes depend on which end of the patient is closer to. Primer skin lesions are generally asymmetric, alopecic, annular, sharply defined and broad platelets with the appearance of “Swiss cheese” where clinically normal skin islets are found.

5.4. Borderline lepromatous leprosy

The immune system is weak enough to stop bacterial proliferation but sufficient to suppress inflammation that causes tissue damage [59]. Clinical findings are considerably diverse. Lepromatous lepra-like weak-edged and tuberculoid lepra-like sharp-edged plaques providing a classical dimorphic annular appearance are seen in only one of three patients [54]. Large plaques with sharp or weak edges and normal papules and nodules on which normal skin islands are visible can also be observed. The number of lesions varies from solitary to multiple. While the annular plaque lesions show asymmetrical placement, the nodules localized symmetrically. Neurological involvement is common and severe sensorimotor damage can be observed.

5.5. Lepromatous leprosy

Extensive disease is seen due to the inadequate cellular immune response. Classical lesions are characterized by multiple, diffuse, often symmetric, sharply defined papules, plaques and nodules. Involvement areas are usually the face, the hip and the lower extremity (**Figure 1**). The infiltration of the forehead skin leads to generation of lion face, which is a characteristic facial appearance (**Figure 2**). Hair loss is widespread, especially in the eyebrows (madarosis) and lashes [60].

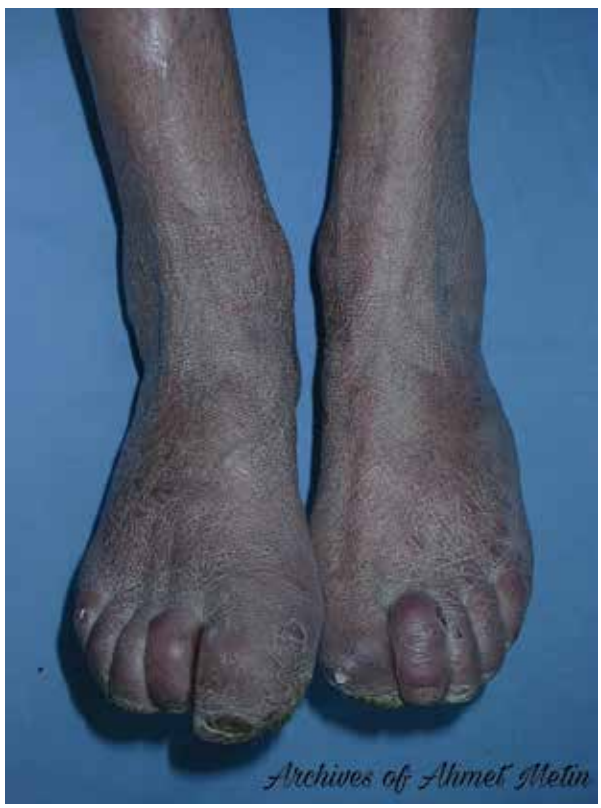

Figure 1. Lower extremity involvement.

Figure 2. Characteristic facial appearance (lion face).

6. Complications

Corneal dryness, abrasion and ulceration are very common in patients with leprosy due to the secretory irregularity and corneal insensitivity. A careful eye examination should be performed in every leprosy patient to prevent serious complications that can result in blindness.

Depending on the perforation and collapse of the nasal septum, saddle nose and rhinitis-like findings can be observed. Snoring due to nodule occurrence in vocal cords and larynx involvement, and gynecomastia, impotence and infertility as a result of decrease in blood testosterone level due to testicular involvement in male patients may be seen [61].

Venous insufficiency due to endothelial involvement of the valves of deep venous vessels may lead to stasis dermatitis and venous ulcers.

In the advanced disease phase, multiorgan involvement (liver, spleen, peripheral lymph nodes, bone marrow) can be observed.

7. Immunologic reactions

Immunological reactions are inflammatory conditions that clinicians and patients may encounter before, during, or months or years after treatment [62]. Approximately 30–50% of patients are involved. There are two types of reactions that are linked to different immunological mechanisms that are not fully understood: Type 1 and type 2 [63]. These immunological reactions may mimic the drug reaction, the clinician should pay attention to that they are not drug reactions and that treatment should not be interrupted. In both types of reactions, general weakness, fatigue and fever can be observed. Other clinical findings differ according to the developing reaction.

Type 1 reaction: Typically occurs in TT and BT. It is due to an increase in cell-mediated immunity and a delayed-type hypersensitivity reaction to *M. leprae* antigens [55, 57, 63, 64]. Characteristic clinical findings are increased inflammation in existing lesions, formation of new lesions, pain and sensitivity in nerves (neuritis), progressive neurological failure.

Type 2 reaction: Typically, BL and LL patients develop when treatment begins. Pregnancy and pyogenic infections may induce. The type 2 reaction due to the formation of immune complex with hyper humoral immunity represents cutaneous and systemic small vessel vasculitis [55]. Characteristic clinical findings; painful nodular lesions (erythema nodosum leprosum) that occur suddenly, severe swelling and pain in the joints, iridocyclitis, orchitis, sensitive lymphadenopathy, glomerulonephritis, and hepatosplenomegaly [57, 63, 64].

Lucio phenomenon is a rare complication characterized by sudden-onset, necrotizing cutaneous small vessel vasculitis in diffuse, untreated LL patients in the Mexican and Caribbean region [65]. Lesions that are painful but have no increase in temperature can be cured by scarring. Ulceration especially may be observed in knees.

8. Differential diagnosis

Although reduction and absence of sensory perception distinguish leprosy lesions from other diseases, this finding may not always be detectable. With the reason that a wide variety of cutaneous lesions are present, leprosy can be confused with many diseases. In suspected patients, the exact diagnosis is made by skin biopsy.

Hypopigmented lesions may mimic pityriasis alba, pityriasis versicolor, mycosis fungoides and sarcoidosis [66].

Figured erythematous plaques may be confused with fungal infections, annular psoriasis, sarcoidosis, mycosis fungoides, lichen planus, systemic lupus erythematosus [66].

Infiltrated plaques and nodules generate definitive diagnosis with cutaneous leiomyoma, sarcoidosis, syphilis, keloid, cutaneous lymphoma, granuloma annulare [66].

In addition, definitive diagnosis of type 1 reaction includes acute lupus erythematosus, drug reactions, cellulitis; the definitive diagnosis of the type 2 reaction should be considered to include other conditions that may cause vasculitis and panniculitis.

9. Diagnosis

For diagnosis, leprosy must first come to mind. Although diagnosis is made substantially by clinic examination, diagnosis must be supported by laboratory for classification and treatment. Microbiological and pathological tests should be performed after history and clinical evaluation [67]. WHO recommends that individuals with one of the two cardinal findings in endemic regions are considered to be leprosy [68].

- Lesions compatible with leprosy with sensory loss (with or without nerve thickening).
- Positive skin smear.

Hypoesthesia skin lesion is the most important diagnostic factor because it is not expected in another skin disease other than leprosy. After evaluation of skin lesions, peripheral nerves should be palpated for thickening, and nerve examination of lesions and distal extremities should be performed [69]. Conjunctiva and corneal examination should also be made.

9.1. Sampling

Skin smear, a rapid diagnostic method, requires experience. The skin compressed between the thumb and index finger is cut with lancet with a width of 5 mm and a depth of 3 mm. The collected dermal fluid is spread on the lame. Tissue fluid should not be bloody. The most preferred regions are the earlobe, elbow and knee extensor faces. It may require three to six repetitions. The result is generally negative in less bacillar and TT. Nasal sampling is not recommended, especially because of fragility in LL cases. A 4 mm punch biopsy is the ideal method for sampling. The biopsy should be taken from the most erythematous, contagious and expanding area. Nerve biopsy may be required to support diagnosis, especially in cases of pure nerve involvement [67].

9.2. Microscopic examination

In vitro *M. leprae* culture is not possible. Demonstration of acid-resistant bacilli in material taken by skin smear or biopsy is standard diagnostic technique. With Ziehl-Neelsen staining, acid-resistant basils are colored fuchsia in blue background [67]. Bacteriological index (BI) is determined by rating between 1+ (1 bacteria in every 100 area) and 6+ (min. 1000 bacillus in every area) with the amount of bacteria in each microscope area. Patients with BI scores lower than 2 are considered as PB, whereas BI scores above 2 are considered MB [69].

9.2.1. Molecular methods

One of the molecular diagnostic methods, PCR, is the detection of *M. leprae* DNA. Biopsy material, tissue fluid, blood, urine, nerve tissue, oral and nasal mucosa swab and ocular lesions can be used for PCR [70]. While the specifity can be 100%, the sensitivity ranges from 34% to 80% in cases with PB forms to greater than 90% in cases with MB forms of the disease. The support of diagnosis is an important diagnostic tool in treatment follow-up, transmission surveillance of the immediate surroundings of leprosy individuals and in cases characterized with particularly pure nerve involvement which is difficult to diagnose or atypical lesions [71].

9.2.2. Hystopathology

The main pathologic feature is a granulomatous reaction. Epithelioid cells, macrophages, lymphocytes, plasmocytes and rarely neutrophil and mast cells are observed. Different granulomatous reactions occur according to the immune response of the host. While epithelial cells are mostly observed in TT and BT cases, foamy macrophages are observed mostly in LL and BL cases.

- *TL:* The reaction is mostly multifocal, periadnexal and perineural. Infiltrate is in dermis. The epidermis is usually atrophic. Giant langerhans cells are pathognomonic. Multinuclear giant cells can be seen while plasma cells are not expected to be observed. Perineurium is intact and is surrounded by lymphocytes. It can even infiltrate with granuloma structures. There is marked edema in the nerve tissue.
- **BT:** Findings are similar to those for TT. Epithelioid cells are less maturated, giant cells are undifferentiated and small. Epithelioid granulomas are less organized and tubercular structures are less prominent.
- **BB:** Epithelioid cells are immature. Organized epithelial granuloma structures are absent. Lymphocyte spread is diffuse and macrophages are quite over. Nerve tissue is not edematous. It has been infiltrated and partially destroyed by epithelial cells and lymphocytes.
- *BL:* Macrophage and lymphocyte predominant infiltration is present. Epithelioid cells are rare. Infiltration can be diffuse, nodular, perivascular and periadnexal. The epidermis and dermis are separated from each other by a narrow zone formed by the collagen. While macrophages contain more or less foamy cytoplasm, the formation of large vacuoles is not a feature of BL. The nerves have the onion skin perineurium.
- *LL:* The dermis is also characterized by diffuse macrophage invasion. There are no epithelioid cells. The epidermis is atrophic and has a very apparent grenz zone. Skin attachments are surrounded by macrophages and are atrophic. Macrophages contain gray cytoplasm with foamy changes. Large vacuolarizations can be seen. Perineural macrophage accumulation is present and perineum appears like onion skin. There is no sign of significant infiltration, and even the nerves can be quite normal. The nerves can be hyalinized or fibrotic [72].

9.2.3. Lepromin test

About 0.1 ml of lepromin antigen is administrated intradermally on to the forearm. The test is interpreted twice, first 24–48 hours and then 21 days. The first reaction is indicative of susceptibility, but may cross-react with other mycobacteria. The second reaction is resistance indicator to bacillus. Nodule >5 mm is considered positive. The most important point for the lepromin test is that it is not a diagnostic test, it should be used for classification and prognostic purposes.

9.3. Serology

M. leprae is stimulating cell-mediated abnormal response. Although the Ig that are formed are not protective, the importance of detection of the IgM formed towards PGL-1 (phenolic

glycolipid-1)-the only accessible test-is increasing day by day [67]. Search for effective diagnostic tests is accelerated by shifting the focus of the leprosy control strategy to early diagnostic and rapid treatment. Several studies have shown a correlation between serological titer and BI. The skin smear, the gold standard for classification, is thought to be a test that can be used as a support for clinical findings when histology is not possible [73, 74]. There was also a relationship between serology and reaction and relapse risk. Patients with a high PGL-1 Ig M level had a high risk of developing type 1 reactions [75]. In another study, post-treatment reactions were found to be more likely to develop in patients with positive serology after treatment [76]. In another important meta-analysis, seropositive healthy contacts were observed to develop three times more leprosy when compared to negatives [77]. In the light of these findings; additional studies are needed to determine serology, classification, early diagnosis, follow-up of disease, detection of individuals at risk, and determination of who should take prophylaxis among these individuals.

10. Management

10.1. Medical treatment

MDT is the key point of disease control. Dapsone, rifampicin and clofazimine are the first line drugs. Because of the increased drug resistance due to monotherapy and the ineffectiveness of each one on *M. leprae*, the use of multiple drugs was initiated in 1981 in line with WHO's recommendation. The use of dapsone, rifampin, and clofazimine (MB-MDT) is recommended for MB leprosy (BB, BL, LL), while dapsone and rifampin (PB-MDT) is recommended for TL, BT for 6 months since 1982. However, the duration of treatment for MB leprosy has changed over the years. The use of 12 months of MDT independent of smear is currently recommended, although formerly it was suggested that the treatment be continued until two consecutive negative skin smears are obtained [78, 79]. A single dose combination of rifampin 600 mg, ofloxacin 400 mg and minocycline 100 mg is recommended for patients with low baseline leprosy with a single lesion [80, 81]. Recommended doses are presented in **Table 1**.

Minocycline, clarithromycin and ofloxacin can be used as second-line drugs in MDT, where first-line drugs cannot be tolerated. Minocycline 100 mg/Daily can be used instead of dapsone and clofazimine, ofloxacin 400 mg/day instead of clofazimine, clarithromycin 500 mg/daily can be used where dapsone, clofazimine or rifampin cannot be tolerated [82].

WHO recommends that cases with a skin smear test +, or those without a definite diagnosis, are definitely treated with MB-MDT. Furthermore, attention should be paid to the fact that MB leprosy cases should not be treated with PB-MDT [81].

On the other hand, the National Hansen's Disease Programs (NHDP)—in the USA—involves different regimen. Treatment is recommended 12 months for PB leprosy and 24 months for MB leprosy. Furthermore, unlike the current regimen of WHO, rifampin is used daily rather than monthly [82]. Recommended doses are presented in **Table 1**.

	WHO recommended treatment regimens			NHDP recommended treatment regimens		
	Agent	**Dose**	**Duration**	**Agent**	**Dose**	**Duration**
	Tuberculoid (TT and BT)(paucibacillary)			*Tuberculoid (TT and BT) (paucibacillary)*		
Adult	Dapsone	100 mg/daily	6 months	Dapsone	100 mg/daily	12 months
	Rifampicin	600 mg/a month		Rifampicin	600 mg/daily	
Child*	Dapsone	50 mg /daily		Dapsone	1 mg/ kg/daily	
	Rifampicin	450 mg/a month		Rifampicin	10–20 mg/kg/daily (not >600)	
	Lepromatous (LL, BL, BB) (multibacillary)			*Lepromatous (LL, BL, BB) (multibacillary)*		
Adult	Dapsone	100 mg/daily	12 months	Dapsone	100 mg /daily	24 months
	Rifampicin	600 mg/a month		Rifampicin	600 mg/daily	
	Clofazimine	50 mg/daily and 300 mg/a month		Clofazimine	50 mg /daily	
Child$^{\delta}$	Dapsone	50 mg/ daily		Dapsone	1 mg/kg/daily	
	Rifampicin	450 mg/a month		Rifampicin	10–20 mg/kg/daily (not >600)	
	Clofazimine	50 mg/daily and 150 mg/a month		Clofazimine	1 mg/kg/daily	

*Adjust dose appropriately for child less than 10 years. For example, dapsone 25 mg daily and rifampicin 300 mg given once a month under supervision.

$^{\delta}$Adjust dose appropriately for child less than 10 years. For example, dapsone 25 mg daily, rifampicin 300 mg given once a month under supervision, clofazimine, 50 mg given twice a week, and clofazimine 100 mg given once a month under supervision.

Table 1. Recommended treatment doses of leprosy.

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Moxifloxacin, pefloxacin, sparfloxacin, levofloxacin, and rifapentine that is a rifampin derivate are other agents with demonstrated efficacy [83, 84]. Clinical trials are needed in the long term.

Another treatment regimen studied is Uniform-MDT. It is the use for 6 months of dapsone, rifampin and clofazimine for patients with both PB and MB. However, the need for using additional clofazimine in PB patients and whether the treatment will be sufficient in MB patients are important questions present. This treatment is believed to set zero the risk of abduction of MB leprosy patients who received insufficient treatment by introducing a PB group [85]. In a study in which MB leprosy relapse was assessed in particular, relapse was found to be well below the targeted 5-year relapse rate of 5% [86]. Existing studies promises hope although further studies are needed [87, 88].

10.2. Treatment of immunological reactions

10.2.1. Type 1 reaction

Supportive therapies such as parol, non-steroidal anti-inflammatory drugs (NSAID), can be administered if there are no neuritis findings such as pain, function, or sensory loss. If nerve involvement is present, prednol 0.5–1 mg/kg/day peros is the first choice treatment. When the reaction is relieved, the dose is slowly reduced so that it remains above the dose of 0.25 mg/kg/day for at least 3–6 months. In ongoing process, dose reduction is continued with careful follow-up of nerve functions [89]. Cyclosporin is another option when steroids are not usable [90, 91]. In a study in which azothioprine was assessed, efficacy in type 1 reaction was not demonstrated [92].

10.2.2. Type 2 reaction

The incidence of type 2 reaction has decreased after addition of clofazimine to MDT with anti-inflammatory effect [93]. If there is no findings of neuritis, supportive treatment care such as NSAID (aspirin, indomethacin) and paracetamol may be administered. Prednol (0.5–1 mg/kg/day) is the first choice if neuritis is present. Once the reaction is decreased, the dose should discontinued by reducing the dose in time. However, frequently the reaction relapse during dose reduction. In this case, thalidomide, clofazimine and pentoxifylline can be used as adjuvants. WHO recommends to start by using clofazimine 3 * 100 mg (300 mg/day) and then reduces to 100 mg/day. It should not be used for type 2 reaction as a single agent and for more than 12 months. Reaction can be rapidly controlled by using thalidomide 300–400 mg/day. Dosage may be reduced to 100 mg/day for extended periods of usage. Since it is teratogenic, attention must be paid to the use in women who has childbearing potential [81]. Pentoxifylline is used as 3 * 400 mg. In a study that compares the efficacy of clofazimine and pentoxifylline, pentoxifylline effectively reduced the initial severity, while clofazimine showed a slow but sustained effect [94]. Resistant erythema nodosum leprosum cases that treated successfully with methotrexate, infliximab and etanercept have been reported [95–97].

11. Follow-up

WHO aims to follow the patients up monthly by 28-day drug supply. In regions where monthly follow-up is difficult, it is recommended that more than a monthly dose be given and

train family members or nearby people for observation purposes [81]. On the other hand, the NHDP recommends first follow-up on 1st month and then with 3 months periods. Reaction therapy should be followed up closely. Disease progression is largely due to incompatibility to treatment. Complete information about possible reactions, complications, drug side effects should be provided and the patient and his/her relatives compliance to treatment should be ensured. At each follow-up, laboratory tests for drug toxicity are recommended with full clinical evaluation including nerve examination. While complete blood, urea, creatinine, AST, ALT follow up is adequate, it is advisable to check glucose 6 phosphate dehydrogenase level for once for dapsone usage before treatment, if possible. A yearly skin smear or biopsy is recommended to follow the bacteria burden. After treatment, it is advisable to follow up at least 5 years of PB cases and 10 years of MB cases [82, 98].

Existence of persister strain, insufficient/inappropriate treatment, monotherapy, drug resistance, high BI at diagnosis, number of lesions and lepromin negativity are factors that increase relapse risk. The risk of relapse after MDT has decreased considerably. In a study of 3248 patients followed for 16 years, cumulative relapse rates after MDT were 1.78% [99]. The relapse rate was 6.1/1000 person years in one of the last current studies in which 2177 patients are followed up [100]. To reduce the risk of relapse, individualization of treatment duration is recommended in individuals predicted to be at high risk for relapse [101].

11.1. Drug resistance

Drug resistance is the result of leprosy-resistant transmission (primary resistance) or mutation-induced secondary resistance which develops under treatment [102]. *FolP1, rpoB, and* gyrA gene mutations are associated with dapsone, rifampicin and ofloxacin resistance. Gene sequencing by PCR is a rapid and sensitive method to demonstrate drug resistance [103]. Due to the lack of effective second-line drugs; resistance to first-line drugs is a matter of concern to the WHO Global Leprosy Program. In 2008, drug resistance global surveillance was initiated to assess the efficacy and drug resistance level of the current leprosy control strategy. Eighteen reference centers have been established in 18 pilot countries and mutation detection by PCR has begun. Obtained data suggest that resistant *M. leprae* does not pose a risk for current disease management [104, 105].

12. Elimination/prevention

With MDT, in 1985, with 5.1 million leprosy cases falling to 3.1 million in 1991, WHO aimed to eliminate leprosy worldwide in 2000. Elimination target was 1/10,000. Countries like India and Brazil have been under the target even though the target has been reached substantially around the world. Although Brazil is one of the last countries to reach the goal today, endemic areas like Chhattisgarh, Pará and Madura are still far behind the target. On the other hand, in spite of successful elimination program, WHO declared in 2013 that leprosy control is faltered due to the plateau of the incidence of leprosy until 2005, the new diagnosis of leprosy cases with G2D remain constant between 2010 and 2013, and the number of new cases in children is not decreasing. In addition, late-diagnosis-related disability-ending stigmatization of leprosy patients continued. Epidemiological data showed that the

prevalence-based elimination program could not stopped the spreading. The meeting was held in Brazil in 2015 and the strategy was changed. A strategy based on incidence instead of prevalence was identified. Early diagnosis and prompt inclusion of all patients to treatment were aimed [30, 106, 107].

Living in the same house with a leprosy person increases the risk of spreading by 2–10 fold. Most of the new cases constitute subclinical infection cases in contact and no diagnosed cases. For this reason, for eliminating leprosy, it is very important to detect new cases with effective contact monitoring. The development of diagnostic tests that can detect subclinical infection and separate exposure to and infection with *M. leprae* is very important [101, 108].

12.1. Immunoprophylaxis/chemoprophylaxis

In fact, BCG, which is tuberculosis specific, is the only vaccine used for leprosy protection. The level of protection, as well as the protection against leprosy is evidenced, varies between 20% and 90% in the literature. Protection level of the vaccine, when vaccinated in first decade, was higher in women and lower socioeconomic individuals [109]. With age, the level of protection decreases [110]. Although it has been shown that revaccination of leprosy patients and contacts with them have been shown to reinforce the protection, this approach is still not common and there is a need for studies evaluating efficacy [39, 111].

Protection of post-contact chemoprophylaxis (PEP) has been shown in clinical trials in a range of 35–57% in patients with asymptomatic contact. Although it is not included in WHO's official recommendations, PEP evaluations rapidly continue with a single dose of rifampin (SDR). One study has shown that the protection of PEP with BCG combination is better in distant-contact individuals [112]. With the cooperation of Novartis agency and Netherlands Leprosy Relief (NLR), Leprosy Post-Exposure Prophylaxis (LPEP) program has been established. A large-scale study was started to investigate the applicability of the use of SDR as a PEP by the program and the impact on the number of new diagnosed cases. It is expected that the first data will be obtained in 2019 [113].

12.2. Prevention of disability

Permanent nerve damage is a part of the natural process of the disease, and the risk is also quite increased with leprosy reactions. Reducing the stigmatization of leprosy patients and providing mental well-being is possible by preventing nerve damage. Delay in recognition of leprosy and leprosy reactions, and therefore of delay in treatment is the most important factor on permanent nerve damage. Follow-ups should be done in leprosy centers whenever possible. It is important to ensure compliance with treatment and follow-up. Patients should be educated on issues such as care of existing wounds, proper shoe selection, self-examination of hands and feet. Another important point is that the leprosy does not come to mind because of the rare occurrence in some areas and the patients are followed up with false diagnoses. The implementation of information programs for healthcare professionals is also important in this context [114–116].

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Chapter 4

Molecular and Biotechnological Approaches in the Diagnosis of Leprosy

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Abstract

Leprosy is a worldwide health problem, which needs the development of new and innovative strategies to be controlled. Early diagnosis of leprosy is an important contribution to reducing the incidence of the disease; thus, the development of biotechnology platforms, which include the mapping of antigens with potential to be used in immunodiagnostic and molecular methods for the detection of *Mycobacterium leprae*, is an important tool to confirm the clinical diagnostic. Molecular biology and biotechnological methods have been used to assist in the diagnosis of this disease, each one with its advantages and drawbacks. Enzyme-linked immunosorbent assay (ELISA) is the used method for leprosy diagnosis, and it allows the detection of infection-related antigens. Alternatively, due to their versatility to perform the same functions as the protein and non-protein natural antigens, mimetic peptides are considered an important tool. On the other hand, lateral flow assay (LFA) and optical and electrochemical biosensors are rapid and portable methods, capable of performing diagnosis in the field without sample preparation. This chapter presents such techniques, their uses in the diagnosis and detection of M. leprae, as well as the potential for the development of new techniques and strategies that can help to control and understand mycobacteriosis.

Keywords: *Mycobacterium leprae*, immunoassays, molecular tests, biomarkers

1. Introduction

Leprosy is a chronic infectious disease, dermato neurological and incapacitating, which has *Mycobacterium leprae* as its causal agent [1]. Even with the worldwide effort to eliminate this disease as a public health problem [2], countries such as India and Brazil still present a higher number of cases than the World Health Organization (WHO) recommended [3]. In this sense, this organization defined as a global strategy the reduction in the incidence of new cases as a priority [4], highlighting the importance of early diagnosis, which aims to reduce the transmission of the disease in the community [5], which includes correctly diagnosing cases with suspicion of the disease and to identify subclinical infection.

The clinical and epidemiological diagnosis of leprosy represents the gold standard for confirmation of the disease [6]. Bacilloscopy and Mitsuda's reaction are important tests to identify the etiological agent [7, 8], but there is a need to use complementary tests that allow a more accurate diagnosis with high sensitivity and specificity. In this context, stand out the standardization of serological and molecular tests, important for the understanding of the epidemiological profile of the disease.

The use of *M. leprae* specific antigens in serological tests has been the subject of research. The use of phenol-glycolipid-1 (PGL-1) [9], lipoarabinomannan (LAM) [1] and heat shock proteins (GroES and GroEL) [10] as antigens for the enzyme-linked immunosorbent assay (ELISA) and in immunosensors can be validated as methods for detecting new cases of the disease and for early diagnosis [11]. In addition, molecular tests aid in the identification of specific *M. leprae* sequences in clinical samples, which can be amplified through the polymerase chain reaction (PCR) technique, allowing DNA detection of the infectious agent [12] and/or through the use of real-time PCR technology that allows the evaluation of bacterial load [13] and also the monitoring of drug resistance [14].

Thus, this chapter will present an overview of the laboratory diagnosis of leprosy in the world. Initially, we will present a review of the main tests traditionally used in clinical routine and regulated by the WHO, in addition to the complementary tests that have been focus of research as a future perspective for the early diagnosis of the disease. These diagnostic approaches may contribute to a reduction in the number of cases of the disease, since they allow the monitoring of populations and endemic and hyperendemic areas.

2. Leprosy diagnosis: traditional exams

The diagnosis of leprosy includes clinical observation of the patient, dermato-neurological clinical exams and complementary laboratory tests. Therefore, identify lesions and damaged nerves and analyze the life history of the patient are essential tools in the identification and detection of disease. These practices combined with other tools and methodologies are able to assist in epidemiological and disease control strategies, helping to map index cases and individuals who may develop leprosy, such as household contacts.

2.1. Dermato-neurological clinical exam, bacilloscopy, histopathology and Mitsuda test

For the diagnosis of leprosy, it is essential to perform clinical-dermatological exams that search for lesions in the epidermis, areas with changes in sensitivity (may be thermal, painful and tactile) and motor impairment-searching for thickened nerve trunks-which are classic signs of the disease [7]. In addition to clinical examination, there is a class of diagnostic exams and tests that are widely standardized and used in reference networks and centers [7, 15].

Bacilloscopy is the most commonly used exam in clinical practice and, together with the dermato-neurological clinical test, is the most useful methodology for diagnosis [16]. The test presents advantages such as reduced cost and aid in the confirmation of new cases and patients with relapse [15]. The methodology used to perform the exam is a dermal smear of sites, these being: ear lobes (LO), elbows (C) and active lesions, where it is possible to analyze the presence of the bacillus using a specific staining and optic microscopy [17, 18].

As a result, a Bacilloscopy Index (BI), proposed by Ridley and Joplin, is provided, where there is a logarithmic scale ranging from 0 to 6 [19–21]. Thus, after an average of the fields analyzed, the result may vary from BI = 0, associated with patients of the tuberculoid (TT) pole and BI = 3+ to 6+, associated with lepromatous patients (LL). It is still possible to analyze the morphological index (MI) of the bacilli arranged on the slide, describing aspects of their morphological integrity [20]. Thus, bacilli may present three aspects related to their structure, classified as integral, fragmented or granular [21, 22]. Integral bacilli are considered viable, that is, they are related to host susceptibility to the parasite. These bacilli exhibit cell structure with preserved ends and uniform staining and are seen in smears of patients who are either non-treated or have relapse. Both fragmented and granular bacilli present flaws in the cell wall structure, being considered unviable or dead and more observed in post-treatment patients [17–22].

Bacilloscopy is effective when associated with the results of clinical exams [7, 15]. However, it is an exam that presents low sensitivity, since 50% of the smears of the sick individuals are negative. In addition, the exam requires adequate laboratory infrastructure and trained professional apparatus, factors that are not always a reality in hyperendemic regions and where medical and financial resources are reduced [7].

Histopathology is commonly performed for the diagnosis of diseases caused by obligate intracellular parasites and has good indices of sensitivity and specificity in the detection of leprosy [23]. However, the method encounters issues related to cost, time of analysis and false-negative results, besides of being an invasive exam [24]. Exactly for these reasons, histopathology is only recommended for individuals where it is impossible to assess degrees of cutaneous sensitivity, such as in children, elderly and mentally handicapped people; when it is not possible to classify the dimorphic clinical form; or when there is uncertainty whether the diagnosis is leprosy or other diseases that cause local hypoesthesia [23]. The diagnosis using this exam also depends on the association with the clinical aspects and bacilloscopy. In addition, biopsies of peripheral nerve branches are not recommended and should only be performed in the last instance [23, 24].

The Mitsuda test or Mitsuda reaction does not present diagnostic value, but it is an alternative prognostic tool that assists in assessing susceptibility to lepromatous forms [27]. It is an exam based on the use of heat-killed bacilli (lepromin), derived from extract of the inactivated "leprosy bacillus" under the skin of LL patients. The test consists of inoculating the Mitsuda antigen intradermally and reading them between days 21 and 28 after the challenge in order to analyze the late cellular response of patients [8, 25].

Mitsuda's reaction has good agreement when related to bacilloscopy. Typically, individuals with a diameter reaction greater than 10 mm are considered resistant; they do not get sick or develop the TT shape, being Mitsuda positive [24]. While reactions with a diameter between 3 and 5 mm are indicative of dimorphic leprosy and, below this value, the test indicates anergy of the host's immune system to the bacillus, associated with patients in the LL pole, being Mitsuda negatives [8, 24, 26]

3. Complementary immunological tests

The discovery of the lipid apparatus present in the bacillus capsule and the characterization of a range of important lipidic and proteic components in the immunogenicity [30] allowed innovations in the leprosy serology [27].

Serological techniques are based on the detection of specific antibodies against the bacillus, since immunoglobulin production occurs in response to the antigenic signal of stimulation. These tests are important because they represent a class of complementary tests capable of detecting leprosy cases, besides the possibility of diagnosis recommendation, disease prevalence determination, infection evaluation in endemic and hyperendemic areas, and household contacts monitoring.

3.1. ELISA with native PGL-1 and its synthetic molecules

Many researches have used natural *M. leprae* antigens for the immunodiagnosis of leprosy [19, 28]. The elucidation of the structure of the PLG-1-the first *M. leprae* specific antigen to be isolated and the main antigenic glycolipid of the bacillus [29]-is a clear example of the widespread use of these molecules.

ELISA (enzyme-linked immunosorbent assay) has been widely used as a research tool for the detection of anti-PGL-1-native antibodies [29–32]. The technique consists of a quantitative test based on the IgM class antibodies response. In this scenario, important aspects in the biology and epidemiology of the disease, such as the determination and comparison of the positivity of patients and home contacts in several areas, besides the fluctuations in the reactivity profile in individuals from the hyperendemic area [33, 34] have been described from the studies with PGL-1.

Anti-PGL-1 antibodies are present in large numbers in untreated multibacillary patients, but paucibacillary patients naturally have low circulating antibody concentrations. For this reason, some of these patients present negative results against the diagnosis, even showing positive clinical signs [35, 36].

In addition, there's still a great limitation in obtaining the native molecule, restricted to the growth of *M. leprae* in armadillos. As an alternative, several synthetic analogous molecules associated with the tri or disaccharides of PGL-1 have been produced from the conjugation of these elements with BSA (Bovine serum albumin) and Phenol (P) or Octyl (O). The literature shows several semisynthetic analogues, among which the most well known are: monosaccharide-octyl-BSA (MO-BSA), disaccharide-BSA (D-BSA), natural disaccharide-octyl-BSA, natural octyl-HSA (ND-O -BSA and ND-O-HSA), natural trisaccharide-phenol-BSA (NT-P-BSA) which are used as antigens in immunodiagnosis [30, 36].

A study carried out in the hyperendemic region of the Brazilian Amazon points to the potential of synthetic molecules to identify new cases of leprosy and, similar to glycolipid, they have good detection rates in multibacillary patients. In addition, research shows that the molecules exhibit behavior related to the spectral immunology of the disease, where the LL pole has a higher antibody titer that decays at the boderline and tuberculoid poles [35]. When comparing two molecules derived from PGL-1 in the region, it was possible to observe that NT-P-BSA was very effective in the monitoring of home contacts and MB patients, whereas ND-O-BSA obtained better sensitivity and specificity indices in paucibacillary individuals [37].

ELISA requires skilled labor and specific equipment which is not always available. Therefore, the use of serological tests of both the native molecule and synthetic derivatives is important to validate increasingly adequate methods for the seroepidemiology of both endemic and hyperendemic regions, besides helping to standardize the positivity indices of the clinical forms that can vary intensely from one area to another.

3.2. ELISA with recombinant proteins

If the characterization of PGL-1 was important for the synthesis of several derived molecules with similar immunological aspects, the decoding of the *M. leprae* genome was essential for the identification of proteins and peptides with applicability in the laboratory detection of the disease [38]. Several advantages are associated with the use of these molecules, especially for reducing the cost of the assays and reflecting the spectral character of leprosy immunology. Thus, assays using recombinant proteins indicate high levels of antibodies in LL patients that decay in patients of the boderline and tuberculoid poles [38–43].Currently, there are a large number of *M. leprae* recombinant proteins, normally identified as ML, and several studies have evaluated the potential of these proteins [44]. In 2007, a survey conducted in Brazil, the Philippines and Japan evaluated the cellular and humoral response to 33 recombinant proteins across a broad population spectrum and identified three proteins (ML0405, ML2055 and ML2331) capable of inducing the humoral response in multibacillary (MB), production of IgG, as well as the cellular response in PB. Comparison between sites identified differential response patterns between populations, however, in all locations ML0405 and ML2331 showed similar results to PGL-1 serology in MB33 patients [40].

Due to the potential of these two proteins, the Leprosy IDRI Diagnostic protein (LID-1) was generated, resulting from the fusion between the two MLs. LID-1 was produced in order to maintain the reactivity profile of both proteins and was subsequently evaluated in several populations of Japan, Brazil, Venezuela, the Philippines and Nepal. The results pointed to the

potential of early detection of the disease using this protein, besides the possibility of its immobilization in different platforms [40].

Also in this scenario, in 2009 a chimeric protein with multiple epitopes (PADL), from the fusion of epitopes of recombinant proteins (ML0405, ML0049, ML0050, ML0091, ML0411, ML2055 and ML2311) was designed. The chimeric molecule was tested in serum from pauci and multibacillary patients living in Brazil and the endemic controls from the Philippines. The results demonstrated that all the portions that formed the protein have specific binding capacity to antibodies and the same showed great effectiveness in the diagnosis of MB patients and no specific response to the serum of the endemic controls, showing promise in the diagnosis of the disease [45].

3.3. Lateral flow tests

In addition to the ELISA immunoassays, leprosy serology may also be performed with the lateral flow test, known as ML-flow. The development of this immunochromatographic semiquantitative assay was due, in particular, to the possibility of field use. The test was developed by Burker-Sékula et al. [46], aiming the detection of IgM antibodies against several antigenic molecules such as PGL-1 and its synthetic derivatives, recombinant proteins and peptides [30].

The ML-flow test is not a diagnostic method, but assists in the classification of patients and presents low cost and easy execution, making its use possible in health services routine, especially in regions where laboratory resources are not available [30, 35, 46, 47]. Therefore, ML-flow is a methodology widely used in hyperendemic areas, especially in Brazil, where populations of Maranhão, São Paulo, Pará and Minas Gerais states were tested and reaffirm the importance of detection and control of disease cases through simple but reliable methodologies [32, 48–52].

In the search for increasingly fast and accurate tests, in 2012 the Brazilian Institute of Infectious Research together with the company OrangeLife developed a test capable of offering a diagnosis in only 10 minutes using only one drop of blood of the patient. The tool has received approval from the Brazilian National Sanitary Surveillance Agency (ANVISA) and has been field tested to evaluate the potential of the new platform. The assay is based on immunochromatography aspects, where recombinant proteins like LID-1, used as antigen, are immobilized on nitrocellulose membranes. Detection can be performed by the presence of IgM and IgG antibodies from various samples such as whole blood, plasma and serum.

Among the advantages of the test, it is mentioned the low cost of the tool, the possibility of early detection, agile and minimally invasive. In addition, it is associated with software stored on smartphones, further facilitating the interpretation of the exam and ensuring the availability of information in databases. Parallel analyzes of the rapid detection tool show the ability to diagnose, in most cases, the presence of the infection before clinical symptoms appear, contributing to the generation of accurate diagnoses and quality.

The interdisciplinary researches related to leprosy provided an amount of laboratory tools used as alternative methodologies for the more accurate and efficient diagnosis of the disease. Although there are still difficulties linked to the detection of paucibacillary forms, subclinical infections and contact monitoring, all research reinforces the importance of the search and use of efficient platforms and able to ally reduced cost and good indexes of sensitivity and specificity for the disease.

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4. Complementary molecular tests

After the advent of the genome sequencing of the *M. leprae* bacterium [53], species-specific genetic sequences have been searched in order to standardize diagnostic tests based on DNA analysis. These sequences can be amplified through the PCR technique, which allows the detection of bacillus DNA from small amounts of *M. leprae* cells [12].

The first works using the PCR technique were performed a little over 20 years, but the data were not satisfactory for the identification of *M. leprae* DNA in paucibacillary (PB) patients [54]. Thus, the methodology of molecular biology began to be used as an alternative method to traditional diagnostic methods. These data stimulated the search for new specific sequences for the identification of the bacillus, as well as the use of several clinical samples [55].

Many studies have been carried out involving different sequences and target genes, with the aim of increasing sensitivity and specificity in the identification of bacillus, especially in patients with low bacillary load. The literature reports the use of sequences that amplify gene regions encoding the 36 kDa [56], 18-kDa [57] and 65-kDa antigens [58], complex 85 [59], 16S rDNA [60] as well as for repetitive sequences of *M. leprae* (RLEP) [61]. By comparing these sequences, RLEP has been shown to be more sensitive and more specific than the bacilloscopic index. This could be explained by the number of copies, estimated to be at least 28 units, of the RLEP sequence in the *M. leprae* genome. In addition, this sequence generates a 130pb amplicon, which is considerably small compared to the sequences mentioned above, that is an important factor in the best efficiency in conventional PCR. Having a specific sequence is of great importance, since the PCR technique may be useful in the differential dermatological diagnosis [62].

A significant advance in increasing bacillus identification occurred with the use of real-time PCR technology. This methodology has been used in the follow-up of leprosy patients undergoing treatment [63] evaluation of bacterial load [13] viable bacterial load [60] and determination of resistance to treatment [14].

In clinical practice, detection of *M. leprae* by PCR in patients with negative bacilloscopy or inconclusive histopathology is of great value to define the correct diagnosis and treatment scheme [64]. In the same way, the methodology can be useful, for those patients with the pure neural form (PNL), who usually do not have cutaneous lesions and because of this they have deficient treatment scheme [65, 66]. Further, the PCR technique may be useful in early identification, since a considerable number of studies have addressed the positivity of *M. leprae* DNA in contacts of leprosy patients [48, 67–69].

In a study carried out in a hyperendemic area in cases of leprosy, it was possible to identify DNA from the bacterium in buccal and nasal swab samples in individuals with subclinical infection with multibacillary or paucibacillary index cases [48]. The identification of DNA from the Hansen bacillus in buccal and nasal swab raises considerations about the participation of this risk group in the transmission chain, besides the route of infection of the bacillus [70]. Although PCR can be a useful tool for identification, few studies associate the presence of *M. leprae* DNA to the development of the disease [71, 72], highlighting the importance of the use of serological tools and the follow-up of patients with subclinical infection [73, 69]

5. New biotechnological tools in the diagnosis of leprosy

5.1. Mapping of new markers

Many studies have used post genomic procedures for the discovery of new antigens that can be used in the diagnosis of leprosy [71–75]. These studies have explored sequences of *M. leprae* for the identification of proteins or peptides that can be used in the serodiagnosis of the different clinical forms of leprosy [76].

The antigens ML0405, ML2331 and ML2055—the first two of previously unknown function and the latter a membrane protein - were used for serological tests in in multibacillary patients of the clinical forms boderline lepromatous (BL) and LL untreated [77]. The ML0308 and ML2498 proteins, a conserved hypothetical protein and an enoyl-CoA hydratase respectively, showed humoral and cellular immunogenicity and can be used in the diagnosis of tuberculoid and lepromatous forms [78]. These antigens were used in the production of fusion proteins, such as LID-1 (leprosy IDRI diagnostic-1) [42] and PADL [45, 79].

The tools of bioinformatics, genomic analysis and proteomics are also being used for mapping in silico of important antigenic targets of *M. leprae* [80]. This type of analysis was used to define a group of 50 potential antigens in mycobacteria, some being restricted to *M. leprae* [78].

Peptides derived from specific and immunogenic proteins of *M. leprae* have also been tested in patients with leprosy and controls [81]. Peptides obtained of proteins from *M. leprae* were promising as indicators of exposure [82].

The peptides are small in size and can be expressed on the surface of bacteriophage to select peptides that mimic different targets (pathogens, cellular receptors or antibodies) [83]. Mimetic peptides may have important applications in the diagnosis of leprosy, mimicking antigens such as PGL-1 [84] or other *M. leprae* antigens [85, 86]. Alternatively, due to their versatility to perform the same functions as the protein and non-protein natural antigens, mimetic peptides are considered an important tool in immunodiagnostic of infectious disease.

5.2. Biosensors as platforms for the diagnosis of leprosy.

The post-genomic, the identification and obtainment of hundreds of molecules with immunogenic potential have broadened the versatility of detection platforms and contributed to an optimal diagnostic test, especially for tropical diseases [87]. In recent decades, biosensors have been gaining more space in scientific research and diagnosis of various diseases [88].

Biosensors are analytical devices that have specific reactions and/or specific interactions mediated by a diversity of components (antigens, antibodies, enzymes, DNA fragments, organelles, receptors and even mimetic peptides) that, in contact with a transducer, have the conversion of a biological signal-a result of the interaction between specific components-in a measurable signal proportional to the analyte concentration [89]. These platforms can be electrochemical, piezoelectric, thermal, optical and based on surface plasmon resonance, depending on the

type of transducer used [90]. There are still specific classes of biosensors such as immunosensors [91], which evaluate interactions between antibodies and antigens, and genosensores, based on the hybridization of DNA-specific ribbons [87].

There is a wide range of studies showing the efficacy of biosensors for the detection of various diseases such as leishmaniasis, bacterial diseases, cystic fibrosis, dengue and leprosy itself [92–96]. In Brazil, a genosensor for *M. leprae* was constructed using the immobilization of a bacillus single-stranded DNA (ssDNA) on functionalized graphite electrodes. The interaction between the immobilized sequence and *M. leprae* double-stranded DNA (dsDNA) is measured electrochemically by reductions in the peak oxidation current and using ferrocenecarboxyaldehyde as the hybridization indicator. The result was very promising, showing efficient detection in only 3 minutes [87, 88, 96].

Currently, Brazilian research groups are betting on the use of electrochemical biosensors as an indispensable tool in the diagnosis and control of diseases. This innovation is mainly because these sensors are sensitive, reliable, fast response and operate in conditions that pretreat the samples. In addition, these techniques are capable of providing exceptionally low detection limits.

In this scenario, mimetic peptides of proteins and glycolipids present in the bacillus capsule have been validated for the immunogenic potential and immobilized in these electrochemical detection platforms. Thus, the proposals consist of using different biological fluids such as blood, secretion and saliva, ensuring a less invasive and more comfortable test to the patient and the manipulator. Subsequently, these platforms will be tested in hyperendemic areas, in order to evaluate their detection potential and help in the epidemiological control of the disease.

6. Conclusion

The early diagnosis of leprosy is one of the goals of the WHO for the control and reduction of new cases of the disease. This strategy will be implemented with the development of new diagnostic tools more sensitive and can be applied in large-scale monitoring. Molecular techniques and new biotechnological approaches can be used as complementary tests.The qualitative PCR, RLEP and real time PCR have been used for the detection of *M. leprae* in samples of different tissues of patients or of household contacts.

Immunodiagnosis can be done using different native *M. leprae* antigens such as PGL-1, LAM or their synthetic derivatives. Post-genomic technologies can be used for the production of recombinant chimeric proteins, peptides obtained in silico or mimetic peptides. Immunodiagnosis can be performed by ELISA, lateral flow tests and biological sensors.

Biotechnology and molecular biology have contributed to the development of research and improve the diagnosis of leprosy. Significant advances in laboratory diagnosis contribute to improving clinical practice.

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Chapter 5

Leprosy Reactions

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Additional information is available at the end of the chapter

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Abstract

Sudden changes in immune-mediated response to *Mycobacterium leprae* antigen are referred to as leprosy reactions. The reactions manifest as acute inflammatory episodes rather than chronic infectious course. There are mainly two types of leprosy reactions. Type 1 reaction is associated with cellular immunity and particularly with the reaction of T helper 1 (Th1) cells to mycobacterial antigens. This reaction involves exacerbation of old lesions leading to the erythematous appearance. Type 2 reaction or erythema nodosum leprosum (ENL) is associated with humoral immunity. It is characterized by systemic symptoms along with new erythematous subcutaneous nodules.

Keywords: leprosy, type 1 reaction, reversal reaction, type 2 reaction, erythema nodosum leprosum

1. Introduction

Sudden changes in immune-mediated response to *Mycobacterium leprae* antigen are referred to as leprosy reactions. The reactions manifest as acute inflammatory episodes rather than chronic infectious course [1]. These reactions account for about 30–50% of cases with leprosy [2]. Both patients with low and high load of leprosy bacilli are at risk of developing leprosy reactions. Leprosy reactions can occur at any time before, during, or after the treatment. Patients with fewer skin lesions and without nerve involvement are less likely to develop leprosy reactions. The presence of multiple lesions in close proximity to peripheral nerves, facial involvement, and presence of nerve thickening without functional impairment are risk factors for the development of leprosy reactions. Patients developing leprosy reactions are more likely to develop sequelae or deformities [3]. There are mainly two types of leprosy reactions. Type 1 reaction involves exacerbation of old lesions leading to the erythematous appearance. Type 2 reaction is an immune complex-mediated reaction. It is characterized by systemic symptoms along with new erythematous subcutaneous nodules [4].

2. Type 1 reaction

2.1. Introduction

Type 1 reaction is a delayed hypersensitivity reaction. It mostly occurs in borderline patients as well as in patients with lepromatous leprosy (LL) and those with tuberculoid leprosy (TL) receiving therapy. Reaction can be the first sign of the disease and it often persists for a few weeks or months [5]. Classically, two subtypes of type 1 reactions have been described; first subtype is called reversal reactions, false exacerbation reaction or upgrading reactions and this type of reaction is reversible. Second subtype is called downgrading or downgrading reaction and it is associated with disease worsening. Upgrading (reversal) reactions occur in patients receiving therapy, and downgrading reactions occurs in patients who do not receive therapy. Due to decrease in bacterial load, borderline patients receiving therapy progress to tuberculoid phase of the disease spectrum. Bacterial load increases in patients who do not receive therapy and clinical appearance shifts to the lepromatous phase of the disease spectrum due to impaired cellular immunity [6].

2.2. Pathogenesis

These reactions are associated with cellular immunity and particularly with the reaction of T helper 1 (Th1) cells to mycobacterial antigens. It has been demonstrated that cytokines derived from Th1 cells such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), interleukin-2 (IL-2), and interferon gamma-Y (IFN-Y) play a more prominent role. High levels of TNF-α, soluble IL-2 receptors, and adhesion molecules also reflect severity of local inflammation. Borderline leprosy patients with type 1 reaction show increased expression of TNF-α mRNA in peripheral nerves and skin. Type 1 reactions are mediated by Th1 lymphocytes and secreted proinflammatory cytokines IFN-Y and IL-12, and free oxygen radicals [4, 5]. It was demonstrated that macrophages could initiate neural inflammatory process even in the absence of bacilli in the neural tissue [7].

2.3. Clinical features

Reversal reaction episodes often occur within the first 6 months of multidrug therapy (MDT) [8]. After initiation of therapy, skin lesions with manifestations of regression or lesions appearing as hypochromic macules become erythematous and edematous, and these lesions, then, become scaled and rarely become ulcerated [9]. The existing lesions show signs of inflammation, but no new lesions occur. Previously unnoticed or invisible patches may become prominent. This may give the impression of the development of new lesions. The lesions are often painless, but tenderness may sometimes be found. The lesions are often accompanied by edema and neuritis in the extremities [6]. Edema in the hands and feet may be sometimes the main symptom of reversal reaction. There may be burning pain in the lesions, pain in the face and extremities, and decrease in muscle strength. Isolated neuritis is commonly observed within the first 12 months of therapy. Nerve thickening and pain may occur and preexisting peripheral neuropathy may become prominent (sensory, motor, or autonomic). Ulnar, median, posterior tibial, fibular, radial, and facial nerves are the most

commonly involved nerves. The patients may present with the symptoms of neural dysfunction such as loss of sensation, facial palsy, claw hand, and drop-foot. Hyperesthesia may occur in palmar and plantar areas, associated with widespread nerve damage [1]. The ability to close eyelids is lost due to damage in the facial nerves (lagophthalmos) [10]. Neural damage is important, as it is considered the main cause of deformities and sequelae in the course of reversal reactions. Neuritis episodes may be severe; however, it sometimes has an insidious and even painless course, which is called silent neuritis. Silent neuritis is defined as sensory or motor dysfunction in the absence of skin lesions observed in type 1 and type 2 reactions [11]. It may cause inflammatory eye diseases, including iritis and scleritis, and it may even result in blindness. Systemic symptoms such as weakness, fever, bone pain, lymphadenomegaly, joint pain, and generalized edema are rarely observed and these symptoms indicate the severity of clinical condition. Furthermore, systemic symptoms are minimal in patients close to the TL pole of the spectrum and more commonly observed in patients close to the LL pole. Fever is usually absent and patients' general condition is good [6, 10].

2.4. Risk factors

The risk of type 1 reaction may increase with vaccination, MDT, pregnancy, puerperality, infections, stress, trauma, and oral contraceptive use. The extensiveness of skin lesions has been described as an important risk factor both in patients with low and high bacilli load [1]. It has been shown that the risk of developing neural damage, along with the risk of developing reversal reaction, is 10-fold higher in patients in whom three or more body segments are affected [11]. Facial involvement is a risk factor for the development of reversal reaction, as it is for lagophthalmos [12]. Although factors which can induce type I reactions are not clearly known, recent studies have pointed to genetic factors [5]. Identification of the risk factors, therefore, allows more meticulous follow-up of patients and early treatment [1].

2.5. Histopathology

The type 1 reaction is characterized by edema in the upper dermis and disorganized granulomas. The foreign body giant cells, Langhans giant cells accompanied by epidermal erosion and spongiosis, and fibroplasia appear in the dermis. The necrosis, ulcer and inflammatory infiltration by neutrophils may be observed in severe reaction [13]. The cytology of preexisting granulomas is differentiated by the presence of large epitheloid cells and decreased number of bacilli. Inflammatory cells often infiltrate epidermis and increased neural destruction is observed. The edema inside and around the granulomas results in the damage of surrounding tissues and nerves [1].

2.6. Treatment

The main goal of treatment in type 1 reaction is to suppress the cellular immunity. Prevention of nerve damage required early diagnosis and early institution of anti-inflammatory medications. MDT must be continued during the reactions. Corticosteroids are the most effective drugs used to treat reversal reaction. Their main effects are to inhibit activation of cellular immune response and suppress inflammatory response against *M. leprae* antigens in the skin

and nerves. Corticosteroids increases vasodilation by inhibiting the release of mediators such as arachidonic acid (prostaglandins) metabolites and platelet activating factor (PAF), vasoactive amines, neuropeptides, interleukin-1 (IL-1), TNF-α, and nitric oxide (NO). They inhibit adhesion of neutrophils, eosinophils, and lymphocytes to the endothelial cells, their migration to the inflammation site, and decrease vascular permeability. They inhibit phagocytosis and production of oxygen-free radicals [1].

Clinically, corticosteroids change the course of reversal reactions in many ways. They decrease intraneural and cutaneous edema and promote rapid recovery of the symptoms [1]. Earlier initiation of corticosteroid treatment can eliminate the risk of permanent neural dysfunction [3]. Corticosteroids must be continued at immunosuppressive doses for prolonged period. A prednisolone dose of 40 mg has been suggested as the start dose to control many of the type I reactions. However, patients with neural involvement require a dose of 1 mg/kg (60 mg) or sometimes higher doses (2 mg/kg). [14] Prednisolone dose must be reduced only after observing clinical recovery and tapering the dose to 20 mg/day. Recovery is often occurs within 3 months but may sometimes exceed 6 months. Intravenous methylprednisolone pulse therapy has been used to control reactions. Pulse therapy is indicated in severe reversal reactions and in cases of acute or chronic neuritis who have previously received oral corticosteroid therapy for prolonged period. The therapy involves administration intravenous methylprednisolone at a dose of 1 gr/day for consecutive 3 days in the first week and this is followed by a dose of 1 gr/week for consecutive 4 weeks, and finally 1 gr/month for consecutive 4 months. Prednisone 0.5 mg/kg/day is administered between the cycles of pulse therapy [15]. The treatment should be modified with a return to the previous dose in case worsening of clinical condition. Correct start dose and dose tapering regimen for prednisone must be determined on a patient basis, and this decision must rely on the follow-up of the loss of sensory functions and motor examination findings. The recommended duration of treatment is often 4–9 months in patients with borderline tuberculoid (BT) leprosy, 6–9 months in patients with borderline-borderline (BB) leprosy, 6–18 months in patients with borderline lepromatous (BL) leprosy; however, the treatment may last 24 months or longer. Patients with recent neural lesions and particularly those with less than 6-month duration better respond to therapy compared with patients in whom therapy is initiated in late periods [1].

Immunosuppressive medications such as azathioprine and cyclosporine can be used alone or in combination with corticosteroids [16]. Thalidomide is an effective drug used as an alternative to corticosteroid therapy and it allows long-term disease control [17]. Nerve decompression surgery has a limited place and it is recommended for patients with permanent pain after corticosteroid therapy. Surgery can be performed in patients with TL and BT leprosy with neuralgia and nerve abscesses in whom therapy with immunosuppressive is not feasible [1].

3. Type 2 reaction

3.1. Introduction

Type 2 reaction or erythema nodosum leprosum (ENL) occurs in patients with high bacilli load as in patients with multibacillar type leprosy (BL and LL) [5]. Type 2 reaction is considered to

be more complicated than type 1 reaction due to systemic nature and recurrent episodes [4]. The differences between type 1 and type 2 reactions are summarized in **Table 1** [1, 3, 4, 6, 10, 13]. Type 2 reaction course is 1–2 weeks, but may occur multiple recurrences over several months [5]. ENL is identified by Pocaterra et al. as single acute (one ENL episode lasting less than 6 months, recurrence is not), multiple acute (repeated discrete episodes) or chronic (an episode lasting for more than 6 months, continuous episodes) [18].

Parameter	Type 1 reaction	Type 2 reaction
Immunological response	Type 1 helper cells	Type 2 helper cells
Pathogenesis	Type IV hypersensitivity reaction (delayed cell-mediated)	Type III hypersensitivity reaction (immune complex formation and deposition)
Type of reaction	Reversal reaction Downgrading reaction	Erythema nodosum leprosum Lucio's phenomenon Erythema multiforme-like reaction
Clinical phenotype	Tuberculoid, borderline tuberculoid, borderline-borderline Previous treatment (except in downgrading reactions)	Borderline lepromatosis, lepromatosis Previous treatment or not
Cutaneous features	Acute onset of erythema and swelling of previous lesions No new lesions	New painful subcutaneous nodules in previously unaffected skin Necrotic areas Polymorphous erythematous plaques
Neurological features	Painful neuritis with or without loss of nerve function Pain or tenderness in one or more nerves Muscle weakness in the hands, feet, or face	Painful neuritis with or without loss of nerve function Pain or tenderness in one or more nerves Muscle weakness in the hands, feet, or face
Systemic manifestations	Rarely	Common (Fever, weakness, lymphadenitis, iridocyclitis, neuritis, arthritis, dactylitis, orchitis)
Risk factors	Multidrug therapy Vaccination Pregnancy Puerperality Oral contraceptive Infection Stress Trauma	Lepromatous leprosy Vaccination Pregnancy Puerperality Puberty Infection Stress
Recurrence	Less likely	Most likely
Histopathology	Tuberculoid granuloma Superficial dermal edema Dermal fibroplasia Disorganized granuloma and necrosis or ulceration in severe reactions	Neutrophilic infiltrate in the mid-deep dermis and subcutaneous tissue Leukocytoclastic vasculitis of the small and medium vessels
Treatment	Nonsteroidal anti-inflammatory drug Systemic corticosteroids	Acetylsalicylic acid, pentoxifylline Systemic corticosteroids Clofazimine Thalidomide

Table 1. The differences between type 1 and type 2 reactions [1, 3, 4, 6, 10, 13].

3.2. Pathogenesis

Type 2 reaction is associated with humoral immunity. This is a type 3 hypersensitivity reaction associated with the deposition of immunocomplexes produced by binding of antigens released by the destruction of bacilli with antibodies [6]. Immunocomplexes cannot be phagocytosed by the macrophages, cleared by the kidneys, and they are deposited on the vessel walls [19]. This reaction is also associated with increased levels of proinflammatory cytokines. Release of inflammatory cytokines and followed by neutrophilic infiltration contribute to the development of variable characteristic clinical findings depending on the involved organ. In type 2 reaction, vasculitis and/or concurrent panniculitis occurs with inflammatory infiltration by neutrophils [5].

3.3. Clinical features

Type 2 reaction may occur in the early periods of therapy and even after completion of therapy, as it takes long time for the body to eliminate dead bacilli in the macrophages. It often occurs in the first three years after initiation of leprosy treatment. Sudden deterioration in clinical condition may be observed in patients with LL and rarely in patients with BL leprosy [6]. This reaction can involve multiple organs and systems. Immunocomplexes accumulate in the circulation and they are deposited in the skin, eyes, joints, lymph nodes, kidneys, liver, spleen, bone marrow, endothelium, and the testes. The lesions are multiple, bilateral, erythematous, firm, painful, subcutaneous nodules resembling erythema nodosum that are distributed symmetrically. Pustular, bullous ulcerated, and necrotic types have also been reported. Some nodules may persist as a chronic painful panniculitis and lead to scar. The target lesions of erythema multiforme may occur in any region [4, 6]. The lesions more often occur in external surfaces of the body [20]. General symptoms such as fever, weakness, edema, myalgia-arthralgia, dactylitis, bone tenderness, and lymphadenomegaly are observed prior to the occurrence of or concurrent with ENL lesions. Iridocyclitis, episcleritis, eye pain (photophobia), orchitis, liver, or kidney damage can be observed. Neuritis, painful enlarged nerves and nerve function impairment may occur [4, 5]. Necrosis can occur as a result of vascular thrombosis and ischemia. Vascular occlusion is probably associated with vasculitis caused by immunocomplex deposition on the vessel wall and leukocytoclasia. This should not be confused with Lucio's phenomenon observed with classical LL. In Lucio's phenomenon, the majority of the bacilli infect capillary endothelium, leading to endothelial proliferation, thrombosis, and vascular occlusion [21]. Laboratory tests show elevated levels of acute phase reactants such as C-reactive protein (CRP), α1-antitrypsin, α1-acit glycoprotein (AGP), and γ-globulins [22].

3.4. Risk factors

Lepromatous leprosy forms with high bacilli load, vaccination, infection, puberty, pregnancy, puerperality, with significant hormonal changes occurring in women are risk factors for the development of type 2 reaction. Emotional and psychological stress and associated immunological and hormonal changes have been regarded to trigger these reactions; however, this has yet to be confirmed [4, 10].

3.5. Histopathology

Two different histopathological variants have been described in ENL. First variant has been reported by Ridley as "the pink nodule type" or classical ENL (or mild ENL form). Typically, there are clusters of neutrophils accumulated around the foamy macrophages at the center of small granulomas. Eosinophils, plasma cells, and mast cells are present. Classical characteristics of vasculitis affecting small- or medium-sized vessels, necrotizing changes, and thrombosis formation have been reported in almost 25% of the patients. Indeed, vasculitic changes mostly occur in early lesions. Vasculitic changes involving neutrophilic infiltration, hemorrhage, and thrombus formation may be severe in necrotizing ENL. Necrosis in epidermis and dermis, collagen degeneration can be observed and this may result in dermal fibrosis [13]. Intact acid resistant bacilli (ARB) are found in the lesions of untreated patients, whereas granular and fragmented ARB are often found in patients receiving therapy. Lucio's phenomenon must be histopathologically differentiated from real erythema nodosum, Sweet syndrome, pyoderma gangrenosum, and deep micotic infections [13, 23].

3.6. Treatment

Type 2 reaction often regresses with addition of clofazimine to the MDT. After the use of clofazimine-containing MDT, type 2 reaction prevalence has decreased in leprosy patients under therapy. Suppression of inflammation is the basis of therapy. Bed rest and drugs such as acetylsalicylic acid, corticosteroids, nonsteroidal anti-inflammatory drug (NSAID), chloroquine, antimony compounds, pentoxifylline, and thalidomide are used in the treatment [4, 24, 25].

Corticosteroids and thalidomide are still considered the mainstay of therapy in severe cases of type 2 reaction presenting with orchitis, iridocyclitis with glaucoma, and neuritis that cause neural dysfunction [14]. Administration of high doses of corticosteroids with pulse therapy and rapid dose tapering within 2–3 weeks have been deemed appropriate as type 2 reaction is an episodic disease. If maintenance therapy must be avoided particularly in patients with chronic recurrent ENL, as long term therapy with prednisolone causes dependence to corticosteroid therapy and side effects. Thalidomide seems to be the choice of drug in maintenance therapy. Action mechanism of thalidomide is not clear. It is thought to be effective in the inhibition of TNF-α. It has some side effects which do not necessitate discontinuation of therapy. Neuropathy has been reported in approximately 20–30% of patients. It is often masked by leprosy neuropathy [26]. It is well tolerated at a dose of 100–300 mg/day in cases with recurrent disease and it provides prolonged remission [4]. Clinical trials have shown that thalidomide rapidly controls ENL and it is superior to acetylsalicylic acid and pentoxifylline therapy. On the other hand, thalidomide is teratogenic when used in early periods of pregnancy [25]. Thalidomide analogs chemically resemble thalidomide, but side effects are not the same. Revlimid and aktimid are promising drugs in this category [27].

Clofazimine is recommended in the treatment of chronic recurrent reactions. Clofazimine is administered for 12 weeks together with corticosteroids at doses of 100 mg tid, 100 mg bid, or 100 mg/day. Clofazimine is less effective than corticosteroids and it often takes 4–6 weeks to be fully effective. Addition of clofazimine to the therapy is extremely beneficial in reducing corticosteroid doses or discontinuation of corticosteroid therapy in patients who have become

dependent on corticosteroids. The total duration of clofazimine therapy should not exceed 12 months [18].

Corticosteroids and thalidomide are the mainstay of therapy in the control of type II reaction. Selective cytokine inhibitors and phosphodiesterase type-4 inhibitors with potential TNF-alpha activity but without T-cell activating effect are new drugs [17].

4. Differential diagnoses

In general, cutaneous drug reactions, local skin infections, relapses, diabetes, Bell's palsy, rheumatoid arthritis, rheumatic fever, and disc prolapse must be taken into consideration in differential diagnosis. It may manifest as various cutaneous drug reactions such as urticarial, lichenoid, exanthematous reactions, erythema nodosum, erythema multiforme, Stevens-Johnson syndrome and toxic epidermal necrolysis. The patients usually suffer from itching and burning in some of these lesions, whereas these symptoms are not observed in patients with leprosy. Furthermore, new skin lesions do not resemble preexisting lesions. Localized skin infections developing in patients with leprosy are often confined to a particular body site. The lesions do not occur bilaterally and medical history is often remarkable for trauma or insect bites that could cause an infection. New lesions appear if relapse occurs, and this often has an insidious course rather than a severe clinical course. Reaction often occurs within the first 3 years after initiation of leprosy therapy and old lesions exhibit acute pain and tenderness. Diabetic patients are prone to infections and development of peripheral neuropathy. Furthermore, regulation of blood glucose is impaired upon administration of corticosteroids. All patients must be screened for diabetes and referred to an advanced facility if diabetes is diagnosed. Bell's palsy may mimic facial paralysis caused by leprosy reactions. These patients do not have nerve thickening, sensory loss along the nerve projection, and hypopigmented skin lesions. This condition is better evaluated by the ophthalmologists. In Bell's paralysis, widening of palpebral fissure is not associated with the drop of lower eyelid. It occurs in women at childbearing age with rheumatoid arthritis, joint pain, joint deformities, fever, skin rash, and multiple organ involvement. Rheumatoid factor is almost always found to be elevated. However, referral to an advanced facility may be sometimes required to differentiate rheumatoid arthritis from leprosy reaction. Patients with rheumatic fever are usually young patients with fever, joint pain, and skin rash for a short period. These patients have high antistreptolysin O titers and valvular involvement can be found that cause murmur on auscultation. Patients with disc prolapse may present with acute onset of neuropathy in the extremities. Patients often report weight lifting in the early periods or stretching in the back. These patients do not show skin lesions or nerve thickening [23, 28].

5. Conclusion

The reactions can contribute to further deterioration of the quality of life in leprosy. Early diagnosis of reactions can prevent nerve damage and provide early intervention to systemic complications.

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Chapter 6

Demyelination in Leprosy

Dhelya Widasmara and Sri Linuwih Menaldi

Additional information is available at the end of the chapter

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Abstract

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* that has a predilection for peripheral nerves, especially Schwann cells (SCs). Leprosy medications may only eradicate the bacteria without preventing or recovering peripheral nerve damage. Early nerve damage detection is necessary. The expression of Krox-20 in Schwann cells will be examined immunohistochemically, and the level of neuron growth factor (NGF), neuregulin 1 (NRG1), protein 0 (P0), and peripheral myelin protein 22 (PMP22) will be examined in the blood plasmas. A significant decrease was noticed in Krox-20 and NGF, NRG1, P0, and PMP22 level ($p < 0.05$) in disability degree 1 compared to degree 0. Studies proved that markers have shown promising results; Krox-20, NGF, NRG1, P0, and PMP22 could be useful diagnostic tools for early peripheral nerve damage detection in leprosy.

Keywords: leprosy, disability, nerve damage detection, marker

1. Introduction

1.1. Background

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* and has predilection for the skin and peripheral nerves, especially in Schwann cells (SCs) [1–4]. During infection, *M. leprae* causes peripheral nerve damage and even causes disability and deformity in patients. Although the anti-leprosy drug treatment can already eradicate the bacteria, disability and deformity that occur cannot be restored and neither can the function of the nerve that has been lost. By understanding the mechanism of nerve damage caused by *M. leprae*, it is expected that nerve damage can be prevented [5].

Until now leprosy treatment is only for eradication of bacteria, but it cannot prevent or cure peripheral nerve damage and its components, so early detection to determine the presence of bacteria in Schwann cells is important [6, 7]. Several studies in early detection of nerve damage by invasion of *M. leprae* have been conducted, namely, using ultrasound to detect peripheral nerve damage [8], vasomotor reflex and sympathetic skin responses [9], electrophysiology of the peripheral nerves involved [10], and examining axonal markers on skin lesions [11]. There is Ridley-Jopling classification of leprosy based on clinical, bacteriological, histological, and immunological symptoms. Based on bacteriological examination, there are two types of leprosy: paucibacillary (PB) and multibacillary (MB). PB types include tuberculoid type (TT) and borderline tuberculoid (BT) in the Ridley-Jopling classification, whereas MB types include borderline-borderline (BB), borderline-lepromatous (BL) leprosy, and lepromatous leprosy (LL) types [12].

According to the WHO, the levels of disability in leprosy patients are divided into three degrees, ranging from the absence of symptoms to apparent damage or disability [13]. In general, according to Seddon, peripheral nerve damage is divided into three, namely, neuropraxia, axonotmesis, and neurotmesis, whereas in leprosy nerve damage occurs as a result of demyelination of peripheral nerves [14].

In the central nervous system (CNS), myelin is formed by oligodendrocyte. Myelin in the CNS has a spiral structure such as peripheral nerve myelin which has an inner mesaxon and an outer mesaxon that ends in a loop or cytoplasmic junction. The cytoplasm of glia in the CNS is confined to only a portion of the myelin sheath. The glia junction continues with the oligodendrocyte plasma membrane through a layered process. One oligodendrocyte can eliminate about 40 or more axons, whereas in the peripheral nervous system (PNS), the myelin deposition of an axon can reach up to 100 layers of myelin [15].

Schwann cell differentiation is governed by the expression of certain transcription factors. After receiving signals from axons, immature Schwann cells including NRG1 will increase the expression of some transcription factors such as NFκB, Oct-6, and Brn2. These factors will stimulate initiation of the promyelination stage whereby the Schwann cell will interact with the axon and begin to express the initial myelination marker. An increase in the Krox-20 gene requires Schwann cells to initiate the myelination process and express the specific protein of myelin. In mature nerves, Schwann cells that do not express Krox-20 will remain nonmyelinated cells. In the injury condition, c-Jun and Sox-2 will increase rapidly. This will lead to a decrease in Krox-20 and Schwann cell differentiation. Cross-resistance Krox-20 and c-Jun will stimulate the switch of complex transcription. Promyelination signals from axons such as neuregulin will result in Krox-20 expression via the phosphatidylinositol-3 kinase (PI3K) pathway. The activation of the Janus kinase (JNK) pathway during the injury period will stimulate c-Jun expression. However, the signals that activate these pathways in Schwann cells are still not known [15].

The study evaluated the Krox-20 expression on Schwann cells in the skin biopsy of leprosy patients, and it was observed that there was a significant difference of Krox-20 expression among patients with degree of disability 0 and 1. (T-tailed test shows $F = 8.881$ with $p = 0.000$ ($p < 0.05$)). Findings summarized a significant decline in Krox-20 expression in degree of

disability 1 compared to degree of disability 0 (T-test, F = 8.881, p = 0.000 (<0.005)). Sensitivity and specificity can be 100%. It means that if the Krox-20 expression is more than 8, the degree of disability will be 0 and vice versa. ROC curve showed that area under the curve is 1.0 (100%) with p = 0.000. On the other hand, in patients with degree of disability 1, it can be seen that the expression of Krox-20 is minimal [16].

The function of Schwann cells is to synthesize the myelin sheath. When these cells are infected by *M. leprae*, the consequence is a demyelination resulting from neuritis. It is suspected that *M. leprae* infection in Schwann cells is a direct cause of Schwann cell dysfunction that can cause demyelination in leprosy patients [2].

Leprosy is one of the diseases that can cause nontraumatic nervous system disorder and is most commonly found in the world. Diagnosis of this disease can be established relatively easily because it does not require sophisticated equipment. The problem is the number of disabilities even though *M. leprae* has been eradicated in accordance with the applicable protocol. Early identification of the occurrence of disability is also the constraint in establishing diagnosis of disability as a sequel of leprosy. Nerve cell damage by leprosy is the result of the demyelination of peripheral nerve cells. Demyelination is caused by the entry of *M. leprae* into Schwann cells as the main target. The entry of these bacteria can cause the demyelination of Schwann cells suspected through the activation of the c-Jun pathway. When damage occurs in Schwann cells, automatically as a form of defense, Schwann cells will repair the damage that occurs, namely, by remyelination. The process of remyelination is influenced by NRG1 and NGF as a neurotrophic factor, as well as the availability of PMP22 and P0 as specific basic materials of myelin in peripheral nerves. Until now, based on the literature review we have read, the factors used to determine early disability in leprosy patients are unknown. Meanwhile, the use of WHO criteria to determine the degree of disability is still rough because it involves only three sensory organs: feet, hands, and eyes. This study is aimed to determine the early markers of nerve damage, namely, demyelination and remyelination in leprosy patients with degrees of damage 0 and 1 based on WHO criteria.

2. Literature review

2.1. Leprosy in general

2.1.1. Definitions

Leprosy is a chronic granulomatic infection caused by *M. leprae.* This disease attacks the skin, nasal mucous membranes, and peripheral nerves [17].

2.1.2. Etiology

The cause of leprosy is *M. leprae* which is transmitted by droplet from nasal secretions and received by the nasal mucosa and other respiratory tracts. This bacterium mainly attacks

Schwann cells in the peripheral nervous system and can cause peripheral nerve functional disabilities as well as disability [18].

2.1.3. Epidemiology

Multidrug therapy (MDT) program has greatly reduced the prevalence of leprosy to less than 1 case per 10,000 people in 90% of endemic countries where leprosy is considered as a public health problem. However, the leprosy case detection rate is still high [19].

2.1.4. Diagnosis

Based on physical examination, according to the WHO, there are three special physical signs for leprosy which can already be used to make the diagnosis. The three special signs are:

1. Redness or hypopigmented skin lesions with loss of sensation (especially sensation of touch and temperature)
2. Peripheral nerve involvement, such as peripheral nerve thickening with loss of sensation (especially touch and temperature)
3. Acid-resistant bacteria in the skin smear of the patient in a certain place

The diagnosis of the degree of disability in leprosy patients is based on the criteria published by the WHO as shown in **Table 1**.

The main classification according to WHO leprosy disease consists of paucibacillary (PB) and multibacillary (MB). If five hypopigmented patches can be found in the patient's skin and there is no BTA in the skin smear, it can be classified as PB type. However, if more than five leprosy hypopigmentations are spotted, and/or BTA are found in skin smear, it may be considered as MB leprosy patients [19].

	Symptoms
Hands and feet	
Grade 0	No anesthesia, deformity, or structure damage
Grade 1	There is anesthesia, no deformity
Grade 2	There is anesthesia and deformity
Eyes	
Grade 0	No problems with the eyes
Grade 1	Eye problems due to leprosy, visus is not worse than 6/60 or finger count on 6 m
Grade 2	Some severe disorders (visus < 6/60, unable to count fingers, and lagophthalmos, iridocyclitis, and opacity of the cornea)

Table 1. Assessment of the degree of disability and deformity index (hands, feet, and eyes) according to the WHO [20].

2.1.4.1. Monofilament test

One of the techniques which is used to assess neuronal function in leprosy patients is monofilament test (MFT). The test is performed using five different single filaments, i.e., 200 mg, 2 g, 4 g, 10 g, and 300 g. Each site is then assessed by the perceived filament, which is the heaviest monofilament obtained and the highest value with a total value of 15 for the ulnar, median, and radial (four filaments) nerves and a total value of 12 for the foot (three filaments). The normal threshold value is 200 mg for the hand and 2 g for the foot (other than the heel) [10, 11]. This test is considered positive if the MFT value is 3 for each nerve. "Fixed" or "unchanged" test result is when the test score has one- or two-point difference from the previous test score (basal value). If the value increases by three points or more, it is said to be "damaged" or "broken." If the value decreases three points or more, it is said to be "improved." If the patient's examination value improves by three points or more, and the total value of the neural examination is reduced by two points or less when compared to the current patient condition, it is said to be "cured."

For the ulnar, median, and posterior tibial nerves, the same examination as the INFIR study is performed, except for the median nerve on the tip of middle finger rather than the little finger (**Figure 1**).

2.1.5. Therapy

Patients with PB type can be given two types of drugs which are already available in the package. The first is for 6 months, i.e. In PB case, in patients over 15 years old, the drug is given in multidrug treatment (MDT) form with rifampicin 600 mg and dapsone 100 mg on the first day, the second day, and until the 28th day and so on for up to 6 months. The second is for 1 year, i.e., MB case. In patients over 15 years, rifampicin 600 mg, clofazimine 300 mg, and dapsone 100 mg on the first day of the first month can be given. In the second and subsequent days, they are given dapsone 100 mg and clofazimine 50 mg [19].

2.1.6. Prognosis

In general, the prognosis of leprosy after getting the correct treatment is good. Healing in complications of neurological disorders is very limited, which means they are difficult to heal. Lesions on the skin can usually be healed in the first year of treatment, while color disorder of the skin and skin damage usually persist. Physical therapy, reconstructive surgery, nerve and tendon transplantation, and surgical operations to correct contractures can improve the patient's quality of life [20].

2.1.7. Complications

Good care and attention to the possibility of reversal reactions due to leprosy treatment will minimize long-term neurological sequelae. Here are some possible leprosy complications:

2.1.7.1. Reaction type 1:

Slow hypersensitivity reaction occurs when BL leprosy shifts into LL leprosy during treatment. This reaction is a description of good immune response and formation of IFN-γ and

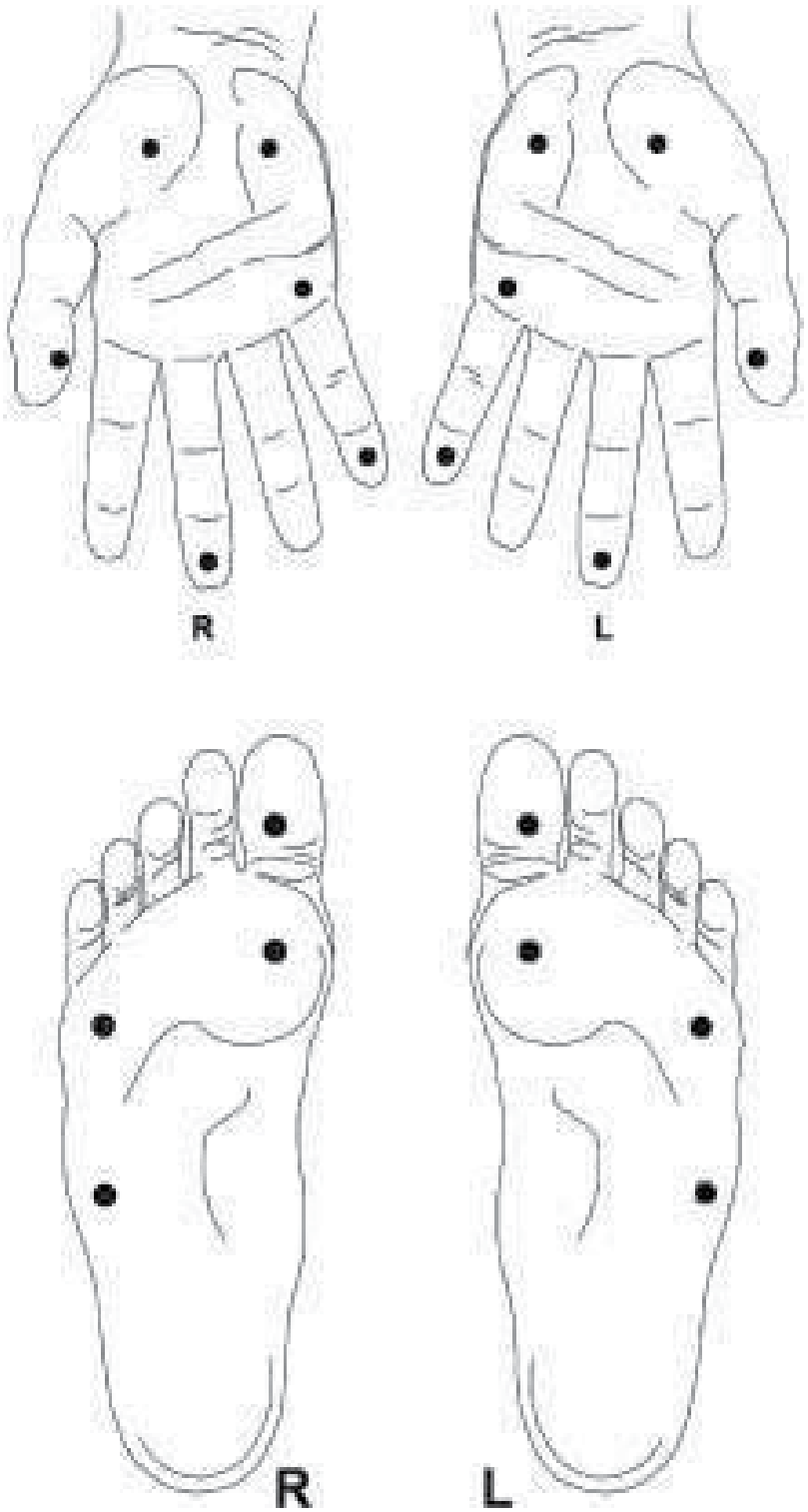

Figure 1. Semmes-Weinstein monofilament testing points [21].

TNF-α locally. This reaction is characterized by edema and erythema in skin lesions, the formation of skin lesions, neuritis, and loss of sensation and motor. The incidence of type 1 reactions in leprosy patients with BL is about 30%. Nonsteroidal anti-inflammatory drugs (NSAIDs) and high-dose steroids can be given in the treatment of type 1 reactions.

2.1.7.2. Reaction type 2:

It is often referred to as erythema nodosum leprosum (ENL), which is the complication of LL type. This reaction is characterized by the formation of subcutaneous inflammatory nodules with fever, lymphadenopathy, and arthralgia. High levels of TNF-α and deposition of the immune complex are suspected to be closely related to ENL.

In addition, a complication that may also occur is Lucio phenomenon, which is a severe complication in MB-type leprosy characterized by bluish bleeding plaque and necrotic ulceration. In this phenomenon, leprosy bacteria may have spread to endothelial cells along with the appearance of necrotic and vasculitic epidermis with thrombus formation and endothelial proliferation [6, 20].

2.1.8. Immunoprophylaxis and chemoprophylaxis

Bacille Calmette-Guerin (BCG) vaccine has been widely used in leprosy endemic countries to provide protection against leprosy. The first dose of BCG provides protection between 14 and 80% against leprosy, while the second dose provides protection between 0 and 50%. Single dose of rifampicin administered to close contacts from newly diagnosed leprosy patients shows an efficacy of 57% in reducing new cases of leprosy [22]. Other studies show the protective effect of immunoprophylaxis and chemoprophylaxis in controlling leprosy. This is done by giving BCG vaccine at infancy in combination with rifampicin given to leprosy contacts. However, the effect of this combination is more useful for PB form contacts rather than MB form [23].

2.2. The peripheral nerve as a target of *M. leprae*

The Schwann cell (SC) of the peripheral nervous system (PNS) is essential for the survival and function of neurons, that is, to enclose axons. SCs were discovered by Theodore Schwann who investigated peripheral nerves. The function of this SC is to assist the myelination of axons and to direct the neurons. SC develops after birth from immature cells to become myelin or without myelin. The first immature cells go through the myelination path at birth and then go through the non-myelin path later in its development [24].

Both SCs which are myelin and without myelin produce an extracellular matrix that forms basal lamina containing collagen around axons. These neurons consist of units of axon cells—Schwann and some neurons (fascicles) surrounded by solid fibrous tissues enveloping the perineurium. Furthermore, these fascicles are also grouped and create neural stems surrounded by other tissues called the epineurium.

Myelinated SCs form myelin that surrounds large axons to increase the conductivity of neurons. The unmyelinated SC surrounds several small axons separated by the cytoplasm. Myelin is formed by the differentiation of the plasma membrane of SC. Myelin consists of multilayered membrane that encloses the axon in both the CNS and PNS. This helps in increasing the

speed of nerve impulses along the axon. Myelin contains 80% fat and 20% different proteins between the PNS and CNS [25, 26]. The major lipid classes found in other membranes are also found in myelin such as neutral lipids, phosphoglycerides, and sphingolipids. However, myelinated PNS has more sphingomyelin (10–35%); higher content of monogalactosylsphingolipid, cerebroside [Gal-C] (14–26%), and sulfatide [SGal-C] (2–7%); and less galactolipid and cholesterol than CNS myelin. The main fatty acid in myelinated PNS is oleic acid [C18: 1 (n − 9)] (30–40% of total fatty acids). Myelin is also characterized by a very high long-chain fatty acid (>18 carbon). The long-chain fatty acids contained in sphingolipids are mostly saturated fatty acids. Myelinated PNS proteins are enriched in glycoproteins and basic proteins [25, 27].

The main proteins of the PNS are 28 kD myelin Protein-0 (P0) 50–60% and 100 kD myelin associated glycoprotein (MAG); both proteins presented only with myelinated SC to maintain the myelin solid structure and the integrity of the axons-myelin. Peripheral myelin protein-22 (PMP-22 kD), myelin basic protein (MBP, 15%), and myelin P2 (10%). Both MBP and P2 myelin are located in the cytoplasm. Other heavy molecular mass of glycoproteins such as 170 kD periaxin are present in small amounts. Myelin P0, an immunoglobulin-like immune cell protein, is immutable among species, with various posttranslational modifications such as phosphorylation, acylation in amino acid regions 110–119, and glycosylation with single, N-linked nine asparagine sugar chains 93 [25]. MBP in the PNS has four polypeptide bands ranging from 14 to 21 kD. This structure has various posttranslational modifications such as phosphorylation and methylation, and this is one of the major autoantigens in multiple sclerosis disease. Myelin P2 or fatty acid-binding protein 8 is a small protein (14 kD) with a high positive charge and is concentrated mainly in thick myelin sheaths. This structure is a member of the family of fatty acid-binding proteins with a high affinity for oleic acid, retinoic acid, and retinol. This structure function is to assemble and maintain myelin lipids. P2 is also an autoantigen in autoimmune peripheral neuropathy, Guillain-Barre syndrome (GBS). Its function is related to stabilizing the dynamics of myelin membranes and transport to and from lipid membranes [26, 27].

Myelin is only produced if the cell is in contact with several types of axons. Axons send signals that are important to identify the SC. Examples of axon signals are beta-neuregulin-1 (NRG1) and glial growth factor (GGF). SC can be activated to enter proliferation via axonal signaling NRG1 to bind and activate the ErbB2/ErbB3 receptor complex on SC to activate MAPK for cell proliferation. The factors governing myelination of SC include transcriptional factors Krox-20, Oct-6, and Sox-10; they also inhibit cell death and proliferation. Special myelin protein, P0 for diminished myelinated SC if immature cells are not associated with axons. If SC loses interaction with an axon, there can be dedifferentiation into an immature SC. If the cells associate with their axon again, they will become myelinated or nonmyelinated depending on the stimulating signal. SC has the ability to block apoptosis through the effects of growth factors such as insulin-like growth factors (IGFs), platelet-derived growth factor-BB (PDGF-BB), and neurotrophin-3 (NT-3) on autocrine circuit [24].

One of the events associated with SC pathogenesis is nerve injury and demyelination. This can be due to axonal damage and axon-SC signal interference. Another cause is the immune stimulation (autoimmunity) that targets myelin as in multiple sclerosis and Guillain-Barre syndrome (GBS). As with autoimmune disorders, *M. leprae* causes peripheral nerve demyelination that begins with damage to the myelin sheath and decreases the speed of the action-potential conduction [28].

2.3. Nerve damage in leprosy

Nerve damage is a major characteristic of leprosy pathogenesis. The first stage of nerve damage in leprosy is the localization of *M. leprae* in peripheral nerves. A recent research about leprosy in experimental animal armadillos successfully revealed that after *M. leprae* successfully penetrate the skin barrier, these bacteria gather in epineurial lymph and blood vessels around the nerve and then go into the endoneurium compartment through the blood supply. However, if *M. leprae* can enter SC freely through the only way through the Schwann cells exposed to the dermis; the only way to prevent transmission of leprosy is to prevent the attachment of leprosy to Schwann cells [2].

In a study by Harboe et al., it is declared that *M. leprae* binds to the G domain of the α2-laminin chain (LN-α2) expressed by the Schwann cell axons. In addition, it is shown that $\alpha\beta$-dystroglycan (DG) in the basal lamina acts as complex receptor of LN-α2/*M. leprae* bonds. Presumably, there are other receptors that play a role in Schwann-*M. leprae* cell interactions because blocking of these receptors has not been successful in completely preventing the attachment of *M. leprae*. The third stage is the role of LBP-21 protein (LPS-binding protein, 21 kDa) which is surface antigen of *M. leprae* which acts as adhesin molecules to interact with Schwann cells. In addition, other *M. leprae* surface antigens sphingoglycolipid-1 (PGL-1) are also shown to bind to laminin-2. Therefore, PGL-1 is also involved in the Schwann cell invasion via the basal lamina via laminin-2-dependent pathway. It is suspected that PGL-1 serves as a second receptor to *M. leprae* where the combination of PGL-1 and LBP-21 provides sufficient energy in binding to Schwann cells so that *M. leprae* can enter Schwann cells safely [6].

The reversal reaction in leprosy is closely related to the increase of immense cellular immune reactions against mycobacterial antigens. Histologically, the lesions are invaded by mononuclear cells and result in edema and hyperemia. These events are the basis of immunosuppressant administration in leprosy reactions, while antimycobacterial drugs should be continued as well. In the study, TNF-α and TNF-α mRNA levels were very high. This suggests a strong immunological reaction to *M. leprae* antigens.

Contact-dependent demyelination induced by *M. leprae* in nerve culture in the absence of immune cells also shows the role of nonimmune mechanisms during early infection and nerve involvement in leprosy infection. During the development of acute ENL with type 2 cytokine pattern in leprosy patients, there is an increase in IL-6, IL-, and IL-10 as well as the persistent expression of IL-4 and IL-5 mRNAs in the lesions. Chronic ENL often leads to nerve damage, which is possible due to induction of local immune complex deposition with granulocytes that cause tissue damage and complement activation.

The c-Jun molecule is a major component of complex transcription of transcription factors and forms JunB and JunD in the Jun mammalian protein family. The c-Jun molecule is involved in cellular functioning and dependent on N-terminal phosphorylation performed by the Jun N-terminal kinase (JNK) enzyme. Thus, JNK can affect protein content of c-Jun. The levels of c-Jun in Schwann cell culture are high despite the simple culture medium. The c-Jun proteins are present in immature Schwann cells on embryonic and neonate nerves, but their presence is suppressed in individual cells as transcription factors of Krox-20 premyelination are activated and the myelinating begins. In Schwann cell culture, the addition of Krox-20 expression is sufficient to suppress the expression of c-Jun protein. Krox-20 is also involved

in c-Jun suppression in vivo, as c-Jun levels remained high in the Krox-20 null nerve where myelination is discontinued. In vitro experiments indicate that c-Jun suppression is absolutely necessary for myelination processes since Schwann cells with high c-Jun expression will be inhibited in the myelination process of the axon in which the induction of Krox-20 or cAMP myelin genes is inhibited. In contrast, in Schwann c-Jun null cells, it has increased myelinated gene expression [29].

c-Jun will be upregulated rapidly after nerve injury. This is a procedure that triggers the dedifferentiation of Schwann cells. To determine the function of c-Jun under these conditions, Arthur-Farraj et al. made the Schwann cell without c-Jun. The process of myelination at the stage of development is not so affected on the specimen. However, it turns out that c-Jun is normally suppressed as the myelination process begins. However, after the process of injury passed, there is a delayed degradation of the myelin sheath. This presumably occurs because of decreased ability of c-Jun null cells in digesting myelin. In addition, there is a delay in the inactivation of myelin genes and the failure to activate the necessary molecules of demarcated cells including L1, p75NTR, and N-cadherin. All of these molecules are important because after the injury, c-Jun protein required Schwann cells to differentiate and adjust the molecular phenotype as it is immature. One of the properties that can be known when the cell is deficient or when there is disruption of c-Jun in Schwann cells is the loss of regeneration ability dramatically and the loss of recovery ability after injury [30].

The key role of axon integrity in controlling switches from c-Jun negative, positive Krox-20 which functions in maintaining myelin differentiation, to positive c-Jun; negative Krox-20 in dedifferentiated cells can be clearly observed in experiments using Wild mice. In these mice, axonal degeneration and myelin degradation that occur after neural cuts are delayed for up to 2–3 weeks. It is presumed that during the period of maintenance of myelin after an axotomy, the expression of Krox-20 is maintained and c-Jun is inhibited. At the time when axons degenerate and the myelin sheath begins to break down, c-Jun is expressed, while Krox-20 is no longer expressed [31].

2.4. Immunopathogenesis nerve damage in leprosy

2.4.1. Immune-mediated damage

2.4.1.1. The role of cellular immunity

SC can take, process, and present the specific antigen of *M. leprae* to T cells resulting in the production of Th1 modulatory immune cytokines such as TNF-α and INF-γ [32]. SC can present mycobacterial antigen to MHC class I, CD8+ cytotoxic T cell, and also can present mycobacterial antigen to class II MHC, CD4+ CTLs. SC was found to express co-stimulatory molecules and adhesions to T cells. As a result of this stimulation, SC will be killed by cytotoxic granules (granulysin, granzyme, and perforins) produced by CTLs [33]. SC can express TLRs (TLR1 and TLR2) which are activated by LAM *M. leprae*, a lipoprotein such as 19 and 33 kD. SC activation will lead to cytokine production (TNF-α, IL-12) and apoptosis [34]. TLR expression was found to be greater in TT patients than LL patients [35]. The large amount of cytokines released by Th1 cells, especially IL-12, IL-2, and TNF-α, will cause apoptosis of infected cells and decrease in bacterial load and increase granuloma formation such as lesions in TT type [36].

Macrophages also play role in the stimulation of immunity in leprosy. Infected macrophages by *M. leprae* can present its antigens to T and B cells and release cytokines including TNF-α [1]. Both of these cells can produce reactive oxygen intermediate (ROI) which results in further nerve damage at the site of granuloma [26]. *M. leprae* stimulates macrophages to produce TGF-β which is responsible for decreased nerve regeneration [26]. Other cells may also present antigens which are dendritic cells, also found to effectively present the *M. leprae* antigen and stimulate CD4+ and CD8+ cytotoxic T cells. Th1 cells are thought to be involved in CMI-DTH reactions (cell-mediated immunity-delayed-type hypersensitivity) and are important in response to intracellular pathogens. In contrast, Th2 cytokines stimulate the production of antibodies. C-type lectin DC-SIGN shows binding to LAM and triggers the production of IL-10 and TGF-β and inhibits the production of IL-12 and TNF-α [37].

Scollard proposes that *M. leprae* interacts with the vascular endothelial cells and perineurium before successfully infecting the SC. This leads to the possibility that *M. leprae* infects the peripheral nerve tissue through the bloodstream [2]. This mechanism is thought to play an important role in SC immunopathogenesis and peripheral nerve damage to leprosy.

2.4.1.2. The role of humoral immunity

High levels of antibodies (IgM and IgG) are usually found in LL-type leprosy patients. Th2 (IL-4, IL-5, and IL-10) cytokines found in LL patients decrease TLR2 expression and stimulate activation of B cells. Activated B cells can make IgM, IgA, and IgG antibodies ineffective in killing intracellular bacteria. Therefore, *M. leprae* is able to survive and spread, causing various nerve damages [38]. After chemotherapy, there is usually a decrease in antibody levels. There is a strong correlation between bacterial load and humoral immune response. Analysis of antibody response has also been proposed as a tool for leprosy classification [39].

The antibodies produced against *M. leprae* play a role in the uptake of *M. leprae* by phagocyte cells and initiate the pathogenesis of the disease. For example, the discovered antibodies are binding to complement, which then binds PGL-I and binds C3 complement to *M. leprae*. The binding of this complement will mediate the uptake of bacilli through complement receptors in phagocytes [40]. In addition, secreted antibodies can form immune complexes with *M. leprae* antigen or with cross-reactive host molecules. This complex can then be recognized by antigen-presenting cells through specific receptors and delivered to T cells. In LL patients an excess of immune complex antibodies is found. However, if the antibodies are taken by APC cells (macrophages), they fail to activate T cells. Specific mature B-cell markers (CD20, CD79, CD138) that produce antibodies are found to be higher in skin lesions of BL/LL patients than BT patients [41]. In addition, when compared to gene expression of leprosy skin lesions of LL and TT types, it showed upregulation of B-cell-specific gene. Immunohistology of LL and TT skin lesions showed that IgM and IgA are more common in LL-type leprosy lesions, correlated with Th2 immunity and increased IL-5. LL is associated with an increase in systemic humoral response [42].

Another proposed mechanism which can cause neuropathy in leprosy and is also associated with stimulation of the immune response during infection is autoimmunity. The concept and general criteria of autoimmunity in leprosy have been established since 1969. It was found that in leprosy, an increase in immune complex (1gG-IgM, IgG-IgA, and complement components) is similar to other autoimmune diseases such as systemic lupus erythematosus (SLE),

Guillain-Barre syndrome (GBS), and rheumatoid arthritis. The formation of antigen-antibody complexes and complement stimulation as well as the recruitment of PMN cells can cause tissue damage and injury to the vessel wall as seen in autoimmune and leprosy diseases [42].

T cells in leprosy lesions may be produced either against specific antigens of *M. leprae* or autoantigens such as HSP-66. This may cause other mechanisms of nerve damage caused by autoimmune damage such as from tissue (neuropathy). In LL-type leprosy patients, there was a decrease in the specific antigen level of T cells against *M. leprae* antigen 65 kD, but at the same time, there was an increase in anti-antibody levels of 65 kD IgG. The similarity between bacterial proteins and host components or the presence of molecular mimicry is an important aspect for host-pathogen interactions. By using this mechanism, pathogen can avoid detection by the immune system or may cause autoimmunity. The monoclonal antibodies that arise against *M. leprae* antigens such as 65 kD antigen react with host antigens such as peripheral axons found in the skin.

In addition, the protein sequence of myelin PNS P0 is compared to the *M. leprae* protein sequence (leproma) and also other genomic databases for protein sequences and structural equations in other pathogens involved in neurodegeneration. This resulted in 11 hits with the right pair ranging from six to seven P0 myelin residues in the *M. leprae* genome, but not in other genomes of mycobacteria. Among these, two suitable on *M. leprae* are special proteins including ferredoxin NADP reductase (62%) and conserved membrane protein (36%) (ML2453, ML1504). Comparisons to other pathogen databases show that P0 has similar sequences with polio virus receptors (23.4%) and herpes virus (4%). In addition, searching for the myelin P0 sequence on the whole genomic database revealed that it has similar sequence to the immunoglobulin superfamily. This family plays an important role in the interactions of proteins-proteins and protein-ligands. The similarity between bacteria and host is that they can cause autoimmunity and neurodegeneration (demyelination) as can be seen in leprosy transmission. For example, anti-neural antibodies from serum of leprosy patients were found to bind the myelin protein P0.

Some autoantibodies found to be important in leprosy patients from western India with 50% detected in LL, 44.4% in BL, and 54.8% in BT. These autoantibodies are antinuclear antibodies (ANA), anti-double-stranded DNA (dsDNA) and anti-single-stranded anti-DNA (ssDNA), and antinuclear antigens (anti-ribonucleoprotein (nRNP), anti-Smith and anti-histone antigen (AHA)).

Since leprosy is one of the differential diagnoses of rheumatic diseases, some autoantibodies can be examined in leprosy serum using ELISA techniques and correlated with joint involvement. For example, in Brazil, most leprosy patients have no active reaction. Therefore, the frequency of IgM-rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP) antibody, antinuclear antibody (ANA), and antineutrophil cytoplasmic antibody (ANCA) is low in leprosy patients. However, the prevalence of anticardiolipin (aCL) antibodies and anti-β2 glycoprotein I (β2GPI) antibodies is significantly higher in leprosy patients than in the control group.

Glycolipids and glycosphingolipids expressed as determinants of myelin surfaces in SST are important for the function and stability of myelin itself. The resistance to these molecules (glycolipids) by autoantibodies can cause demyelination and nerve damage as found in leprosy patients. Therefore, many scientists study the autoantibody and its relationship to nerve

damage found in leprosy. Glycolipid or glycosphingolipid neural antibodies such as ceramide have been studied in leprosy patients in India. The anti-ceramide antibody IgM titers were found to be significantly higher in MB-type leprosy patients than both control and PB leprosy patients (96% of MB-type and 60% of PB-type patients). Groups in Brazil found elevated levels of anticardiolipin (aCL) and anti-β2-glycoprotein I (anti-β2-GPI) in leprosy patients, especially in LL-type patients with IgM-isotype dominant. The percentage of antiphospholipids in leprosy can be due to exposure to phospholipid antigen by tissue damage during the infection process. Another explanation is that the homology between PL bacteria and host results in the production of antibodies against cross-reactive heterological sequences.

Other anti-glycolipid antibodies are anti-sulfatide (cerebroside) which has also been reported in various cases of demyelinated peripheral polyneuropathy. Sulfatide, a glycolipid with single sulfate saccharide, is associated with the myelin membrane of nerve cells. Antibodies inhibit sulfatide synthesis expressed on myelin as a surface determinant, resulting in demyelination. IgM-subtype antibodies against sulfatide were increase in MB- or LL-type leprosy compared to PB and control. IgM anti-sulfatide is positively correlated with the patient's bacterial index. The similarity between mycobacterial sulfolipid with trehalose sulfate and host tissue (sulfatide) can stimulate autoantibodies against host sulfatide with galactose sulfate.

Variations in the number of autoantibodies found in leprosy patients can be attributed to the genetic background of the study population, the presence of infectious diseases, and the techniques used to detect autoantibodies. The hypothesis proposed for the development of such antibodies during infection is due to adaptive immune responses and activation of polyclonal B cells. Bacterial or viral antigens, with homologous sequences to tissue hosts, will be presented to T lymphocytes that stimulate B lymphocytes to produce antibodies against heterologous sequences.

In addition, autoantibodies to the antigenic epitope of myelin protein are reported in various chronic demyelinating diseases. For example, in chronic inflammatory polyradiculoneuropathy (CIPD), in which both humoral and cellular immunities are involved, there are antibodies to the myelin protein. The major antigenic components of myelin are myelin P0, P2 myelin, and peripheral myelin protein 22 (PMP22). These proteins are also associated with induced experimental autoimmune neuritis (EAN). Such autoantibodies may be produced during tissue damage and then continue to exacerbate further tissue damage during the disease process.

2.4.2. Demyelination without immunological process

Beside inflammatory process, axon demyelination can be caused by the presence of *M. leprae*. Rambukkana et al. have proved this both in vitro (in schwannn cell coculture) and in vivo (in $Rag1^{-/-}$ mice) had low levels of T cells and B lymphocytes. In this model *M. leprae* is able to induce demyelination within 24 hours postinfection without apoptosis or toxic effects on cells. The *M. leprae* component such as PGL-1 cell wall is also associated with the demyelination of SC in the model. Therefore, Rambukkana concluded that the survival of *M. leprae* was unnecessary in nerve demyelination induction in vitro and in vivo [5]. In addition, in his study *M. leprae* was found to be capable of inducing extracellular signals regulated by the Erk1/Erk2

kinase signal via the MAPK-MEK-dependent pathway. This activation caused by contact dependent between M. leprae and primary SC without apoptosis or cell death in SC. In this scenario, *M. leprae* is found to bind and induce the phosphorylation of ErbB2 receptors in SC other than laminin receptors located closely to ErbB2. As a result of this signal activation, *M. leprae* succeeds in inducing SC proliferation and demyelination [5]. Rambukkana stated that the mechanism of nerve damage in the absence of an immunological process played a role in the early stages of the disease. However, it is an immune system-mediated element that eventually causes nerve damage. When the *M. leprae* antigen is presented by myelinated and unmyelinated SC, both types of cells are subjected to attack by macrophages, T cells, and cytokines that are released as a result of the cell's inflammatory response. This inflammatory process will produce two SC phenotypes and sensoric-motoric damage subsequently [5].

Another mechanism that is suspected to cause nerve damage can be grouped in the form of nonimmune mediated, and it is the biochemical and metabolic changes in the nerve compartment. The examples of these mechanisms are axonal atrophy due to hypophosphorylation of myelin proteins and axonal neurofilament. Many proteins in PNS are phosphorylated such as myelin P0, MAP, and neurofilament proteins. An experiment which studied the phosphorylation of PNS proteins in leprosy nerves compared to normal nerves found that decrease in protein phosphorylation protein levels of 25 kD in leprosy patients' nerves. The phosphorylated (25 kD) protein is thought to be the myelin glycoprotein P0 [43]. Later studies by the same group also found that *M. leprae* could bind these myelin P0 (25 kD) glycoproteins and inhibit phosphorylation in vitro. The outer binding of the myelin can help *M. leprae* to reach the SCs target for invasion [43].

The neurofilament protein belongs to the filament intermediate (IF) found together with the microtubules and microfilaments in the cytoskeleton structure. Other proteins that make up IF in vimentin, peripherin, internexin, and nestin neuron. The NFS protein (neurofilament) in the axon consists of triplet proteins, namely, the molecular weight of NF-H (high), NF-M (medium), and NF-L (low) neurofilament proteins. The neurofilament protein contains an amino-terminal head domain, which is central-helical domain, and terminal-carboxyl-tailed domain in various lengths. Increasing the total number of NF proteins in axon results in an increase of axonal diameter. In addition, the phosphorylation of NF proteins is important to determine the axonal caliber. NF-M and NF-H proteins are highly phosphorylated in the C-terminal tail domain on the replication of KSP (lysine-serine-proline) in myelinated axons (Chung-Ho Liang, 1996). Several studies have shown that NF-H migrates more rapidly in SDS-PAGE after extensive dephosphorylation by alkaline phosphatase. Several studies have demonstrated the important role of NFS in the growth of myelinated radial axon using NFS protein knockout gene.

In leprosy patients, a decrease in axonal diameter was found to be associated with the loss of sensoric and motoric function. Therefore, the relationship between neuropathy and phosphorylation of NF protein was then investigated. The technique used was Western blot and immunohistochemistry to examine the phosphorylated NF epitope (SMI 31) on leprosy patients' nerves. In addition, Shetty et al. found the decrease or loss of SMI 31 in staining of infected nerve fibers. The NF protein band migrates faster (lower) than expected and decreases the

levels of NF protein content in the infected nerve. These results indicate the presence of hypophosphorylated NF subunits during leprosy infection, which are thought to lead to increasing susceptibility to proteolytic degradation of NFS. This result is consistent with the previous research, where phosphorylation was found to protect NFS against nonspecific proteolysis by calpain. Several studies by Shetty et al. found that lipoarabinomannan (LAM) *M. leprae* could inhibit the protein kinase C (PKC) enzyme which is responsible for the phosphorylation of the neurofilament protein. Recently, Save et al. also found that hypophosphorylation of NFS proteins by measuring the activity of enzyme kinases is responsible for NFS phosphorylation in the nerves of infected mice. The authors point out that as long as NFS loses its reactivity to specific NF-phosphate (SMI 31) antibodies, there is a decrease in KSPXK kinase activity from cyclin-dependent kinase (CDK) and MAPK in *M. leprae*-infected nerves. Decreases in NFS phosphorylation and NFS degradation may subsequently result in decreasing in interfilamentary distances that affect axonal growth and result in axonal atrophy.

In addition, *M. leprae* was found to induce upregulation of metalloproteinase matrix (MMP-2 and MMP-9) in SC which causes demyelination and damage to the blood-nerve barrier (Teles, 2010). The MMP protein family consists of proteolytic enzymes that participate in remodeling the extracellular matrix and the regulation of leucocyte migration. The function of MMP-2 is to degrade the type I collagen (gelatin), and the function of MMP-9 is to degrade the type IV collagen, which is a major component of the basal membrane. Increased MMP secretion is associated with tissue damage and can be used as a biomarker in many inflammatory disorders. During mycobacterial infection there was an increase in MMP-9 secretion that correlated with TNF-α production. It was found that during tuberculoid leprosy and type I (RR) lesion reactions, MMPs increased in the central region of granuloma, where dominant macrophages and epithelioid cells were obtained.

Nerve damage in leprosy infection is divided into two stages: (1) early stage which has no inflammation cells. This phase is initiated by contact between *M. leprae* with SC in SST and causes nerve damage. This phase often occurs on the entire spectrum of leprosy. It is characterized by sub-perineural edema, axonal atrophy, and demyelination with loss of myelinated nerve fibers. (2) The second phase is the phase which is mediated by inflammation with lymphatic cells in the form of tuberculoid and macrophage cells in lepromatous leprosy lesions. In this stage, the presence of autoantibodies against nerve components is reported in leprosy as another mechanism of nerve damage. The presence of common antigenic determinant between *M. leprae*, the skin, and nerves such as heat-shock proteins leads to the production of autoantibodies [26].

The presence of *M. leprae* in the nerves can also cause leprosy neuritis which has no skin manifestation, but nerve damage can be detected. Nerve damage can be caused by a full inflammatory inflammation of macrophages that produce foam cells in granulomas. This inflammatory process causes the stimulation of cytotoxic T-cell activity, axonal degeneration after SC death, and demyelination. Using different SC and axonal markers on the immunohistochemical slide of leprosy neuritic nerve, there was a decrease in immunoreactivity of NF200 as a result of the loss of myelinated fibers. In addition, the S-100 protein staining of the myelinated fibers was reduced due to loss fiber after the onset of demyelination. NGFr staining of the neuritic

nerve is also reduced in SC and/or small fiber axons. A decrease in myelinated fibers results in a decrease in MBP [44].

2.5. Schwann cells and their interactions with NGF, NRG, P0, and PMP22

2.5.1. Nerve growth factor (NGF)

Nerve growth factor (NGF) is firstly discovered in neurotrophin family. NGF is essential in the development and maintenance of phenotypic peripheral nerve cells and for the functional integrity of the cholinergic nerves in the central nervous system. The mature form of NGF (from ProNGF precursors) has an important role in development and in adult life and also has proapoptotic and neurotropic properties.

A study by Chan et al. about NGF in controlling axon receptivity on myelination by Schwann cells revealed that NGF is an axonal signal regulator that controls the myelination of nerve cells in the dorsal ganglia that expresses TrkA. NGF triggers the myelination by Schwann cells, but it inhibits myelination by oligodendrocyte cells. This reinforces that NGF plays a role in the Schwann cell myelination in the PNS.

2.5.2. Neuregulin 1 (NRG1)

In early life, Schwann cells are made of cells in neural plates and undergo massive migration, proliferation, and maturation before finally undergoing differentiation. During this period, Schwann cells are constantly in contact with axons and axonal signals, particularly neuregulin-1 (NRG1). In fact, NRG is a signaling protein that mediates the interactions of cells in the nervous system, heart, breast, and other organ systems. Neuregulin is also ligand for the ErbB family of tyrosine kinase receptors. Neuregulin itself has four families, namely, NRG1, NRG2, NRG3, and NRG4. However, until now, the biological functions of NRG2, NRG3, and NRG4 are still not widely known [45]. NRG1 is a growth factor that is very influential on the development of cells in the neural plate during the early stages of embryonic development. NRG1 is also involved in migration, axon growth, and synapse formation [46, 47]. In addition, NRG1 is a strength for Schwann cells to differentiate. This is proven in cell cultures where many aspects of Schwann cells are administered by NRG1, which are:

1. NRG1 serves to suppress the neuronal differentiation of the neural stem cell plate but to stimulate the differentiation of glial cells.
2. NRG1 is required for Schwann cell progenitor survival.
3. NRG1 also serves to stimulate proliferation and migration of Schwann cell precursors.
4. NRG1 gives an important signal to myelinate.

In vivo, the early stages of Schwann cell development depend on NRG1/ErbB signaling, as in mouse-fed animals without Schwann cell progenitors in peripheral neuronal development such as ErbB2, ErbB3, or NRG1.

2.5.3. Protein 0 (P0)

P0 or MPZ is one of the major protein components of myelin nervous system. P0 protein is transmembrane glycoprotein belonging to the immunoglobulin superfamily. It is the largest part of protein in SST myelin and is thought to be responsible for the adhesion of the outer surface of the cell and the myelin plasma membrane [48].

2.5.4. Peripheral myelin protein 22 (PMP22)

Peripheral myelin protein 22 (PMP22) is a major component of myelin (Snipes et al., 1992). This is evidenced by the discovery of PMP22 mRNA in Schwann cells and the PMP22 protein in the solid part of the myelin sheath. Assessment of the presence of PMP22 as a peripheral nerve myelin protein is also supported by the finding that the regulation of PMP22 expression during neural development and after neuronal trauma is identical [16].

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Chapter 7

Immunogenetics of *MHC* and *KIR* in the Leprosy

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Additional information is available at the end of the chapter

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Abstract

Several genetic polymorphisms in immune response genes have been associated to leprosy. This fact converges on the main hypothesis that genetic factors are involved in the disease susceptibility in two distinct steps: leprosy *per se* and their clinical forms. These genes play an important role in the recognition process, in the activation of the main metabolic pathway of the immune response and in the evolution of the disease. The scope of this project was to highlight the role of the immune response genes in the context of leprosy, emphasizing the participation of some of them in the signaling and targeting processes in response to bacillus infection and on disease evolution, such as HLA, *KIR* and *MIC* genes. Some environmental and genetic factors are important when the exposure to the bacillus occurs, leading to cure or not. Factors that favor a cellular or humoral immune response may influence the clinical manifestations after the infection inducting to one of extreme poles. Furthermore, some genetic factors were associated to the type of reaction that some individuals present during the disease development. Thus, it is very important to highlight the participation of some genetic factors in the immunopathogenesis of leprosy.

Keywords: leprosy, *HLA* genes, *MICA* genes, *KIR* genes, genetic predisposition, genetic polymorphism

1. Introduction

Leprosy is a chronic infectious granulomatous disease caused by the obligate intracellular bacillus *Mycobacterium leprae* (*M. Leprae*). Dermatoneurological signs and symptoms, such as skin and peripheral nerve lesions, are common manifestations of the disease and occasionally, it may affect respiratory tract, eyes, lymph nodes, nasal structures, testicles and internal organs [1, 2].

Leprosy is an important endemic disease, considered as a serious public health and social problem worldwide, as it leads to neural impairment or physical disability. Thus, special attention is needed, due to the consequences in the socioeconomic life of the patients or even their possible sequels in those who are cured. Worldwide, leprosy cases spread across more than 140 countries, with 22 countries accounting for 95% of global leprosy. These countries such as India, Brazil, Indonesia, Democratic Republic of Congo, Ethiopia, Nepal, Bangladesh and others have a high detection rate [3].

Bacillus has a high infectivity and low pathogenicity, that is, it infects many people, but only few become ill [1]. Leprosy is influenced by host genetic and mycobacterial factors, and environmental factors such as nutritional status and rate of exposure to bacillus. The immune response is of fundamental importance for the body's defense against exposure to the bacillus, but in some individuals, leprosy can lead to changes in the immune response and to the development of distinct clinical forms. Among those who fall ill, the degree of immunity varies by determining the clinical form and course of the disease [4].

The immune response to the *M. leprae* is a task of the T lymphocytes responsible for adaptive immunity. CD4+ T lymphocytes can be divided into two subpopulations, which exert different functions in the defense of the organism mainly against intracellular bacterial infections, such as leprosy. These lymphocytes have the ability to induce the cellular or humoral immune response that is related to the types of secreted cytokines and the development of Th1 or Th2 responses [5, 6].

The predominance of cellular or humoral immune response may influence the evolution of the leprosy and the clinical characteristics observed in the tuberculoid (TT) and lepromatous (LL) clinical forms. The patients with the TT form have a strong cellular immune response, with a predominance of Th1 cells, activation of macrophages and Th1 cytokines secretion, such as interleukin (IL)-2, IL-6, IL-12, IL-15, IL-18, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), limiting the disease to few localized lesions of the skin and peripheral nerves. Patients with the LL form present a humoral response and lack of cellular response, with a predominance of CD8+ suppressor T cells and Th2 standard cytokines, such as IL-4, IL-5, IL-10 and IL-13, which inhibit the activation of macrophages. Here there is a proliferation of bacillus and presence of many lesions in the skin and peripheral nerves [5–7].

The disease can be classified into three forms: (i) Madrid (1953) classification, based on clinical and bacteriological criteria [8]; (ii) Classification of Ridley and Jopling (1966) that emphasizes clinical, bacteriological, immunological and histological aspects [9] and (iii) World Health Organization (WHO) (1982) operational classification with therapeutic purpose, based on the bacilloscopic index, which is related to the clinical forms [10]. In 1988, this operational classification was updated and clinical criteria were also established, considering paucibacillary (PB) patients such as those with less than five cutaneous lesions and/or one affected nerve trunk and multibacillary (MB) such as those with more than six lesions and/or more than one affected nerve trunk. It is still considered MB when the bacilloscopy is positive, regardless the number of lesions [11]. The classifications adopted for clinical forms of leprosy such as Madrid, Ridley and Jopling and WHO are summarized and listed in **Table 1**.

WHO	Paucibacillary (PB)		Multibacillary (MB)	
MADRID	Indetermined (I)	Tuberculoid (T)	Dimorph (D)	Virchowian (V)
Ridley and Jopling		TT	BT BB BL	LL

TT: tuberculoid-tuberculoid, BT: borderline tuberculoid, although presenting characteristics of the paucibacillar form, it has been operationally classified as multibacillary, BB: borderline borderline, BL: borderline lepromatous, LL: lepromatous-lepromatous.

Table 1. Correlation between the classifications of Madrid [8], Ridley and Jopling [9] and WHO [10, 11] adopted for leprosy.

At present, it is known that there are several factors influencing the control and appearance of the disease, such as immune response, time of exposure to bacillus, virulence of the pathogen, environmental factors, genetic variation of the bacillus and, mainly, the immunogenetic variability of the host leading to susceptibility or resistance to leprosy *per se* [12–17], clinical forms [18–20] of the disease and leprosy reactions [21, 22] (**Figure 1**).

The selection of candidate genes in disease pathogenesis is usually based on two criteria: functional genes with a critical role in the pathogenesis of the disease and the location in the genomic region that may be involved in disease control; and yet a combination of the both.

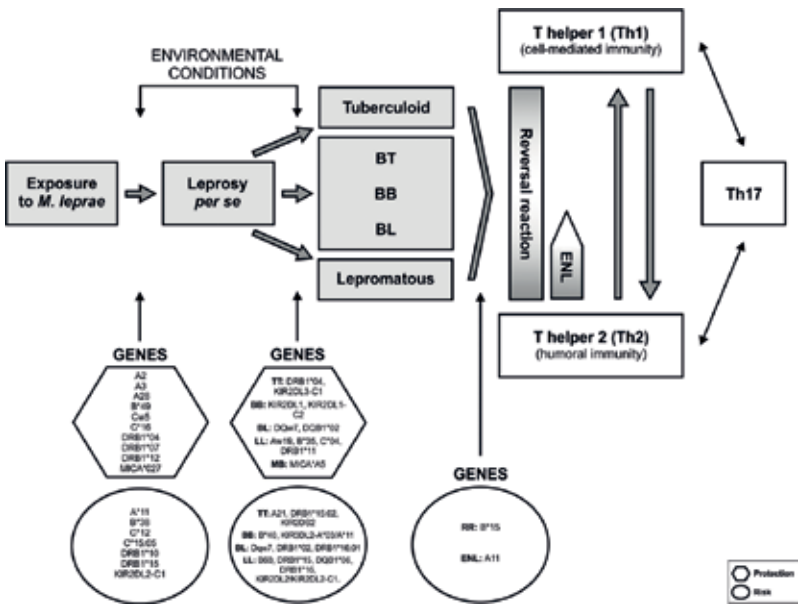

Figure 1. Schematic representation of the clinical spectrum of leprosy suggesting the participation of different genes (*HLA*, *MICA* and *KIR*) in the control of the pathogenesis of the disease. Susceptibility or resistance to leprosy *per se*, clinical forms and leprosy reactions were showed. After exposure, most individuals are resistant to leprosy. Susceptible individuals may present the infection *per se* or develop one of the clinical forms and reactional types of leprosy, which are dependent on the host's immune response pattern. MB: multibacillary, BB: borderline borderline, BL: borderline lepromatous, BT: borderline tuberculoid, LL: lepromatous leprosy, TT: tuberculoid leprosy; *per se*: leprosy independent of specific clinical manifestation. RR: type 1 or reversal reaction. ENL: type 2 reaction or erythema nodosum leprosum.

These genes are generally those that participate in the immune response in leprosy, such as cytokine genes, *HLA* (human leukocyte antigen) genes, *MICA* (major histocompatibility complex class I chain-related protein A) and *KIR* (killer cell immunoglobulin-like genes receptors), among others.

The two types of studies with molecular genetic markers are those of binding and association. The binding studies are related to the genetic mapping that allows the tracking of chromosomal regions linked to the disease. Gene-susceptibility/disease resistance studies are based on the comparison of the allelic frequencies of a genetic marker in populations (affected and unaffected individuals) [23].

Recently, a new approach to identify genes involved in human diseases is being carried out; it is the so-called genome-wide association study (GWAS). This is an association study of the entire genome in which many single nucleotide polymorphisms (SNPs) are tested in healthy controls and patients, allowing the analysis of hundreds or thousands of these polymorphisms at the same time. Genetic markers are considered to be associated with disease phenotypes when there is a significant difference in the frequencies observed between these two groups [24]. These works with genetic markers are performed aiming to contribute to the early diagnosis, prognosis, understanding of pathophysiology and improvement in the treatment of the disease.

Thus, the proposal of this chapter is to evidence the participation of some innate immune response genes, specifically, *HLA*, *MIC* and *KIR* genes, on overall leprosy and on evolution to the various clinic forms of disease.

2. Major histocompatibility complex

2.1. Introduction

The major histocompatibility complex (MHC) is composed of several genes, some of which are capable of encoding molecules that will display antigenic peptides on the cell surface for recognition by T cells. Other genes encode heat shock proteins, some cytokines and complement factors and approximately 40% of them have some function in the immune system [25, 26].

In relation to antigen presentation on the cell surface, the antigenic peptides originate from several sources, such as intracellular bacteria and viruses, products of cellular metabolism or proteins and lipids own or foreign to the organism [26].

In humans, a MHC sub region, called human leukocyte antigen (HLA), is located on the short arm of chromosome 6 and gives rise to HLA class I and II molecules. The HLA is polymorphic and each locus has many alleles contributing to human diversity as well as meeting the need for presentation of a wide range of antigens. The set of *HLA* alleles present on each chromosome is called haplotype, so all heterozygous individuals have two codominant *HLA* haplotypes [25, 27].

Understanding the mechanism of the presentation of antigens is of great importance for immunology, since it is able to explain events such as transplant rejection, autoimmune diseases, tumor immunity and response to infection, such as leprosy [28].

2.2. Structural characteristics

Each HLA molecule consists of a peptide-binding cleft, immunoglobulin (Ig)-like domains and transmembrane and cytoplasmic domains. Class I HLA has the α-chain encoded by MHC genes and the β2-microglobulin chain encoded by a non-MHC region. Class II HLA has both the α- and β-chain encoded in the MHC (**Figure 2**). The cleavage site is the site where the peptides are established during their presentation to the T lymphocytes. In addition, cleft are the polymorphic residues, that the amino acids responsible for differentiating the HLA from each other, as well as making the presentations more antigenic specific. The Ig domains are non-polymorphic and are responsible for binding between HLA and T cell: class I HLA molecules bind to CD8+ T cells and HLA class II molecules bind to the helper T cells CD4+ T cells [29, 30].

2.3. Nomenclature

The convention for the use of a four-digit code to name *HLA* alleles and distinguish them from the nomenclature given to coded proteins was introduced by the Nomenclature Report 1987. Currently, an allele name can be composed of four, six or eight digits, depending on its sequence. The first two digits describe the allele family. The third and fourth digits refer to the way in which DNA sequences were discovered.

Alleles that are different in the initial four digits have differences in nucleotide substitutions, which alter in protein coding. The fifth and sixth digits are used to distinguish alleles that differ by the synonymous substitutions of nucleotides in the coded sequence. The seventh and eighth digits are used when the alleles differ by sequence polymorphisms in introns or in 5′ and 3′ untranslated regions.

Each HLA allele name has a unique number, corresponding to up to four sets of digits, separated by a colon. The first two sets of digits are assigned to all alleles and the other two only for longer names and when needed (**Figure 3**) [31].

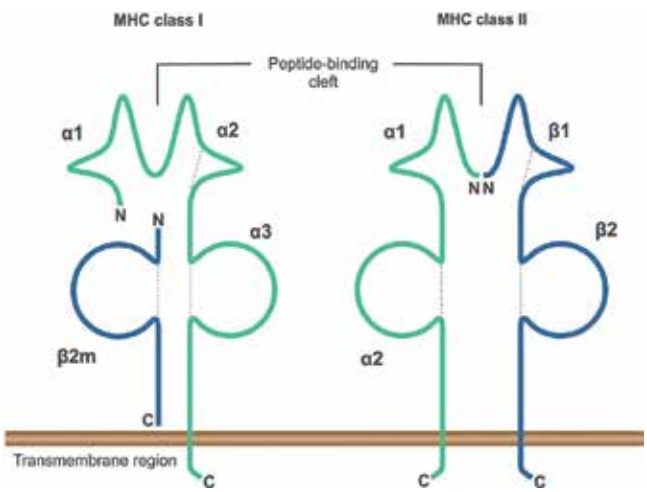

Figure 2. Structure of the class I and II MHC molecules.

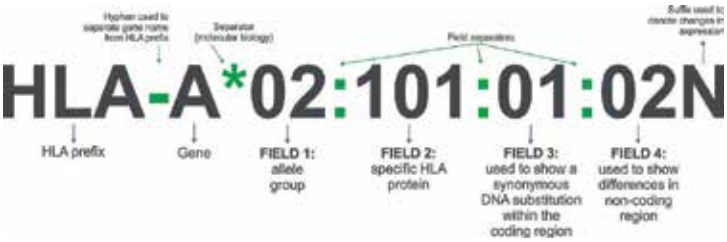

Figure 3. Schematic example of the meanings for each code in the HLA nomenclature [31].

2.4. HLA classical

2.4.1. HLA class I

There are three classical *loci* belonging to MHC class I: *HLA-A*, *HLA-B* and *HLA-C*. They encode molecules that have the same name as their respective genes. HLA class I molecules are expressed in all nucleated cells and platelets, as these molecules present the antigenic peptides for CD8+ T lymphocytes, which kill infected cells or with tumor antigens. The *HLA-E*, *HLA-F* and *HLA-G loci* also belong to *HLA* class I, but are considered non-classical (**Figure 3**) [32]. They are expressed at low levels when compared to classical *HLA* class I as well as do not have as many polymorphisms, and their functions in the immune system are limited [29, 30].

2.4.2. HLA class II

HLA class II molecules are expressed in dendritic cells, B lymphocytes, macrophages and other cell types, and present the antigenic peptides to the virulent CD4+ helper T lymphocytes, which recognize the antigens in the secondary lymphoid organs. Differentiated CD4+ helper T cells activate other cells, together with B lymphocytes, so that the extracellular microorganisms are eliminated. The three *HLA* class II *loci* are called *HLA-DP*, *HLA-DQ* and *HLA-DR*. The two chains of each molecule of class II are encoded by two different MHC genes. Thus, the extracellular parts of α and β chains are subdivided into two segments, A1 and A2, or B1 and B2, both of which are polymorphic chains, that is, each of the DP, DQ and DR *loci* contain separate genes designated as A or B, which encode α and β chains, respectively, in each copy of chromosome 6. Each individual has one HLA-DRA (DRA1), one to three DRB (DRB1 and DRB3, 4 and/or 5), one DQA (DQA1), one DQB (DQB1), one DPA (DPA1) and one DPB (DPB1) [25, 29, 30].

2.5. *MICA* and *MICB* genes

The human MHC class I chain-related genes (*MICA* and *MICB*) are located in the *HLA* class I region in chromosome 6, but are not part of the classical *HLA* (**Figure 4**). These genes show about 30% of homology to HLA class I, but the transcribed molecules do not present antigenic peptides on the cell surface. These genes are mainly transcribed into fibroblasts and epithelial cells. The MIC molecules bind to NKG2 receptors, activating NK cells and also modulate the function of CD8+ T cells. Studies have related associations of polymorphisms in *MICA* and *MICB* genes with several diseases (ankylosing spondylitis, psoriasis, dengue and tuberculosis) [32–36], one of them being leprosy, which will be discussed in a next topic in this chapter.

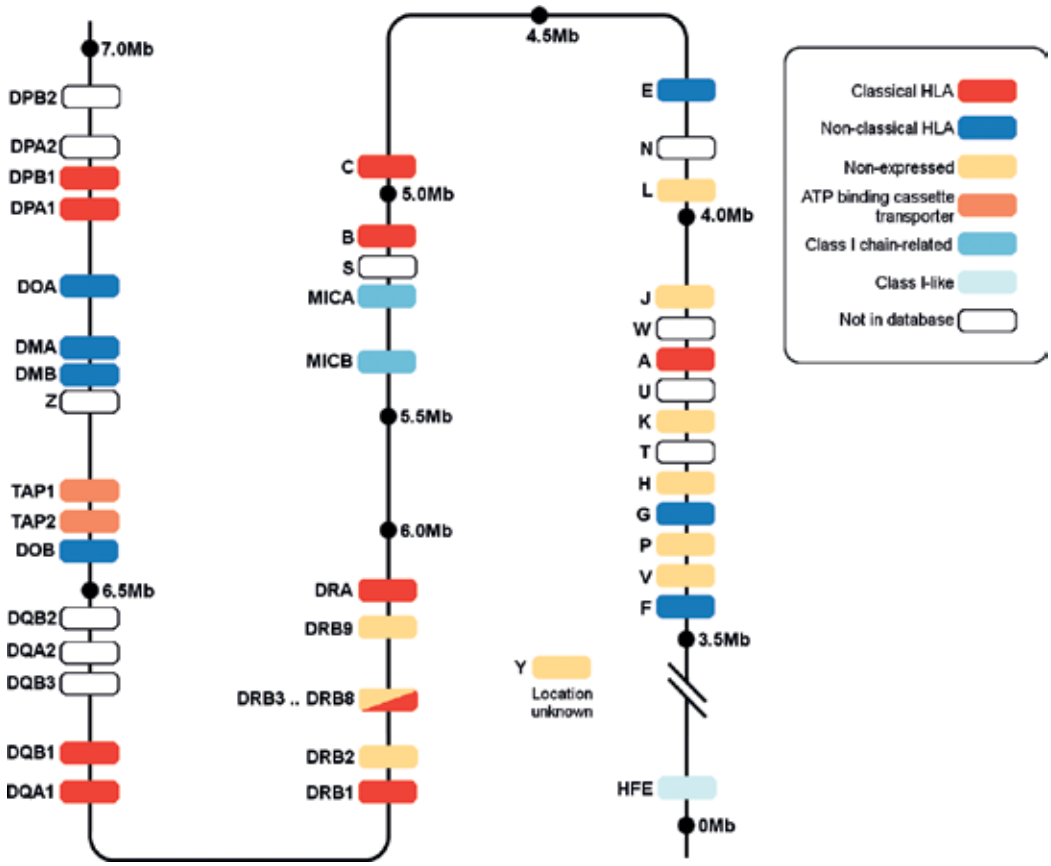

Figure 4. Schematic map of the human *MHC* gene [32].

2.6. HLA polymorphism

The immune system has the complex task of responding to different types of pathogens that come in contact with the human organism. Adaptation that ensures antigen protection and increased immune system efficiency can occur through life-long genetic recombination, such as antibody formation, or the different HLA molecules in the population. HLA molecules are responsible for presenting a fraction of the antigenic peptide (epitope) for T cells; however, the choice to determine which epitope will be presented according to the *HLA* genes and their alleles in each individual. Thus, the regions responsible for the antigenic presentation in the HLA molecules present high polymorphism rates. This means that with the advancement of diagnostic methodologies, the discovery of allelic variations of HLA has increased exponentially (**Figure 5**) [27, 29].

The evolutionary success in the amplification of the HLA repertoire may explain why it is difficult to associate a specific HLA phenotype with the susceptibility or protection against a particular disease, since the change of a single amino acid in the sequence of the HLA molecule can affect the adaptive immune response of the individual [32]. Despite this difficulty, studies have shown associations among several HLA and autoimmune and infectious diseases [27, 29].

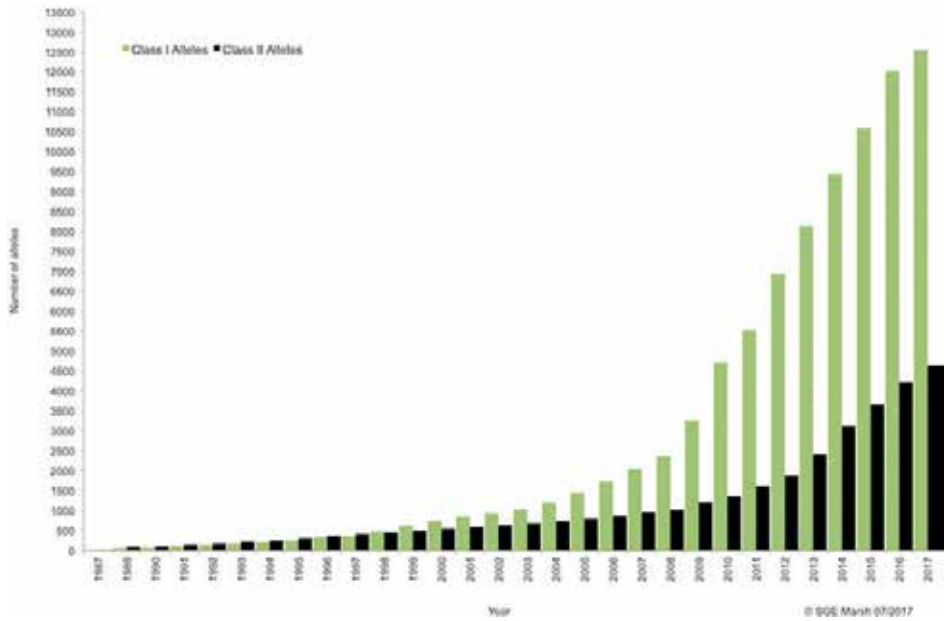

Figure 5. Advances in the findings of allelic variations in *HLA* class I and II *loci* over the past 30 years. Class I *HLA* alleles are represented in green and class II *HLA* alleles in black [32].

2.7. Influence of HLA on leprosy

The role of HLA molecules in leprosy is to present epitopes of the bacillus to T lymphocytes. However, polymorphisms in *HLA* genes or incorrect presentation of the antigenic peptide may interfere or contribute to the success of the response of the host against the pathogen. In view of this, several studies have indicated genes associated with susceptibility or protection against leprosy in different populations (**Tables 2** and **3**).

Allele, haplotype	Population	Population size	Phenotype	Association
*A*02:06*	Southern Indian	32 leprosy patients and 67 healthy individuals	Leprosy *per se*	Susceptibility [37]
*A*02:06*	Mumbai/Indian	103 leprosy patients and 101 healthy individuals	Leprosy *per se*	Susceptibility [14]
*A*11*	Brazilian	225 leprosy patients and 450 healthy individuals	Leprosy *per se*	Susceptibility [38]
*A*11:02*	Southern Indian	32 leprosy patients and 67 healthy individuals	Leprosy *per se*	Susceptibility [37]
*A*11:02*	Mumbai/Indian	103 leprosy patients and 101 healthy individuals	Leprosy *per se*	Susceptibility [14]
*B*15*	Brazilian	202 leprosy patients and 478 healthy individuals	RR	Susceptibility [22]
*B*18:01*	Mumbai/Indian	103 leprosy patients and 101 healthy individuals	Leprosy *per se*	Susceptibility [14]

Allele, haplotype	Population	Population size	Phenotype	Association
*B*18:01*	Southern Indian	32 leprosy patients and 67 healthy individuals	Leprosy *per se*	Susceptibility [37]
*B*35*	Brazilian	225 leprosy patients and 450 healthy individuals	LL	Protection [38]
*B*38*	Brazilian	225 leprosy patients and 450 healthy individuals	Leprosy *per se*	Susceptibility [38]
*B*51:10*	Southern Indian	32 leprosy patients and 67 healthy individuals	Leprosy *per se*	Susceptibility [37]
*B*51:10*	Mumbai/Indian	103 leprosy patients and 101 healthy individuals	MB	Susceptibility [14]
*C*04*	Brazilian	225 leprosy patients and 450 healthy individuals	LL	Protection [38]
*C*04:07*	Southern Indian	32 leprosy patients and 67 healthy individuals	Leprosy *per se*	Susceptibility [37]
*C*04:07*	Mumbai/Indian	103 leprosy patients and 101 healthy individuals	MB	Susceptibility [14]
*C*04:11*	Mumbai/Indian	103 leprosy patients and 101 healthy individuals	MB	Protection [14]
*C*04:11*	Southern Indian	32 leprosy patients and 67 healthy individuals	Leprosy *per se*	Protection [37]
*C*05*	Brazilian	202 leprosy patients and 478 healthy individuals	B	Protection [22]
*C*07*	Brazilian	225 leprosy patients and 450 healthy individuals	Leprosy *per se*	Susceptibility [38]
*C*07:03*	Southern Indian	32 leprosy patients and 67 healthy individuals	Leprosy *per se*	Susceptibility [37]
*C*07:03*	Mumbai/Indian	103 leprosy patients and 101 healthy individuals	MB	Susceptibility [14]
*C*12*	Brazilian	225 leprosy patients and 450 healthy individuals	Leprosy *per se*	Susceptibility [38]
*C*16*	Brazilian	225 leprosy patients and 450 healthy individuals	Leprosy *per se*	Protection [38]
*C*15:05*	Indian	364 leprosy patients and 371 healthy individuals	Leprosy *per se*	Susceptibility [15]
*C*15:05*	Vietnamese	198 families	Leprosy *per se*	Susceptibility [15]
*C*15:05*	Vietnamese	292 families	Leprosy *per se*	Susceptibility [15]
*A*11-B*40*	Mumbai/Indian	103 leprosy patients and 101 healthy individuals	ML	Susceptibility [14]

MB: multibacillary, PB: paucibacillary; B: borderline leprosy, BB: borderline borderline, BL: borderline lepromatous, BT: borderline tuberculoide, LL: lepromatous leprosy; TT: tuberculoid leprosy, *per se*: Leprosy independent of specific clinical manifestations, ENL: type 2 reactions or erythema nodosum leprosum, RR: Type I or reversal reaction.

Table 2. Associations between ***HLA*** class I and leprosy.

Allele, haplotype	Population	Population size	Phenotype	Association
*DQA1*01:02*	Indian	93 leprosy patients and 47 healthy individuals	LL	Susceptibility [39]
*DQA1*01:03*	Indian	93 leprosy patients and 47 healthy individuals	LL	Susceptibility [39]
*DQA1*02:01*	Indian	93 leprosy patients and 47 healthy individuals	LL	Susceptibility [39]
*DQA1*03*	Japanese	93 leprosy patients and 114 healthy individuals	Leprosy *per se*	Protection [40]
*DQB1*02*	Brazilian	202 leprosy patients and 478 healthy individuals	B	Protection [22]
*DQB1*02:01*	Brazilian	76 families (1166 individuals)	TT	Protection [41]
*DQB1*02:01*	Brazilian	76 families (1166 individuals)	Leprosy *per se*	Protection [41]
*DQB1*02:01*	Argentinean	89 leprosy patients and 112 healthy individuals	LL	Protection [42]
*DQB1*02:02*	Argentinean	89 leprosy patients and 112 healthy individuals	LL	Protection [42]
*DQB1*02:03*	Argentinean	89 leprosy patients and 112 healthy individuals	LL	Protection [42]
*DQB1*04:01*	Japanese	93 leprosy patients and 114 healthy individuals	Leprosy *per se*	Protection [40]
*DQB1*05:01*	Brazilian	76 families (1166 individuals)	TT	Susceptibility [41]
*DQB1*05:01*	Brazilian	76 families (1166 individuals)	Leprosy *per se*	Susceptibility [41]
*DQB1*05:03*	Indian	93 leprosy patients and 47 healthy individuals	TT	Protection [39]
*DQB1*06:01*	Indian	93 leprosy patients and 47 healthy individuals	TT	Susceptibility [39]
*DQB1*06:01*	Indian	93 leprosy patients and 47 healthy individuals	LL	Susceptibility [39]
*DRB1*02*	Japanese	79 leprosy patients and 50 healthy individuals	BL/LL	Susceptibility [43]
*DRB1*04*	Brazilian	578 leprosy patients and 691 healthy individuals	Leprosy *per se*	Protection [44]
*DRB1*04*	Euro-Brazilian	578 leprosy patients and 691 healthy individuals	Leprosy *per se*	Protection [44]
*DRB1*04*	Vietnam	194 single-case families	Leprosy *per se*	Protection [44]
*DRB1*04*	Argentinean	89 leprosy patients and 112 healthy individuals	TT	Protection [42]
*DRB1*04:05*	Japanese	93 leprosy patients and 114 healthy individuals	Leprosy *per se*	Protection [40]
*DRB1*04:05*	Taiwanese	65 leprosy patients and 190 healthy individuals	MB	Protection [45]
*DRB1*07*	Brazilian	76 families (1166 individuals)	Leprosy *per se*	Protection [41]
*DRB1*07*	Euro-Brazilian	578 leprosy patients and 691 healthy individuals	Leprosy *per se*	Protection [44]

Allele, haplotype	Population	Population size	Phenotype	Association
*DRB1*07*	Brazilian	578 leprosy patients and 691 healthy individuals	Leprosy *per se*	Protection [44]
*DRB1*07*	Brazilian	202 leprosy patients and 478 healthy individuals	B	Protection [22]
*DRB1*07:01*	Indian	93 leprosy patients and 47 healthy individuals	LL	Susceptibility [39]
*DRB1*08*	Brazilian	169 leprosy patients and 217 healthy individuals	LL	Susceptibility [46]
*DRB1*08:08*	Argentinean	71 leprosy patients and 81 healthy individuals	Leprosy *per se*	Protection [47]
*DRB1*09*	Southern Indian	230 leprosy-affected sib-pair	TT	Protection [48]
*DRB1*09*	Chinese	305 leprosy patients and 527 healthy individuals	Leprosy *per se*	Protection [49]
*DRB1*10*	Brazilian	578 leprosy patients and 691 healthy individuals	Leprosy *per se*	Susceptibility [44]
*DRB1*10*	Afro-Brazilian	578 leprosy patients and 691 healthy individuals	Leprosy *per se*	Susceptibility [44]
*DRB1*10*	Vietnam	194 single-case families	Leprosy *per se*	Susceptibility [44]
*DRB1*11*	Brazilian	70 leprosy patients and 77 healthy individuals	LL	Protection [50]
*DRB1*11:03*	Argentinean	71 leprosy patients and 81 healthy individuals	Leprosy *per se*	Protection [47]
*DRB1*12*	Brazilian	578 leprosy patients and 691 healthy individuals	Leprosy *per se*	Protection [44]
*DRB1*12*	Japanese	79 leprosy patients and 50 healthy individuals	Leprosy *per se*	Protection [43]
*DRB1*14*	Brazilian	85 leprosy patients and 85 healthy individuals	TT	Susceptibility [20]
*DRB1*14:01*	Argentinean	71 leprosy patients and 81 healthy individuals	Leprosy *per se*	Susceptibility [47]
*DRB1*14:06*	Argentinean	71 leprosy patients and 81 healthy individuals	Leprosy *per se*	Susceptibility [47]
*DRB1*15*	Afro-Brazilian	578 leprosy patients and 691 healthy individuals	Leprosy *per se*	Susceptibility [44]
*DRB1*15*	Brazilian	578 leprosy patients and 691 healthy individuals	Leprosy *per se*	Susceptibility [44]
*DRB1*15*	Chinese	305 leprosy patients and 527 healthy individuals	Leprosy *per se*	Susceptibility [49]
*DRB1*15*	Indian	93 leprosy patients and 47 healthy individuals	TT	Susceptibility [39]
*DRB1*15*	Indian	93 leprosy patients and 47 healthy individuals	LL	Susceptibility [39]
*DRB1*15*	Indian	54 leprosy patients and 44 healthy individuals	TT	Susceptibility [51]

Allele, haplotype	Population	Population size	Phenotype	Association
*DRB1*15:01*	North Indian	113 leprosy patients and 111 healthy individuals	BL/LL	Susceptibility [52]
*DRB1*15:01*	Indian	93 leprosy patients and 47 healthy individuals	LL	Susceptibility [39]
*DRB1*15:02*	Southern Indian	230 leprosy-affected sib-pair	TT	Susceptibility [48]
*DRB1*15:02*	Indian	93 leprosy patients and 47 healthy individuals	TT	Susceptibility [39]
*DRB1*15:02*	Indian	85 leprosy patients and 104 healthy individuals	TT	Susceptibility [53]
*DRB1*15:02*	Asian Indian	27 leprosy patients and 19 healthy individuals	TT	Susceptibility [54]
*DRB1*16*	Brazilian	85 leprosy patients and 85 healthy individuals	LL	Susceptibility [20]
*DRB1*16:01*	Brazilian	169 leprosy patients and 217 healthy individuals	BL	Susceptibility [46]
*DRB5*01:01*	Indian	93 leprosy patients and 47 healthy individuals	LL	Susceptibility [39]
*DRB1*15:01-DRB5*01:01-DQA1*01:02-DQB1*05:02*	Indian	85 leprosy patients and 104 healthy individuals	TT	Protection [53]

MB: multibacillary, PB: paucibacillary; B: borderline leprosy, BB: borderline borderline, BL: borderline lepromatous, BT: borderline tuberculoide, LL: lepromatous leprosy; TT: tuberculoid leprosy, *per se*: Leprosy independent of specific clinical manifestations, ENL: type 2 reactions or erythema nodosum leprosum, RR: type 1 or reversal reaction.

Table 3. Associations between ***HLA*** class II and leprosy.

3. *MIC* genes

The findings of new immune response genes are occurring in order to clarify their possible participation in the occurrence or severity of a disease. Among them, we can highlight *MIC* (MHC class I chain-related genes) that were discovered during a search for new coding sequences, located near the *HLA-B* gene [55].

MIC constitutes a second lineage of non-classical *MHC* class I genes and correspond to the *MICA, MICB, MICC, MICD, MICE, MICF* and *MICG loci* (**Figure 6**). *MICA* genes are located on the short arm of chromosome 6 (6p21.3), about 46.5 kb from HLA-B toward the centromere. Only *MICA* and *MICB* are expressed in proteins that belong to the immunoglobulin superfamily (IgSF) [56–58].

Like classical HLA genes, *MICA* also shows a high polymorphism in humans, whereas *MICB* appears to be less polymorphic, although it has been little explored. Since the discovery and characterization of NKG2D as its corresponding receptor in NK cells and in subsets of T cells, these genes have received increasing attention in the context of organs and stem cell

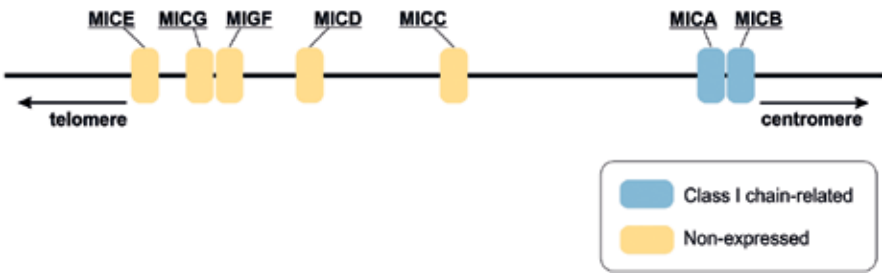

Figure 6. Genes and pseudogenes of the *MIC* family on region class I of the human MHC. Functional genes are represented in blue and the pseudogenes in yellow.

transplantation. *MICA* and *MICB* encode glycoproteins, which are stress induced and can be recognized by receptors such as NKG2D (C-type lectin-like activating immunoreceptor). They are capable of inducing immune responses involving Tγδ cells and NK cells, independently of the processing of conventional class I MHC antigens [57, 59, 60].

3.1. Structure of the MIC molecule

MICA molecules are codominantly expressed and are polypeptides of 383–389 amino acids with a size of 43 kDa in length [56, 57] and the MICB molecules are also polypeptides with a similarity of 83% amino acids with MICA. The structure of the MICA molecule is similar to HLA class I antigens, with three extracellular domains (α1, α2 and α3), a transmembrane domain and a cytoplasmic tail. MICA molecules have an extremely flexible rod connected to the platform formed by the α1/α2 domains and the α3 domain. Four α-helices are arranged under eight pleated β-strands forming a reduced slit that it would not be possible to attach a peptide composed of more than three or four amino acid residues (**Figure 7**) [61].

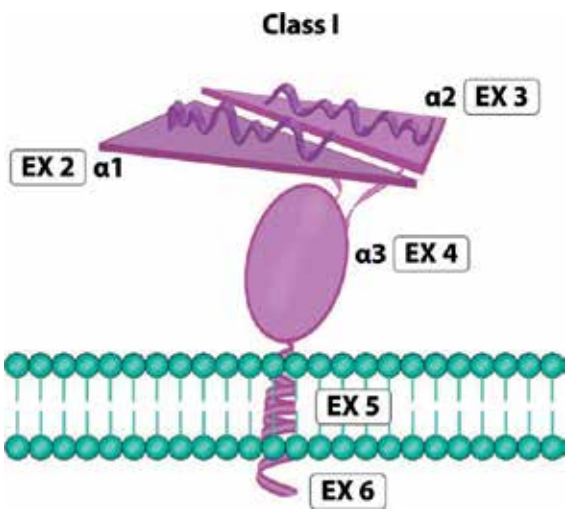

Figure 7. The structure of the *MICA*. Exon 2 encodes a leader peptide, exons 2–4 encode three extracellular domains, exon 5 a transmembrane domain and exon 6 a cytoplasmic tail [61].

In exon 5, there is a short tandem repeat sequence (STR) at position 304 consisting of GCT nucleotide breaks, which encode the amino acid alanine in the transmembrane region (TM). STR is absent in *MICB*. Based on the number of GCT, the alleles are named as A4, A5, A5.1, A6, A7, A8, A9 and A10. A5.1 differs from A5 by the insertion of a guanine nucleotide in the GCT (GGCT) [62], leading to a change in the reading matrix causing a terminus premature codon within the exon that encodes the transmembrane domain [33, 63, 64]. Thus, A5.1 is a 35–40 kDa truncated glycoprotein that eventually reaches the cell surface, but not at its physiological site. This is another characteristic of the *MICA* polymorphism: several alleles have identical extracellular domains but differ in the TM region. The identification of the polymorphism in the TM region is essential to avoid ambiguities [65].

The expression of the *MICA* gene was recognized in gastrointestinal and thymic epithelial cells in isolated endothelial cells, fibroblasts and keratinocytes. MICA molecules are ligands of the NKG2D receptors and Tγδ cell receptors (TCRγδ). The recognition of the MICA molecules by Tγδ Vδ1 cells through the interaction with the α1 and α2 domains was confirmed later in another study [66].

Tγδ cells constitute a small population of T cells expressing antigenic receptor proteins that resemble those of CD4+ and CD8+ T cells, but are not identical. Tγδ cells recognize many different types of antigens, including some proteins and lipids, as well as small phosphorylated molecules and alkyl amines. These antigens are not presented by MHC molecules [25]. It is not known whether there is a need for a particular cell type or distinct antigen presentation system for the presentation of antigens to these cells. MICA molecules are also recognized by their NKG2D receptors present on the surfaces of NK cells, associated with DAP10 molecule. This NKG2D-MICA complex activates phosphorylation of the tyrosine residues of the DAP10 molecule, triggering a cascade of cell signaling that enhances the cytotoxicity of NK cells. This complex also enhances the production of IFN-γ by NK cells, participating as a co-stimulator factor in the immune response against *Mycobacterium* [67].

Therefore, MICA is a stress-induced MHC class I molecule that binds to NKG2D receptors, primarily NK cells, stimulating NK cells, T CD8+ cells and some Tγδ cells [68]. Previous studies have suggested that HLA-B *loci* alleles were associated with some diseases caused by pathogens and, as there is strong linkage disequilibrium between the two genes due to the proximity of *MICA*, this could indirectly contribute to this response.

3.2. Association of *MICA* and *MICB* genes with leprosy

Some infectious and noninfectious diseases such Behçet's disease, ankylosing spondylitis, Reiter's syndrome, Kawasaki disease, psoriasis vulgaris and Chagas disease have been associated to *MICA* genes. These studies suggest that allelic variants of *MICA* may be directly related to NKG2D receptor binding of Tγδ and NK cells affecting the effects of cells activation [35, 69–74].

In the first study of association between the *MICA* gene and leprosy, the *MICA**A5 allele was found associated with protection against MB form in Chinese patients [19]. In India, the *MICA*5A5.1*, *MICB*CA16* and *MICB*CA19* alleles were associated with susceptibility to leprosy *per se* and *MICB*CA21* allele with protection [48]. Recently, in a study in Brazil, the *MICA*010* and *MICA*027* alleles were associated with protection against the MB form and *MICA*027* was associated with protection to leprosy *per se* [16].

4. Killer cell immunoglobulin-like receptors (KIRs)

4.1. Natural killer cells

Natural killer (NK) cells make up about 10–15% of the lymphocytes in human peripheral blood, with an important participation on the innate immune response. In addition, they are sources of type I cytokines, IFN-γ, as well as TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF) and other cytokines and chemokines [75]. In their original lineage, repertoire of receptors and effector functions, the NK cells appear to be a transitional cell type, which would be a bridge between the innate and adaptive immune system. The name is derived from two aspects: (*i*) NK cells are able to mediate their effector function (lysis of target cells) spontaneously in the absence of prior sensitization and are then called "killer" and "natural" and (*ii*) another aspect is that they perform their function with a very limited repertoire of receptors encoded in progenitor lines that do not undergo somatic recombination. The absence of previous sensitization and the absence of gene rearrangement for the formation of receptors for target cells indicate that NK cells are part of the innate immune system [76]. The major surface markers associated with NK cells are CD16 and CD56, while the T cell receptor (TCR) is absent [77].

The function of NK cells is to remove abnormal cells from the host, as infected cells or tumor cells, by exocytosis of lytic proteins (perforin/granzyme pathway) and by FasL or TRAIL (factor-apoptosis inducing linker of tumor necrosis) expression. Chemokines secreted by NK cells, such as IFN-γ and TNF-α, can mediate cytotoxic effects, activate dendritic and T cells, and influence the individual's immune response [78].

NK cells perform their task using two sets of receptors: activators and inhibitors present on their surface that interact with binding molecules on the surface of the target cell. The balance of these interactions determines whether or not the NK cell will be activated [9]. The major activation receptors expressed on NK cells include FcγRIIIA (CD16), DNAM-1 (CD226), NKG2C (KLRC2: killer cell lectin-like C2 receptor), NKG2E (KLRC3: killer cell lectin-like C3 receptor), NKG2D (KLRK1: killer cell lectin-like receptor K1), KIR-activating forms (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1), natural cytotoxicity receptors (NCRs) called NKp30 (natural cytotoxicity triggering receptor 3), NKp46 (NCR1: natural cytotoxicity triggering receptor 1), NKp65 (KLRF2: killer cell lectin-like F2 receptor) and NKp80 (KLRF1: killer cell lectin-like F1 receptor). The inhibitory receptors are KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5, KIR3DL1, KIR3DL2, KIR3DL3, NKG2A (KLRC1: killer cell lectin-like C1 receptor), LILRB1 (leukocyte immunoglobulin-like B1 receptor), KLRG1 (NKR2B4: natural killer cell receptor 2B4), NKp44 (NCR2: natural cytotoxicity triggering receptor 2) and KIR2DL4 (NKR2B4: natural killer cell receptor 2B4) [75].

4.2. KIR molecules

KIRs are members of a group of regulatory molecules found on the surface of NK cells and T cell subpopulations. They were first identified for their ability to confer some specificity in cytolysis mediated by NK cells [79, 80]. This specificity occurs through the interaction of isotypes of KIR with HLA class I molecules, protecting unaltered cells from the destruction caused by NK cells. Different types of KIRs can be expressed on the surface of NK cells,

which may be activators or inhibitors [79], with a combinatorial selection of receptors to be expressed by the cell.

Thus, in an individual, NK cells can randomly express a different set of activating and inhibitory receptors, and not all NK cells in an individual have the same receptors. This differential expression between NK cells and certain KIR/HLA interactions may contribute to heterogeneity in NK cell activation levels, observed both among different individuals and among distinct NK cell subpopulations of the same individual [81].

NK cells become responsible for tolerance when their inhibitory KIRs identify class I HLA surface molecules as self-antigens, and trigger inhibitory signaling through the tyrosine kinase phosphorylation of intracytoplasmic inhibition motifs based on tyrosine immunosorbent (ITIM) [82]. Even with the presence of activating receptors, the inhibitory signal is translated into tolerance, absence of cytotoxicity and cytokine production by NK cells when the target cell is normal. When the cell is infected with a virus or transformed into a tumor cell, this tolerance environment is altered, especially by the low or no expression of HLA class I molecules, which is known as part of the escape mechanism of tumor cells to the adaptive immunity [83].

NK cells are activated to produce cytotoxicity and cytokines, precisely due to the escape mechanism of altered ITIM cells; but alternatively there are positively charged transmembrane residues, which facilitate the physical association with DAP12 accessory proteins, releasing the activating signal via immunoreceptor tyrosine-based activation motifs (ITAM) [75].

4.3. *KIR* genes

The *KIR* genes are located on chromosome 19 (19q13.4) in a 1 Mb gene complex called the leukocyte receptor complex (LRC) which is shown in **Figure 8**. There are several gene families in the LRC region, among them leukocyte Ig-like receptors (LILRs); Ig-like transcripts (ILTs); killer cell Ig-like receptors (KIRs); platelet collagen receptor glycoprotein VI (GPVI); Fc IgA receptors, FcGammaR; natural cytotoxicity triggering receptor 1 (NRC1); leukocyte-associated Ig-like receptors (LAIRs); sialic acid-binding immunoglobulin-like lectins (SIGLECs); members of the CD66 family, such as the carcinoembryonic antigen (CEA) genes and the genes encoding the transmembrane adapter molecules DAP10 and DAP12 [84, 85].

The *KIR* gene family has 15 genes (*KIR2DL1, KIR2DL3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2D5, KIR3DL1, KIR3DL2, KIR3DL3* and *KIR3DS1*) and 2 pseudogenes (*KIR2DP1* and *KIR3DP1*). They are divided into two functional groups: inhibitors that prevent lysis of the target cell and the activators that cause lysis of the target cell. The inhibitory group has eight genes that are *KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5A, KIR2DL5B, KIR3DL1, KIR3DL2* and *KIR3DL3;* the activator group has genes such as *KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5* and *KIR3DS1*; while *KIR2DL4* may be an activator or inhibitor. Between them, there are four *KIR* genes that are called structural (framework) genes, since they are present in almost all individuals: *KIR3DL3, KIR3DP1, KIR2DL4* and *KIR3DL2* [85, 86].

4.4. Structure and nomenclature of KIR

The naming of *KIR* genes is responsibility of the HUGO Genome Nomenclature Committee (HGNC) [87]. The designation of the *KIR* gene system considers the structure of the KIR protein.

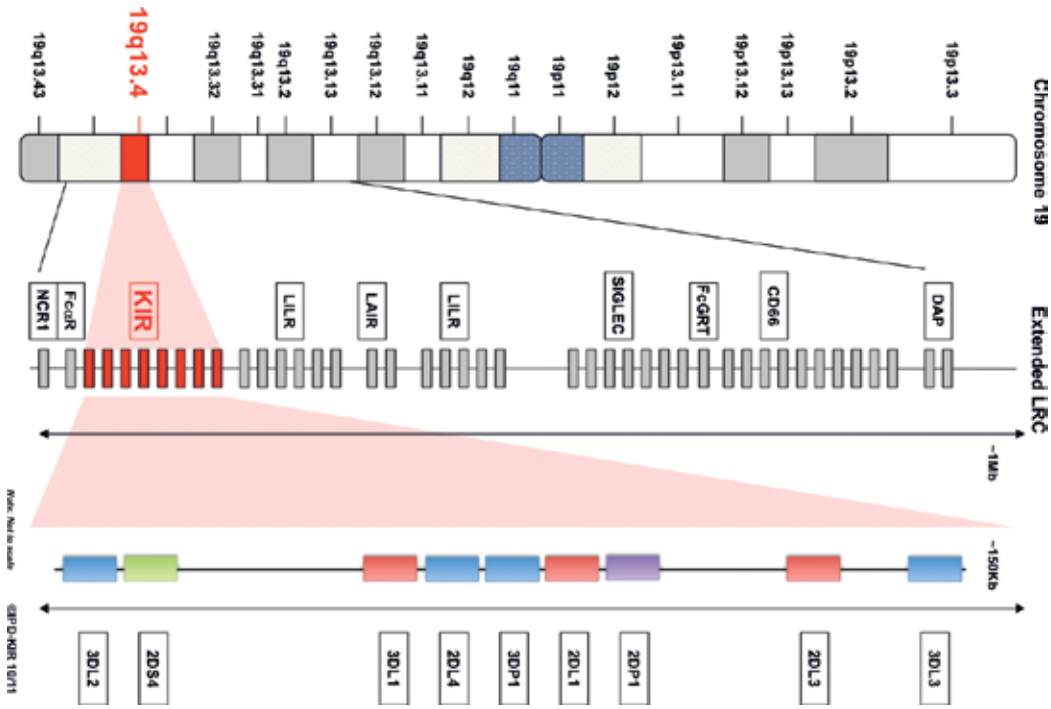

Figure 8. Diagram showing the cluster genes of the extended leukocyte receptor complex located (LRC) on chromosome 19 with highlight to *KIR* A haplotype at position 19q13.4 (in red). Among the molecules encoded by the extended LRC set of genes are the DAP adaptor proteins, CD66 antigens, SIGLEC, FcGRT, LILR, LAIR, FcAlphaR and NCR1 receptors. Within the *KIR* A haplotype are the framework genes (blue boxes), pseudogenes (purple box), inhibitory *KIR* (red boxes) and activating *KIR* genes (green box). *KIR2DL4* can be an inhibitory or an activating gene and *KIR3DP1* is also considered as framework gene [86].

They are classified based on two characteristics: number of extracellular Ig domains (2D or 3D) and characteristics of the cytoplasmic tail of the KIR protein, being S for short tail and L for long tail [88]. KIR3D is formed by the domains D0, D1 and D2, while KIR2DL1, KIR2DL2, KIR2DL3 and all KIR2DS have the D1 and D2 (Type I) domains; and KIR2DL4 and KIR2DL5 have the domains D0 and D2 (Type II) [89]. The long cytoplasmic tail (L) is associated with ITIM motifs that release a signal of inhibition to the cell. This signal of inhibition is due to the phosphorylation of a tyrosine residue that promotes the recruitment of (SHP-1 and SHP-2), which promote the dephosphorylation of protein substrates of tyrosine kinases related to the activation of NK cells. On the other hand, short tail (S) activation receptors have ITAM motifs in their transmembrane domain that associate with the adapter molecule DAP-12. The interaction of these receptors with their ligands results in the recruitment of SyK and ZAP-70 tyrosine kinases by ITAMs, resulting in the reorganization of the cytoskeleton to release granules and also in the transcription of cytokine and chemokine genes [90]. The structural characteristics of KIR that define their nomenclature are represented in **Figure 9**.

The *KIR* pseudogenes are identified by the letter "P" just after the digit corresponding to the domain type, as in the pseudogenes: *KIR2DP* and *KIR3DP*.

KIR genes follow a basic organization structure with 4–9 exons. Exons 1 and 2 encode the protein leader sequence; exons 3, 4 and 5 encode extracellular domains (D0, D1 and D2, respectively);

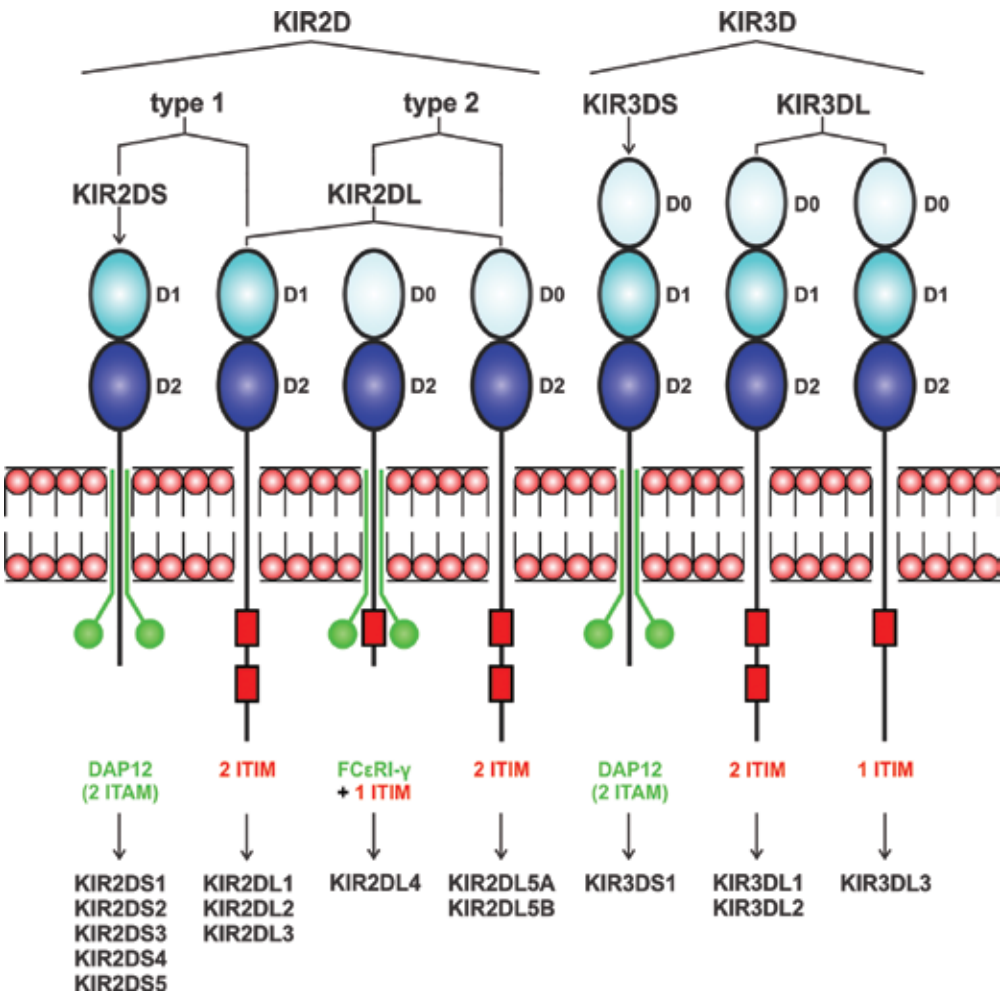

Figure 9. Domain structure of the KIR molecules. The structural characteristics of two and three Ig-like domain KIR proteins are shown. The association of activating KIR to adaptor molecules is shown in green, whereas the ITIM of inhibitory KIR are shown as red boxes. KIR2DL4 contains signature sequences of both activating and inhibitory receptors [86].

exon 6 encodes the tail, which lies between the extracellular domain and the membrane; exon 7, the transmembrane portion; and exons 8 and 9 encode the cytoplasmic tail [91].

4.5. *KIR* haplotypes

The *KIR* genes in the LRC form haplotypes on the same chromosome passed in blocks from generation to generation. There are two groups of *KIR* haplotypes: A and B, differentiated mainly by the number of activator *KIR* genes [92].

The A haplotype has seven *KIR* genes, predominantly the genes that encode the inhibitor receptors, such as *KIR2DL1*, *KIR2DL3*, *KIR2DL4*, *KIR3DL1*, *KIR3DL2* and *KIR3DL3*, with only

one activator gene, *KIR2DS4*. The B haplotype has a greater diversity of genes: *KIR2DL5*, *KIR2DL2*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5* and *KIR3DS1*, with the activation signals predominating. A and B haplotypes have the frameworks genes [86, 93].

The KIR Nomenclature Committee considered that the distinction between A and B haplotypes is useful in biological and clinical terms, and thus developed a consistent and logical set of criteria to distinguish them. Therefore, a haplotype can, for example, be called KH-001A or KH-022B [86]. The haplotypic diversity of *KIR* genes varies in different populations, suggesting that there may be variable effects of the receptors on several diseases, offering protection against one particular pathology or predisposition to the other.

4.6. KIR ligands

NK cells perform the recognition of foreign cells in the body through the interaction of KIRs on own cell surface with ligands on target cells surface: classical class I HLA-specific molecules (HLA-A, HLA-B and HLA-C) and non-classical (HLA-E and HLA-G) [94]. The activity of NK cells requires the interaction between a given class I HLA antigen expressed on the surface of the cells and a specific KIR, inhibitor or activator.

HLA-C molecules are the major ligands of KIR and can be distinguished in two groups of ligands (C1 and C2). All HLA-C carry a valine (V) at position 76 and a dimorphism in the position 80, which may be asparagine (N) or lysine (K). The alleles that have asparagine at position 80 are called C1 group (codifying by *C*01*, *C*03*, *C*07*, *C*08*, *C*12*, *C*13*, *C*14*, *C*16:01*, *C*16:03* and *C*16:04*) and are the ligands of KIR2DL2/KIR2DL3 and KIR2DS2. On the other hand, the molecules that possess lysine at position 80 (K80) belong to the C2 group (codifying by *C*02*, *C*04*, *C*05*, *C*06*, *C*15*, *C*16:02*, *C*17* and *C*18* genes) and bind to KIR2DL1 and KIR2DS1 [95].

Some HLA-B molecules express Bw4 epitopes that are also present in some HLA-A molecules encoded by HLA-A*09, HLA-A*23, HLA-A*24, HLA-A*24:03, HLA-A*25 and HLA-A*32. The KIR3DL1 and the KIR3DS1 interact with HLA-Bw4, which differs from Bw6 due to a polymorphism at position 77 and 80. Bw4 molecules may have multiple amino acids at the position 77, either asparagine or aspartic acid or serine, and a dimorphism at the position 80, which may be isoleucine or threonine. The allotypes containing Bw4 with Isoleucine (Bw4-80I) generally exhibit strong inhibition, while Bw4 alleles with Threonine (Bw4-80 T), such as those encoded by HLA-B*13, HLA-B*27, HLA-B*37:01 and HLA-B*44, appear to be better ligands for certain KIR3DL1 subtypes. Other KIRs have less defined specificities, such as KIR3DL2, which recognizes HLA-A variants (A3 and A11), KIR2DL4 recognizing HLA-G and KIR2DS4 recognizing C*04. The ligands for KIR2DL5, KIR2DS3, KIR2DS5, KIR3DS1 and KIR3DL3 have not been identified to date [95, 96].

Although KIR activators exhibit a ligand recognition structure very similar to inhibitory receptors, as in the 2DL1/2DS1-C2 group pair and the triad of 2DL2/2DL3/2DS2-C1 group, the binding affinity of the activating variants is strongly reduced in comparison to the inhibitory variants. Therefore, when there are binding of inhibitory and activating receptors at the same time, it is believed that the inhibitory signal prevails [96].

4.7. Influence of *KIR* genes and ligands on leprosy

It is known that the interaction of KIRs and their HLA ligands can result in activation or inhibition of NK cells and the occurrence of different immunological and clinical responses to various types of diseases, such as infectious diseases (AIDS, malaria, tuberculosis, Chagas disease, dengue fever and leprosy) [97–101], autoimmune and inflammatory diseases (psoriasis, rheumatoid vasculitis and Crohn's disease) [102–104] in different populations and ethnicities.

The pioneering studies of *KIR* genes in leprosy were carried out in Brazil. The first study was performed in the southern region of Brazil, where the *KIR2DL1* inhibitor gene with its C2 group ligand was shown to be protective for BB and its homozygous ligand (*KIR2DL1*-C2/C2) was associated with the clinical form TT. Another inhibitory gene and its ligands (*KIR3DL2*-A*03/A*11) were associated with susceptibility to borderline leprosy. The activating genes *KIR2DS2* and *KIR2DS3* were shown to be a risk factor for TT form, compared to the more widespread form LL. Thus, TT patients with both activating genes (*KIR2DS2* and *KIR2DS3*) may develop better activation of NK cells and a competent cellular immune response with a more localized manifestation of the disease. The inhibitory *KIR2DL3*-C1 and *KIR2DL3*-C1/C1 were associated to protection against TT form, when compared to the control group and other clinical forms [105].

The second study of *KIR* genes with leprosy was performed in a hyperendemic region of Brazil, and the *KIR2DL1* inhibitory gene was a protective factor for leprosy *per se* and its BB form. The frequency of the homozygous *KIR2DL2* gene in the presence of the C1 group (*KIR2DL2*/*KIR2DL2*-C1) was higher in leprosy patients *per se* and in clinical forms TT and LL, when compared to the control group. The *KIR2DL2*/*KIR2DL3* haplotype with its homozygous C1 ligand (C1/C1) was associated with protection for leprosy *per se* and TT and LL forms [17].

The inhibitory effect of *KIR2DL2*/*2DL2*-C1 may contribute to the development of leprosy, mainly to a worse prognosis in *M. leprae* infections. The activating *KIR2DS2* gene with its C1 ligand was a risk factor for leprosy *per se* and the clinical form TT. In this same study, it was observed that higher frequency of inhibitory genes may favor the susceptibility of the development of the disease [17]. Thus, this study confirmed the influence of *KIR* genes and their HLA ligands on the immunopathology of leprosy.

Activating and inhibitory *KIR* genes in the presence of their HLA ligands may have an impact on the development of leprosy and its clinical forms. The balance between these genes may interfere with the progression of the disease to a more localized (TT) or disseminated (LL), or to maintain an intermediate pattern between the two poles (BB), thus highlighting the role of NK cells and the production of cytokines.

5. Conclusions

This chapter outlined the contribution of the innate and adaptive immune genes to leprosy pathogenesis, highlighting the *HLA*, *KIR* and *MIC* polymorphism genes contribution for clinical forms and reactions of leprosy. Immune responses against the *M. leprae* vary considerably between populations, which can be partly attributed to the genetic variation of the immune response to ensure the survival of populations. HLA and non-HLA genes should act together

affecting the susceptibility to leprosy, resulting in different clinical manifestations or reactions. Hence, for a complete understanding of the genetic mechanisms of leprosy susceptibility, it will be necessary to join efforts to present a pattern of genes that would in fact be important to predict a clinical form or more severe reaction of the disease.

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Chapter 8

Genetic Variation in Pattern-Recognition Receptors and Association with Leprosy

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Additional information is available at the end of the chapter

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Abstract

Mycobacterium leprae is a highly infectious and low pathogenic microorganism that is the causal agent of leprosy. The differences in vulnerability to leprosy, the spectral immune response, and the clinical manifestations of this disease are related to different genetic backgrounds among individuals. In this sense, genetic variants, especially in genes related to mycobacteria recognition and host immune response, may be key factors to explain individual susceptibility and resistance to leprosy and their conditions. In this chapter, studies regarding association of genetic variants in pattern-recognition receptors (PRRs) and leprosy will be reviewed revealing the importance of molecules such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2) in leprosy initiation and maintenance.

Keywords: polymorphisms, pattern-recognition receptors, *mycobacterium leprae*, leprosy

1. Introduction

Leprosy is caused by *Mycobacterium leprae*, which is an intracellular bacterium with high infectivity and low pathogenicity. It means that there are a large number of people exposed to this pathogen; however, the majority of them are naturally resistant. On the other hand, some people develop leprosy once challenged with *M. leprae*. The people who develop disease may present different clinical forms of leprosy. Some of them develop a localized disease, named tuberculoid leprosy, with a strong host response, which does not avoid development of nerve injury and physical disabilities. Some patients develop a more severe form of leprosy, named lepromatous leprosy, whereas other patients present intermediate and instable clinical forms

(borderline tuberculoid, borderline borderline, and borderline lepromatous). Besides, there are different levels of susceptibility to leprosy reactions. The differences among *M. leprae* strains are not sufficient to explain this variable outcome of leprosy. There is a variable spectrum of host immune response that depends on the genetic characteristics of the infected individual. The immunological and genetic basis underlying *M. leprae* infection remains largely unknown.

In this sense, several studies have been conducted aiming to explore the molecular basis of leprosy, and they are mainly focused on aspects of host-pathogen interaction and modulation of host immune response against *M. leprae*.

Recognition of *M. leprae* by the host innate immune system is the first step in dealing with the invading bacteria and is crucial to initialize the adaptive immune response to infection. Microorganisms are recognized by host through germline-encoded pattern-recognition receptors (PRRs). The PRRs are able to sense highly conserved motifs from the invading microorganisms. These motifs are called pathogen-associated molecular patterns (PAMPs) [1].

One family of PRRs is the Toll-like receptors (TLRs) that are expressed on cell surface of different cells from innate immune system or endocytic vesicle membrane [2]. These receptors have an extracellular domain that recognizes different bacterial agonists. The activation of TLRs mediate host immune response regulating phagocytosis and antimicrobial activity or initiating signaling cascades (through activation of transcription factors NF-κB and IRF) culminating in modulation of cytokines and chemokines release [3].

There is another family of PRR, called nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) that are cytoplasmic receptors, and they are able to recognize bacterial agonists inside the host cells. Some members of this family activates NF-κB, interferon regulatory factors (IRFs), and mitogen-activated protein kinases (MAPKs), which control the expression of mediators of immune response, such as cytokines, chemokines, and type I interferons. On the other hand, other members lead to activation of caspase-1 acting through inflammasomes [4].

Genetic variants leading to functional changes in PRRs, such as TLRs and NLRs, may be involved with differences in host immune response modulation and consecutive susceptibility or resistance to leprosy and their clinical forms as well leprosy reactions.

In this chapter, studies about genetic association of PRRs, specifically TLRs and NOD2, with leprosy will be reviewed.

2. Genetic variations in toll-like receptors (TLRs)

TLRs were first described in the mid-1990s after genome sequencing of *Drosophila melanogaster*. Toll protein from *D. melanogaster* was identified as an important molecule in initiating innate immune response in this organism in response to fungal infection and Gram-positive bacteria. This molecule was also described as important in embryonic dorsoventral patterning [5]. After this first description, proteins similar to Toll protein from *D. melanogaster* were identified in mammalian cells, including humans, and they are referred as Toll-like receptors. TLRs are members of superfamily of interleukin receptors, and they act as PRRs recognizing PAMPs [6, 7].

The TLRs are also type 1 transmembrane receptors and may be expressed at cell surface or endocytic vesicle membrane. The receptors TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed at cell surface, whereas TLR7, TLR8, and TLR9 are expressed at endosomes and lysosomes. TLR3 may be expressed either at cell surface and endosomes or lysosomes [2]. The intracellular TLRs recognize viral and bacterial nucleic acids [8].

There are ten human TLRs already described (TLR1–TLR10), and they recognize different kinds of ligands [2, 9]. TLR1, TLR2, and TLR6 are mainly stimulated by lipoproteins from Gram-positive bacteria; TLR2 also recognizes lipoteichoic acid (Gram-positive bacteria), zymosan, β-glucan from fungi, and GPI anchors; TLR3 recognizes dsRNA from viruses; TLR4 is activated by lipopolysaccharides (Gram-negative bacteria) and GPI anchors; TLR5 recognizes bacterial flagellin; TLR7 and TLR8 recognize ssRNA from viruses; TLR9 is activated by DNA; and ligands for TLR10 are still not known [10]. TLRs are also able to sense and signal tissue damage through recognition of damage-associated molecular pattern molecules (DAMPs) [11].

After recognition of ligands by the specific TLR, a dimerization of TLRs as homodimers or heterodimers may occur. Ligation of TLR and a ligand may also lead to conformational changes in dimers. The differential association in heterodimers causes a diversification in ligand recognition. For example, the heterodimer TLR1-TLR2 recognizes triacylated bacterial lipopeptides, whereas TLR2-TLR6 heterodimer recognizes diacylated lipopeptides [12, 13].

Dimerization and conformational changes will activate intracellular signaling cascade and innate immune response against the pathogen, produce acute inflammation, lead to modulation of adaptive immune response, and induce antimicrobial pathways [3, 12, 14].

The important roles played by TLR in recognizing PAMPs, especially PAMPs from *M. leprae* and initiation of immune response, highlight its potential involvement with leprosy susceptibility and development. In the following sections, studies about association between polymorphisms in TLR1, TLR2, and TLR4 leprosy will be presented. The genetic association studies between TLRs and leprosy are summarized in **Table 1**. The other TLRs do not interact with *M. leprae* or do not have genetic association with leprosy.

2.1. Genetic variations in TLR1

The heterodimer formed by TLR1 and TLR2 recognizes killed *M. leprae* through triacylated lipoproteins leading to cell activation. These two receptors are expressed in a higher level in localized tuberculoid form than in disseminated lepromatous form, evidencing the role of TLRs in host defense. Type-1 cytokines IFN-γ, GM-CSF, IL-12, and IL-18 stimulate TLR1 and TLR2 activation, while type-2 cytokines IL-4 and IL-10 impair its activation. Although TLR1 response is important to fight infection, exacerbated activation of TLR can lead to immunopathology, tissue damage, and, in the case of leprosy, nerve damage [15].

The involvement of TLR1 in leprosy has been highlighted by several association studies.

Some polymorphisms in TLR1 were studied in different populations revealing their association with conditions related to leprosy. One of them is the SNP rs5743618 (T/G I602S) which presents a variable frequency depending on the population. Hawn et al. (2007) showed that

Gene	Polymorphism	Associated Condition	Reference
TLR1	Rs5743618 (T/G – I602S)	Leprosy susceptibility TLR1 trafficking to the cell surface	[17]
		Leprosy susceptibility	[18]
		NF-κB activation Reduced cytokines response Reversal reaction protection	[19]
	Rs4833095 C/T (N248S)	Leprosy susceptibility Leprosy reactions	[21]
		Leprosy susceptibility TNF/IL-10 ratio	[22]
		IL-12p40 and IL-17 levels	[23]
TLR2	Rs121917864 (C/T – R677W)	IL-12 level	[26]
		Lepromatous leprosy susceptibility	[28]
		NF-κB activation	[29]
		IL-2, IL-12, IFN-γ, TNF-α and IL-10 levels	[30]
	Rs3804099 (C/T – N199N)	Leprosy susceptibility IL-17 and IL-6 levels	[23]
		Leprosy reversal reaction	[33]
	Rs7656411 (G/T)	CXCL-10 level	[23]
	VNTR CT and TG Upstream to TLR2 start codon	Leprosy reversal reaction	[33]
	VNTR GT Intron 2 TLR2	(13 repeats) Leprosy protection (longer repeats) increased *TLR2* expression and decreased IL-10 expression; leprosy susceptibility	[34]
TLR4	Rs1927914 (A/G)	IL-17 and IL-1β levels	[23]
	Rs4986790 (G/A – D299G)	Leprosy protection	[39]
		Leprosy susceptibility	[42]
	Rs4986791 (C/T – T399I)	Leprosy protection	[39]
TOLLIP	Rs3793964 (C/T)	Leprosy susceptibility TOLLIP and IL1-Ra levels	[47]

Table 1. Association studies of genetic polymorphisms in TLRs and leprosy.

rs5743618 in *TLR1* is involved with the regulation of innate immune response to triacylated lipopeptides (ligands of TLR1/TLR2 heterodimer), and it is also related to the differential response to *M. tuberculosis* extract. The 602I variant was shown as able to induce an increased level of NF-κB signaling after induction by lipopeptides in a Vietnamese population. As expected, there is also an association with the cytokine profile as the 602I genotype presents a higher production of IL-6 in whole blood stimulated with lipopeptides supporting the role of TLR receptors in activating innate immune response against *M. leprae* [16]. In the same year, Johnson and coll. (2007) noted an association of this same SNP rs5743618 with surface trafficking of TLR1 and response of blood monocytes to bacterial ligands. More specifically, the variant 602S is associated with an impairment of TLR1 expression in cell surface leading to a loss of cellular responses. Corroborating these evidences, the variant 602S was shown to be a protective factor for leprosy [17]. Wong and coll. (2010) have analyzed the data from more than 1500 individuals from different studies and regions and identified *TLR1* and *HLA-DRB1DQA1* as the main genes related to leprosy susceptibility. Besides that, the protective variant 602S is rare in Africa but is the most frequent among European descendants, which suggests the selection pressure over this locus from mycobacteria [18].

Investigating the possible involvement of TLR1 with adaptive immune response affecting the clinical manifestations of leprosy, Misch and coll. (2008) evaluated rs5743618 polymorphism demonstrating a decrease of NF-κB activity related to the 1805G allele. In a Nepalese sample, the 1805G allele was protective against reversal reaction, which is characterized by an exacerbated Th1 cytokine response [19]. Another polymorphism able to interfere with TLR1 activity is rs4833095 (C/T N248S) as the 248 N variant impairs TLR1 functioning and sensing of microbial cell wall components [20]. In a Bangladeshi study, the homozygous 248SS genotype was associated with leprosy, but 248 N is homogeneously distributed among subjects. The 248 N allele is associated with erythema nodosum leprosum, while patients with reversal reaction are more likely to have the 248S allele [21]. These results were corroborated in a Brazilian sample, in which an association effect of 248S allele with leprosy susceptibility was found. In this same study, no association was identified between rs5743618 and leprosy diverging from previous results. However, rs4833095 was shown to be in a moderate linkage disequilibrium with rs5743618, suggesting a higher effect of the last one among Brazilians. The susceptibility allele 248S leads to a lower tumor necrosis factor (TNF)/IL-10 ratio after stimulation with *M. leprae*, and this same allele influences on TLR1 structure which may explain the functional alterations [22]. The association of 248S allele was not confirmed by Santana and coll. (2017) in another study with a Brazilian sample. However, they found association of rs4833095 with differential production of IL-17 and IL-12p40 [23]. These variations in TLR1 highlight the functional role of these genetic determinants in modulating the immune response during *M. leprae* infection.

2.2. Genetic variations in TLR2

TLR2 can form heterodimer with TLR1, as mentioned before, and with TLR6. These different forms of presentation allow TLR2 to recognize different cell wall components, such as lipopeptides, peptidoglycan, glycosylphosphatidylinositol-linked proteins, and zymosan [24].

TLR2 is responsible by recognition of cell wall fractions from *M. leprae* and mediate proinflammatory signaling by stimulating TNF-α production in macrophages in a Toll-like receptor-dependent manner [25]. Moreover, TLR2 is able to stimulate NF-κB signaling and subsequent induction of inflammatory cytokines [26]. As described to TLR1, type-1 cytokines IFN-γ, GM-CSF, IL-12, and IL-18 lead to enhancement of TLR2 activation, and type-2 cytokines IL-4 and IL-10 inhibit activation of TLR2 [15]. TLR2 is also expressed at surface of Schwann cells. Recognition of lipoproteins from *M. leprae* by TLR2 is required to stimulate apoptosis of these cells and may be related to nerve injury in leprosy [27].

In this way, variations in *TLR2* could be associated with a differential response against mycobacteria and be involved with individual susceptibility to leprosy. Looking for genetic variants in TLR2 in an association study about leprosy, Kang and coll. (2001) performed a screening in intracellular domain in leprosy patients. They detected the polymorphism rs121917864 (C/T R677W) in a conserved region of TLR2 present only in lepromatous patients, which suggests a role in susceptibility to this form of the disease [28]. After this, the same research group published a study demonstrating a role of rs121917864 in innate immune response activation, specifically the response by monocytes. Patients with the variant 677 W present a decrease in serum levels of IL-12 after stimulation with cell lysate of *M. leprae* compared to the patients with no amino acid substitution confirming that TLR2 is involved with immune response against *M. leprae* [26]. Another study also showed the relation of TLR2 with innate immune response studying the polymorphism rs121917864. In this study, the *M. leprae*-dependent activation of NK-κB signaling was impaired with 677 W variant in response to cell wall fractions of *M. leprae* and *M. tuberculosis*. These results allow to hypothesize that this variation in *TLR2* may be involved with the poor cellular immune response in leprosy patients [29]. The profiles of different cytokines are also affected by rs121917864 substitution. Peripheral blood mononuclear cells (PBMC) from lepromatous leprosy patients carrying this transition showed a lower level of expression of IL-2, IL-12, IFN-γ, and TNF-α after stimulation with *M. leprae*. On the other hand, levels of IL-10 increased, while there is no change in IL-4 production. This alteration in cytokines profiles in lepromatous patients can also be involved with a low level of cellular immune response in these patients during *M. leprae* infection [30]. Despite of what was shown in Korean population by Kang and coll. (2001), Malhotra and coll. (2005) did not identified the rs121917864 substitution in an Indian population. According to authors, rs121917864 is in fact a variation in a duplicated region 93% homologous located in exon 3 of *TLR2*. This duplicated region is a pseudogene [28, 31]. The rs121917864 polymorphism was also not identified in Japanese leprosy patients [32]. However, although such conflicting results may be related to the fact occurring in a duplicated region, these different results may also be explained by different genetic backgrounds among different populations.

Another important SNP in *TLR2* in the context of leprosy is the silent mutation rs3804099 (C/T N199 N). Santana and coll. (2017) found that the T allele is associated with an increased risk to leprosy and patients carrying this allele produce a high level of IL-17 and IL-6. In this same study, an intronic transition of SNP in *TLR2* rs7656411 (G3724 T) presented no association with leprosy in Brazilian population. However, individuals carrying G allele presented high levels of CXCL10 production [23]. Although no association of rs3804099 (C/T N199 N) with leprosy

was found, Bochud and coll. (2008) have already demonstrated that T allele has a protective effect against reversal reaction [33].

In addition to SNPs, microsatellites are important polymorphisms that can be markers of disease susceptibility, and there are studies trying to identify microsatellites in *TLR2* and investigating their association with leprosy conditions. In an case-control study with leprosy patients from Ethiopia, a microsatellite upstream to *TLR2* start codon containing two variable nucleotide tandem repeats (VNTR) (CT and TG) is associated with leprosy reversal reaction which is mainly characterized by a Th1 immune profile [33]. TLRs are able to modulate the immune response inducing a Th1 or Th2 profile; in this way, changes in these receptors may lead to a differentiation in immune profile from patients and involve with development of leprosy reactions. The microsatellite characterized by GT repeats on intron II of TLR2 is associated with protection against leprosy with 13 repeats being related to resistance. On the other hand, longer GT repeats are associated with a decrease in *TLR2* expression and increase in IL-10 production being associated with leprosy susceptibility [34].

2.3. Genetic variations in TLR4

TLR4 has as main ligand LPS from Gram-negative bacteria. However, studies have already demonstrated that TLR4 also recognizes ligands from *M. leprae* [35, 36]. TLR4 acts through MyD88-dependent and MyD88-independent (TRIF) signaling [37, 38].

The association of polymorphisms in *TLR4* with leprosy susceptibility was investigated in some studies reinforcing the involvement of this gene in response against leprosy. Bochud and coll. (2009) identified two polymorphisms rs4986790 (G/A D299G) and rs4986791 (C/T T399I) with the minor alleles presenting a protective effect against leprosy in an Ethiopian population. Eventually, these variations may have a role in *TLR4*-induced effects in immune response against leprosy [39]. Polymorphism rs4986790 was also investigated in a population from Malawi revealing borderline association with leprosy. There is a increased frequence of heterozygosity in control group which is contradictory with another study that demonstrated that heterozygosity of rs4986790 was associated with hyporesponsiveness after LPS inhalation [40, 41]. However, the Malawi study was corroborated by a family-based and case-control study, in which the AA genotype was associated with leprosy susceptibility and GA genotype associated with leprosy protection in an Indian population [42]. Aiming to understand the effect of polymorphisms in TLR4 signaling, Figueroa and coll. (2012) compared cells of human embryonic kidney 293/CD14/MD2 complemented with wild-type *TLR4* and mutant variants. No differences in *TLR4* expression were identified after induction by LPS. However, the *TLR4* signaling was altered since MyD88 and TRIF recruitment were impaired with *TLR4* carrying the rs4986790 polymorphism. These results were also observed with polymorphism rs4986791, however, in a lesser extent [43].

Changes in non-translated regions of *TLR4* gene may also be able to induce changes in immune profile. The A > G substitution in an intergenic upstream region (rs1927914) and A > G substitution in an intronic region (rs1927911) were associated with both leprosy per se and leprosy reactions. Although rs1927911 induces no differences in cytokines and chemokines level of production, the

allele A of rs1927914 is associated with a higher expression of IL-17 and IL-1β, which is highly produced in multibacillary leprosy patients [23].

2.4. Genetic variations in toll-interacting protein (TOLLIP)

The activity of TLRs must be under tight regulation to avoid an exacerbated response that would be harmful to the host. Toll-interacting protein (TOLLIP) is an adaptor protein which impairs the NF-κB and JNK signaling induced by TLR2 and TLR4, among other signaling pathways, playing a role in immune response regulation. TOLLIP is able to suppress IRAK-1 activity facilitating IL-1R/TLR-induced cell signaling during inflammation [44, 45]. After TLR2 stimulation, TOLLIP regulates the pro-inflammatory and anti-inflammatory balance by inhibiting IL-6 and TNF and stimulating IL-10 [46].

In this way, changes in TOLLIP activity may be related to leprosy susceptibility. In a Mexican population, the possible association of TOLLIP polymorphisms with leprosy susceptibility was analyzed. The following four polymorphisms were investigated in lepromatous leprosy patients and control individuals: promoter −526 C > G (rs5743854), silent mutation rs3750920 (C/T P139P), rs5744015 (C/A A222S), and 3′UTR rs3750919 (G/A). However, no association was identified between the cited polymorphisms and lepromatous leprosy, but a trend of protective association to lepromatous leprosy was identified in homozygous CC genotype of rs3750920 [44].

Six haplotype-tagging SNPs were also analyzed regarding association with leprosy in a population from Nepal. Among the tested SNPs, the intronic rs3793964 (C/T) exhibited association with leprosy being the TT genotype associated with leprosy susceptibility. Moreover, TT genotype and T allele were associated with increased expression of TOLLIP and IL-1Ra in the skin tissue. The study also demonstrates that TOLLIP induces IL-1Ra in monocytes that inhibit IL-1R activating helping *M. leprae* to evade from host immune response in early stages of leprosy development [47].

3. Genetic variations in nucleotide-binding oligomerization domain-containing protein 2 (NOD2)

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) is a family of intracellular PRR that is able to recognize bacterial molecules. Humans have 23 different NLRs that present two characteristic domains: NOD and leucine-rich repeats (LRRs). The first one is required for oligomerization, while the second one is required for ligand binding. There are different subfamilies of NLRs [48]. Some NLRs activate the protease caspase-1, so they act through inflammasomes leading to pyroptosis. The inflammasome-related NLRs are NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, and NAIP. However, other components of NLR family (NOD1, NOD2, NLRP10, NLRX1, NLRC5, and CIITA) act through activation of NF-κB, mitogen-activated protein kinases (MAPKs), and interferon regulatory factors (IRFs) leading to innate immune response stimulation [4].

There are some PAMPs exclusively recognized by NLRs, such as muramyl dipeptide (MDP) that is a constituent of peptidoglycan cell wall present in Gram-positive and Gram-negative bacteria [49]. *M. leprae*, in turn, presents a distinct MDP structure, and some studies have evidenced the role of NOD2 in recognizing *M. leprae* through MDP [50]. NOD2-mediated recognition of MDP from *M. leprae* induces bacterial killing activating NF-κB signaling cascade, production of IL-32, differentiation of dendritic cells, and autophagy [51–53].

Due to its role in immune response modulation, variations in *NOD2* gene is involved with different inflammatory diseases, such as Crohn's disease [54], Blau's syndrome [55], arthritis [56], and others. In this sense, several studies have investigated the association of *NOD2* genetic variants with leprosy susceptibility and leprosy reaction.

In a genome-wide association study from Zhang and coll. (2009) about susceptibility to leprosy in a Chinese population, variants in six genes of innate immune response were identified as associated with the disease (*CCDC122*, *C13orf31*, *NOD2*, *TNFSF15*, *HLA-DR*, and *RIPK2*). Specifically, in *NOD2* gene, two variants were identified in association with susceptibility to the disease: rs9302752 (A/G) and rs7194886 (G/A), being the first one with a stronger association with multibacillary leprosy. The two other evaluated SNPs in *NOD2*, rs8057341 (A/G) and rs3135499 (A/C), exhibited no association with leprosy in this study [57]. After this genome-wide association study, Grant and coll. (2012), in a family-based study, tried to validate in a Vietnamese population the genetic variants previously associated with leprosy in the Chinese study. They analyzed SNPs in *HLA-DR-DQ*, *RIPK2*, *CCDC122-LACC1*, and *NOD2* in leprosy patients and control individuals. Four polymorphisms were evaluated in *NOD2*: rs9302752, rs7194886, rs8057341, and rs3135499. The G risk allele for the variant rs9302752 was associated with leprosy, as identified in Chinese population. According to this Vietnamese study, some variants identified by Zhang and coll. may not directly associated with leprosy risk, but they are in linkage disequilibrium with another variants, which are the causal ones. This possibility could explain the divergent results found in different studies [57, 58].

In a subsequent study, Marcinek and coll. (2013) genotyped the variant rs9302752 but now in an Indian population. However, this variant was not in Hardy-Weinberg equilibrium in this population. Some other variants in genes involved in signaling pathways mediated by NOD2 were shown to be associated with leprosy in this study. One of them is the haplotype formed by rs40457 G and rs42490 A in *RIPK2* gene that together with *NOD2* is responsible for activate NF-κB signaling. This haplotype gives an increased risk to leprosy in individuals, whereas the haplotype AA has a protective role. In addition, the minor A allele of rs1873613 polymorphism in *LRRK2* is associated with paucibacillary leprosy progression [59].

Other conflicting results about genetic variants in *NOD2* and their relation with leprosy emerged in a Brazilian study from Marques and coll. (2014) [60]. In this study, authors aimed to validate the results found in the previous Chinese study regarding SNPs in four genes: *CCDC122-LACC1*, *NOD2*, *TNFSF15*, and *RIPK2* [57]. The A allele of variant rs8057341 in *NOD2* was associated with leprosy resistance in Brazilian population. This result is also different from that identified in Vietnamese study, in which rs8057341 has no association with leprosy. Other variants from *NOD2*, besides rs8057341 A, were related to leprosy protection being under-transmitted to the affected offspring in Brazilian population: rs2111234 G and

rs3135499 C [58, 60]. These conflicting results may be, at least partially, related to different genetic backgrounds among different populations.

The previous study conducted by Zhang and coll. was realized in a Han Chinese population, which is the main ethnic group from China [57, 61]. A second study was realized in a Chinese population evaluating the genetic association of *NOD2* (*C13orf31* and *CCDC122*) with leprosy but now in a population from Yi ethnic group that represents a minority group. The following polymorphisms in *NOD2* were investigated: rs9302752, rs7194886, rs8057341, and rs3135499. Only the SNP rs3135499 was differentially distributed between leprosy patients and healthy control individuals [62]. This result is different from that found for rs9302752 by Zhang and coll., Marcinek and coll., and Grant and coll. and rs7194886 and rs8057431 by Zhang and coll. [57–59].

Even with some discrepant results, the cited studies agree in associate *NOD2* with leprosy, however, with different specific variants. These differences may be due to the influence of different ethnicities or linkage disequilibrium between different SNPs.

In addition to association with susceptibility to leprosy, variation in *NOD2* may be related to susceptibility to development of leprosy reactions. Leprosy reactions are acute inflammatory reactions that may occur before leprosy diagnosis, during the treatment or after the treatment. Some factors are able to initiate these reactions, like intercurrent infections, pregnancy, and physical and emotional stress, among others [63]. Leprosy reactions are classified into two main types: type 1—reversal reaction (RR) and type 2—erythema nodosum leprosum (ENL). RR occurs mainly in earlier stages of treatment but can also occur after the treatment, in borderline clinical forms. It is characterized by an exacerbated Th1 response. The patient with RR presents an exacerbation of preexisting lesions, which became edematous and erythematous and may develop ulceration. RR is the leading cause of nerve damage in leprosy and consequent physical disability [63–65]. ENL, in turn, mainly occurs in lepromatous and borderline lepromatous leprosy and may happen during treatment but is more frequent after the treatment. Patients present subcutaneous nodules that are painful and erythematous and also may be ulcerative. In this kind of reaction, immune complexes are related to their initiation, but cell-mediated immunity also plays a role in ENL. There is an increase in $CD4^+$ T cells, TNF-α, and IFN-γ [63, 66, 67]. Several studies have already evidenced the possible functional mechanisms by which NOD2 plays a role during development of leprosy reactions by pro-inflammatory activities. The stimulation of Th1 and Th2 is one of these mechanisms [68, 69], as well as negative regulation of TLR2-mediated Th1 response [33, 70, 71].

Some of the previously mentioned polymorphisms in *NOD2* (rs7194886, rs9302752, and rs8057341), besides the additional rs751271 (G/T) and rs2066843 (C/T), were investigated for their association with leprosy inflammatory reactions in a Brazilian population. The variant rs751271 is associated with leprosy, being the genotype TT related to faster reaction development, whereas the genotype GT and G allele carriers are protection factors against leprosy reactions. The authors also suggest that rs751271-GT genotype produces lower levels of IL-6 in patients without reaction, so the hypothesis is that individuals with this genotype present a better balance in cytokine production which could be related to protection against leprosy reactions [72].

Polymorphism rs751271 were already investigated in a Nepal population regarding its association with reactive states of leprosy showing no association with type 1 reversal reaction (RR) neither with erythema nodosum leprosum (ENL). In this study, 32 polymorphisms in *NOD2* gene were evaluated. Four out of 32 polymorphisms were associated to leprosy susceptibility when the allele frequencies were compared between leprosy patients and healthy control: rs12448797, rs2287195, rs8044354, and rs1477176. When the genotype frequencies were compared, eight SNPs were associated with leprosy: rs2287185, rs8044354, rs8043770, rs13339578,

Gene	Polymorphism	Associated Condition	Reference
	Rs9302752 (A/G)	Leprosy susceptibility Multibacillary leprosy	[57]
		Leprosy susceptibility	[58]
	Rs8057341 (A/G)	Leprosy protection	[60]
	Rs7194886 (G/A)	Leprosy susceptibility	[57]
	Rs2111234 (A/G)	Leprosy protection	[60]
	Rs3135499 (A/C)	Leprosy protection	[60]
NOD2	Rs751271 (G/T)	Leprosy reactions	[72]
	Rs12448797 (C/T) Rs1477176 (C/T) Rs13339578 (A/G)	Leprosy susceptibility	[73]
	Rs8043770 (C/G) Rs4785225 (A/C/G) Rs751271 (G/T)	Leprosy susceptibility, RR	
	Rs2287195 (A/G) Rs8044354 (A/G)	Leprosy susceptibility, ENL, RR	
	Rs7194886 (C/T) Rs1861759 (G/T)	ENL, RR	
	Rs17312836 (A/C) Rs6500328 (A/G) Rs18611758	ENL	

*ENL = erythema nodosum leprosum, RR = reversal reaction

Table 2. Association studies of genetic polymorphisms in NOD2 and leprosy.

rs4785225, rs751271, rs12448797, and rs1477176. The variants rs12448797 and rs1477176 are located in genes adjacent to *NOD2*, *SL1C1*, and *CYLD*, which are regions in linkage disequilibrium with *NOD2* that may influence the expression of *SL1C1* or *CYLD* and *NOD2*. Regarding to RR, five SNPs showed association of their allele frequencies (rs2287195, rs8044354, rs8043770, rs7194886, and rs1861759), and seven SNPs exhibited association of their genetic frequencies in a dominant model (rs2287195, rs8044354, rs8043770, rs7194886, rs1861759, rs4785225, and rs751271). Four SNPs were associated with ENL in an allelic level (rs8044354, rs17312836, rs1861758 and rs1861759), while seven SNPs at the genotypic level (rs2287195, rs8044354, rs7194886, rs6500328, rs17312836, rs1861759, and rs18611758) [73].

The association of polymorphisms in *NOD2* gene with leprosy reactions highlights the potential role of this gene in susceptibility and development of RR and ENL. The genetic association studies between NOD2 and leprosy are summarized in **Table 2**.

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Hansen's Disease - The Forgotten and Neglected Disease provides concise information on the relevant aspects of the disease of leprosy, including immunological, epidemiological, clinical and molecular studies that are of great importance in the study of an often-ignored disease, which remains a great challenge to humanity.

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