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New insights in leprosy (Hansen's disease)

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New insights in leprosy (Hansen's disease)

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Editorial: New insights in leprosy (Hansen's disease)

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Editorial on the Research Topic
New insights in leprosy (Hansen's disease)

Leprosy, also known as Hansen's disease, has a long and complex history dating back to ancient times. Traditionally, it has been viewed through fear and isolation, perpetuating social stigmas that have endured for centuries. The quest to understand and combat leprosy is ongoing, with significant advancements in the 20th century. The development of multidrug therapy has proven effective in treating and critically reducing the disease burden worldwide (1). Recently, the scientific community has witnessed a resurgence in interest in leprosy, leading to a cascade of discoveries. This editorial delves into recent strides in leprosy research, shedding light on novel perspectives and potential solutions.

Leprosy transmission has long been scrutinized and recent studies have provided a more nuanced understanding of how the mycobacterium spreads. Studies exploring environmental reservoirs in leprosy-endemic regions have detected *Mycobacterium leprae* DNA in soil and water samples, and animals (ex. armadillos). This finding challenges the conventional belief that the bacterium resides exclusively in humans and prompts a reconsideration of the factors contributing to leprosy transmission (2). Information regarding the role of ticks in the transmission of leprosy is scarce. An interesting study by Krausser et al. weakened the hypothesis that ticks may be involved in leprosy transmission, sustained by the lack of *M. leprae* DNA found in ticks from Eastern Africa. Despite these findings, further studies are required to clarify the roles and interactions between vectors and *M. leprae*.

Accurate and early diagnosis is crucial for effective management of leprosy (3). Our Research Topic includes two outstanding papers that used specialized active searching in the Brazilian Amazon. Costa et al. investigated the occurrence of leprosy among children residing on Caratateua Island and found a high number of new cases in the pediatric population compared to the local baseline records. Bouth et al. focused on the unique genetic characteristics of the identified strain and its implications for drug resistance in leprosy cases. These findings contribute to our understanding of the genetic diversity of *M. leprae* in this region and provide insights into the challenges posed by drug resistance in leprosy control. By shedding light on the specific challenges faced in these areas and the evidence of a high hidden prevalence, both studies underscore the urgency of implementing targeted interventions and healthcare infrastructure, including complementary laboratory tests, to effectively combat and mitigate the spread of Hansen's disease.

Recent innovations in diagnostic tools have paved the way for precise and timely disease identification. Molecular techniques such as polymerase chain reaction have enhanced the sensitivity and specificity of leprosy diagnosis, enabling healthcare professionals to intervene at earlier stages and prevent disease progression (4). Point-of-care diagnostics have also been a game changer, particularly in resource-limited settings. Portable and rapid diagnostic tests allow healthcare workers to conduct on-the-spot assessments, facilitating quicker treatment initiation, and reducing the burden of leprosy on affected individuals and communities (4). Our Research Topic included two studies that underscored this exciting topic. Pierneef et al. discuss a field-friendly serosurvey conducted in Bihar, India, focusing on anti-phenolic glycolipid (PGL)-I antibodies to monitor *M. leprae* transmission in children. This study aimed to develop an efficient and practical method to assess leprosy transmission in a resource-limited setting. The second study by Lima et al. explored the clinical significance and performance of serological testing, focusing on IgA, IgM, and IgG antibodies against Mce1A. The Mce1A protein is part of the Mammalian cell entry (Mce) operon in *M. leprae*, which is involved in entering the mycobacteria into host cells. Mce1A proteins have been studied for their potential significance in leprosy, including research to understand host-pathogen interactions better and, remarkably, as a potential target for leprosy vaccines. Moreover, antibodies against the Mce1A protein have also been investigated as diagnostic and disease progression markers. The study performed by Lima et al. assessed the utility of these antibodies as biomarkers for detecting Hansen's disease and provided valuable insights into leprosy diagnosis.

The integration of artificial intelligence (AI) and machine learning in leprosy research has immense potential. These technologies can be employed in accurate and rapid diagnosis through image recognition technologies aiding in the early identification of leprosy cases and may assist in personalized treatment plans by analyzing individual patient data and optimizing therapeutic outcomes. Additionally, AI can also assist in analyzing vast datasets, identifying patterns, and predicting disease trajectories (5). In this regard, de Andrade Rodrigues et al. explored the application of an AI probabilistic modeling approach based on Bayesian networks to assess the likelihood of leprosy patients experiencing reactions, providing valuable insights into predicting and addressing potential leprosy complications. These applications highlight the promising role of AI in enhancing the efficiency and precision of leprosy research and management strategies.

In the search for predictors of leprosy progression, we also highlight the work of Bezerra-Santos et al., who examined the potential correlation between sTREM-1 and TNF-α, and the severity or progression of leprosy. The roles of these biomarkers are part of a broader network of immune responses to *M. leprae* infection. Research suggests that sTREM-1 is associated with activating myeloid cells and releasing pro-inflammatory cytokines, contributing to the inflammatory processes observed during Hansen's disease. TNF has also been implicated in leprosy immunopathogenesis, associated with granuloma formation, contributing to tissue damage in cutaneous and nerve lesions. The authors' findings suggest that the levels of these biomarkers are linked to clinical outcomes and are promising markers for monitoring and predicting the disease course.

The study of inflammatory reactions to leprosy presents several challenges. The delicate balance between controlling the infection and preventing excessive nerve inflammation is a critical challenge in managing leprosy-associated neuritis. In cases of severe inflammation, immunomodulatory treatments, such as corticosteroids, may be considered to modulate the immune response and mitigate nerve damage; however, using such treatments requires careful consideration of potential side effects and monitoring by healthcare professionals.

Variability in individual responses to reactions, the lack of standardized diagnostic criteria, and the absence of universally accepted treatment guidelines contribute to the complexity of managing these episodes. Our study presents two striking case reports: (i) the first report of two cases of leprosy-associated neuritis that received corticosteroid injections as part of the treatment, providing a new and promising option for leprosy neuritis (Spitz et al.) and (ii) a case report on the use of cyclophosphamide pulse therapy for treating chronic and refractory erythema nodosum leprosum, providing evidence of persistent and difficult-to-treat cases of type 2 leprosy reaction (Machado et al.). The identification of novel drugs guided by large-scale clinical trials is critical for optimizing treatment strategies.

Contact evaluation, a crucial aspect of leprosy control, traditionally relies on identifying and monitoring individuals in close contact with affected patients. Recent advancements in this field have refined contact tracing and assessment strategies, including molecular diagnostic tools and serological tests, leading to a better ability to identify asymptomatic carriers and individuals with early signs of infection. dos Santos et al. focused on identifying and diagnosing neural complications at an early stage among individuals living near patients with leprosy. This study underscores the significance of timely detection in initiating appropriate interventions and highlights the valuable insights gained from the practices of a reference center in Brazil.

Although these insights have marked significant progress in leprosy research, challenges persist in eradicating this disease. Limited funding, regional disparities in healthcare infrastructure, and the need for international collaboration are formidable hurdles. Addressing these challenges requires concerted efforts from governments, non-governmental organizations, and the scientific community to ensure that the momentum gained in leprosy research translates into tangible benefits for those affected. As reported by Montezuma et al., the evidence available in the field of leprosy, even that proposed by leading world references, has very low certainty. Thus, we reinforce the need for more robust data in the field of leprosy to apply the finest evidence-based care during daily assistance to our patients.

Recent strides in leprosy research signify a turning point in the battle against this age-old disease. From diagnostic innovations to active search interventions, a multifaceted approach to understanding and managing leprosy is beginning to yield promising results. As we stand on the cusp of a new era in leprosy research, we must harness collective knowledge and resources to propel these insights into practical solutions, ultimately paving the way for a world free from the shackles of leprosy.

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Early diagnosis of neural impairment in seropositive leprosy household contacts: The experience of a reference center in Brazil

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Introduction: Leprosy is an infectious disease that remains with a high number of new cases in developing countries. Household contacts have a higher risk for the development of the disease, but the neural impairment in this group is not well elucidated yet. Here, we measured the chance of occurrence of peripheral neural impairment in asymptomatic leprosy household.

Methods: Contacts who present anti-PGL-I IgM seropositivity, through electroneuromyography (ENMG) evaluation. We recruited 361 seropositive contacts (SPC) from 2017 to 2021, who were subjected to an extensive protocol that included clinical, molecular, and electroneuromyographic evaluations.

Results: Our data revealed a positivity of slit skin smear and skin biopsy qPCR of 35.5% (128/361) and 25.8% (93/361) respectively. The electroneuromyographic evaluation of the SPC showed neural impairment in 23.5% (85/361), with the predominance of a mononeuropathy pattern in 62.3% (53/85). Clinical neural thickening was observed in 17.5% (63/361) of seropositive contacts, but among the individuals with abnormal ENMG, only 25.9% (22/85) presented neural thickening in the clinical exam.

Discussion: Ours results corroborates the need to make the approach to asymptomatic contacts in endemic countries more timely. Since leprosy in its early stages can present an indolent and subclinical evolution, serological, molecular, and neurophysiological tools are essential to break the disease transmission chain.

KEYWORDS

leprosy, Hansens' disease, mycobacterium leprae, household contacts, neural impairment, peripheral neurophaty

1. Introduction

Leprosy is a chronic disease due to infectious by *Mycobacterium leprae (M. leprae)* that remains an important health problem in developing countries, such as India and Brazil, because of the late diagnosis and a high number of new cases (1). This bacillus has a slow replication rate with a long incubation period and infects especially peripheral nerves and skin (2).

The World Health Organization classifieds leprosy into paucibacillary (PB) or multibacillary (MB) forms according to the number of skin lesions and the slit skin smear aiming treatment protocols (3). In clinical practice, the classification of Ridley and Jopling is also used (4), classifying patients into five clinical forms: tuberculoid, borderline-tuberculoid, borderline-borderline, borderline-lepromatous and lepromatous.

Besides these clinical forms and the operational classification of WHO, a major challenge in this chronic disease is the definition of subclinical infection or latent leprosy. Even in the absence of symptoms, *M. leprae* is replicating and invading the host tissues (5), and biomarkers for the infection as anti-phenolic glycolipid-I (PGL-I) IgM antibodies have been recommended to detect a risk of infection in asymptomatic patients, especially household contacts. Some studies have also suggested a relation between infection in these patients and other biomarkers such as IL-6 and nutritional status (6), serum levels of IgA antibodies against NDO-HSA (7), CCL2 chemokine associated with IFN-γ (8), and IgM profile against NDO-HSA, LID-1, and NDOLID antigens, and monocytes and CD4+ lymphocyte frequency (9), beyond arginase activity (10) as a protective marker against this infection.

Leprosy household contacts present a risk for the development of the disease (11) and could maintain the spread of the *M. leprae* even if the index case is treated since some studies have shown positive PCR for *M. leprae* DNA in samples as nasal swabs, nasal turbinate biopsies, and/or peripheral blood in asymptomatic cases (12–15). Considering that positive results for anti-PGL-I IgM in these household contacts are associated with a higher risk of becoming ill, the evaluation and serology anti-PGL-I IgM of these individuals are recommended (5).

In Brazil, which ranks second worldwide in the number of leprosy's new cases, MB is the most prevalent form and is associated with neural disabilities in the diagnosis (1). In contrast, the neural involvement in the subclinical infection in these household contacts is still not well elucidated and its evaluation is relevant, especially for the future establishment of chemoprophylaxis protocols.

This study aimed to evaluate the clinical and laboratory predictors of subclinical neural impairment in leprosy household contacts.

2. Methods

It is a cross-sectional observational study, from 2017 to 2021, in which we recruited leprosy household contacts from the National Reference Center of Sanitary Dermatology and Leprosy in Brazil, under the approval of the Ethics Committee of the Federal University of Uberlandia. A written informed consent was obtained from all participants for research participation. Some participants were minors and their parents provided written consent on behalf of them.

At this center, leprosy contacts are followed up for a period of at least 7 years, annually, when they are evaluated by a multidisciplinary team and submitted to dermatoneurological examination and serological analyses by Enzyme-linked immunosorbent assay (ELISA) anti-phenolic glycolipid I (anti-PGL-I) Immunoglobulin M (IgM).

From 2017 to 2021, 741 new cases of leprosy were diagnosed in this service and 3,128 household contacts were notified, totaling an average of 4.2 contacts per patient. A proportion of 77.8% (2,502/3128) of these attended the initial evaluation, when all were submitted to anti-PGL-I serology collection. A total of 21 contacts had clinical signs of leprosy at baseline and 25% (620/2481) were seropositive. In this study, 361 seropositive contacts were submitted to all complementary exams at the time when seropositivity to the anti-PGL-I ELISA was confirmed (Figure 1). We excluded those who showed clinical evidence of leprosy or had any type of neurological symptoms and those who presented other etiologies of peripheral neuropathies, such as: chronic alcoholism, diabetes mellitus, thyroid disease, hormonal dysfunctions, malnutrition, hereditary neuropathy, hepatitis B or C, HIV, autoimmune diseases.

2.1. Clinical characterization

Epidemiological and clinical data were recorded. All patients underwent a rigorous dermatoneurological evaluation by two expert professionals (neurologist and dermatologist/leprologist).

2.2. Laboratory analyses

Identification of acid-fast bacilli (AFB) – This analyses were performed on slit skin smears from six sites (two ear lobes, two elbows, two knees), as well as skin and/or nerve biopsy samples.

ELISA anti-PGL-I IgM serology – It was performed on all household contacts. Serum anti-PGL-I IgM antibodies were detected by ELISA performed against the purified native PGL-I from the *M. leprae* cell wall. The reagent was obtained through BEI Resources, NIAID, NIH: Monoclonal Anti-*Mycobacterium leprae* PGL-I, Clone CS-48 (produced *in vitro*), NR-19370. The titration of anti-PGL-I antibodies was expressed as an ELISA index according to the proportion between the bacillary load of the sample in relation to the cutoff point. Values above 1.0 were considered positive (16).

DNA Extraction and Real Time Quantitative Polymerase Chain Reaction (qPCR) of the following samples: 1- slit skin smear (one sample) from six sites (two ear lobes, two elbows, two knees); 2- elbow skin biopsy. The qPCR assay targeting *M. leprae* DNA was performed by targeting the bacillus-specific genomic region (RLEP) in a real-time PCR system (ABI 7300, Applied Biosystems, Foster City, CA, United States) (13, 17, 18).

2.3. Electroneuromyography

Electroneuromyographic studies were carried out utilizing the MEB 4200 K (NIHON-KODHEN) device. In the sensory conduction study, the median, ulnar, radial, lateral antebrachial cutaneous, median antebrachial cutaneous, sural and fibular superficial were examined bilaterally. In the motor conduction study, the median, ulnar, common fibular, and tibial bilaterally nerves were examined, supplemented by techniques for focal impairment identification at compression sites often affected in leprosy neuropathy, such as median nerve at the wrist, ulnar nerve at the elbow, fibular nerve at the fibular head and tibial nerve at the ankle. The electroneuromyography (ENMG) was used to define the number of affected nerves and also the pattern of neural impairment (mononeuropathy or multiple mononeuropathy). Basically, reduced compound muscle action potential and sensory nerve action potential amplitudes suggest an axonal impairment of peripheral nerves, while prolonged latencies and/or reduced conduction velocities suggest a demyelinating pattern. All examinations were performed by the same neurologist, with expertise in electroneuromyography and leprosy.

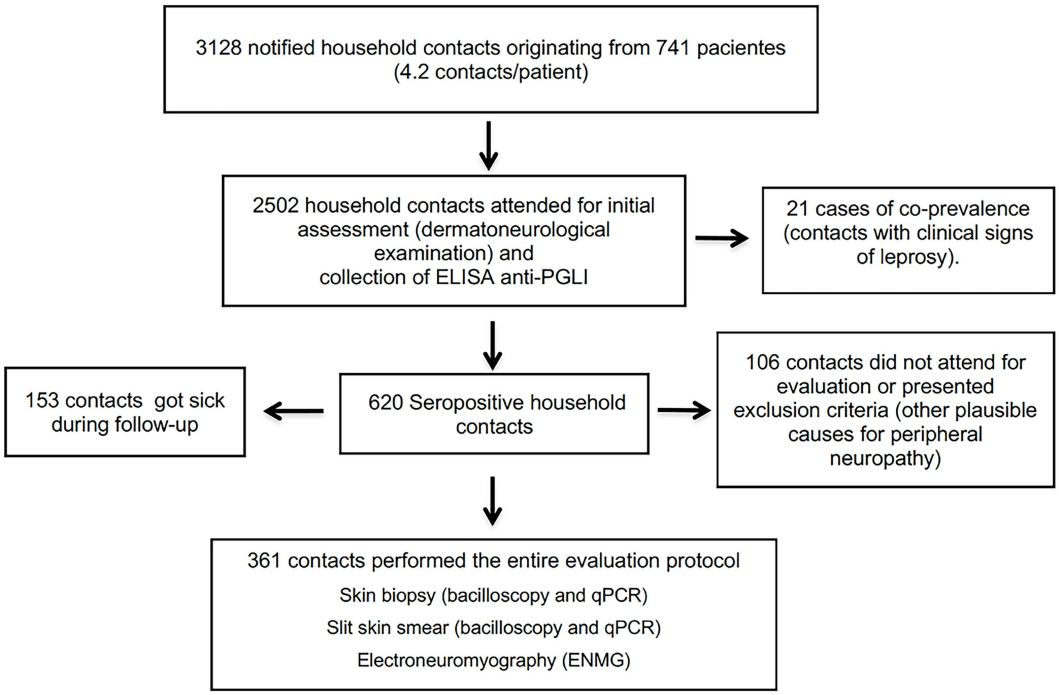

FIGURE 1
Algorithm proposed for household leprosy contacts selection.

2.4. Skin biopsy

All of the leprosy contacts selected did not present any skin lesion. For this reason, the biopsy of a small elbow skin fragment was performed, considering that it is a cold region with possible intradermal neural impairment, and therefore a site often altered in leprosy neuropathy. A wedge-shaped incision was made using a scalpel blade, and a fragment of approximately 1 cm along its greatest length that reached the hypodermis was removed. One part of the skin sample to be sent to the molecular pathology and biotechnology laboratory was wrapped in sterilized aluminum paper and immersed in liquid nitrogen. The other part was sent to the institution's pathology laboratory in a flask containing 10% buffered formalin, for histopathological evaluation. Fite-Faraco staining was used to investigate *M. leprae*.

2.5. Statistical analysis

The Shapiro Wilk test was used to test data normality within groups. The Wilcoxon-Mann–Whitney U Test was carried out, and the Binomial Test was applied for the Study of Dichotomous Variables, with significance defined as $p < 0.05$. To assess the level of agreement between the presence of electromyographic abnormalities and the existence of neural thickening, the kappa coefficient analysis was performed. The value of the Kappa coefficient close to 1 indicates that there is agreement between the evaluations and values below 0.60 indicate inadequate agreement. The statistical software used was GraphPad Prism version 7 (La Jolla, CA, United States).

3. Results

In this study, 361 seropositive contacts (SPC) were evaluated, with a mean age of 35.7 years (± 18.1) and with a female predominance (66.2%; 239/361). In relation to the type of exposure, 83.1% (300/361) reported intradomiciliary contact with leprosy patients. The mean anti-PGL-I IgM ELISA index was 2.31(±1.03). In the slit skin smear and skin biopsy analysis, the evaluation by the qPCR showed positivity of 35.5% (128/361) and 25.8% (93/361) respectively, all with negative bacilloscopy (Table 1).

Only 14.4% (52/361) of the patients were positive in the molecular evaluation by qPCR of RLEP of slit skin smear and skin biopsy and among the 128 patients with positive results in the slit skin smear, 59.4% (76/128) were negative in the skin biopsy.

Regarding the electroneuromyographic evaluation, 23.5% (85/361) presented neural impairment identified by ENMG. 62.3% (53/85) presented a mononeuropathy pattern and 37.7% (32/85) multiple mononeuropathy. The detailed pattern of the ENMG findings is described in Table 2.

The mean number of nerves affected was 2.1 per household contact. The most affected sensory nerves were the ulnar, followed by the superficial fibular and sural and among the motor nerves were the common fibular and ulnar. The nerves most frequently affected are described in Table 3.

Regarding the proportion of electroneuromyographic impairment according to the ELISA index, SPC with values above 4.0 showed a higher proportion of neural impairment (Table 4).

The presence of clinical neural thickening was observed in 17.5% (63/361) of SPC and among the 85 household contacts with abnormal ENMG, only 25.9% (22/85) presented neural thickening in the clinical

TABLE 1 Epidemiological, clinical, and laboratory characteristics among the household contacts of leprosy patients.

	Seropositive household contacts *n*=361
Age	35.7 ± 18.1
Sex	
Male	122 (33.8%)
Female	239 (66.2%)
Type of contact	
Intradomiciliary	300 (83.1%)
Extradomiciliary	61 (16.9%)
Index case	
Multibacillary	306 (84.8%)
Paucibacillary	55 (15.2%)
ELISA index	2.31 ± 1.03
Slit skin smear qPCR	128 (35.5%)
Skin biopsy qPCR	93 (25.8%)
Bacilloscopy	0

TABLE 2 Distribution of the electroneuromyographic pattern in seropositive household contacts of leprosy patients.

Electroneuromyographic pattern	*n*	%
Sensory axonal mononeuropathy	29	34.1
Focal demyelinating mononeuropathy	24	28.2
Asymmetrical sensory and motor demyelinating neuropathy	15	17.6
Asymmetrical sensory and motor axonal neuropathy with focal slowing of conduction velocity	8	9.4
Asymmetrical sensory axonal neuropathy with focal slowing of conduction velocity	5	5.9
Asymmetrical sensory axonal neuropathy	4	4.8
Total	85	100

TABLE 3 Distribution of peripheral nerves most affected in the electroneuromyographic evaluation of the seropositive household contacts of leprosy patients.

Peripheral nerves	*n*	%
Sensorial nerves		
Ulnar	41	22.5%
Superficial fibular	24	13.2%
Sural	16	8.8%
Median	6	3.3%
Superficial radial	6	3.3%
Medial antebrachial cutaneous	4	2.2%
Lateral antebrachial cutaneous	2	1.1%
Motor nerves		
Common fibular	37	20.3%
Ulnar (elbow)	29	15.9%
Tibial	14	7.7%
Median	3	1.6%
Total		182 2.1 nerves/contact

TABLE 4 Proportion of electroneuromyographic impairment according to the ELISA index.

ELISA index	Abnormal ENMG
1.1–2.0	22.5% (41/182)
2.1–3.0	19.8% (27/136)
3.1–4.0	23.4% (11/47)
> 4.1	30.0% (6/20)

evaluation and the agreement between these methods was weak (Table 5).

For the group of SPC with abnormal ENMG, a higher neural thickening frequency was observed. The positivity of the qPCR in slit skin smears and skin biopsy was also higher in this group (Table 6).

4. Discussion

In this study, we measured the prevalence of peripheral neural impairment in asymptomatic SPC, through ENMG evaluation.

From 2014 to 2016, we conducted a study in which 175 seropositive and 35 seronegative contacts were recruited and subjected to an extensive protocol that included clinical, molecular, and electroneuromyographic evaluations (19). This study showed that seropositive contacts presented a 4.0-fold higher chance of neural impairment. Since then, electroneuromyographic evaluation has become routine and has been performed in asymptomatic SPC. This study is a continuation of the previous results presented, but carried out in a more timely manner, reaffirming the importance of neurophysiological assessment of this neglected population.

Regarding other clinical forms, primary neural leprosy is the only one that presents with neural impairment without skin lesions or other clinical manifestations. An electrophysiological study is more sensitive than the clinical exam and previous studies showed that abnormalities in ENMG might be present in a high proportion of asymptomatic leprosy patients (20, 21).

The classical neural impairment of leprosy, defined by a sensory impairment with neural thickening before muscle weakness and deformities (20, 22–24), was observed in these SPC. Sensory nerve conduction impairment was the most frequent and the earliest parameter in ENMG evaluation (22–24). In contrast, neural thickening does not show agreement with the electrophysiological evaluation, confirming the need for a combined assessment, since the electrophysiological evaluation does not substitute a detailed clinical examination.

TABLE 5 Comparison between clinical examination for detection of neural thickening and electroneuromyographic evaluation of seropositive household contacts of leprosy patients.

		Electroneuromyography							
		Normal		Abnormal		Total			
		n	%	*n*	%	*n*	%	Kappa	*p*-value
Neural thickening	Normal	235	65.1	63	17.5	298	82.6	0.132	0.011
	Abnormal	41	11.3	22	6.1	63	17.4		
		276		85		361	100		

TABLE 6 Distribution seropositive household contacts of leprosy patients according to the electroneuromyographic pattern, and comparisons of proportions.

Parameters	Abnormal ENMG *n* =85	Normal ENMG *n* =276	*p*-value
ELISA anti-PGL-1 index	2.41 ± 1.20	2.28 ± 0.98	0.52
Neural thickening	22 (25.9%)	41 (14.9%)	0.0192
Slit skin smear qPCR	40 (47.0%)	88 (31.9%)	0.0106
Skin biopsy qPCR	32 (37.6%)	61 (22.1%)	0.0042

The screening of household contacts with anti-PGL-I is well established in the literature and other biomarkers have been evaluated to assess the risk of developing the disease (6–10). Furthermore, neural thickening and/or qPCR of slit skin smear and skin biopsy show a significant association with neural damage and could be used as biomarkers to initiate the treatment in these asymptomatic patients.

This study corroborates the need to make the approach to asymptomatic contacts in endemic countries more timely. Despite the numerous evidence obtained so far, there is no effective recommendation for chemoprophylaxis or for the treatment of asymptomatic contacts who have evidence of subclinical infection using molecular tools. One of the limitations of the study and a point to be observed in the next ones is the prospective evaluation of asymptomatic contacts submitted to chemoprophylaxis, to prove a reduction in neural damage after its implementation. Therefore, as is already done in other chronic infectious diseases, such as tuberculosis, it is necessary to transform these studies into public health policies, since the only way to advance in leprosy control is through early diagnosis.

Clinical evaluation is undoubtedly very important in the clinical approach to patients and household contacts. However, in a disease as complex as leprosy, which in its early stages can present an indolent and subclinical evolution, serological, molecular and neurophysiological tools are essential to break the disease transmission chain.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Federal University of Uberlandia. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

DS and IG contributed from the research design to analysis of the results and the writing of the manuscript. LG, IB, and TO collected data and organized the database, and wrote the draft of the manuscript. DA and AL performed the statistical analysis and wrote the draft of the manuscript. All authors revised the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Serological testing for Hansen's disease diagnosis: Clinical significance and performance of IgA, IgM, and IgG antibodies against Mce1A protein

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Hansen's disease (HD) is an infectious, treatable, and chronic disease. It is the main cause of infectious peripheral neuropathy. Due to the current limitations of laboratory tests for the diagnosis of HD, early identification of infected contacts is an important factor that would allow us to control the magnitude of this disease in terms of world public health. Thus, a cross-sectional study was conducted in the Brazilian southeast with the objective of evaluating humoral immunity and describing the accuracy of the immunoassay based on IgA, IgM, and IgG antibodies against surface protein Mce1A of *Mycobacterium*, the predictive potential of these molecules, the clinical significance of positivity, and the ability to segregate new HD cases (NC; n=200), contacts (HHC; n=105), and healthy endemic controls (HEC; n=100) as compared to α-PGL-I serology. α-Mce1A levels for all tested antibodies were significantly higher in NC and HHC than in HEC (p<0.0001). The performance of the assay using IgA and IgM antibodies was rated as highly accurate (AUC>0.85) for screening HD patients. Among HD patients (NC), positivity was 77.5% for IgA α-Mce1A ELISA, 76.5% for IgM, and 61.5% for IgG, while α-PGL-I serology showed only 28.0% positivity. Multivariate PLS-DA showed two defined clusters for the HEC and NC groups [accuracy=0.95 (SD=0.008)] and the HEC and HHC groups [accuracy=0.93 (SD=0.011)]. IgA was the antibody most responsible for clustering HHC as compared to NC and HEC, evidencing its usefulness for host mucosal immunity and as an immunological marker in laboratory tests. IgM is the key antibody for the clustering of NC patients. Positive results with high antibody levels indicate priority for screening, new clinical and laboratory evaluations, and monitoring of contacts, mainly with antibody indexes ≥2.0. In light of recent developments, the incorporation of new diagnostic technologies permits to eliminate the main gaps in the laboratory diagnosis of

HD, with the implementation of tools of greater sensitivity and accuracy while maintaining satisfactory specificity.

KEYWORDS

Hansen's disease, testing, serological, diagnosis, Mce1A, antibodies

1. Introduction

Hansen's disease (HD) is an infectious and contagious disease that mainly affects the skin, the peripheral nerves, mucosa of the upper respiratory tract, and the eyes, being caused by bacilli of the *Mycobacterium leprae* complex, which includes *M. leprae* and *M. lepromatosis* (1). HD is the most common treatable cause of peripheral neuropathy; however, it can progress to physical disabilities and deformities in the absence of an early diagnosis and the implementation of effective multidrug therapy (MDT) (2). HD is classified as a major public health issue and in 2019, with more than 200,000 new cases of HD reported worldwide and 27,864 reported in Brazil, a value equivalent to 93% of all cases in the Americas region and to 13.7% of the global cases registered. The heterogeneous distribution and the epidemiological indicators of Brazil at the global level reveal a scenario of continued transmission, with the disease representing a priority among the health problems of the country (3). According to the World Health Organization (WHO), as a result of the impact of the COVID-19 pandemic, more than 120,000 new cases were reported in 2020, with a 37% reduction compared to 2019 (4).

The incorporation of new laboratory technologies for an early diagnosis of HD and the identification of infected individuals will allow the control of the transmission chain and the global magnitude of the disease, as proposed by the WHO strategies (3). Thus, the absence of high performance diagnostic platforms for the diagnosis of patients across the clinical spectrum of the disease and of oligosymptomatic household contacts (HHC) are gaps in health units that do not allow early case detection, accurate diagnosis, or prompt treatment. Currently, anti-phenolic glycolipid-I (α-PGL-I) serology is the most widespread tool for the complementary diagnosis of the disease and contact with *M. leprae* based on antibody research. However, due to the low and variable sensitivity and negative predictive value of this test, as well as its low ability to detect early cases, paucibacillary patients, and macular and neural forms, its accuracy is not satisfactory for use as a diagnostic laboratory tool (5–7). Parallel to this, the slit-skin smear and the anatomopathological examination of the skin biopsy, despite having high specificity, are also techniques that depend on the bacillary load of the host and are of low sensitivity for effective detection and screening of HD cases and their HHC (6, 7). More recently, the introduction of molecular biology to identify bacillus DNA in clinical samples (skin, nasal swab, and intradermal scraping) has increased the probability of detecting new cases while maintaining high specificity and has shown that the polymerase chain reaction (PCR) may be used to confirm most field cases (8). On the other hand, PCR is an expensive method not available to all laboratories for the diagnosis of HD, in addition to the absence of a gold standard laboratory test (7).

To validate new biomarkers for the diagnosis of all clinical forms of HD, infected individuals, and characterization of these molecules in the population residing in an endemic region, antibodies against the mammalian cell-entry protein 1A (Mce1A) of *Mycobacterium* were evaluated. Mce1A is reported to mediate bacillus entry into cells in the host's reticuloendothelial system cells and to induce their survival (9, 10). Despite the presence of the Mce1A protein in the *Mycobacterium* genus, preliminary studies have shown that conditions such as bacillus Calmette-Guérin (BCG) vaccination and latent tuberculosis infection (LTBI) do not interfere with the levels of anti-Mce1A antibodies (α-Mce1A) in HD patients (11, 12). Previously published studies have reported the potential of α-Mce1A antibodies for the detection and monitoring of HD, also indicating its role in the identification of asymptomatic contacts (11, 12). However, the present study is the first one carried out in a state of low endemicity in the Brazilian southeast, including patients with macular forms and mainly neurological signs and symptoms, representing the largest sample tested for the proposed serological assay. Thus, determining the most appropriate test cut-off value for each region and each biomarker. On this basis, our study aimed to describe the accuracy of an immunoassay based on α-Mce1A IgA, IgM, and IgG antibodies, as well as the predictive potential of these molecules, the clinical significance of positivity and their ability to segregate HD patients, contacts, and healthy endemic controls.

2. Materials and methods

2.1. Design and study population

A cross-sectional study was conducted at the National Referral Center in Sanitary Dermatology and HD, University Hospital of the Ribeirão Preto Medical School (HCFMRP-USP), University of São Paulo, Brazil, from 2020 to 2022. The study population ($N = 405$) was classified into three groups: new HD cases without MDT (NC), household contacts of HD patients (HHC), and healthy endemic controls (HEC).

2.1.1. New HD cases (NC)

NC ($n = 200$) were diagnosed by clinical evaluation according to the Brazilian Ministry of Health and WHO guidelines using recommended cardinal signs (13). The dermatological and neurological evaluation of the patients was the confirmatory exam performed by dermatologists and leprologists for the diagnosis of HD. Auxiliary tests to the clinical diagnosis were used, such as assessment of tactile sensation with a Semmes-Weinstein esthesiometer, ultrasound of peripheral nerves, and electroneuromyography, besides complementary exams such as serology, molecular exams, and bacilloscopy. Considering that none of

the classifications for HD include all of the clinical manifestations of HD, particularly those involving macular and pure neural forms, we classified the patients considering the guidelines adapted by Madrid (Congress of Madrid 1953) and the Indian Association of Leprology (IAL 1982) classifications as follows: indeterminate (I), polar tuberculoid (TT), borderline (B), borderline lepromatous (BL), polar lepromatous (LL), and pure neural (N); and PB (I and TT clinical forms) and MB (B, BL, LL, and N forms) according to the WHO operational criteria. Considering the classification by Frade et al. (14), patients with atypical hypochromatic macules and with altered sensation and neurological findings were classified as having the B and MB forms. All newly diagnosed patients were referred to a health unit for standard MDT.

2.1.2. Household contacts (HHC)

HHC ($n = 105$) were defined as individuals residing or having resided in the same household with an HD patient in the last 5 years at the time of diagnosis (3). All HHC were clinically screened for signs and symptoms of HD and subjected to laboratory analysis with serological and molecular exams. Clinical examinations were performed by dermatologists and leprologists at HCFMRP-USP.

2.1.3. Healthy endemic controls (HEC)

HEC ($n = 100$), representing community contacts, were defined as healthy individuals residing in the Ribeirão Preto region, SP, Brazil. During the last 5 years (2018 to 2022), the state was classified as having low endemicity. The Ribeirão Preto municipality was classified as having very high endemicity in 2021 for the first time during the study period, according to the new case detection rate of the disease. All participants reported that they had no history of diagnosis or contact with an HD, were test-negative for human immunodeficiency virus (HIV), had no diseases, and did not use immunosuppressive drugs.

2.2. Anti-PGL-I serology

Indirect ELISA was used to measure the α-PGL-I IgM titer of every serum sample and the cut-off was based on the OD average among healthy subjects multiplied by 2.1 plus 10%, according to a previously reported protocol (7, 12, 15). Serology was performed with an ND-O-BSA (PGL-I)-based glycoconjugate of bovine serum albumin (NR-19346. BEI Resources).

2.3. Molecular diagnosis of *Mycobacterium leprae* DNA

Total DNA extraction from a skin biopsy and/or earlobes and at least one elbow, knee and/or lesion slit-skin smear sample was performed with the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, cat: 51306) according to the manufacturer's protocol. DNA was used to perform quantitative PCR-RLEP according to a previously reported protocol (7, 16). The quantitative PCR (qPCR) result was considered positive for the detection of *M. leprae* DNA with amplification up to a 40.0 cycle threshold (Ct) and melting temperature at 87.5°C. The maximum number of cycles used was 40.0.

2.4. Anti-Mce1A serological testing

Quantitative evaluation of IgA, IgM, and IgG antibody α-Mce1A protein was performed by indirect ELISA according to a previously reported protocol (7, 11, 12). Purified recombinant Mce1A protein was provided by Dr. LW Riley (University of California, Berkeley, CA, USA). The respective index was calculated by dividing the optical density (OD 450 nm) of each sample by the cut-off, with indexes above 1.0 being considered positive. The cut-off point was based on mean OD between healthy controls compared to samples from patients with HD. The OD data were analyzed by receiver operating characteristic (ROC) curves to determine the cut-off point highest and matched sensitivity, specificity, and likelihood ratio, as previously described (7, 11, 12). For all assays, negative control samples from healthy individuals with no history of diagnosis or contact with HD, positive samples for α-Mce1A antibodies from patients diagnosed with HD, and wells considered blank without the addition of specific antibodies and with peroxidase-linked second antibody for each immunoglobulin tested were added. The OD values of the blank wells were used for subtraction in the respective results obtained in each well with the tested samples.

2.5. Statistical analysis

Data were analyzed with GraphPad Prism v. 9.0 software (GraphPad Inc., La Jolla, CA, USA). Study population characteristics were analyzed by the t test and Chi-squared test. Antibody level variations were analyzed by the Kruskal–Wallis test, followed by Dunn's test. The ability of immunoglobulin levels to discriminate NC and HHC from HEC was evaluated by ROC curves. The accuracy classification was based on Bowers et al. (17). The level of statistical significance was set at $p < 0.05$. The combined performance of the antibodies in distinguishing the groups was determined using Python 3.9.12 in the Jupyter Notebook environment. The libraries used were Numpy 1.21.5, Pandas 1.4.2, Matplotlib 3.5.1, Scipy 1.7.3, Sklearn 1.0.2, and Shap 0.40.0. Data were first anonymized and all patient identification was excluded from the database. For multivariate analysis, the dataset variables were transformed using the Partial Least Square method and the two latent variables that explained most of the variance were used to construct the graphs. The Mahalanobis distance and the Chi-square distribution with a threshold of 0.95 were used to detect outliers. Partial Least Square-Discriminant Analysis (PLS-DA) was implemented with a stratified cross-validation of 10 divisions and 20 repetitions and a variable importance in projection (VIP) score plot for important antibody identified by PLS-DA analysis was evaluated. The VIP score value closest to or greater than 1 is the of rule thumb for selecting relevant variables. Thus, to investigate the importance of the variables, the Shapley values of each individual for each of the antibodies were obtained and the mean of the module of these values was then calculated. Spearman's correlation was used to compare the antibody levels and classification was based on Akoglu (18). Finally, Hierarchical clustering was performed using Euclidean distance and Ward's linkage algorithms were performed using MetaboAnalyst 5.0. The analyzes were carried out with the antibody indexes corresponding to each group under study, and all input data have been normalized and transformed into logarithm. Two parameters were considered to perform hierarchical clustering. The first one is similarity measure—Euclidean distance,

Pearson's correlation, and Spearman's rank correlation. The other parameter is clustering algorithms, including average linkage (clustering uses the centroids of the observations), complete linkage (clustering uses the farthest pair of observations between the two groups), single linkage (clustering uses the closest pair of observations), and Ward's linkage (clustering to minimize the sum of squares of any two clusters). Heatmap was presented as a visual aid in addition to the dendrogram also showing distance measure using Euclidean and clustering algorithm using ward.D, where dendrogram data values are transformed to an average color scale displaying high values in red and low values in blue. The study was developed with pre-specified tests and considering α-PGL-I ELISA and PCR as reference standard and α-Mce1A ELISA as index test.

3. Role of the funding source

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

4. Results

4.1. Clinical and demographic findings

The spontaneous demand for care at the health unit did not permit the recruitment of a population with no statistically significant difference in terms of age, which on average ranged from 41.1 to 58.5 years ($p < 0.0001$) among the groups. Female sex was predominant among all individuals evaluated and ranged from 55.5 to 63.8% ($p = 0.35$). 96.5% of NC were classified as MB and the most diagnosed clinical form was B (78.5%). Molecular diagnostic comparison (PCR-RLEP) showed 94.9% negative results for HHC and 43.7% positivity for *M. leprae* DNA in NC ($p < 0.0001$) (Table 1).

TABLE 1 Study population characteristics (N=405).

	HEC n=100	HHC n=105	NC n=200	p-value
Age, years, mean (SD)	58.5 (16.7)	41.1 (17.7)	54.2 (17.2)	**<0.0001**[a]
Sex, *n* (%)				
Male	40 (40.0)	38 (36.2)	89 (44.5)	0.35[b]
Female	60 (60.0)	67 (63.8)	111 (55.5)	
Operational Classification, *n* (%)				
PB	-	-	7 (3.5)	
MB	-	-	193 (96.5)	
Clinical Form, *n* (%)				
I	-	-	1 (0.5)	
TT	-	-	6 (3.0)	
B	-	-	157 (78.5)	
BL	-	-	5 (2.5)	
LL	-	-	10 (5.0)	
N	-	-	21 (10.5)	
PCR-RLEP, *n* (%)				
Negative	-	75 (94.9)[c]	80 (43.7)[d]	**<0.0001**[b]
Positive	-	3 (3.8)	61 (33.3)	

[a]Comparison by the Kruskal–Wallis test.
[b]Comparison by the Chi-square test.
[c]Data not available for 14 HHC.
[d]Data not available for 53 NC.
HEC, healthy endemic controls; HHC, household contacts of HD patients; NC, new cases of HD; SD, standard deviation; PB, paucibacillary; MB, multibacillary; I, indeterminate; TT, tuberculoid; B, borderline; BL, borderline lepromatous; LL, lepromatous; N, neural; PCR-RLEP, quantitative polymerase chain reaction-specific repetitive element.

4.2. Anti-Mce1A and anti-PGL-I antibodies are biomarkers for the diagnosis of patients and their contacts

The antibody profiles of α-Mce1A protein and α-PGL-I indexes in newly diagnosed HD patients (NC), household contacts of HD patients (HHC), and healthy endemic-control individuals (HEC) are represented in Figure 1 as median and interquartile range (IQR). α-Mce1A IgA levels were significantly higher in the NC [median: 1.39 (IQR: 1.00–2.02), $p < 0.0001$] and HHC [median: 1.17 (IQR: 0.83–1.83), $p < 0.0001$] groups as compared to the HEC group [median: 0.62 (IQR: 0.42–0.81)] (Figure 1A). IgM α-Mce1A was evidently increased in HHC [median: 1.57 (IQR: 0.95–2.47), $p < 0.0001$] and NC [median: 1.51 (IQR: 1.025–2.32), $p < 0.0001$] as compared to HEC [median: 0.63 (IQR: 0.43–0.81)] (Figure 1B). α-Mce1A IgG indexes were higher in the NC [median: 1.14 (IQR: 0.87–1.51), $p < 0.0001$] and HHC [median: 1.070 (IQR: 0.80–1.34) $p < 0.0001$] groups than in HEC [median: 0.80 (IQR: 0.68–0.96)] (Figure 1C). The HHC group had moderate levels of α-PGL-I IgM [median: 0.50 (IQR: 0.30–1.0), $p = 0.0041$] as compared to HEC [median: 0.4 (IQR: 0.2–0.6)]. The NC indexes against PGL-I [median: 0.6 (IQR: 0.22–1.1)] showed significant differences compared to the HEC indexes ($p < 0.0001$) (Figure 1D).

4.3. Performance of anti-Mce1A antibodies and IgM anti-PGL-I for HD diagnosis

ROC curve analysis was performed to evaluate the performance of the three immunoglobulins against the Mce1A protein and IgM α-PGL-I for the diagnosis of NC, and the area under the curve (AUC), cut-off, sensitivity, and specificity values with 95% CI are shown in Table 2. α-Mce1A IgA had the best significant performance with AUC = 0.90 (CI: 0.87–0.93; $p < 0.0001$), with a case detection probability of 77.5% (CI: 71.1%–83.1%), and 89% (CI: 81.2%–94.4%) specificity. IgM showed a performance with AUC = 0.87 (CI: 0.83–0.91; $p < 0.0001$), with a 76.5% chance of correct classification (CI: 70.0%–82.2%) of new cases, and an 88% probability of identifying true negative individuals (CI: 80.0%–93.6%). The performance of the assay using IgA and IgM antibody was rated as having high accuracy (AUC > 0.85) for screening HD patients. The serological test with IgG showed AUC = 0.75 (CI: 0.69–0.80; $p < 0.0001$), 61.5% sensitivity (CI: 54.4%–68.3%) and 96% specificity (CI: 90.1–98.9) and was classified as having a moderate probability of providing correct results (AUC = 0.75–0.85). The α-PGL-I test showed performance with an AUC = 0.67 (CI: 0.61–0.72;

$p < 0.0001$), 34.6% (CI: 28.5%–41.2%) probability of case detection, and 96% (CI: 90.1%–98.9%) specificity. The performance of α-PGL-I serology was classified as having low accuracy (AUC < 0.75). The absence of difference between NC and HHC in all analyses for immunoglobulin levels led to the evaluation of the ELISA performance only for the group of patients compared to controls (HEC).

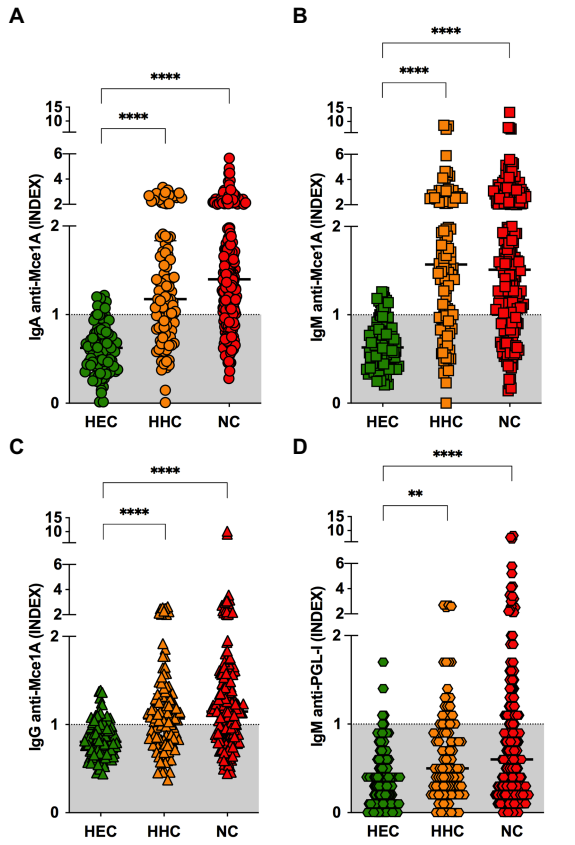

FIGURE 1
Biomarkers for the diagnosis of HD patients and their contacts. IgA **(A)**, IgM **(B)**, IgG **(C)** α-Mce1A, and anti-PGL-I **(D)** antibody indexes in plasma samples from HEC (n=100), HHC (n=105); and NC (n=200). Statistical significance was determined by the Kruskal–Wallis test followed by the Dunn's test; significance was considered at ****p<0.0001 and **p=0.0041. The respective index was calculated by dividing the optical density (O.D. 450nm) of each sample by the cut-off. Indexes above 1.0 were considered positive. HEC, healthy endemic controls; HHC, household contacts of HD patients; NC, new cases of HD; IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G.

4.4. Positivity and evaluation of serological biomarkers in parallel

The performance of the α-Mce1A assay was also evaluated based on the percentages of biomarker seropositivity (Table 3). IgA α-Mce1A ELISA for NC was positive in 77.5% (155/200) of patients, IgM in 76.5% (153/200), IgG in 61.5% (123/200), and α-PGL-I serology in 28.0% (56/200) of positive NC. HHC were 11.8, 6.0, 4.5, and 8.0% less seropositive for the tested antibodies, respectively, as compared to NC. The use of the α-Mce1A immunoassay in NC compared with HEC showed 7.0x more positivity for IgA α-Mce1A, 6.4 for IgM and 2.9 for IgG. None of the assays performed with HEC samples showed antibody indexes ≥2.0. A positive serological test with a ≥2.0 index in HHC and NC, respectively, was obtained in 20.0 and 26.5% for IgA ELISA, in 32.4% and 34.0% for IgM ELISA, in 8.6% and 7.0% for IgG ELISA, and 3.8% and 2.0% for IgM α-PGL-I. The use of the new α-Mce1A IgA, IgM, and IgG biomarkers allowed an increase of 49.5%, 48.5%, and 33.5%, respectively, in the detection of NC as compared to the use of α-PGL-I serology.

Parallel analysis of markers with α-Mce1A ELISA showed results with up to 5.0% seropositivity for all antibodies tested in the HEC group and 14.3 and 17.0% for HHC and NC, respectively. Thus, the combination of positivity for two tested antibodies showed greater overlap for IgM + IgG in the HHC (27.6%) and for IgA + IgG in NC (36.0%), an increase of positivity of 5.7% for HHC and of 19.0% for NC, as compared to the serial evaluation with IgA + IgM + IgG. For all overlaps performed, NC showed better seropositivity results (Table 3).

The low seropositivity and accuracy of the α-PGL-I serology meant that the authors did not use it in the subsequent analyzes of the study.

4.5. Multivariate models employed to distinguish endemic controls, HD patients, and contacts by means of the New serological biomarkers

The comparison of α-Mce1A antibody levels among NC, HHC, and HEC is shown in Figure 2. The performance of the model was evaluated using the intercept coefficient of determination (R^2), predictive relevance (Q^2), and significance of the permutation test (PT). Multivariate PLS-DA [$R^2 = 0.38$ (SD:0.01); $Q^2 = 0.42$ (SD:0.28); PT: $p = 0.009$] showed two defined clusters for the HEC and NC groups [accuracy = 0.95 (SD = 0.008)] and had the highest scores driving the cluster separation (LV1 = 56.99%) (Figure 2A). HEC and HHC [$R^2 = 0.40$ (SD:0.01); $Q^2 = 0.36$ (SD:0.32); PT: $p = 0.009$] also obtained excellent accuracy [accuracy = 0.93 (SD = 0.011)] and scores driving the cluster separation (LV1 = 61.92%) (Figure 2D).

TABLE 2 Comparison of the performance of IgA, IgM, and IgG α-Mce1A protein and IgM α-PGL-I for the diagnosis of new HD Cases (n=200).

Antibody	AUC (95% CI)	*P*-value	Cut-off (O.D)	Sensitivity % (95% CI)	Specificity % (95% CI)	LR+
α-Mce1A IgA	0.90 (0.87–0.93)	<0.0001	0.189	77.5 (71.1–83.1)	89.0 (81.2–94.4)	7.045
α-Mce1A IgM	0.87 (0.83–0.91)	<0.0001	0.146	76.5 (70.0–82.2)	88.0 (80.0–93.6)	6.375
α-Mce1A IgG	0.74 (0.69–0.80)	<0.0001	0.172	61.5 (54.4–68.3)	79.0 (69.7–86.5)	2.929
α-PGL-I IgM	0.67 (0.61–0.72)	<0.0001	0.295	34.6 (28.5–41.2)	96.0 (90.1–98.9)	8.662

AUC, area under the curve; CI, confidence interval; O.D., optical density; LR+, positive likelihood ratio; Mce1A, mammalian cell-entry protein 1A; IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G; PGL-I, phenolic glycolipid-I.

TABLE 3 Positivity profile of serological biomarkers in HD diagnosis.

Seropositivity *n* (%)	HEC (*n* =100)	HHC (*n* =105)	NC (*n* =200)
α-Mce1A IgA			
>1.0 < 2.0	11 (11.0)	48 (45.7)	102 (51.0)
≥2.0	0 (0)	21 (20)	53 (26.5)
Total	11 (11.0)	69 (65.7)	155 (77.5)
α-Mce1A IgM			
>1.0 < 2.0	12 (12.0)	40 (38.1)	85 (42.5)
≥ 2.0	0 (0)	34 (32.4)	68 (34.0)
Total	12 (12.0)	74 (70.5)	153 (76.5)
α-Mce1A IgG			
>1.0 < 2.0	21 (21.0)	51 (48.6)	109 (54.5)
≥ 2.0	0 (0)	9 (8.6)	14 (7.0)
Total	21 (21.0)	60 (57.2)	123 (61.5)
α-PGL-I IgM			
>1.0 < 2.0	4 (4.0)	17 (16.2)	39 (19.5)
≥ 2.0	0 (0)	4 (3.8)	17 (8.5)
Total	4 (4.0)	21(20.0)	56 (28.0)
α-Mce1A IgA + IgM + IgG			
>1.0 < 2.0	1 (1.0%)	10 (9.5)	26 (13.0)
≥ 2.0	0 (0)	5 (4.8)	8 (4.0)
Total	1 (1.0%)	15 (14.3)	34 (17.0)
α-Mce1A IgA + IgM			
>1.0 < 2.0	2 (2.0%)	15 (14.3)	44 (22.0)
≥ 2.0	0 (0)	6 (5.7)	16 (8.0)
Total	2 (2.0%)	21 (20.0)	60 (30.0)
α-Mce1A IgA + IgG			
>1.0 < 2.0	5 (5.0%)	1 (0.9)	61 (30.5)
≥ 2.0	0 (0)	8 (7.6)	11 (5.5)
Total	5 (5.0%)	9 (8.5)	72 (36.0)
α-Mce1A IgM + IgG			
>1.0 < 2.0	4 (4.0%)	23 (21.9)	48 (24.0)
≥ 2.0	0 (0)	6 (5.7)	10 (5.0)
Total	4 (4.0%)	29 (27.6)	58 (29.0)

HEC, healthy endemic controls; HHC, household contacts of HD patients; NC, new cases of HD; Mce1A, mammalian cell-entry protein 1A; IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G.

The analysis performance for the HHC and NC groups [$R^2 = 0.01$ (SD:0.004); $Q^2 = -2.03$ (SD:4.3); PT: $p = 0.56$] was not satisfactory [accuracy = 0.56 (SD = 0.024); LV1 = 37.7%] (Figure 2G). The ROC curve for model performance in discriminating the groups showed that IgM had the best accuracy in discriminating between HEC and NC (AUC = 0.87) (Figure 2B) and HEC and HHC (AUC = 0.86) (Figure 2E). The α-Mce1A IgG antibody showed the lowest accuracy among these groups (AUC = 0.75 and 0.72, respectively). α-Mce1A antibodies showed a low performance of IgA (AUC = 0.54), IgM (AUC = 0.51) and IgG (AUC = 0.50) in segregating HHC and NC due to the absence of difference in immunoglobulin levels in these groups (Figure 2H).

The ranking of the evaluated antibodies indicated that, in the discrimination among the groups after multivariate analysis, IgA α-Mce1A obtained a VIP score higher than 1 (VIP: 1.22; 1.13; 1.51), being the biomarker most responsible for the clustering of these groups (Figures 2C,F,I). The IgM antibody was the second relevant biomarker distinguishing between NC and HHC versus HEC (VIP: 0.96; 0.99, respectively). However, the IgG antibody was found to be the second most ideal biomarker only for the analyses between HHC and NC (VIP: 0.74) (Figure 2I).

4.6. Anti-Mce1A antibodies associated with HD diagnosis by means of Shapley values

Comparative assessment of α-Mce1A antibody levels in HHC and NC had preferably positive Shapley values, suggesting that these conditions always tended to diagnose infection and/or disease. The values were represented as group means and as minimum and maximum values of individuals. Figures 3A,C,E plotted Shapley values for each individual while Figures 3B–F the average of the absolute values (modules). The IgA antibody showed the highest positive Shapley value in the analyses between HEC and NC (Figures 3A,B) i.e., 0.173 (range: −0.348-1.017), a value of 0.169 (range: −0.329-0.707) between HEC and HHC (Figures 3C,D), and a lower value of 0.039 (range: −0.096-0.215) between HHC and NC (Figures 3E,F). The Shapley values of IgG α-Mce1A for HHC and NC as compared to HHC and HEC appear clustered and partially negative [0.017 (−0.301–0.032)], thus suggesting that antibody positivity in these groups had less potential for association with the diagnosis of HD due to their similar response. On the other hand, the IgG antibody ranked better than the IgM α-Mce1A antibody in the evaluation of the difference between HHC and NC. IgM was found to be clustered and with most positive Shapley values (Figures 3E,F) [0.013 (−0.019–0.112)], thus being the marker that, after IgA, showed a positive impact on HD diagnosis between HHC and NC. In light of these results, the values obtained with the IgA and IgM α-Mce1A antibodies ranged from negative to positive for all group comparisons, thus suggesting that these conditions were always leaning toward HD diagnosis (Figures 3A–F).

Thus, the higher the IgA value, more PLS-DA tended to classify the individual as NC, and the lower its value or negative as HEC. The same is true for IgM. For IgG, the higher its value, the more the model tended to classify as HEC. This behavior was caused by the association of the IgG antibody with treated patients and low seropositivity in the diagnosis. Figure 3B shows that IgA contributed more than IgM, which contributed more than IgG. In Figure 3C, the higher the Shapley value, the more the model tended to classify as HHC. In Figure 3E, the higher the Shapley value, the more the model ranked the individual as NC.

4.7. Correlation of immunoglobulins against Mce1A protein

Matrix correlation of α-Mce1A antibody levels among the study groups was calculated and the values are shown in color scale. NC and HEC showed a fair correlation between IgA and IgM ($r = 0.46$; $p < 0.001$) and between IgA and IgG ($r = 0.50$; $p < 0.001$). IgM and IgG showed a moderate positive correlation ($r = 0.66$; $p < 0.001$) between these two groups (Figure 4A). All positive correlations were fair for HHC and HEC, with $r = 0.42–0.59$ ($p < 0.001$) (Figure 4B). The correlation between

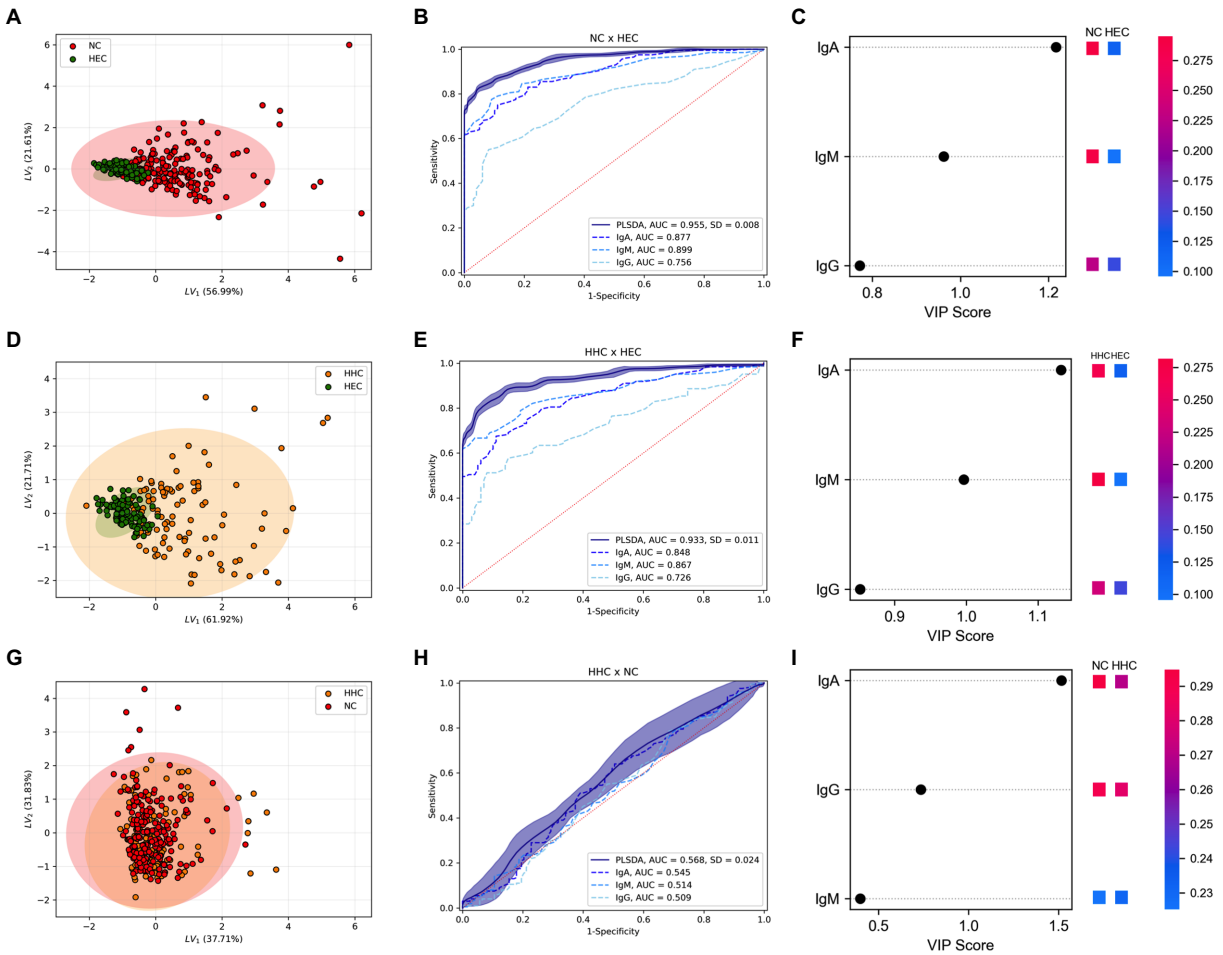

FIGURE 2
Simultaneous analysis of α-Mce1A antibodies in the clustering of groups. Partial least squares-discriminate analysis (PLS-DA) plot of IgA, IgM and IgG α-Mce1A combined from HEC, HHC, and NC. PLS-DA score scatter plots for HEC (green) and NC (red) **(A)**; HEC and HHC (yellow) **(D)**; HHC and NC **(G)**. Rank of the different immunoglobulins identified by PLS-DA according to the Variable Importance in Projection (VIP score) on the x-axis. The colored boxes on the right indicate the relative levels of the corresponding antibody (OD) in each group under study **(C,F**, and **I)**. Receiver operating characteristic (ROC) curve for schematic performance of PLS-DA classifiers over the validation set for combined antibodies and isolated levels in the NC and HEC **(B)**, HHC and HEC **(E)**, HHC and NC **(H)** groups. HEC, healthy endemic controls; HHC, household contacts of HD patients; NC, new cases of HD; IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G.

HHC and NC for IgA and IgM was poor ($r = 0.074$; $p = 0.186$) and the correlation for IgA versus IgG and for IgM versus IgG was classified as fair (r = 0.42; $p < 0.001$) and moderate ($r = 0.59$; $p < 0.001$), respectively (Figure 4C). Further analyses demonstrated that α-Mce1A IgA correlated poor ($r = 0.15$; $p = 0.04$), IgM, and IgG ($r = 0.37$; $p < 0.0001$) fair with α-PGL-I indices. The proposed assay with Mce1A was able to detect different individuals in comparison with PGL-I serology.

4.8. Anti-Mce1A serology was able to provide hierarchical clustering for the individuals evaluated

We combined these plasma antibodies indexes with the group's classification in HEC, HHC, and NC to apply machine learning using hierarchical methods of cluster analysis and as the main objective of the algorithm to provide the level of importance of each biomarker for each group through the heatmap. The following results were obtained: α-Mce1A IgA and IgM serology yielded essential results for NC identification as compared to HEC (Figure 5A); positivity for ELISA IgA was responsible for the clustering of HHC, while IgG ELISA was responsible for the clustering of HEC (Figure 5B), showing a very low involvement of IgM serology in the clustering of these two groups (HHC and HEC); positive samples for IgA and IgM distinguished NC from HHC, with IgA being the most intense antibody in terms of clustering performance in the HHC group (Figure 5C).

5. Discussion

The present results confirm the biomarker potential of α-Mce1A antibodies in the diagnosis of patients with HD, the screening of their contacts, and the assessment of exposure to the bacillus in endemic regions (Figure 6). The published results (11, 12) of the analysis with antibody levels in the different clinical forms and operational classification do not show differences between these groups for levels of α-Mce1A immunoglobulins. Also, there is no correlation or association between PCR positivity and bacillary load with positivity or higher levels of α-Mce1A antibodies in the tested samples. Thus,

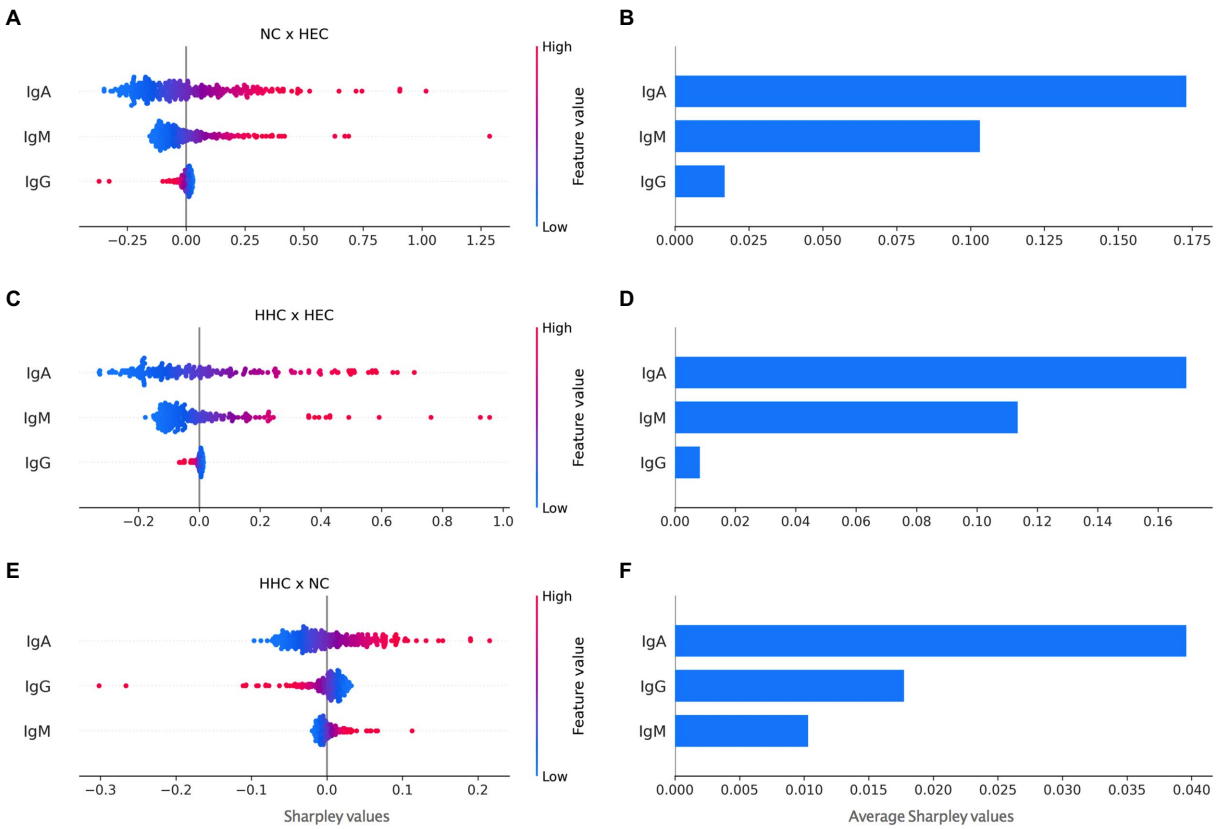

FIGURE 3
Contrasting Shapley values for impact on HD diagnosis for all antibodies against Mce1A protein. Each marker in the scatter plots corresponds to an individual and red to blue shades correspond to negatives to positive Shapley values **(A, C, E)**. The scatter plots expose not only the importance of a potential risk factor for HD diagnosis but also its range of effects over the NC and HEC **(A)**, HHC and HEC **(C)**, HHC and NC **(E)** groups. Scatter plots **(B,D**, and **F)** showed average Shapley values for the respective comparisons. HEC, healthy endemic controls; HHC, household contacts of HD patients; NC, new cases of HD; IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G.

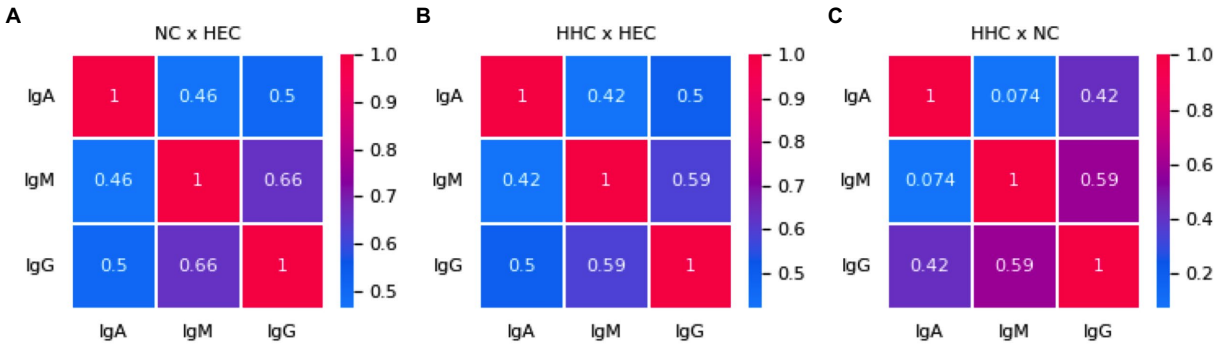

FIGURE 4
Immunoglobulins against Mce1A protein correlate weakly and moderately. Correlation matrix of α-Mce1A antibodies for NC and HEC **(A)**, HHC and HEC **(B)**, HHC and NC **(C)**. Spearman's correlation coefficients between two pairs of variables are shown in the heatmap. Red to blue shades correspond to increasing values of Spearman's correlation coefficient, as shown in the color bar. HEC, healthy endemic controls; HHC, household contacts of HD patients; NC, new cases of HD; IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G.

α-Mce1A serology differs from the α-PGL-I tool, which has been consolidated in the literature for correlation with bacillary load, operational classification, and multibacillary clinical forms. Therefore, the work analysis strategies aimed to identify patients with HD regardless of clinical classification and laboratory results for PCR, bacilloscopy, and α-PGL-I serology.

Serological testing for IgA is presented as an additional tool for the diagnosis and classification of HD, with potential utility for exposure monitoring of household contacts. In agreement with our data, Silva et al. (19) reported greater IgA reactivity against the conjugated antigen formed by natural octyl disaccharide linked to human serum albumin (NDO-HSA) among household contacts of PB

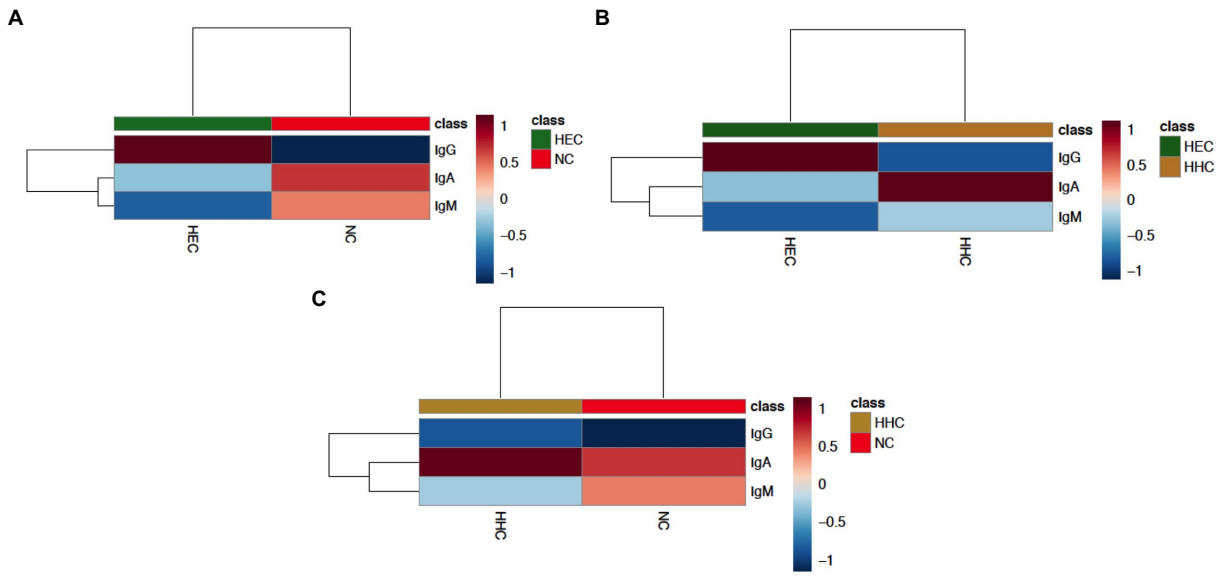

FIGURE 5
The indexes of anti-Mce1A biomarkers can cluster hierarchically. The Clustering result is shown as a dendrogram and heatmap (distance measure using similarity measure - Euclidean distance, and algorithm using clustering to minimize the sum of squares of any two clusters - Ward's linkage). Hierarchical cluster analysis was performed using normalized and transformed antibody indexes. Each sample begins as a separate cluster and the algorithm proceeds to combine them until all samples belong to one cluster for HEC and NC **(A)**, HEC and HHC **(B)**, and HHC and NC **(C)**. The heatmap shows the dendrogram data values transformed into an average color scale with high values in red and low values in blue. HEC, healthy endemic controls; HHC, household contacts of HD patients; NC, new cases of HD; IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G.

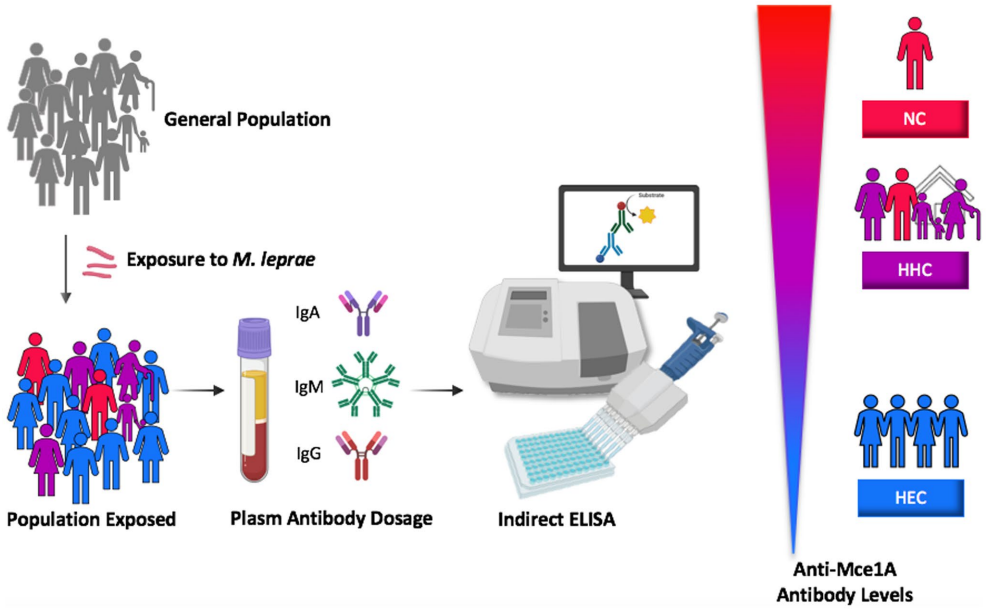

FIGURE 6
Proposed model of the stimulus and utility of specific antibodies against Mce1A protein in the laboratory diagnosis of HD based on the level of seropositivity for patients, household contacts and endemic controls. HEC, healthy endemic controls; HHC, household contacts of HD patients; NC, new cases of HD; Mce1A, mammalian cell-entry protein 1A.

and MB patients than among non-endemic controls. Accordingly, α-Mce1A IgA showed satisfactory accuracy (AUC 0.90) with 77.5% sensitivity and 89.0% specificity and revealed greater seropositivity of the immunoglobulins tested in patients, with 77.5% for new cases and 65.7% for contacts. In parallel, our analyses show that IgA was the antibody most responsible for clustering contacts between patients and endemic controls.

IgA is an antibody associated with the mucosal response, the main gateway of the bacillus in the establishment of infection, participating in the early stages of HD and in subclinical infection

(19–21). The importance of IgA for host mucosal immunity is well established and, although its role in the systemic circulation has not been fully elucidated (22), its usefulness as an immunological marker in laboratory tests has been confirmed.

Most published studies use IgM as a target molecule in serological assays in view of the fact that the seroprevalence of α-PGL-I IgM is higher than the seroprevalence of IgA and IgG in endemic areas (23). IgM-seropositive individuals are at higher risk of developing the disease (24); however, IgM seropositivity is not predictive of the disease, as demonstrated with α-PGL-I IgG (5, 25). The findings using IgM and α-PGL-I IgG corroborate the data obtained with α-Mce1A serology. In the evaluation of previous *M. leprae* infection as a risk factor for diagnosed transmission through IgM α-PGL-I serology, IgM represents a biomarker of greater sensitivity than IgG since it can be detected in many individuals already infected with the bacillus despite the absence of disease (23, 26, 27). The diagnostic performance of α-PGL-I ELISA was only 28.0% for new cases of HD and 20.0% for their contacts, with a 34.6% probability of case detection, and 96.0% specificity. Thus, 48.5% fewer positives were identified in comparison with IgM α-Mce1A serology.

IgM α-Mce1A had high accuracy (AUC 0.87) with a chance of correct classification of 76.5% of new cases and 88.0% specificity; 76.5% of newly diagnosed patients with the disease and 70.5% of household contacts were seropositive in ELISA.

Positive results with a high index indicate the priority for screening, new clinical and laboratory evaluations, and monitoring of contacts, mainly with indexes ≥2.0. These results were obtained here in 32.4% of contacts and 34.0% of new HD cases with positivity in the IgM α-Mce1A immunoassay. Thus, IgM is the key antibody for the clustering of new cases in relation to the other groups evaluated (HHC and HEC).

The ELISA results for all tested immunoglobulins were negative for an index ≥2.0 in healthy individuals from the endemic region, emphasizing the importance of serologies with a high value of seropositivity (≥ 2.0) in patients and their contacts. IgA serology with values ≥2.0 was positive in 26.5% of patients (NC) and 20.0% of contacts. On the other hand, rates higher than ≥2.0 for IgG α-Mce1A were only detected in 7.0% of the cases and 8.6% of the contacts. Thus, having positive serology for the contact and the case demonstrates the need for greater clinical surveillance of these individuals and the differentiation between these groups will be based on the clinical diagnosis, which remains the confirmatory evidence and gold standard for the diagnosis of HD.

Disease control and protective immunity in HD are associated with effective cellular immunity of T-cell responses. Studies evaluating antibody responses are primarily focused on their utility as a serological diagnostic tool. Rada et al. (28) showed that IgG responses decrease in MB and PB patients during treatment with MDT when using IgG against *M. leprae* antigens such as ML0405, ML2331, and LID-1 aiming to monitor the treatment of patients with the non-reactive LL form. However, data from assays targeting the detection of α-Mce1A IgG show variable seropositivity according to endemicity, showing that there may be a lower frequency of positives (61.5%) in less endemic regions and a high seroprevalence in new cases in a hyperendemic region (84.0%), as reported by Lima et al. (12). Patients treated with MDT had the highest rate of IgG positivity, which was detected in 89.5% of the patients evaluated in the study.

Recently, different cases of patients treated at an emergency unit in the Brazilian southeast were clinically diagnosed with HD and characterized by presenting hypoanesthetic skin lesions and thickened nerves, with peripheral nerve ultrasound demonstrating asymmetric and focal multiple mononeuropathy, and also with a positive molecular diagnosis in all patients tested by RLEP-PCR. Confirming the potential and innovative aspect of the new markers proposed for HD serology, 71.4, 100, and 42.8% of patients were positive for IgA, IgM, and IgG α-Mce1A, respectively. However, 100% were negative for α-PGL-I IgM (29).

Laboratory assays using α-PGL-I and α-LID-1 by ELISA and rapid test platforms with NDO-LID show low sensitivity and accuracy and are not recommended for isolated use in the diagnosis of HD, considering the complexity of the immunological presentations and the clinical aspects of the disease. A study by Frade et al. (30) demonstrated 48%–62% sensitivity and 70% specificity for α-PGL-I and α-LID ELISA and 40% specificity for NDO-LID. Other reports evaluating different studies with protocols using the PGL-I antigen demonstrated an average sensitivity of 63.8% and an average specificity of 91% as a diagnostic method in HD but are indicated mainly in MB cases, due to low positivity in PB cases (6).

The development of serological tests using antigens shared by a genus of a pathogen requires the evaluation of potential factors that can cause cross-reactivity in the results, such as vaccination with BCG, which is widespread in Brazil (> 90%) (31). The response to α-Mce1A antibodies was evaluated by Lima et al. (2017) in individuals with one or two BCG scars. However, in this study, we did not evaluate the response in newly vaccinated contacts and the proposal of the serological diagnosis during the clinical investigation of the patients and contacts before any prophylactic and/or therapeutic method for HD. Levels of antibodies against *M. tuberculosis* proteins (32) and LTBI did not induce distinct levels of α-Mce1A antibodies in the diagnosis of HD patients (11, 12, 33). A linear immunodominant epitope KRRITPKD (residues 131 and 138 in Mce1A) is highly conserved in *M. tuberculosis*, which is a possible explanation for the difference in response between patients with tuberculosis and HD, despite the homology between the mce1 gene (12, 34). Thus, allowing less chance of cross-reactions between individuals infected with both species of mycobacteria. However, it is a limitation of the α-Mce1A antibody assay for diagnosing HD in patients also diagnosed with or with a recent history of active tuberculosis. We sought to use three different antibodies (IgA, IgM, and IgG) to minimize bias and ensure the different proposed interpretations, such as diagnosis, potential subclinical infection, contact with *M. leprae*, and patients already treated for HD.

In parallel, for the determination of the cut-off value in populations with different endemic profiles, we need to know the pretest probability of the disease of interest as well as the costs incurred by misdiagnosis. Accordingly, the cut-off value is not universal and should be determined for each region and each disease condition according to endemicity (35). We still do not have commercially available serological tests capable of detecting cases with high sensitivity and accuracy. Thus, more exploratory studies to characterize new molecules capable of providing an immunological signature with high sensitivity and maintaining specificity are implemented as an advance in the search for new technologies to aid in the diagnosis of HD and the screening of contacts. Currently, α-Mce1A serology has not been able to distinguish contacts and patients with active disease, requiring further studies to understand whether seropositivity for the markers among household contacts is a predictor of the development of active disease or will only allow the identification of the contact with the bacillus regardless of disease progression. The seropositivity pattern of contacts for the tested immunoglobulins, similar to that found in patients and absent in healthy endemic controls,

contributes as one more alert test for better clinical follow-up of positive contacts. α-Mce1A serology corrects the main shortcomings in accuracy of the previous serology (PGL-I), as it demonstrates greater sensitivity, regardless of the clinical form or bacillary load.

The PCR-RLEP technique proved to be a methodology for identifying patients at diagnosis due to its positive rate (33.3%). The molecular technique showed performance with a positivity rate of 3.8% in household contacts. Thus, it represents another high-specificity diagnostic platform assisting with the diagnosis and screening of potential subclinical cases. Sensitivity can range from 51% to 91%, and specificity from 46% to 100% (6). A study published in the same endemic region identified a PCR positivity rate of 41.0% and sensitivity and specificity of 41.0 and 100% for HD patients, respectively (7). The evaluation of cases using complex neurological assessment techniques permits a better classification of patients into MB forms (2). In the present study more than 70.0% of the cases were diagnosed with the B clinical form and 96.5% with the MB form, mainly in patients with atypical hypochromatic macules with altered sensation, neurological findings on hands and feet, and lower bacillary load.

In line with the search for new tools for an early diagnosis such as ELISA α-Mce1A, the treatment of these cases is the next step toward achieving the goal of eliminating the disease in the community. As reported by the WHO, case detection and treatment with MDT alone are insufficient strategies to interrupt transmission. Thus, to boost the prevention of HD, the current recommendation is an active search of the household and social contacts of each patient, accompanied by the offer of preventive chemotherapy (3).

In summary, the present data suggest that combined serological testing based on IgA, IgM, and IgG α-Mce1A antibodies should be performed in order to ensure an interpretation of the three possibilities proposed for the new markers: positive IgA and/or IgG indicative of contact with the bacillus due to the strong positive correlation between these antibodies; positive IgM for diagnosis or priority for further clinical follow-up of contacts; Negative IgM and positive IgG as a form of therapeutic monitoring after MDT use. Serological assays are complementary diagnostic platforms, clinical correlation is always necessary and the region's endemicity is considered. Finally, the incorporation of new diagnostic technologies makes it possible to eliminate the main gaps in the laboratory diagnosis of HD with the implementation of tools of greater sensitivity and accuracy while maintaining satisfactory specificity. This procedure contributes to the goals of the WHO for the identification of initial and infected cases and for the interruption of bacillary transmission in the family environment, effectively reaching zero disability and eliminating the stigma of Hansen's disease.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board for Human Research of HCFMRP-USP (Protocol number 4.142.534/2020). The patients/participants provided their written informed consent to participate in this study.

Author contributions

FL and MF substantially contributed to study conception and design, acquisition of data, and/or analysis and interpretation of data. HL, FP, NF, and MF contributed to the clinical care of patients. VA, GF, and GD provided acquisition of clinical data. FL and NP executed and interpreted the laboratory tests. FL, MS, and DT contributed to statistical analysis and interpretation of the data. LR and SA provided scientific guidance and advice. MF gave final approval of the final submitted version and provided supervision and orientation of the study. All authors contributed to the interpretation of the results and to critical revision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Effectiveness and safety of multidrug therapy containing clofazimine for paucibacillary leprosy and clarithromycin for rifampicin-resistant leprosy: a systematic review and meta-analysis

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Introduction: The present study aimed to evaluate leprosy cure and relapse rates as primary outcomes related to two additional strategies for leprosy treatment: clofazimine for paucibacillary (PB) leprosy patients and clarithromycin for patients with rifampicin-resistant leprosy.

Methods: We conducted two systematic reviews (protocols CRD42022308272 and CRD42022308260). We searched the PubMed, EMBASE, Web of Science, Scopus, LILACS, Virtual Health Library and Cochrane Library databases, registers of clinical trial databases and gray literature. We included clinical trials evaluating the addition of clofazimine to PB leprosy treatment and the use of clarithromycin for treating patients with rifampicin-resistant leprosy. Risk of bias (RoB) in randomized clinical trials was assessed by the RoB 2 tool and that in non-randomized clinical trials was assessed by the ROBINS-I tool; and the certainty of the evidence was assessed by the Grading of Recommendations Assessment, Development and Evaluation (GRADE) system. A meta-analysis of dichotomous outcomes was performed.

Results: For clofazimine, four studies were included. Cure and relapse rates were not different with the addition of clofazimine to PB leprosy treatment and demonstrated very low certainty of evidence. For clarithromycin, six studies were included. Considerable heterogeneity resulted from the difference between comparators, and studies showed no difference in the assessed outcomes with the addition of clarithromycin to rifampicin-resistant leprosy treatment. Mild adverse events were reported for both drugs but did not significantly impact treatment.

Discussion: The effectiveness of both drugs still needs to be determined. Adding clofazimine to PB leprosy treatment may reduce the repercussions of an incorrect operational classification with no apparent relevant side effects.

Systematic review registration: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42022308272; https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42022308260, identifier: CRD42022308272; CRD42022308260.

KEYWORDS

leprosy, meta-analysis, drug resistance, microbial, GRADE approach, systematic review

1. Introduction

Leprosy is a chronic infectious granulomatous disease caused by *Mycobacterium leprae* and *Mycobacterium lepromatosis* that predominantly affects the skin and peripheral nerves (1). Regrettably, leprosy is still one of the most neglected diseases worldwide, impacting more than 120 countries, mainly in underdeveloped settings; more than 200 thousand new cases were reported in 2019 (2). Early diagnosis and treatment are crucial for reducing the burden of this disease and avoiding long-term irreversible consequences such as deformities and mutilations (3, 4).

Although leprosy is one of the oldest known diseases of humankind, effective leprosy treatment only began in 1941 with the discovery of sulfone (5, 6). The historical management of leprosy involved compulsory isolation, leading to permanent social stigma (7). Dapsone toxicity has always been a concern, joined by reports of resistance (8, 9). In this scenario, the World Health Organization (WHO) (5) recruited a group of specialists, called "THELEP." Despite the lack of proper evidence in those days, the problem was too urgent for a solution to be delayed; thus, in 1981, THELEP recommended multidrug therapy (MDT) (5) to solve the dapsone resistance problem and to make shorter treatment periods possible.

Although new cases are registered annually, the incidence of leprosy has dramatically reduced since the introduction of MDT; however, leprosy persists in some countries with endemic pockets such as Brazil, India and Indonesia. Despite the success of MDT, new challenges still arise (2, 10). Recent reports of rifampicin-resistant *M.* leprae (11, 12) and the inherent difficulty in properly classifying patients as having the multibacillary (MB) or paucibacillary (PB) forms are also threats to leprosy control (13, 14). Considering the significant gap in the literature, the WHO relies on expert opinions. In 2018, the "Guidelines for the Diagnosis, Treatment and Prevention of Leprosy" (15) recommended the use of clofazimine for patients with PB leprosy and clarithromycin for leprosy cases resistant to rifampicin (Figure 1).

The present study aimed to evaluate leprosy cure and relapse rates as primary outcomes related to two additional strategies for leprosy treatment: (I) clofazimine for PB leprosy patients and (II) clarithromycin for patients with rifampicin-resistant leprosy. In addition, as secondary outcomes, adverse events bacteriological and morphological index reductions, quality of life and treatment adherence were also assessed.

2. Methods

2.1. Protocol and registration

Two separate review protocols were recorded to analyse the two recent WHO therapeutic recommendations for leprosy treatment. The protocols were registered in the International Prospective Register of Systematic Reviews (PROSPERO): CRD42022308272 (clofazimine review) and CRD42022308260 (clarithromycin review). The reviews strictly followed the recommendations of the Cochrane Handbook for Systematic Reviews of Interventions (16) and were reported following the Reporting Items for Systematic Review and Meta-Analyses (PRISMA) statement (17).

2.2. Eligibility criteria

2.2.1. Population, intervention and comparator studies eligible for the clofazimine review

For the clofazimine review, studies targeting individuals of any age diagnosed with PB leprosy that addressed leprosy treatment using clofazimine in combination with dapsone and rifampicin for 6 months (WHO-PB-MDT) were eligible. For eligibility, dapsone and rifampicin must have been used for 6 months in a comparator group.

2.2.2. Population, intervention and comparator studies eligible for the clarithromycin review

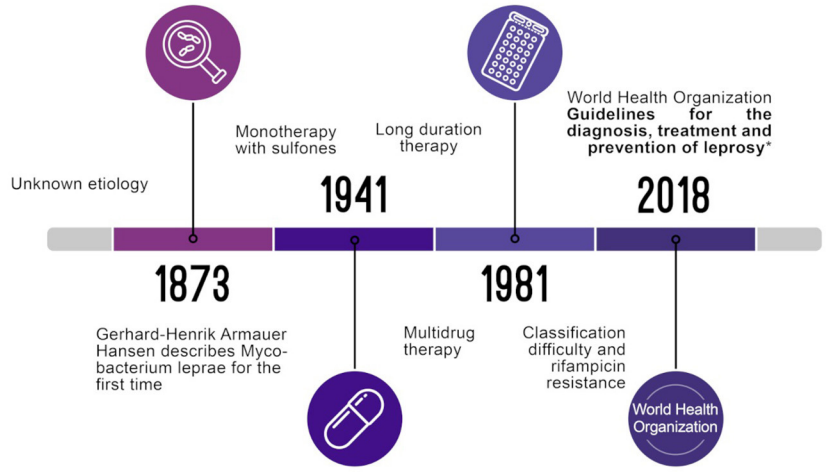
For the clarithromycin review, studies targeting individuals of any age diagnosed with PB or MB leprosy that addressed leprosy treatment with the use of clarithromycin alone or in combination with another drug were eligible. One of the following drug combinations must have been used in a comparator group: dapsone, rifampicin, quinolone, minocycline, clofazimine, ofloxacin and/or sparfloxacin. The presence of rifampicin resistance was assessed by subgroup analysis.

2.2.3. Outcomes and study designs eligible for the clofazimine and clarithromycin reviews

Regarding eligibility, all studies must have evaluated at least one of the following outcomes: efficacy/effectiveness (cure and relapse rates and bacteriological and morphological index reductions), safety (any adverse event or serious adverse event), quality of life or treatment adherence. Eligible study designs included randomized clinical trials (RCTs), non-randomized clinical trials, and observational studies with comparator groups (cohort or case–control studies). Systematic reviews, narrative reviews, experimental animal studies, cross-sectional studies, or case reports were excluded. There were no restrictions regarding the study follow-up time, language or year of publication.

2.3. Sources of information and search strategy

For both reviews, literature searches were conducted on April 1, 2022, in the PubMed, EMBASE, Web of Science, Scopus, LILACS, Virtual Health Library (BVS) and Cochrane Library databases. PubMed, EMBASE and Cochrane Library alerts were set up to provide a weekly update of new literature until August 13, 2022. A search was performed for ongoing studies in clinicaltrials.gov and the International Clinical Trials Registry Platform (ICTRP). The thesis and dissertation databases were manually checked, and gray literature was accessed in the Opengrey.eu database. The reference lists of the relevant studies were searched by the "backwards snowballing" method (Supplementary Tables 1A, 2A, respectively).



Topic*	Therapeutic Changes
Misclassification of multibacillary patients as paucibacillary cases	Inclusion of clofazimine for the treatment of paucibacillary patients.
Increasing reports of leprosy cases with rifampicin resistance	Treatment with at least two second-line drugs (clarithromycin, minocycline or a quinolone) plus clofazimine daily for 6 months, followed by clofazimine plus one of these drugs for an additional 18 months. When ofloxacin resistance is also present, the regimen of choice consists of 6 months of clarithromycin, minocycline and clofazimine followed by clarithromycin or minocycline plus clofazimine for an additional 18 months.

FIGURE 1
This systematic literature review evaluated clinical pathways and the World Health Organization recommendations.

2.4. Selection of studies

In both reviews, the references were exported to EPPI-4 (EPPI Centre, London, UK), and duplicates were removed using the automatic tool. The titles and abstracts were screened by two independent reviewers: CG and SV for the clofazimine review and CG and TM for the clarithromycin review. Disagreements were evaluated by a third reviewer: TM or SV. The full texts of the selected studies were evaluated in the same way.

2.5. Data collection process

Data were extracted using a standardized form developed by a leprosy specialist (CG). Two reviewers extracted the data independently, and disagreements were resolved through consensus: GC + TM for the clofazimine review and CG + SV for the clarithromycin review. The extracted information is disclosed in Supplementary material B.

2.6. Risk of bias assessment of the included studies

The risk of bias of RCTs was assessed at the outcome level using the Cochrane 2.0 Risk of Bias tool (RoB 2), and that of non-randomized clinical trials was evaluated using the Risk of Bias In Non-randomized Studies of Interventions (ROBINS-I) tool by two independent evaluators with subsequent consensus (CG and TM).

2.7. Data analysis

Effect sizes are presented as relative risks (RR) for dichotomous outcomes and by the mean difference (MD) for continuous outcomes. Meta-analysis of the dichotomous outcomes was performed using a random-effects model with the Mantel–Haenszel method in Review Manager software version 5.4 (The Cochrane Collaboration, 2020) if at least two comparable studies were identified. Heterogeneity was verified by forest graphs, chi-squared values ($p < 0.05$) and I^2 statistics (>50%). Regression models were used to assess publication bias if at least ten studies were included.

2.8. Analysis of certainty in the final set of evidence

The certainty in the set of evidence was analyzed using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) system (18).

3. Results

3.1. Results of the clofazimine review

3.1.1. Selected studies (clofazimine review)

The database search resulted in 8,841 references (11 RCT registers and 8,830 records identified in the databases, with 4,796 duplicate records removed), and 4,045 titles and abstracts were screened. Ultimately, 65 full texts were analyzed, and four studies were included in the review [eight publications (19–26), with four included in the main study by de Sá Gonçalves et al. (22) identified as a clinical trial for uniform multidrug therapy for leprosy patients in Brazil - U-MDT/CT-BR (21, 24–26)]. The flow chart of the selected studies (Supplementary Figure 1A) and the excluded studies, including the reasons for exclusion (Supplementary Table 3A), are shown in Supplementary material A.

3.1.2. Characteristics of the included studies (clofazimine review)

Three RCTs (19, 20, 22) and one non-RCT (23) were included; three were conducted in India, and one was conducted in Brazil. Four hundred sixty-four participants were included (the study sample size ranged from 40 to 300 patients with PB leprosy) (Table 1). In all studies, the diagnosis followed the criteria recommended by the WHO.

3.1.3. Risk of bias (clofazimine review)

The studies by Bhate et al. (19) and Katoch et al. (20) were classified as having a high RoB for the cure outcome. The study by Katoch et al. (20) was classified as having a high RoB for the relapse outcome. These studies were evaluated by the RoB 2 tool. The study by Prasad et al. (23) was evaluated by the ROBINS-I tool and was classified as having a serious RoB for the cure outcome. The RoB analysis details are provided in Supplementary material B.

3.1.4. Primary outcomes (clofazimine review)

The cure outcome (clinical inactivity) was assessed in three studies (19, 20, 23) with a 6 month follow-up. The summary effect of the treatment using clofazimine for PB leprosy showed an RR of 1.09 (95% CI: 0.92 to 1.29) compared to that of the control treatment (dapsone and rifampicin). No significant heterogeneity was found. Two studies evaluated the cure outcome within a 12 month follow-up period (19, 23). The summary effect showed an RR of 1.05 (95% CI: 0.78 to 1.40). There was significant heterogeneity among the studies (P-value = 0.04; I^2 = 75%) (Figure 2). Relapse was assessed in only one study (20) at a follow-up time between 2.5 and 3.5 years, with an RR of 0.20 (95% CI: 0.01 to 4.13).

3.1.5. GRADE approach (clofazimine review)

The certainty of the body of evidence was evaluated for the cure outcome and classified as having very low certainty. It was impossible to assess publication bias due to the small number

TABLE 1 Characteristics of the included studies evaluating the use of clofazimine in paucibacillary leprosy treatment.

References	Study design	Country	Follow-up time (years)	N	Age (years)*	Men (N)	Intervention	Comparator	Outcomes evaluated
Bhate et al. (19)	RCT	India	1	80	19 to 45	80	600 mg rifampicin monthly + 100 mg clofazimine every 2 days + 100 mg dapsone daily for 6 months; followed by dapsone monotherapy for 1 year	600 mg rifampicin monthly + 100 mg dapsone daily for 6 months, followed by dapsone monotherapy for 1 year	Cure rate, AEs
de Sá Gonçalves et al. (22)	RCT	Brazil	6	40	6 to 65	NR	WHO-PB-MDT	WHO-PB-MDT without clofazimine	AEs, treatment adherence
Katoch et al. (20)	RCT	India	3.5	300	NR	240	WHO-PB-MDT	WHO-PB-MDT without clofazimine + placebo	Cure rate, relapse rate, AEs
Prasad et al. (23)	CT	India	1	44	10 to 72	32	Rifampicin, clofazimine and dapsone	Rifampicin and dapsone	Cure rate, AEs

RCT, randomized clinical trial; CT, clinical trial; AEs, adverse events; NR, not reported. * Age is presented as the minimum and maximum age.

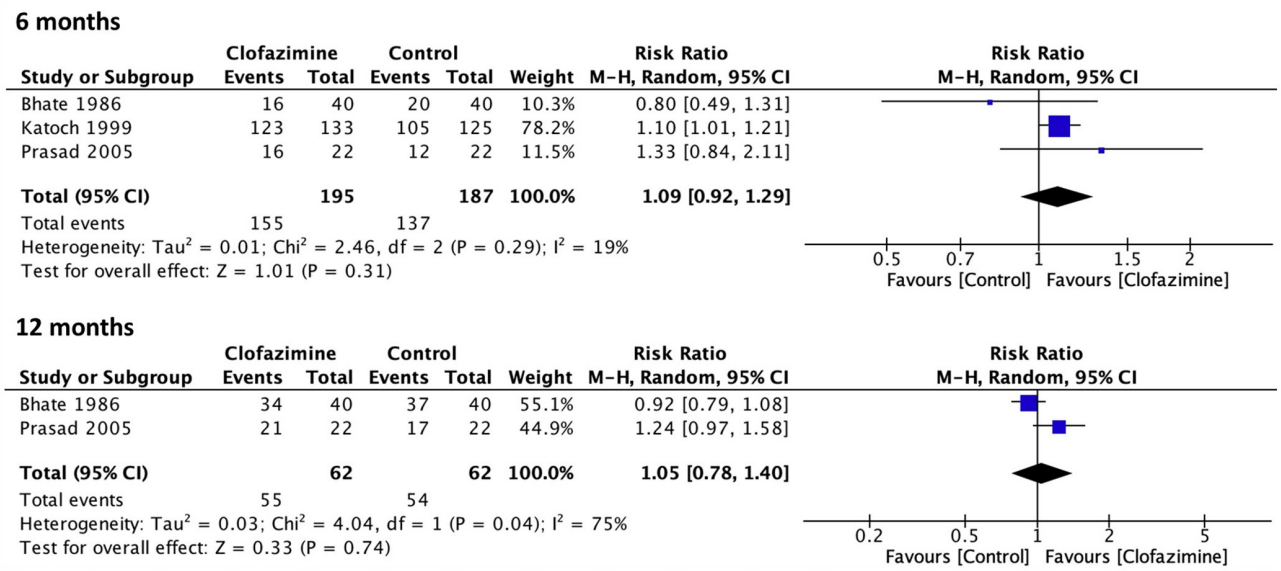


FIGURE 2
Meta-analysis of the studies evaluating clinical cure events comparing World Health Organization paucibacillary multidrug therapy for leprosy with (clofazimine) or without clofazimine (control) at the 6 and 12 month follow-ups.

of studies. In the same way, for the relapse outcome, it was impossible to assess inconsistency because only one study was included (Supplementary material B).

3.2. Results of the clarithromycin review

3.2.1. Selected studies (clarithromycin review)

The initial screening resulted in 2,133 references (two RCT registers and 2,131 records identified in databases, with 632 duplicate records removed), and 1,501 titles and abstracts were screened. Eleven full texts were evaluated, and six studies were included (27–32). The flow chart of the selected studies (Supplementary Figure 2A) and the excluded studies, including the reasons for exclusion (Supplementary Table 4A), are shown in Supplementary material A.

3.2.2. Characteristics of the included studies (clarithromycin review)

A total of 456 participants were included in six RCTs (27–32), and the sample size ranged from 14 to 300 individuals with MB leprosy. In the study by Ji et al. (29, 30), leprosy was diagnosed through skin smears, and in the other four included studies, the diagnosis was defined following the WHO's recommendations [included individuals with positive acid-fast bacilli and more than ten skin lesions (31)].

3.2.3. Risk of bias (clarithromycin review)

The study by Girdhar et al. (27) was classified as having a high risk of general bias, and the study by Ji et al. (29, 30) was assessed as having some concerns for the "cure" outcome. The study by Girdhar et al. (27) was also classified as having a high RoB for the relapse outcome. The RoB analysis details are provided in Supplementary material B.

3.2.4. Primary outcomes (clarithromycin review)

It was impossible to perform a meta-analysis of any of the outcomes due to the heterogeneity of the intervention arms and follow-up times of the included studies. The characteristics of the individual studies are presented in Table 2. The difference between comparators and the variety of associations made this a complex analysis. Only one study compared a modified MB-MDT substituting 600 mg of rifampicin per month with 2 g of clarithromycin per month vs. a classic WHO MB-MDT at 3 months after the start of therapy (28). Analyses of the cure and relapse outcomes of the included studies are presented in Tables 3, 4, respectively. Data on the cure and relapse outcomes at later times were not available.

3.2.5. Analysis of certainty in the final set of evidence (clarithromycin review)

Regarding the heterogeneity of the studies, an analysis of the certainty of the evidence was performed, including the individual outcomes of each study, and the set was classified as having very low or low certainty of evidence (Supplementary material B). Downgraded domains of inconsistency and publication bias could not be evaluated.

TABLE 2 Characteristics of the included studies evaluating the use of clarithromycin in leprosy treatment.

References	Study design	Country	Follow-up time	N	Age (years)*	Men (N)	Intervention	Comparator	Outcomes evaluated
Girdhar et al. (27) Adults	RCT	India	24 months	300	30.9 (16.2)	123	500 mg single dose clarithromycin + 600 mg rifampicin + 200 mg ofloxacin + 100 mg minocycline	600 mg single dose rifampicin + 200 mg ofloxacin + 100 mg minocycline	Cure rate, relapse rate
Girdhar et al. (27) Children							250 mg single dose clarithromycin + 300 mg rifampicin + 200 mg ofloxacin + 100 mg minocycline	300 mg rifampicin + 200 mg ofloxacin + 100 mg minocycline	
Gunawan et al. (28)	RCT	Indonesia	3 months	14	NR	11	2 g clarithromycin monthly + 300 mg de clofazimine; 100 mg dapsone daily + 50 mg clofazimine for 3 months	WHO-MB-MDT for 3 months	AEs, bacteriological and morphological index reductions
Ji et al. (30)	RCT	Mali	56 days	36	31 (9.0)	28	500 mg clarithromycin daily + 100 mg minocycline for 56 days	Comparator 1: 500 mg clarithromycin daily for 56 days Comparator 2: 100 mg minocycline daily for 56 days	Cure rate, bacteriological and morphological index reductions
Ji et al. (29)	RCT	Mali	31 days	50	31.3 (10.5)	37	2 g clarithromycin + 200 mg minocycline single doses on the 1st day + placebo daily for 30 days	Comparator 1: 2 g clarithromycin + 200 mg of minocycline + 800 mg ofloxacin single doses on the 1st day and followed placebo daily for 30 days Comparator 2: 600 mg rifampicin + 300 mg clofazimine single doses on the 1st day + 100 mg dapsone daily + 50 mg de clofazimine for 30 days Comparator 3: 600 mg rifampicin single dose on the first day + placebo daily for 30 days Comparator 4: 300 mg clofazimine single dose on the 1st day + 100 mg dapsone daily + 50 mg clofazimine for 30 days	Cure rate, AEs, bacteriological and morphological index reductions
Tejasvi et al. (31)	RCT	India	48 weeks	30	NR	28	500 mg clarithromycin + 600 mg rifampicin, + 200 mg sparfloxacin + 100 mg minocycline daily for 12 weeks	WHO-MB-MDT for 12 months	AEs, bacteriological and morphological index reductions
Wongdjaja et al. (32)	RCT	Indonesia	12 weeks	26	34.5 (11.56)	19	500 mg clarithromycin daily + 600 mg rifampicin + 400 mg ofloxacin 3x/week for 12 weeks	WHO-MB-MDT for 12 weeks	AEs, bacteriological and morphological index reductions

RCT, randomized clinical trial; AEs, adverse events; NR, not reported. *Age is presented as the mean (standard deviation).

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TABLE 3 Outcome analysis of the inclusion of clarithromycin in multibacillary leprosy treatment in the included studies.

References	Follow-up	Intervention	*N* cure	*N* total	Comparator	*N* cure	*N* total	RR (95% CI)*	Effect direction
Girdhar et al. (27)	6 months	Clarithromycin, rifampicin, ofloxacin and minocycline	117	149	Rifampicin, ofloxacin and minocycline	110	151	1.08 (0.95–1.23)	No difference
	12 months		133	149		135	151	1.00 (0.92–1.08)	
	18 months		133	145		140	148	0.97 (0.91–1.03)	
	24 months		128	140		126	135	0.98 (0.92–1.05)	
Ji et al. (30)	56 days	Clarithromycin and minocycline	11	11	Clarithromycin	12	12	1.00 (0.85–1.17)	No difference
					Minocycline	11	11	1.00 (0.85–1.18)	
Ji et al. (29)	30 days	Clarithromycin and minocycline, followed by a placebo	3	10	Clarithromycin, minocycline and ofloxacin, followed by placebo	2	10	1.50 (0.32–7.14)	No difference
					Rifampicin, clofazimine and dapsone	9	9	0.33 (0.14–0.80)	Favored the comparator
					Rifampicin followed by a placebo	10	10	0.33 (0.14–0.80)	Favored the comparator
					Dapsone and clofazimine	4	10	0.75 (0.22–2.52)	No difference

*RR (95% CI): Relative risk (95% confidence interval), measured by Review Manager version 5.4 software.

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TABLE 4 Relapse outcome analysis of the inclusion of clarithromycin in multibacillary leprosy treatment in the Girdhar et al. (27) study.

References	Follow-up	Intervention	*N* relapse	*N* total	Comparator	*N* relapse	*N* total	RR (95% CI)*	Effect direction
Girdhar et al. (27)	6 months	Clarithromycin, rifampicin, ofloxacin and minocycline	0	149	Rifampicin, ofloxacin and minocycline	0	151	Not estimable	No difference
	12 months		1	149		2	151	0.51 (0.05–5.53)	
	18 months		1	145		0	148	3.06 (0.13–74.55)	
	24 months		0	140		1	135	0.96 (0.06–15.26)	

*RR (95% CI): Relative risk (95% confidence interval), measured by Review Manager version 5.4 software.

3.3. Additional outcomes (clofazimine review and clarithromycin review)

Other outcomes, including bacteriological and morphological index reductions, were appraised for both reviews. Various adverse events were reported; nevertheless, these incidents were usually mild and did not significantly impact treatment feasibility. A detailed description of the other secondary outcomes is provided in Supplementary material B.

4. Discussion

The clofazimine and clarithromycin reviews showed no difference in the outcomes with the addition of clofazimine in PB leprosy treatment and the addition of clarithromycin in rifampicin-resistant leprosy treatment. The studies had methodological limitations, and the certainty of the evidence was very low. Thus, there is uncertainty about the new WHO recommendations for leprosy treatment.

Early diagnosis and treatment are among the most critical actions for leprosy control (8, 33). Treatment success depends on proper prescription of PB- or MB-MDT for 6 or 12 months, respectively, with a further distinction between adults and children. In addition, promoting, supervising and guaranteeing treatment adherence and preventing further reinfection, especially through the systematic assessment and follow-up of household contacts, are also crucial for leprosy control. At the end of MDT, clinical and bacilloscopic results are difficult to interpret since patients' reactional states can clinically worsen and bacillus depuration can be slow. Facing these difficulties, the determination of disease persistence or relapse becomes a challenging task for general physicians, making it impossible to rule out treatment failure and making further investigation of antimicrobial resistance mandatory (34).

The long-term MDT duration and the absence of precise criteria for cure evaluation reinforce that the treatment and follow-up of leprosy patients cannot be separated. Owing to the urgency to provide more effective and accessible therapies, in 2018, the WHO recommended the inclusion of clofazimine for PB patients and the inclusion of clarithromycin for patients with rifampicin resistance.

Considering the addition of clofazimine in PB leprosy treatment, the meta-analysis showed no significant difference in the clinical cure rates compared to the control treatment (dapsone and rifampicin) at the 6 and 12 month follow-ups. However, studies were considerably heterogeneous at the second time point. Only one study evaluated relapse (20) rates and showed that after 3.5 years of treatment, the inclusion of clofazimine in MDT for PB leprosy treatment was not different. Other outcomes, including adverse events, treatment adherence and patient satisfaction, were not different between the two types of PB-MDTs (Supplementary material B), and concerns regarding skin discolouration with clofazimine were discarded.

The inclusion of clofazimine has raised controversies and increased the costs of PB leprosy treatment (35). Although different treatment types for PB and MB leprosy still exist, the more similar the drugs are, the smaller the chance of relapse in MB leprosy patients wrongly identified as having PB leprosy. Moreover, the

treatment scheme simplification facilitated the logistic distribution since only two types of blister drug packs (adults and children) were needed. Studies that tested a 6 month MB-MDT showed that the cure rate is still relevant in some cases (36), although these results should be interpreted carefully. Indeed, it seems that the incorporation of clofazimine into PB-MDT is safe and may mitigate possible relapses resulting from the sometimes tricky differentiation between PB and MB leprosy (37).

The inclusion of clarithromycin in the leprosy treatment arsenal is an interesting option once this drug is proven to be effective against mycobacteria (29, 38, 39). Even though this alternative focuses on antimicrobial resistance to rifampicin, it can also be an option for WHO-MDT in terms of adverse reactions and drug interactions. Unfortunately, most studies have associated this macrolide with rifampicin, meaning that the role of clarithromycin as a possible replacement for rifampicin still needs to be determined. No differences in the assessed outcomes were observed in the various types of multidrug combinations with clarithromycin. The only study that compared clarithromycin with clofazimine and dapsone vs. WHO-MB-MDT showed no difference in the reduction in the bacteriological index after 3 months of therapy (28). However, only a few patients (seven in each group) were included. No safety or adherence issues were detected in the evaluation of the secondary outcomes.

The RoB evaluation is shown in detail in Supplementary material B. Considerable methodological limitations were found in the studies that evaluated the effectiveness and safety outcomes. These biases can lead to overestimation or underestimation of the effect of the intervention. Although an extensive search of the literature was performed, it was impossible to assess publication bias due to the limited number of studies. The strengths of this review, in addition to the careful literature search, were the rigorous process and the full compliance with a previously registered protocol. Finally, the assessment of the certainty of the body of evidence was performed judiciously using the GRADE approach.

The assessment of the certainty of the evidence for the primary outcomes considering the use of clofazimine in PB leprosy treatment was judged to be very low. A similar judgement was made considering the use of clarithromycin in leprosy patients, with the certainty of evidence classified as very low or low. Given this finding, it is possible to determine that there is little confidence in the effect estimate obtained and that the true effect is probably substantially different from the estimated effect. The results also point to the imprecision of studies available in the literature. Thus, new studies with a good methodological quality and an adequate sample size must be carried out to investigate the effect of the inclusion of clofazimine and clarithromycin in leprosy treatment, as recently recommended by the WHO.

5. Conclusion

The addition of clofazimine to PB leprosy treatment helps reduce the negative impact of misclassification with no additional apparent relevant side effects. Although new articles were published after the 2018 WHO recommendations, the effectiveness of this intervention and the inclusion of clarithromycin to substitute for rifampicin in the WHO-MDT still need to be determined. New clinical trials and investment in pharmacovigilance are essential for elucidating these topics.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

TM, SV, and CG: formal analysis, investigation, resources, supervision, validation, visualization, writing—original draft, and writing—review and editing. EA, JB, and GO: formal analysis, investigation, and resources. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1139304/full#supplementary-material

SUPPLEMENTARY MATERIAL A
Complete search strategy, the flow charts of study selection, and the tables of excluded studies, including the reasons for exclusion for both reviews.

SUPPLEMENTARY MATERIAL B
Detailed evaluation of all secondary outcomes included in the clofazimine review, clarithromycin review, and certainty of evidence analysis using the GRADE approach.

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Case report: Injected corticosteroids for treating leprosy isolated neuritis

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One of the main manifestations of leprosy is peripheral nerve impairment. Early diagnosis and treatment are important to reduce the impact of neurological impairment, which can cause deformities and physical disabilities. Leprosy neuropathy can be acute or chronic, and neural involvement can occur before, during, or after multidrug therapy, and especially during reactional episodes when neuritis occurs. Neuritis causes loss of function in the nerves and can be irreversible if left untreated. The recommended treatment is corticosteroids, usually through an oral regimen at an immunosuppressive dose. However, patients with clinical conditions that restrict corticosteroid use or that have focal neural involvement may benefit from the use of ultrasound-guided perineural injectable corticosteroids. In this study, we report two cases that demonstrate how the treatment and follow-up of patients with neuritis secondary to leprosy, using new techniques, can be provided in a more individualized way. Nerve conduction studies in association with neuromuscular ultrasound were used to monitor the response to treatment with injected steroids, focusing on neural inflammation. This study provides new perspectives and options for this profile of patients.

KEYWORDS

leprosy, ultrasound, corticosteroid, case report, neuritis

Introduction

Leprosy remains a public health problem despite efforts to eradicate it. The main manifestations are a result of the skin and peripheral nerve involvement. Neuritis may lead to nerve damage that can occur before, during, or after multidrug therapy (MDT), predominantly in multibacillary (MB) and borderline leprosy, and is commonly associated with type 1 reactions (1). It causes loss of function and can be irreversible if not properly treated. Neuritis has been defined as nerve inflammation, which presents as pain and nerve thickening in conjunction with sensory impairment, associated or not with signs of motor impairment (2–5). Leprosy was initially attributed to *Mycobacterium leprae* as the etiologic agent, but in 2008, *Mycobacterium lepromatosis* was described as a second causative species; however, there is still no consensus on its importance in the epidemiology of leprosy in humans (6). Although MDT targets the causative bacteria and promotes antigenic load reduction, corticosteroids play the most important role in the management of neuritis as

an anti-inflammatory therapy (7). The optimal dose and duration of corticosteroid therapy to treat neuritis is still a matter of debate. The World Health Organization recommends a standard regimen of 12 weeks to treat acute neuritis, starting with 40 mg of prednisolone, the dose of which is reduced over the following 12 weeks. Some studies recommend longer courses for the treatment of type 1 reactions (8). Other studies have compared treatment with 40 and 60 mg of prednisone and both regimens were found to be effective; however, most of the recurrences occurred within a 6-month period after completion of the low-dose regimen (9). Van Brakel et al. (10) concluded that improvement following treatment was directly related to the severity of the nerve damage observed at the beginning of treatment. In patients who did not have neuropathy prior to acute neuritis, steroid treatment resulted in full recovery in 88% of nerves with neuropathy, but only 51% of those with chronic disease or recurrent neuropathy recovered nerve function (11).

Nevertheless, it is known that prolonged therapy and/or high doses of corticosteroids result in a high frequency of side effects, such as arterial hypertension, dysglycemia, skin rashes, and Cushing's syndrome (12, 13). Another treatment option in this patient profile is the use of high-dose intravenous methylprednisolone in the pulse therapy regimen, which can reduce the occurrence of side effects (14). Local corticosteroid injection is a common non-surgical treatment for carpal tunnel syndrome (CTS). Several studies have shown that local corticosteroid injection provides significantly greater clinical improvement of CTS than oral steroids up to 3 months after treatment, as well as an improvement in symptoms compared with a single systemic injection at 1 month follow-up (15, 16). Dammers et al. (16) used a short-acting injectable corticosteroid, 40 mg of methylprednisolone, with 10 mg of lidocaine, for the treatment of CTS. In this study, we report two cases attended at the Ambulatory Souza Araújo (ASA) Leprosy Outpatient Clinic (Oswaldo Cruz Institute—IOC, Fiocruz), a Leprosy Reference Center, in Rio de Janeiro, Brazil, in which corticosteroid injections were used for the management of focal leprosy neuritis.

Case description

The two cases of the present study were diagnosed with leprosy according to the criteria of Ridley and Jopling (17) and were subsequently treated with MDT. They were evaluated by a dermatologist and a neurologist throughout the treatment.

Case 1

A 24-year-old male resident of the metropolitan region of Rio de Janeiro, Brazil, was referred to the Leprosy Outpatient Clinic by the primary care service on account of diffuse skin lesions over the body, which began 10 months previously. Past medical history: yellow fever 4 years previously, was a non-drinker and non-smoker, and said he did not use medications regularly. He said there was no history of leprosy in his family. A dermatological evaluation identified >20 diffuse lesions over the body (face, upper limbs, lower limbs, and trunk) in the form of papules, nodules, and tubercles. A Mitsuda test and bacilloscopy were requested, which were positive (5 mm) and 5.25, respectively, and MDT for MB leprosy was started after classification of the borderline lepromatous form (BL/LL). The patient was assessed by a neurologist at the beginning of treatment despite not having neurological symptoms, and a neurological examination did not show nerve thickening or sensory or motor changes.

After 4 months of MDT, the patient returned to the neurologist claiming he had been suffering from paresthesia in the fourth and fifth fingers of the left hand for ∼1 month. On examination, the patient had pain upon palpation in the region above the left elbow and thickening of the ulnar nerve, associated with tactile hypoesthesia, thermal and pain insensitivity, and grade 4 muscle weakness according to the Medical Research Council (MRC) Scale (18) in the hypothenar region (little finger abductor and first dorsal interosseous muscles). A nerve conduction study (NCS) showed an absence of sensory nerve action potentials (SNAPs) in the left ulnar nerve and a reduced amplitude of compound motor action

TABLE 1 Left ulnar nerve conduction values at the first and control NCS assessment.

Site	Latency (ms)	Amplitude	Segment	Distance	NCV (m/s)
ULNAR L (M)					
First NCS					
Wrist	2.76	1.49 mV	Wrist		
Below elbow	7.71	1.13 mV	Wrist- Below elbow	230 mm	46.5
Above elbow	9.69	770.00 μV	Below—Above elbow	110 mm	55.6
Arm	14.94	650.00 μV	Above elbow—Arm	110 mm	21.0
Control NCS					
Wrist	3.75	3.75 mV	Wrist		
Below elbow	9	3.46 mV	Wrist- Below elbow	240 mm	45.7
Above elbow	11.49	3.02 V	Below—Above elbow	90 mm	36.1
Arm	13.53	3.26 mV	Above elbow—Arm	90 mm	44.1
ULNAR L (S)					
5 finger wrist	0	0 mV	5 finger wrist	120	0

L, left; S, sensitive; M, motor; NCV, nerve conduction velocity; ms, millisecond; mV, millivolt; μV, microvolt; mm, millimeter.

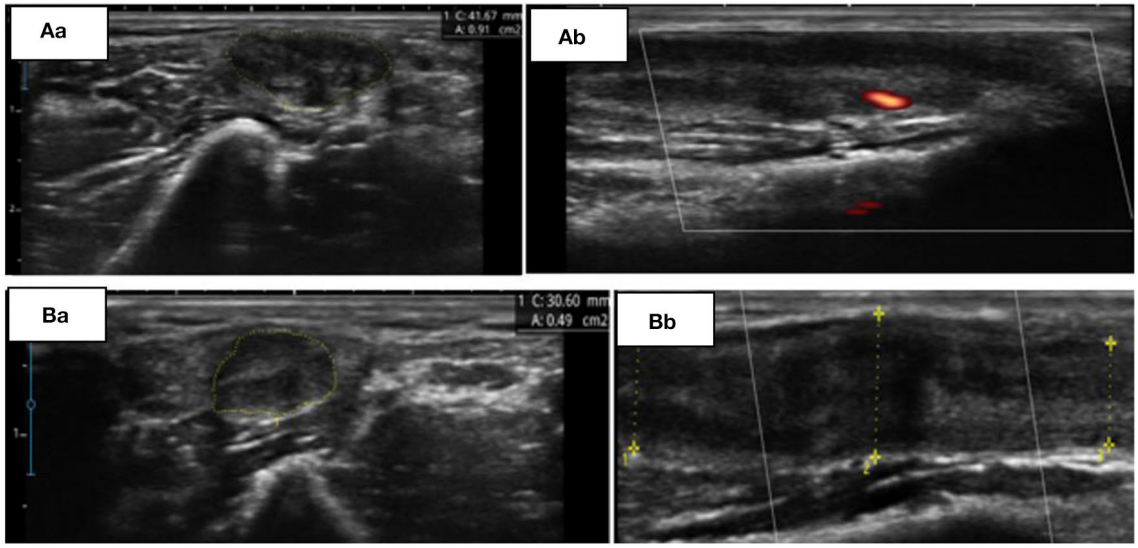

FIGURE 1
(A) Initial findings from ultrasonography (USG). Transverse **(Aa)** and longitudinal **(Ab)** view of the left ulnar nerve at the supraepicondylar region. **(B)** Control USG, 60 days later. Transverse **(Ba)** and longitudinal **(Bb)** view of the same nerve at the supraepicondylar region.

potentials (CMAPs) with a conduction block and reduced motor speed in the elbow segment (Table 1). Other nerves did not show alterations upon clinical examination and an NCS. Acute neuritis of the left ulnar nerve type 1 reaction was diagnosed.

The neuromuscular ultrasound (NMUS) evaluation showed marked ulnar thickening, measuring 24 mm^2 at the epicondylar level and 92 mm^2 at the supraepicondylar level [reference value (RV): 8 mm^2], as well as homogeneous fascicular hypoechogenicity and vascular flow on the Power Doppler (Note: Power Doppler is more sensitive than the Color Doppler for detecting blood flow, but it does not provide information on the direction of flow). Ultrasound-guided local injection of 40 mg methylprednisolone with lidocaine was performed every 2 weeks above the elbow groove where the ulnar nerve was most damaged.

The patient was followed up in neurological appointments every 2 weeks, and after 2 months, the patient was reassessed and there was no complaint of paresthesia or pain upon neurological examination, an MRC grade 5 was determined in all muscles, and a subjective reduction in neural thickening was noted. An NCS was repeated within 3 months after the start of corticosteroid therapy and an improvement in electrophysiological values was observed (Table 1). NMUS showed an improvement in echogenicity and fascicular disarray, a 50% reduction in the cross-sectional diameter at the two levels described above, and no flow in the Power Doppler (Figure 1).

Case 2

A 52-year-old female resident of the metropolitan region of Rio de Janeiro, Brazil, was referred to the Leprosy Outpatient Clinic by the primary care service because of skin lesions and neuropathic pain in her hands for the last year. The patient was under endocrinological follow-up because of insulin-dependent diabetes, for which she was using neutral protamine Hagedorn (NPH) and regular insulin (according to blood glucose measurements) with good control. The patient was dyslipidemic, a smoker (35 packs/year), a non-drinker, and had previous contact with a brother that had leprosy. She said there was no history of leprosy in her family. Physical and dermatological examination showed 11–20 well-defined, erythematous, and hypochromic lesions, with a hypoesthetic lesion in the left upper limb. Bacilloscopy was negative and skin biopsy revealed epithelioid granuloma in the dermis compatible with a reversal reaction (type 1). MDT for paucibacillary (PB) leprosy was started after it was classified as borderline tuberculoid (BT). At this time, the neurological assessment showed bilateral thickening of the ulnar nerves at the level of the elbow, thermal and painful hypoesthesia in the right ulnar nerve, and tactile hypoesthesia and thermal and pain insensitivity in the left ulnar nerve. Furthermore, muscle weakness (MRC grade 4) in the left hypothenar muscles (little finger abductor and first dorsal interosseous muscles) was observed. An NCS was performed, which demonstrated evidence of myelin lesions with secondary axonal involvement in both ulnar nerves. Ultrasonography (USG) showed ulnar nerves with thickening in the epicondylar and supraepincodylar regions and with homogeneous fascicular hypoechogenicity, as well as Power Doppler flow of the right and left ulnar nerves. There was no involvement of other neural territories observed in the initial clinical evaluation, NCS, or ultrasound. Bilateral ulnar neuritis was diagnosed and treatment with oral corticosteroids (1 mg/kg/day) was initiated, with a dose reduction of 0.1 mg/kg/day every 2 weeks until a dose of 0.5 mg/kg/day was reached, after which monthly weaning was performed until withdrawal. The total treatment time was 6 months. During this period, no significant side effects were observed, especially regarding dysglycemia, as the insulin adjustment and control were being monitored by a multidisciplinary team.

TABLE 2 Right median nerve conduction values at the first and control (gray column) NCS assessments.

Site	Latency (ms)	Amplitude (mV)	Distance (mm)	NCV (m/s)	Latency (ms)	Amplitude (mV)	NCV (m/s)
Median R (M)							
Wrist	3.3	6.0			3.27	6.81	
Forearm	4.4	3.2	60	54.1	4.68	4.39	35.5
Elbow	9.9	3.0	180	32.6	10.53	3.72	17.1
Arm	11.1	3.0	90	78.9	12.33	4.07	61.1
Median R (S)							
Site	Latency (ms)		Amplitude		Segment	Distance	NCV (m/s)
3 finger wrist	0		0 mV		3 finger wrist	140	0

R, right; S, sensitive; M, motor; NCV, nerve conduction velocity; ms, millisecond; mV, millivolt; μV, microvolt; mm, millimeter.

The patient was reevaluated with no signs of spontaneous pain or paresthesia, and objective sensory and motor findings were maintained. Electrophysiological examination and USG showed improvements in the ulnar parameters after 6 months.

During the follow-up of ulnar neuritis, a neurological examination suggested the involvement of the right median nerve, which was confirmed by an NCS, wherein a conduction block in the right median nerve of the forearm was observed (Table 2). USG revealed thickening of the median nerve from the level of the middle third of the pronator quadratus muscle up to the main branch of the palmar terminal branches, with a cross-sectional area 2 cm from the carpal tunnel of 32 mm^2 and 18 mm^2 at the carpal tunnel (RV: 9 mm^2), and Power Doppler flow detectable in the carpal region (Figure 2). An increased signal in those regions was detected by MR neurography of the median nerve with uptake by the intravenous contrast; there were no signs of nerve compression.

The diagnosis of right median neuritis was made when the patient was finishing the oral prednisone treatment for ulnar neuritis. Therefore, the patient was started on 40 mg methylprednisolone and lidocaine injected around the perineurium of the median nerve over 3 months. She underwent an NCS after this period, which showed improvement in the nerve conduction values (Table 2). Additionally, there was an improvement in the USG results in terms of the nerve thickening and absence of flow on the Power Doppler. The patient remains in control of the neuropathic pain through quarterly assessments by the neurology team.

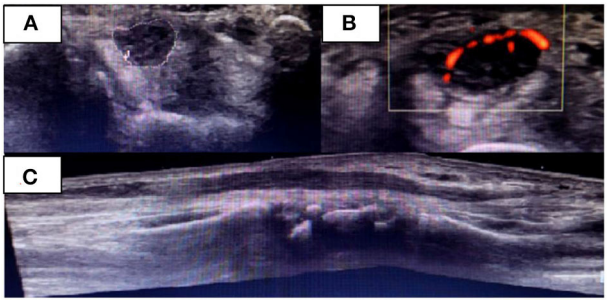

FIGURE 2
Right median nerve on the initial USG examination when median neuritis was diagnosed. Transverse **(A, B)** and **(C)** longitudinal nerve view in the carpal region. Presence of flow in the Power Doppler **(B)**.

Discussion

The clinical form of leprosy depends on the host's immune response to *M. leprae* antigenic determinants (19, 20). The evolution of neurological manifestations in leprosy is related to the clinical forms and the leprosy reactions.

Acute neuritis in MB patients has been described; however, in general, there is little inflammatory response in MB and the symptoms evolve slowly, generating a progressive symmetrical polyneuropathy (19–21), like the neuritis that occurs by complement activation (22). Additionally, silent neuritis has been described and can occur in reactions without clinical manifestations. By contrast, in PB patients, the neural involvement is more limited, occurring as a mononeuropathy or mononeuritis multiplex; patients with borderline forms are the ones who most frequently have neurological damage and complications, as they have an unstable immune response (19–21). The patient of case 2 was PB, diagnosed as having borderline leprosy, and presented median neuritis with the additional involvement of the ulnar nerve, evidencing this immunological instability that generates edema and neural compression above the entrance to the carpal tunnel.

High-resolution USG has been used to evaluate peripheral nerves. Nerves are often enlarged in leprosy patients, especially those with type 1 reactions. Lugão et al. (23) noted that the greater the thickness of the nerve, the greater the flow on the Power Doppler. It is likely that increased blood flow is the first sign of neural injury (24). In the two reported cases, it was possible to see a clear loss of fascicular morphology and neural hypoechogenicity with significant thickening in the affected nerves. The presence of flow on the Power Doppler raises suspicion of the presence of nerve inflammation, and associated with the clinical and electrophysiological findings, neuritis can be diagnosed.

The median nerve of case 2 showed significant thickening proximal to the carpal tunnel (~55% greater than the thickening in CTS) with flow on the Power Doppler. These findings are similar to the USG changes of the median nerve reported in other studies of patients with leprosy. In these studies, morphological changes and fusiform neural thickening occurring 2–5 cm from the wrist were

observed, which is different from what occurs in patients with CTS (25, 26).

Patients diagnosed with leprosy may have to receive high doses of corticosteroids, and perhaps even more than once in cases of recurrent neuritis (1). The use of systemic corticosteroids is limited in patients with comorbidities and other clinical conditions, such as diabetes mellitus, cataracts, hypertension, and immunosuppressed patients. Accordingly, steroids administered at the specific point of neuritis, guided by NCS and ultrasound, can be a safe and successful strategy in these patients. An anesthetic and corticosteroid solution injected around the nerve promotes the hydrodissection mechanism and release of the anti-inflammatory and perineural analgesic medication (27–29). This therapeutic modality may be a promising alternative in cases of leprosy-isolated neuritis.

The use of injectable medications has arisen and evolved with the improvement in clinical, NSC, and imaging parameters, and may be useful for leprosy-isolated neuritis in particular. It is expected that further studies will be carried out on this method to be able to offer these patients more treatment options with the aim of reducing the definitive neurological deficits.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

CS, MJ, and NV: conceived the study. CS, IP, LA, and AS: performed the review of medical records. CS and MJ: analyzed the data. CS: wrote the paper and performed radiology analysis. CS, MJ, IP, LA, and AS: performed clinical evaluation. CS, MJ, IP, and LA: performed neurological and electrophysiological testing. MJ: performed several edits of the draft manuscript and supervised neurological testing. NV: performed procedures. MJ and NV: contributed to the writing of the paper. ES and RP: technical and operational support. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Leprosy among children in an area without primary health care coverage in Caratateua Island, Brazilian Amazon

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Introduction: The detection of leprosy in children is an important epidemiological marker of the disease, indicating the community's early exposure to *Mycobacterium leprae* and active transmission of the infection.

Methods: In order to detect new cases among children by combining clinical evaluation and laboratory tests, we conducted an active case finding among individuals under 15years old on Caratateua Island, located in the city of Belém, in the Pará state, an endemic region in the Amazon. Dermato-neurological examination, collection of 5mL of peripheral blood for IgM anti-PGL-I antibody titration, and intradermal scraping for bacilloscopy and amplification of the specific RLEP region by qPCR were performed.

Results: Out of the 56 examined children, 28/56 (50%) new cases were identified. At the time of evaluation, 38/56 (67.8%) children presented one or more clinical alterations. Seropositivity was detected in 7/27 (25.9%) new cases and 5/24 (20.8%) undiagnosed children. DNA amplification of *Mycobacterium leprae* was observed in 23/28 (82.1%) of new cases and in 5/26 (19.2%) of non-cases. Out of the total cases, 11/28 (39.2%) were exclusively diagnosed by clinical evaluation performed during the active case finding. Seventeen new cases (60.8%) were detected considering the clinical alterations found in addition to positive results for qPCR. In this group, 3/17 (17.6%) qPCR-positive children presented significant clinical changes 5.5months after the first evaluation.

Discussion: Our research detected a number of cases 5.6 times higher compared to the total number of pediatric cases recorded throughout the year 2021 in the municipality of Belém, which shows a critical scenario of underdiagnosing of leprosy among children under 15years old in the region. We propose the use of qPCR technique to identify new cases among children with oligosymptomatic or early disease in endemic areas, in addition to the training of Primary Health Care professionals and the implementation of the Family Health Strategy coverage in the visited area.

KEYWORDS

leprosy, children, RLEP qPCR, anti-PGL-I, active case finding

1. Introduction

Leprosy is an infectious disease that affects mainly peripheral nerves and skin, but also internal organs and the eyes, caused by the *Mycobacterium leprae* bacillus, discovered 150 years ago by the Norwegian doctor Gerhard Armauer Hansen, and by the *Mycobacterium lepromatosis*, described more recently as the foremost causative agent of leprosy in Mexico (1). Despite being one of the oldest known diseases, leprosy continues to affect thousands of people around the world annually, which is related to chronic and historical problems such as lack of diagnosis, misinformation, and social stigma (2).

It is known that the diagnosis of leprosy in childhood, in particular, is closely linked to active transmission foci of the disease in endemic areas (3). In 2021, approximately 9,502 cases among children were reported worldwide, representing a rate equivalent to 4.5 cases per million children (4). In view of these data, one of the main goals proposed by the Global Leprosy Strategy (2021–2030), devised by the World Health Organization (WHO), is to reduce the rate of pediatric leprosy cases by 90% per million children by 2030 (5). However, such a significant reduction goal could also increase underreporting of the disease and hide the true epidemiological scenario of leprosy in the pediatric population.

In Brazil, leprosy continues to be a significant public health problem, affecting children, adults, and elderly individuals of both sexes. Alongside India and Indonesia, Brazil is one of the countries that detects the most cases of the disease in the world. Preliminary data from the Ministry of Health shows that, in 2022, the country recorded 14,962 new cases, with 645 (4.3%) among children under 15 years of age. Pará, located in the Brazilian Amazon and one of the states with the highest number of cases detected per year, diagnosed 1,135 individuals with the disease, considered a highly endemic state, with 13.4 cases per 100,000 inhabitants (6, 7). Among these records, 58 (5.1%) were observed in children under 15 years of age (7).

Belém, the capital of the state of Pará, recorded 158 new cases of leprosy in 2021, of which 5 (3.1%) were detected in children under 15 years of age (8). The Island of Caratateua, also known as Outeiro Island, is located in the city of Belém and is home to approximately 80,000 inhabitants, serving as the headquarters of one of the eight districts of the municipality: the Outeiro Administrative District. This island is an impressive example of explosive population growth, as in 1970 it had only about 1,000 inhabitants, a number that jumped to 15,000 in 1990 and 35,000 in 2010, according to the demographic censuses carried out in the country. With current estimates, the population is around 80 times larger than it was just over 50 years ago (9). Currently, the island represents an important tourist spot in the city, however, it is still characterized by having a socioeconomically vulnerable population (7).

Among the main strategies for combating the disease advocated by the Ministry of Health and WHO is active case finding, which enables the detection of leprosy and immediate treatment of affected individuals (5, 7, 10). Studies have revealed the importance of case screening among schoolchildren as an important mechanism for interrupting the infection transmission chain (11, 12). However, some challenges hinder the establishment and advancement of these, and other strategies related to the disease, including the diagnosis itself. As the diagnosis is predominantly clinical, detecting the disease requires a specialized and experienced professional who recognizes the signs and symptoms that define leprosy. Identifying these characteristics among children can be even more challenging, even among leprologists, given that in many cases, school-age patients do not present a suggestive and evident disease profile, especially in the initial stage. Because they are pediatric patients, the leprologist may also face difficulties in conducting the clinical evaluation of the peripheral nerves (11).

Laboratory techniques have been developed over the past few decades to improve the diagnosis and monitoring of leprosy. Immunological/serological tools, for example, are based on the detection of specific components such as anti-PGL-I antibodies (phenolic glycolipid-I) or the measurement of IFN-γ by T cells of the immune system (13, 14). Previous studies have demonstrated the importance of anti-PGL-I IgM in the serodiagnosis and pathogenesis of leprosy, including the correlation between seropositivity and an increased probability of developing the disease in hyperendemic regions (15, 16).

Another important technique is Real-Time PCR or qPCR (Quantitative Polymerase Chain Reaction), which allows for the amplification of *M. leprae* genetic material in clinical samples. Previous research has demonstrated the efficiency of this method in detecting the pathogen's DNA among sick individuals (17–20). These findings have made qPCR a promising tool for the diagnosis of the disease, considering its high sensitivity and specificity. It is also important for the diagnosis of difficult cases, such as paucibacillary patients, individuals with atypical clinical manifestations or primary neural cases (17, 21). The gene regions used as targets for the method include the RLEP (*M. leprae-specific repetitive element*), rpoT, Sod A, and 16 s rRNA genes, with the first mentioned target being the most sensitive in relation to the others according to previous studies (18, 22).

Therefore, the objectives of this study were: (a) to detect leprosy cases among children under 15 years of age on an island located in an endemic area in the Brazilian Amazon, and (b) to use the qPCR technique in association with clinical examination to define new cases among evaluated children.

2. Materials and methods

2.1. Study area

Pará is a state in the northern region of Brazil that is home to an estimated population of 8.7 million people. It is the second-largest state in the country, with an area of 1,245,870.700 km^2. Belém, the capital of the state, has approximately 1.5 million inhabitants (23), and includes about 39 islands under its administration. Caratateua Island, which has an area of 3.17 hectares, is located 18.8 km from the center of the capital and is characterized by precarious urbanization and higher population density compared to most of the city's islands. The active case finding of the present study was carried out in an after-school program for low-income children conducted in a facility that provided extracurricular activities and meals to enrolled children, located in a neighborhood (Brasília) without coverage of the Family Health Strategy, an important model integrated into Primary Health Care in Brazil, which provides assistance to families through multidisciplinary teams and healthcare services.

2.2. Population and study design

We conducted an active case finding, whose target population consisted of children between 6 and 14 years old enrolled in an after-school program. During the study period, 82 students were enrolled in the location. The active case finding was carried out by a multidisciplinary team in July 2022, with the target population recruited by the program's coordinator, with authorization from the responsible parties. To participate in the study, children were required to be under 15 years old, regularly enrolled in the after-school program, and have written consent from their guardians. The participants underwent a dermato-neurological examination by a leprologist and the collection of 5 mL of peripheral blood for the titration of IgM anti-PGL-I antibodies by ELISA (*Enzyme-Linked Immunosorbent Assay*), as well as an intradermal scraping of the auricular lobes for bacilloscopy and amplification of the specific RLEP region by qPCR.

2.3. Clinical diagnosis

The clinical diagnosis of leprosy was made by a leprologist, based on the recommendations advocated by the World Health Organization, which correspond to the observation of (a) dermatological lesions (hypopigmented or reddish) with loss of sensation and/or (b) thickened or enlarged peripheral nerve with loss of sensation (with or without weakness of the muscles supplied by that nerve); in addition to this, (c) the presence or absence of alcohol-acid resistant bacilli in an intradermal scraping smear was also taken into consideration (24). The simplified modified dermato-neurological assessment form was used to analyze the integrity of neural function, which includes inspection, palpation/percussion, evaluation of sensitivity function and muscle strength associated with nerves, according to the Ministry of Health guidelines (25).

The diagnosed cases were reported, and socioeconomic data were collected through a standardized electronic questionnaire contained in the Hansys software, a system developed by the team at the Dermato-Immunology Laboratory (UFPA) in partnership with the Federal University of West Pará (UFOPA). Individuals registered as cases were notified and referred for treatment with multidrug therapy (MDT) at the nearest health facility.

2.4. Laboratory procedures

Samples of intradermal scrapings from the earlobes were fixed on slides for the bacilloscopy technique and also added to microtubes containing 70% alcohol, which were kept at room temperature for total DNA extraction. The fixed slides were subjected to Ziehl-Neelsen staining adapted for the identification *M. leprae*. The bacterial load and bacillary and morphological indices were calculated according to the guidelines of the Ministry of Health (26). For total DNA extraction, the protocol recommended by the manufacturer (Qiagen DNeasy Blood and Tissue kit, Germantown, MD, United States) was used. Amplification of the RLEP region was performed according to the protocol described in a previous study, using primers LP1 (5′-GTGAGGGTAGTTGTT-3′) and LP2 (5'-GGT GCGAATAGTT-3′) (27).

The collected blood samples were refrigerated at 2° - 4° C, and later centrifuged to obtain the plasma used in the ELISA test. The titration of IgM anti-PGL-I antibodies was performed according to a previously described protocol (28). The cut-off for seropositivity was defined by an optical density (OD) equal to 0.295, based on the mean plus three times the standard deviation of healthy individuals from the same endemic area, according to the protocol described in the cited study. The processing of biological samples was carried out at the Dermato-Immunology Laboratory located in Marituba, Pará.

2.5. Statistical analysis

The Mann–Whitney test (U) was performed to compare the anti-PGL-I antibody titers between study groups. The Pearson Chi-Square test and Fisher's exact test were used to analyze categorical variables. Results were considered significant when $p < 0.05$. Statistical analysis and graphing were performed using GraphPad Prism software version 6.0.

2.6. Ethical process

This study was approved by the Institute of Health Sciences Research Ethics Committee from Pará Federal University (CAAE 26765414.0.0000.0018 CEP-ICS/UFPA). The participants and their guardians signed the Informed Consent Form, authorizing the conduct of the activity.

3. Results

3.1. Active case finding among children under 15years Old

Fifty-six out of 82 (68%) children enrolled in after-school program were evaluated during the active case finding. Among the participants, 29/56 (51.7%) were male, and 50/56 (89.2%) were brown, a self-declared color of skin or ethnic-racial identification, as required by Brazilian laws. Previous DNA studies show a mixed European-Amerindian-African contribution in the formation of Belém population (29). The evaluated children had a mean age of 8.94 years old (±2.28). During the assessment, 28/56 (50%) new cases were diagnosed, of which 15/28 (53.5%) were female, 26/28 (92.8%) were brown, and had a mean age of 9.25 years old (±2.23). Prior contact with individuals diagnosed with the disease was reported in 4/28 (14.2%) of the cases. The presence of the Bacille Calmette-Guérin (BCG) vaccine scar was recorded in 27/28 (96.5%) of the diagnosed children. There were no statistically significant differences observed in the analysis of the variables sex, skin color, presence of BCG scar, and living with leprosy cases (Table 1).

Regarding the diagnosis, primary neural leprosy (i.e., without dermatological lesions, but with involvement of peripheral nerves) and borderline leprosy characterized the clinical form of 20/28 (71.4%) and 8/28 (28.6%) cases, respectively. Ten (35.8%) of the 28 new cases were detected with grade 1 disability and none were detected with grade 2 (Table 2). Six (75%) out of 8 cases classified as borderline leprosy had grade 1 of physical disability. The clinical

TABLE 1 Epidemiological characteristics of children diagnosed with leprosy.

Characteristics	n/n (%)	*p value*
Sex		**0.593**
Female	15/28 (53.5)	
Male	13/28 (46.5)	
Age range		
6–8	11/28 (39.2)	
9–11	12/28 (42.8)	
12–14	5/28 (12.0)	
Race/color		**0.669**
Brown	26/28 (92.8)	
Black	1/28 (3.6)	
White	1/28 (3.6)	
BCG scar		**0.101**
Presence	27/28 (96.5)	
Absence	1/28 (3.5)	
Living with leprosy cases		**0.669**
Yes	4/28 (14.2)	
No	24/28 (85.8)	

The bold numbers are the *p* values (all non-significant) verified by fisher exact test.

TABLE 2 Clinical characteristics of children diagnosed with leprosy.

Characteristics	n/n (%)
WHO operational classification	
Multibacillary	28/28 (100)
Paucibacillary	0/28 (0)
Clinical form	
Primary neural	20/28 (71.4)
Borderline	8/28 (28.6)
Disability grade	
Grade 0	18/28 (64.2)
Grade 1	10/28 (35.8)
Grade 2	0/28 (0)

alterations identified among the new cases can be seen in Table 3. The radial and superficial fibular nerves were altered in 12/28 (42.8%) of the new cases. In addition, loss of muscle strength was observed in 10/27 (37%) cases, with the ulnar nerve being the main affected nerve (37%). Sensory evaluation identified plantar sensitivity alteration in 14/27 (51.8%) of the cases. In 8/28 (28.6%) of the diagnosed children, hypochromic macules with regions of hypoesthesia and/or anesthesia were observed.

3.2. Laboratory results

Figure 1 illustrates the laboratory techniques performed, the quantity of collected samples, and overall results. Considering that some children did not allow blood sample or intradermal scraping collection, the number of samples varied compared to the total number of study participants. Priority was given to qPCR testing over

TABLE 3 Overview of the clinical alterations presented by children diagnosed with leprosy.

Clinical examination	Nerve/region examined	With alteration n/n (%)
	Upper limbs	
Inspection/palpation of nerve	Auricular	4/28 (14.3)
	Radial	12/28 (42.8)
	Ulnar	8/28 (28.6)
	Median	2/28 (7.1)
	Lower limbs	
	Tibial	11/28 (39.3)
	Commom fibular	5/28 (17.9)
	Superficial fibular	12/28 (42.8)
	Upper limbs	
Evaluation of muscle strength*	Radial	2/27 (7.4)
	Ulnar	10/27 (37.0)
	Median	4/27 (14.8)
	Lower limbs	
	Fibular (Extension)	2/27 (7.4)
	Fibular (Dorsiflexion)	0/27 (0)
	Upper limbs	
Sensory evaluation*	Hands	0/27 (0)
	Lower limbs	
	Feet	14/27 (51.8)

*One individual in the group was not submitted to this evaluation.

bacilloscopy testing for intradermal scraping samples from individuals for whom collection was challenging.

Table 4 shows the correlation between the results obtained for anti-PGL-I IgM titration and qPCR amplification of the specific RLEP region. Of the total number of evaluated individuals who underwent serological testing, 12/51 (23.5%) were seropositive, including 7/27 (25.9%) new cases and 5/24 (20.8%) non-cases. Figure 2 shows the distribution of anti-PGL-I IgM levels between both groups. The mean OD between new cases and non-cases corresponded to 0.222 and 0.128, respectively. There was no statistical difference between the groups.

Fifty-four (96.4%) children underwent intradermal scraping collection for qPCR technique and 49/56 (87.5%) also collected material for the bacilloscopy technique. In total, 28/54 (51.8%) individuals tested positive for qPCR, including 23/28 (82.1%) of new cases, which presented an average *ct* (*cycle threshold*) equal to 40.9 cycles (Figure 3). Among non-cases, 5/26 (19.2%) showed positivity for the technique. Double positivity for serological and molecular methods was detected in 6/27 (22.2%) of cases and 1/24 (4.1%) of non-cases. None of the examined children tested positive for the bacilloscopy method.

3.3. Combination of clinical and laboratory aspects

An overview of the application of the qPCR technique for the diagnosis of new cases can be seen in Figure 4. Among the evaluated children, 38/56 (67.8%) presented at least one clinical alteration

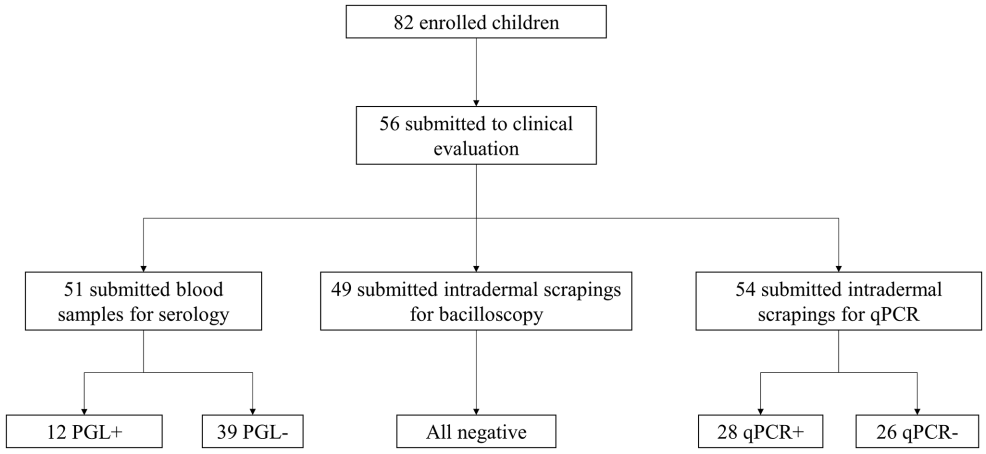

FIGURE 1
Diagram of conducted laboratory examinations, number of samples collected per test, and overall results.

TABLE 4 Correlation between anti-PGL-I IgM titration and qPCR amplification of the RLEP region.

	Anti-PGL-I IgM		qPCR RLEP		PGL/qPCR
	Positive	Negative	Positive	Negative	Double positivity
	n/n (%)	n/n (%)	n/n (%)	n/n (%)	n/n (%)
New cases	7/27 (25.9)	20/27 (74.1)	23/28 (82.1)	5/28 (17.9)	6/27 (22.2)
Non-cases	5/24 (20.8)	19/24 (79.2)	5/26 (19.2)	21/26 (80.2)	1/24 (4.1)
Total	12/51 (23.5)	39/51 (76.5)	28/54 (51.8)	26/54 (48.2)	7/51 (13.7)

during the evaluation. The clinical alterations varied from the unique presentation of pain upon palpation of a peripheral nerve to the loss of muscle strength combined with hypochromic macules and altered sensitivity. Of this group, 20/37 (54%) showed positivity for the qPCR technique. At the time of clinical evaluation by the leprologist, 11/56 (19.6%) children were diagnosed with the disease, of which 6/11 (54.5%) were positive for the qPCR technique. The remaining diagnosed cases, which correspond to 17/28 (60.7%), were defined based on the presence of one or more clinical alterations recorded in the evaluation, added to a positive result in the qPCR technique. Three of these 17 patients (17.6%) did not present clinical alterations during the active case finding carried out in July 2022, and suggestive alterations of leprosy were found approximately 5.5 months after the first dermato-neurological examination, during a reassessment.

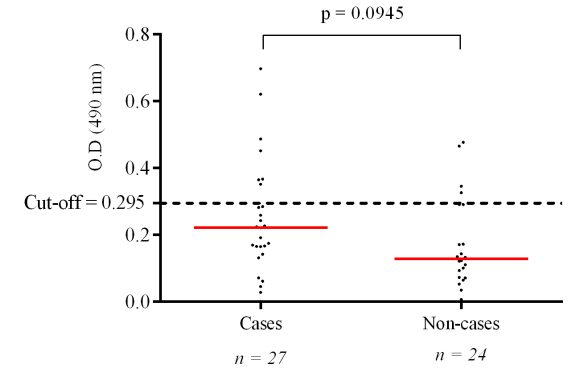

FIGURE 2
Titration of IgM anti-PGL-I antibodies among children under 15years old diagnosed and not diagnosed with leprosy. The *p* value was calculated using the Mann–Whitney test for comparison of two unpaired groups with the aid of GraphPad Prism 6 software.

4. Discussion

Early detection and treatment are essential strategies to break the transmission chain of leprosy, with case finding in the community being the ideal way to achieve them. The present active case finding was carried out after a request from the coordination of an after-school program due to the observation of children with suggestive dermatological lesions and prior knowledge of leprosy cases in families in the region. The Island of Caratateua, like the other inhabited islands in the capital of Pará, is characterized by having a socioeconomically vulnerable population, with the after-school program being a place of shelter and offering extracurricular activities mainly aimed at children from low-income and at-risk families. Historically, the area has experienced intense disorderly occupation by people looking for permanent or holiday housing, which has resulted in substandard housing (9, 30, 31).

During the active search action, 28 out of 56 children under 15 years old were diagnosed with leprosy. This number is 5.6 times higher than the number of pediatric cases registered throughout the year 2021 in Belém, revealing the huge underdiagnosing of the disease

in the municipality, which is classified as highly endemic, with 10.49 cases per 100,000 inhabitants (7, 8).

Previous studies by our research group have demonstrated the hidden prevalence of leprosy cases in hyperendemic municipalities in the state of Pará (12, 18, 27, 32, 33). It is believed that the data found in this research may still be underestimated, given that 67.03% of the population of Belém remains without coverage of primary care services, which is responsible for conducting essential actions to fight leprosy, such as active case finding and assistance to people affected by the disease (33–35). The area where the active case finding was conducted, in particular, is not covered by the Family Health Strategy, the priority model of Primary Health Care in the country (36). In addition, the Covid-19 pandemic has further exacerbated the concerning scenario of underdiagnosing of leprosy in Brazil and worldwide, by hindering patients' access to the health services and consequently negatively impacting the number of new diagnoses (4).

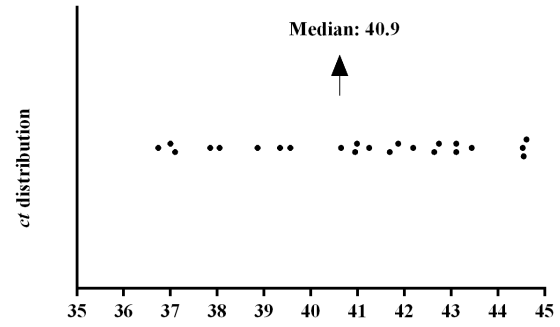

FIGURE 3
Distribution of *ct* (*cycle threshold*) among 23 qPCR-positive children diagnosed with leprosy in active case finding.

It was observed that 10 out of 28 (35.8%) cases had grade 1 physical disability in this study. One of the main characteristics of leprosy is its long incubation period (between 2 and 5 years), which can reach decades in some cases, resulting in a diagnosis that is predominantly made in adults. This can lead to the mistaken idea that diagnosis in children corresponds to early diagnosis. The presented data show that a significant portion of cases was diagnosed late, considering that the detection of physical disabilities is closely linked to delay in leprosy detection. The WHO Global Leprosy Strategy (2021–2030) proposes as a priority to reduce to zero the number of new pediatric patients with physical disabilities by 2030, which will require effective strategies of epidemiological surveillance aimed at early diagnosis and contact tracing in endemic areas (5).

Late diagnosis among children can be multifactorial, including the inability of health professionals to adequately detect the disease. In addition to this fact, a study conducted in tertiary hospitals in India observed that among the main risk factors associated with delayed diagnosis (represented by physical disability and/or positive bacilloscopy), socioeconomic vulnerability was a factor that increased the possibility of delayed diagnosis by 6 times (37). Socioeconomic aspects are deeply associated with the higher risk of development and progression of leprosy, and these factors can also be attributed to the high number of new cases found and the detection of physical disabilities in more than 30% of children diagnosed during this study (38, 39). In addition to facing the disease, patients diagnosed with leprosy are often subjected to social discrimination, historically linked to the infection (2). Regarding leprosy in childhood, deprivation of education, bullying, and rejection due to stigma can occur (40), especially among children with visible disabilities caused by the disease, however, the lack of studies on this aspect makes it difficult to analyze the real impact of the diagnosis on the social life of pediatric patients (41).

Studies conducted in the Comoros Islands, off the southeast coast of Africa, have revealed the persistent hyperendemicity of

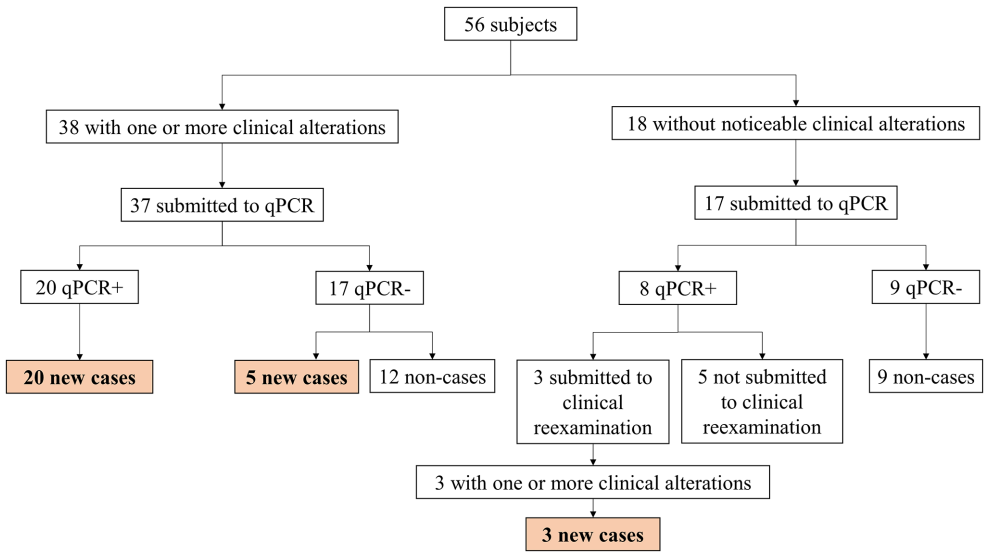

FIGURE 4
Diagram of the use of qPCR technique as a complementary exam of new cases among children under 15years old diagnosed in an active case finding.

leprosy in the population, despite efforts to control the disease over the past 40 years (42, 43). Hasker and colleagues (42) observed that between 2000 and 2015, the trend of increasing numbers of new diagnoses accompanied the period of intensified active case finding activities on the island of Anjouan in the Comoros. Diagnosis among children accounted for an average of 33% of total cases during this period, indicating active transmission of the infection in communities that are marked in part by social inequality and difficulty accessing adequate primary healthcare services (44).

Kiribati, an island nation located in Oceania, achieved the goal of eliminating leprosy as a public health problem in the year 2000 by presenting a prevalence of 0.94 cases per 10,000 inhabitants. However, since then, it has observed a growth in the number of cases above the previously achieved goal, particularly among children, a scenario attributed to increased efforts to detect new cases through active case finding. Of the 2,287 new cases diagnosed in the archipelago between 1988 and 2017, 757 (33%) were registered in individuals under 15 years of age (45). A previous study conducted by our research group on one of the islands in the city of Belém (Mosqueiro Island) identified 65 new cases among 706 (9.6%) schoolchildren evaluated, which evidenced the hidden prevalence of leprosy cases in the area. Like Caratateua Island, Mosqueiro Island also has low coverage of Family Health Strategy services (27).

In addition to clinical aspects, the diagnosis of leprosy can be aided by laboratory tests. Among these, bacilloscopy is considered the gold standard. However, despite its high specificity, the method has low sensitivity, especially in early cases, in paucibacillary cases and in cases of primary neural leprosy (18), which was the predominant form of cases in this study (71.4%). Retrospective studies on the diagnosis of pediatric cases observed positivity in the bacilloscopy method above 50% in Cuba (46) and 80% in Nepal (47), suggesting a concerning dependence on this technique for the diagnosis and detection of cases in more advanced stages in these countries, which have already declared the elimination of leprosy as a public health problem.

As a strategy to improve the ability to detect cases early, laboratory tools such as serological and molecular biology tests have been employed as important biomarkers for the disease. Previous studies have shown an important association between seropositivity for anti-PGL-I antibody titers and a higher risk of developing leprosy in the future in hyperendemic areas of Pará (15). In the present study, 7/27 (25.9%) of the cases tested positive for the serological test. The observed seroprevalence was similar to that found among non-cases (20.8%), which may be attributed to the clinical forms presented by the patients (borderline and primary neural leprosy), which are further from the lepromatous pole, known to be related to high levels of anti-PGL-I IgM antibodies due to the predominance of the humoral immune response.

In addition to the serological method, amplification of the repetitive RLEP region by qPCR showed high ability to detect *M. leprae* genetic material in leprosy patients in previous studies (11, 17, 19, 20), which led to the proposal of submitting individuals who are doubly positive for serological and molecular techniques to treatment for the disease, considering their situation of subclinical infection and potential for maintaining bacillary proliferation in the community (18). Among the new cases in this study, 23/28 (82.1%) tested positive for the qPCR technique. This study emphasizes the use of qPCR as an important biomarker for the diagnosis of leprosy in children in an endemic region, especially in the presence of oligosymptomatic or early disease. Most of the diagnosed cases did not present dermatological lesions, with peripheral nerve changes predominating, highlighting the primary neural character of leprosy.

The positivity in the qPCR technique was also observed among 8/17 (47%) children who initially showed no noticeable clinical alterations in the dermatoneurological examination. The research team proposed the re-evaluation of these individuals. About 5.5 months after the first evaluation, 3/8 (37.5%) of the children attended to be reassessed by the leprologist, who detected important clinical alterations, including pain and tingling when palpating peripheral nerves, loss of hand muscle strength, and hypochromic macules with regions of anesthesia. Given the clinical picture, together with the positivity of qPCR, the children were classified as new cases. The positivity in the molecular biology technique prior to the appearance of noticeable clinical manifestations suggest early detection of leprosy. We intend to carry out the reassessment of the five children who had no previous clinical alterations but tested positive in the qPCR technique (who did not attend the initially proposed reassessment) as soon as possible, in order to investigate whether they have become new cases or not.

Our study demonstrated a high number of hidden cases among schoolchildren on an island located in Belém, capital of Pará state, Amazon Region, where leprosy is endemic. We propose the use of qPCR technique for the definition of new cases based on the association between clinical alterations and positivity for the method among children under 15 years old in endemic areas. In addition, we emphasize the need for training of health professionals for the detection of leprosy and the vitalness of increasing Primary Care coverage in the municipality, which will allow for the enhancement of efforts made for the diagnosis of leprosy in childhood, breaking the chain of disease transmission, and preventing affected children from progressing to physical disabilities.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Institute of Health Sciences Research Ethics Committee from Pará Federal University (CAAE 26765414.0.0000.0018 CEP-ICS/UFPA). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

The study concept was designed by IC, PC, and CS. Clinical examination of sensory-motor functions was performed by SS,

PC, and CS. The datasheet with all clinical and epidemiological information was filled and managed by IC. Serological experiments were performed and analyzed by PC, AG, and JS. Antigens used in serological experiments were generously provided by JS. Molecular experiments were designed, managed, and performed by PC and MS. The manuscript was primarily written by IC and PC with substantial critical revision by CS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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sTREM-1 and TNF-α levels are associated with the clinical outcome of leprosy patients

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Leprosy reaction (LR) and physical disability (PD) are the most significant clinical complications of leprosy. Herein, we assessed the circulating serum-sTREM-1 and TNF-α levels and their genetic polymorphisms in leprosy. Serum-sTREM-1 and TNF-α levels were measured in leprosy patients (LP) before treatment ($n = 51$) and from their household contacts (HHCs; $n = 25$). DNA samples were genotyped using TREM-1 rs2234246 and TNF-α rs1800629-SNP in 210 LPs and 168 endemic controls. The circulating sTREM-1 and TNF-α levels are higher in the multibacillary form. The ROC curve of the serum-sTREM-1 levels was able to differentiate LR from non-LR and PD from non-PD. Similarly, LPs with serum-sTREM-1 levels >210 pg/ml have 3-fold and 6-fold higher chances of presenting with LR and PD, respectively. Genotypes CC+CT of the TREM-1 were associated with leprosy. Taken together, our analyses indicated that sTREM-1 and TNF-α play an important role in the pathogenesis of leprosy and provide promising biomarkers to assist in the diagnosis of leprosy complications.

KEYWORDS

leprosy, soluble TREM-1, inflammatory cytokine, immune markers, leprosy complications

Introduction

Leprosy is a chronic infectious disease caused by the intracellular bacillus *Mycobacterium leprae* and *Mycobacterium lepromatosis* (1). Regardless of the significant reduction in prevalence after the widespread use of multidrug therapy, the new case detection rates have stabilized in the last few years, and leprosy remains endemic in a number of localized regions, such as Brazil, India, and China (2). Note that leprosy reaction (LR) and the occurrence of physical disability (PD) are the most important clinical complications of leprosy (3).

In this regard, several studies have reported the influence of the immunological response on leprosy infection. T helper 1 (Th1) and Th17 cell responses are associated with the control of *M. leprae* while exacerbating the Th1 response, and a high number of $CD8^+$ T cells may be involved with increased disease severity. Alternatively, Th2 and Treg cells are related to the multibacillary presentation, with largely infected macrophages in skin lesions (4).

The triggering receptor expressed on myeloid cells-1 (TREM-1) is a cell-surface receptor constitutively expressed mainly on neutrophils and monocytes. This receptor is involved in the amplification of the inflammatory response by activating transcription factors such as NF-κB (5). Beyond the membrane form (mbTREM-1), TREM-1 can also be found in a soluble form (sTREM-1), which acts mainly negatively, modulating mbTREM-1 receptor signaling (6). Regardless of whether the cellular source of sTREM-1 remains unclear, the role of sTREM-1 related to some infectious diseases has been largely investigated, including several reports showing that sTREM-1 is directly associated with severe disease, as in visceral leishmaniasis (7), pulmonary tuberculosis (8), sepsis (9), and COVID-19 (10).

The TREM-1 gene is present on chromosome 6, and a polymorphism (rs2234246 SNP) has been reported in non-disease individuals to affect the sTREM-1 levels and the expression of messenger RNA to the mbTREM-1. Moreover, the minor allele T was associated with the increased production of this protein (11). Although several genetic studies have been published on leprosy, no studies have been reported on this TREM-1 polymorphism (12–14).

Importantly, the clinical signs of leprosy may be scarce in the early stages of the disease, leading to delayed diagnosis or misdiagnosis (15). Furthermore, patients search for medical support when presenting with some clinical complications (16). Thereby, the identification of some biomarkers to help with the early diagnosis of leprosy and its clinical complications is urgently required. Herein, we reported that circulating sTREM-1 and TNF-α are related to the lepromatous leprosy (LL) form, especially the LR and PD. Thus, these molecules might be promising biomarkers to monitor the occurrence of LR and PD during the clinical follow-up of leprosy treatment.

Materials and methods

Ethics statement

This project was approved by the Ethics and Research Committee of the Federal University of Sergipe (CAAE 0152.0.107.000-07). All subjects or their legal representatives signed a free and clarified term of knowledge contract (IC) agreeing to participate in the study.

Study subjects and data collection

This is a case-control study with two different approaches: first, a case-control study of sera measurements, including 51 leprosy patients and 25 controls recruited from January 2019 to December 2019. Second, a case-control study of genetic markers, including 210 leprosy patients and 168 controls who were enrolled in the study between January 2010 and December 2019.

All leprosy patients included in this study attended the dermatology clinic of the Hospital Universitário at the Universidade Federal de Sergipe, Aracaju City, northeastern Brazil. Leprosy patients were completely examined by dermatologists, and the inclusion criteria were to have a confirmed diagnosis of leprosy prior to starting treatment with conventional multidrug therapy (MDT). In accordance with the Brazilian Ministry of Health, patients were diagnosed by clinical evaluation (dermato-neurological) and histopathological and lymph bacilloscopic examinations (17). Additionally, for the purpose of treatment classification, leprosy patients were classified according to their operational forms: paucibacillary (PB), if they exhibited fewer than five skin lesions and received a negative bacilloscopic examination; or multibacillary (MB), if they presented with five or more skin lesions and tested positive on the bacilloscopic examination. To determine their clinical forms, histopathological examinations of skin biopsies were performed and classified according to Ridley-Jopling's criteria (18) as follows: indeterminate leprosy (IL), tuberculoid leprosy (TT), borderline leprosy (BL), or lepromatous leprosy (LL).

All patients who were invited and included in the study were recruited through convenience sampling at the time of diagnosis and consecutive order. The exclusion criteria were applied to individuals who had diseases known to affect the immune response or that confound the diagnosis of leprosy complications, such as HIV and HTLV-I infections, diabetes, or neurological diseases. The selection of patients did not consider factors such as sex and age as criteria, but efforts were made to match groups to prevent any bias during the analysis.

The control group used in the analysis of serum-sTREM-1 and TNF-α levels was composed of household contacts (HHCs), including any person living in close and prolonged contact with the leprosy patients but not genetically related. These contacts most commonly were the patient's spouses. Moreover, we only included patients who had started MDT. These patients were followed-up monthly during treatment to detect symptoms of LR and neurological disabilities, following the recommendations of the World Health Organization (19), using a specific questionnaire and the neurological simplified evaluation (but this information was not included in this study).

For the genetic analysis, the controls in the study included HHC and an additional population of 115 unrelated individuals living in the same city as the patients, which is an endemic area for leprosy. These two groups were combined to form the "endemic control" group (EC, $n = 154$) in the analyses, representing the control sample. However, owing to a lack of available information and low DNA concentrations, some subjects were excluded, resulting in potential variations in the total number of individuals included in the SNP analysis across the results.

Measurement of serum-strem-1 and TNF-α levels

Sera were obtained from whole blood collected from the leprosy patients before treatment and from HHCs. Serum-sTREM-1

levels were assessed using specific enzyme-linked immunosorbent assay (ELISA) kits (DuoSet-R&D Systems, Abingdon, UK) using the manufacturer's recommended protocol and measured using a microplate reader (Epoch-BioTek, Luzern, Switzerland). A standard curve was generated for each set of samples assayed. Concentrations of the cytokine TNF-α were determined using multiplex assay, according to the manufacturer's instructions, by using a MILLIPLEX– Human Th17 Magnetic Bead Panel kit (Merck Millipore Corporation, USA).

Genotyping TREM-1 rs2234246 and TNF-α rs1800629 SNP

Genomic DNA was extracted from blood samples using the PureLink® Genomic-DNA Kit (Invitrogen™, USA). The concentration and purity of DNA were quantified using NanoDrop™ (Thermo-Scientific™, USA). We genotyped DNA samples using commercial TREM-1 rs2234246 and TNF-α rs1800629 TaqMan® probes (Applied Biosystems™, USA) and TaqMan™ Genotyping Master Mix (Applied Biosystems™, USA) by qPCR, using 7,500 Real-Time PCR (Applied Biosystems™) following the manufacturer's instructions. The results were assessed using TaqMan® Genotyper software version 1.6.0. Information about the analyzed SNPs and assay codes for each probe are presented in Supplementary Table 1.

Statistical analysis

The clinical and demographical data, as well as serum-sTREM-1 and TNF-α levels, were compared across subgroups according to the operational (PB, MB, and HHCs) and clinical forms (IL, TT, BL, and LL) of leprosy and clinical complications (LR or PD). The mean, median, and standard deviation of the groups were calculated. The receiver operating characteristic curve (ROC curve) was used to distinguish groups based on the levels of sera measurements.

D'Agostino–Pearson normality tests were applied to verify if the data exhibited Gaussian distributions. Statistical differences between the groups were determined by the Mann–Whitney U test for two groups or the Kruskal–Wallis test for more groups, followed by the Dunn test for multiple comparisons. Correlations between the cytokine levels were determined using the Spearman correlation test.

For genetic polymorphism analyses, the allelic and genotype frequencies were compared according to the operational and clinical forms of leprosy, and the odds ratio (OR) was calculated using Fisher's exact or the Chi-squared test. The Hard-Weinberg equilibrium (HWE) test was performed using GENEPOP Online 4.2 (20).

All analyses were performed using GraphPad Prism software 8.0.1 (GraphPad Software Inc., USA). To evaluate differences, alpha (∂) was set at 5%, and tests were made using a two-tailed *p-value*.

Results

Clinical and demographic characteristics of subjects

No differences were identified among the mean ages between PB and MB patients or HHCs (Table 1). Nonetheless, the proportion of men presenting with the MB form (57.1%) was significantly higher than the PB form (26.1%; OR = 3.77; $p = 0.02$). Remarkably, the occurrence of LR was significantly higher among MB (71.4%) than PB (17.4%; OR = 11.88; $p < 0.001$). Similarly, patients presenting with a PD degree of 1 or 2 were higher in the MB (60.7%) than in the PB group (10.7%; OR = 3.53; $p = 0.03$).

Serum-sTREM-1 levels are higher in the MB form and in patients presenting with leprosy reactions and physical disability

We observed higher levels of sTREM-1 in MB patients (221 ± 102.2 pg/ml) than HHCs (160.9 ± 107.5 pg/ml; $p = 0.03$; Figure 1A), and no differences were found when comparing all leprosy patients with HHCs (198.9 ± 100.7 pg/ml; $p = 0.15$) or among patients when compared by the Ridley-Jopling classification (Figure 1B). Correspondingly, we observed higher levels of sTREM-1 in patients presenting with LR (228.5 ± 112.1 pg/ml) and PD (247.9 ± 101 pg/ml) than those with no clinical complications (160.1 ± 92.4 pg/ml; $p = 0.04$ and 144.3 ± 87.3 pg/ml; $p < 0.001$, respectively; Figures 1C, D). However, the correlation analysis using the patient classification according to the number of lesions (PB and MB) and sTREM-1 levels resulted in a poor and insignificant result ($r = 0.178$, $p = 0.21$) (Supplementary Figure 1A).

Additionally, using the receiver operating characteristic (ROC) curve, serum-sTREM-1 levels had 69.5% sensitivity and 61.8% specificity for differentiating MB patients from those with HHCs [area under the ROC curve (AUC) = 0.7023; $p = 0.01$; Figure 1E]. Furthermore, the ROC curve of serum-sTREM-1 levels had 54.55% sensitivity and 62.96% specificity for differentiating LR from non-LR patients (AUC = 0.6884; $p = 0.04$; Figure 1F). More importantly, sTREM-1 levels had 77.27% sensitivity and 74.07% specificity for differentiating PD from non-PD patients (AUC = 0.787; $p < 0.001$; Figure 1G).

Considering that serum-sTREM-1 levels increased in patients presenting with MB, LL, LR, and LD, we grouped them into those with serum-sTREM-1 levels > 210 pg/ml and ≤210 pg/ml. Thereafter, we compared the clinical characteristics among those groups (Supplementary Table 2). We used the value of sTREM-1 > 210 pg/ml, as it presented the highest sensitivity and specificity rates for the most severe leprosy outcomes. Interestingly, patients with serum-sTREM-1 levels >210 pg/ml had almost 5-fold higher odds of presenting with the LL form (OR = 4.81; $p = 0.04$). Similarly, leprosy patients have 3-fold higher odds of presenting with LR (OR = 2.81; $p = 0.06$) and 6-fold higher odds of presenting with physical disability (OR = 5.83; $p = 0.004$).

TABLE 1 Demographic and clinical characteristics of patients and household contacts.

Variables	MB ($n = 28$)	PB ($n = 23$)	HHC ($n = 25$)	OR	95% CI	p-value
Age						
Variation	10–68	11–81	20–72	–	–	*0.22
Mean ± SD	43.6 ± 15.9	49.6 ± 18.9	46.9 ± 14.5			
Men *n* (%)	16 (57.1%)	06 (26.1%)	09 (39.1%)	3.77	1.14 to 12.48	**0.02
Number of lesions						
Variation	2–14	1–5	–	–	–	* <0.0001
Mean ± SD	6.58 ± 2.85	1.91 ± 1.59	–			
Leprosy reaction *n* (%)	20 (71.4%)	4 (17.4%)	–	11.88	1.53 to 5.16	**0.0001
Physical disability degree *n* (%)						
Degree 0	11 (39.3%)	16 (50%)	–	3.53	1.09 to 11.36	**0.03
Degree 1 or 2	17 (60.7%)	7 (10.7%)	–			

MB, Multibacillary; PB, Paucibacillary; HHCs, Household contacts; OR, odds ratio; CI, Confidence interval; SD, Standard deviation. *Unpaired test; **Fisher exact test. OR + 95% CI refers to MB × PB. All data were obtained before treatment.

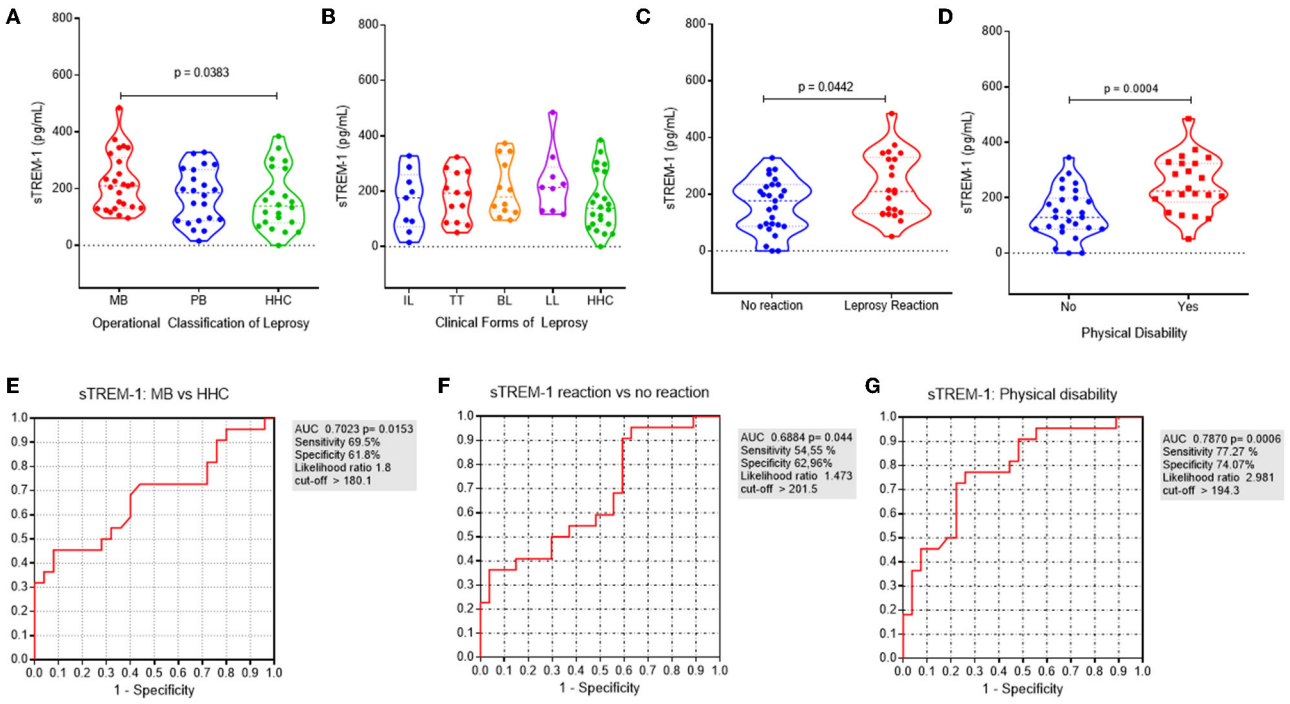

FIGURE 1
sTREM-1 serum levels are associated with MB presentation and clinical complications on leprosy. **(A)** sTREM-1 serum levels according to the operational classification on leprosy: multibacillary (MB-in red), paucibacillary (PB-in blue), and household contacts (HHCs-in green). **(B)** sTREM-1 serum levels according to the clinical forms of leprosy: indeterminate (IL–in blue), tuberculoid (TT–in red), borderline (BL–in orange), lepromatous (LL–in violet), and HHCs (in green). **(C)** sTREM-1 serum levels according to the occurrence of leprosy reaction (LR): no reaction (in blue) and LR (in red). **(D)** sTREM-1 serum levels according to the occurrence of physical disability (PD): no PD (in blue) and with PD (in red). The receiver operating characteristic (ROC) curve was generated to discriminate the levels of sTREM-1 between **(E)** leprosy patients presenting with the MB operational form and HHCs [area under the ROC curve (AUC) = 0.7023; $p = 0.01$]; **(F)** leprosy patients presenting with LR and those with no LR (AUC = 0.6884; $p = 0.04$); **(G)** leprosy patients presenting with some PD (degrees 1 or 2) and those with no PD-degree 0 (AUC = 0.787; $p = 0.0006$).

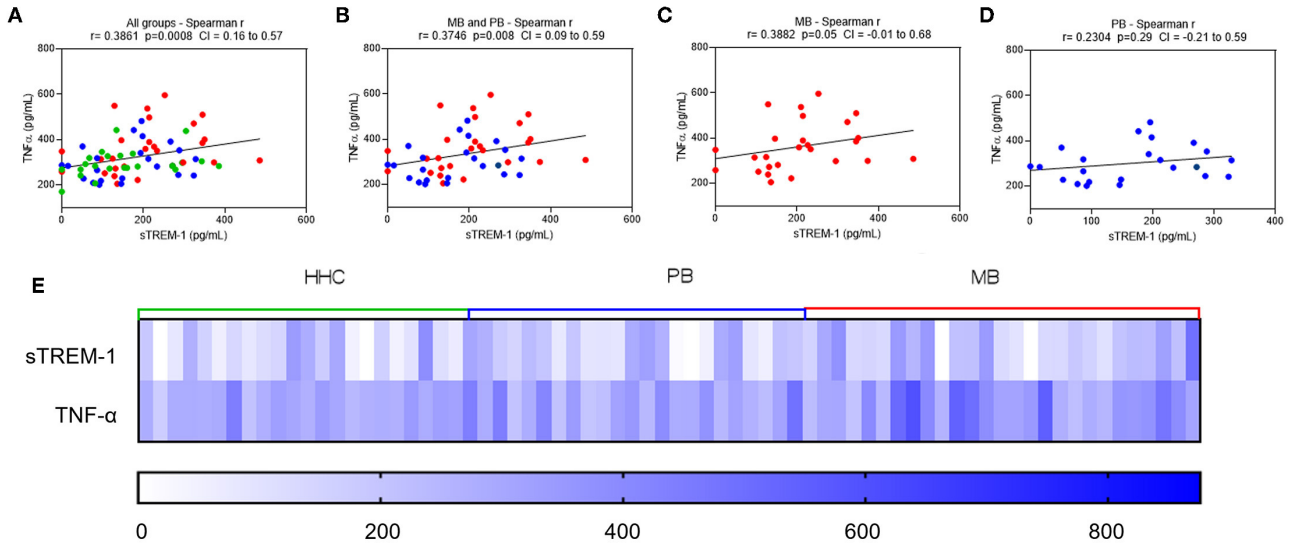

FIGURE 2
Serum levels of sTREM-1 are correlated to the TNF-α. Correlations between serum levels of TNF-α and sTREM-1 in leprosy patients (multibacillary–MB, in red; paucibacillary–PB, in blue) and household contacts (HHC–in green): **(A)** all groups (MB, PB, and HHC); **(B)** MP and PB groups; **(C)** MB group; and **(D)** PB group. Dotted lines on the x-axis represent the values of sTREM-1. Dotted lines on y-axis represent the values of TNF-α. Correlations were analyzed using the Spearman test. **(E)** A heatmap expressing the serum levels of sTREM-1 and TNF-α in MB and PB leprosy patients and HHCs.

Serum-TNF-α levels are higher in MB and LL clinical form

We identified elevated levels of TNF-α in MB (363.2 ± 105.6 pg/ml) than PB patients (300.7 ± 79.1 pg/ml; $p = 0.03$) and HHCs (296.1 ± 60.2 pg/ml; $p = 0.01$; Supplementary Figure 2A), while a comparison of all leprosy patients showed higher but no-significant levels of TNF-α than control patients (334.5 ± 98.6 pg/ml; $p = 0.08$). Similarly, LL patients (427 ± 119.1 pg/ml) also presented higher levels of TNF-α compared to IL patients (304 ± 85.5 pg/ml; $p = 0.03$), TT patients (298.6 ± 78 pg/ml; $p = 0.01$), and HHCs ($p = 0.002$; Supplementary Figure 2B). No differences were observed according to the occurrence of LR or PD (Supplementary Figures 2C, D). In addition, the ROC curve of serum-TNF-α levels had 91.3% sensitivity and 77.8% specificity for differentiating MB patients from HHCs and PB (Supplementary Figures 2E, F). Correspondingly, the ROC curve of serum-TNF-α levels had 91.3% sensitivity and 77.78% specificity for differentiating the LL form from HHCs and IL+TT (Supplementary Figures 2G, H). Complementarily, the correlation analysis among TNF-a levels and the operational classification of patients showed a poor but significant correlation $r = 0.319$ ($p = 0.024$) (Supplementary Figure 1B).

Considering the higher expression of sTREM-1 and TNF-α in MB forms, we performed the Spearman correlation (Figures 2A–D). Interestingly, we observed a weak but significant correlation among sTREM-1 and TNF-α in all groups (Rho = 0.3861; $p < 0.001$; Figure 2A), among PB and MB (Rho = 0.3742; p-value = 0.008; Figure 2B), and only MB (Rho = 0.38; $p = 0.05$; Figure 2C). Similarly, the heatmap showed higher expression of sTREM-1 and TNF-α in the MB forms (Figure 2E).

Association between the TREM-1 rs2234246 and TNF-α rs1800629 SNPs with the occurrence of leprosy

Considering the results in the serum expression of sTREM-1 and TNF-α, we decided to genotype the study population for the TREM-1 rs2234246 and TNF-α rs1800629 SNP and verify whether the differences found could be related to genetic polymorphisms. The characteristics of subjects according to the groups and the leprosy clinical forms are shown in Supplementary Table 3. The frequency of males is higher in leprosy patients than in EC. No deviation was found in HWE. There was a higher frequency of CC+CT genotypes of the TREM-1 rs2234246 than the TT genotype in leprosy patients (OR = 1.598; $p = 0.04$; Table 2). Nevertheless, there were no differences between the allele or genotype frequencies of TREM-1 and TNF-α SNPs, considering the clinical outcomes of leprosy (Supplementary Table 4). Additionally, no differences were observed when the alleles of these SNPs were compared according to the Ridley and Jopling classification (Supplementary Table 5).

Conversely, we observed an association between the presence of a higher producer of sTREM-1 (sTREM-1 > 210 pg/ml) and the TT genotype ($p = 0.03$; Supplementary Table 6). In accordance with this, when we compared the amount of sTREM-1 according to genotypes for the SNP analyzed (TREM-1 rs2234246; Supplementary Figures 3A, B), including all case and

TABLE 2 Frequency and distribution of TREM-1 rs2234246 in leprosy patients and the control group.

TREM-1 rs2234246	Allele and genotype frequencies *n* (%)[a]						
		Case group	Control group				
		LP	HHC	Allele/Genotype	OR	95% CI	*p*-value[b]
		n = 190	*n* = 39				
	C	170 (44.7)	33 (42.3)	C vs. T	1.10	0.67–1.81	0.71
	T	210 (55.3)	45 (57.7)				
	CC	44 (23.2)	6 (15.4)				
	CT	82 (43.1)	21 (53.8)	CC + CT vs. TT	0.87	0.41–1.82	0.85
	TT	64 (33.7)	12 (30.8)				
		LP	EC				
		n = 190	*n* = 154				
	C	170 (44.7)	119 (38.6)	C vs. T	0.77	0.57–1.05	0.12
	T	210 (55.3)	189 (61.4)				
	CC	44 (23.2)	34 (22.1)				
	CT	82 (43.1)	51 (33.1)	CC + CT vs. TT	1.59	1.02–2.45	**0.04**
	TT	64 (33.7)	69 (44.8)				

LP, leprosy patients; HHC, household contacts; EC, endemic control; OR, odds ratio; CI, confidence interval.
[a](%) percentual of the subjects with the specified allele or genotype.
[b]Test for association was performed using the Fisher's exact test. Bold indicate statistically significant results.

control groups, higher quantities of sTREM-1 were detected in the TT genotype patients than in the CT genotype patients ($p = 0.03$).

Discussion

Early identification of the clinical complications of leprosy has a major effect on the clinical management and outcome of these patients (21). Considering this, it is relevant to find new biomarkers to help identify those presenting with LR or PD. Our findings suggest that assessing the sTREM-1 in serum samples from leprosy patients may be a valuable new approach to assist in the diagnosis of LR and PD during the follow-up of these patients.

Herein, we observed higher levels of sTREM-1 in MB patients compared to HHCs. Similarly, higher levels of sTREM-1 were identified in the LL clinical form. Studies assessing sTREM-1 levels in other diseases caused by intracellular pathogens have demonstrated the value of this molecule in differentiating severe from non-severe forms of tuberculosis and leishmaniasis. Feng et al. demonstrated that serum-sTREM-1 levels are significantly increased in pulmonary tuberculosis and are correlated with more advanced involvement in chest x-rays and a higher bacteria burden in sputum (8). More importantly, higher levels of sTREM-1 are independent predictors of on-treatment mortality in tuberculosis.

Furthermore, in a meta-analysis by Wu et al. (22), sTREM-1 had a moderate diagnostic performance in differentiating sepsis from non-sepsis in adult patients. As a result, the authors indicated that a combination of several markers appears to be a useful approach to improving accuracy in diagnosing sepsis. Additionally, Gibot et al. (23), in a prospective study, demonstrated the high performance of a bioscore combining sTREM-1 along with procalcitonin and CD64 on neutrophils index in diagnosing sepsis.

Interestingly, we identified higher serum-sTREM-1 levels in leprosy patients with LR and PD. Additionally, leprosy patients with serum-sTREM-1 levels above 210 pg/ml have almost a 3-fold higher chance of presenting with LR and a 6-fold higher chance of presenting with PD. Notably, those clinical outcomes are the most severe complications of leprosy, and they are causally related to an exacerbated inflammatory response, nerve damage, lack of sensibility, and loss of life quality in leprosy patients (24). Few studies have investigated the role of TREM-1 in neural tissues, and most of them have focused on TREM-2 (25–28). However, previous studies have already demonstrated the role of TREM-1 in amplifying the inflammatory process and protein autophagy that are associated with tissue damage, as already observed in studies with Parkinson's and Alzheimer's diseases (25, 26). Regardless of these findings, the role of sTREM-1 in neural tissues remains unclear, and it is necessary to investigate whether Schwann cells are a source of sTREM-1, attracting and activating neutrophils. Therefore, we could hypothesize that high sTREM-1-serum levels in leprosy may indicate an inflammatory process that occurs in LR and neural damage during *M. leprae* infection. However, experimental and new clinical data are mandatory to confirm this.

Additionally, we identified higher serum levels of TNF-α in MB and LL clinical forms. In previous studies, TNF-α has been extensively described as an important proinflammatory cytokine associated with tissue damage in leprosy (29). Importantly, we have demonstrated that sTREM-1 is positively correlated with TNF-α, although there is a weak but significant correlation. Similarly, Liu et al. (30) confirmed that both serum contents of sTREM-1 and TNF-α are significantly increased in patients with mycoplasma pneumoniae infection. The authors indicated that TREM-1 overexpression enhances the nuclear translocation of NF-kβ and exerts a proinflammatory response, as evidenced

by triggering TNF-α release. When exacerbated, the unregulated inflammatory response can lead to tissue damage, as it usually occurs in LR and as nerve impairment in patients presenting with PD (3, 21, 31).

Concerning the genetic polymorphism analysis, associations between the genotypes CC+CT for TREM-1 rs2234246 and leprosy *per se* or the occurrence of leprosy were observed. The TREM-1 rs2234246 SNP C/T is a functional polymorphism that has been tested in healthy individuals and reported to affect sTREM-1 levels and the expression of mbTREM-1. Furthermore, the T allele is associated with increased levels of this molecule (11). However, the functionality of this SNP and its role in affecting the sTREM-1 levels during active diseases are still inconsistent (32–35). As TREM-1 is a key effector of innate immunity, the presence of CC+CT genotypes associated with a lower expression of mbTREM-1 in the cells increases the odds of developing the disease.

Conversely, our functional data also showed that the TT genotype is related to higher production of sTREM-1, which is associated with clinical complications of leprosy. Altogether, our findings suggest that the TREM-1 SNP may affect the risk of leprosy occurrence, making this an important candidate gene for future studies in more powerful genetic studies. Conversely, the low producers' genotypes are associated with the infection. Moreover, the high inflammatory response associated with clinical outcomes in LR and PD patients is associated with the high production of sTREM-1 in leprosy patients, indicating the importance of a balanced immune response in leprosy.

This study has some limitations that need to be mentioned. All samples were collected and analyzed before the patient's treatment, and we considered only the occurrence of LR and PD at that moment. Notwithstanding, a prospective study evaluating serum-sTREM-1 and other biomarkers before clinical complications is required to assess if these biomarkers can predict these complications. Clearly, the future of biomarkers in leprosy diagnosis requires extensive validation studies of novel biomarkers across heterogeneous groups and evaluation of their power in combination with clinical and laboratory criteria. Moreover, our sample is limited to a small number of patients; thus, new investigations with more participants are required.

In light of the above, our main data showed that higher sTREM-1 levels helped us differentiate multibacillary patients from paucibacillary ones. These data also suggest that this molecule plays an important role in the pathogenesis of the inflammatory response in leprosy and provide a possible novel biomarker to assist in the diagnosis of leprosy's complications and their follow-up, although the mechanism whereby TREM-1 affects the initiation and progression of leprosy warrants further studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Comitê de Ética em Pesquisa da Universidade Federal de Sergipe. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

Concept and design: MB-S, SR, MD, RA, TM, and AJ. Acquisition of data: MB-S, LB, CS, MC, EM, LM, AB, MT, JA, LM-S, and RC. Analysis and interpretation of data: MB-S, LB, CS, MC, LM, AB, LM-S, RC, TM, and AJ. Drafting of the manuscript: MB-S, LB, CS, MT, JA, TM, and AJ. Statistical analysis: MB-S, CS, LM-S, RA, TM, and AJ. Obtained funding: SR, MD, ML, TM, and AJ. Critical revision of the manuscript for important intellectual content and final approval of manuscript: All authors.

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Conflict of interest

Authors SR and MD are employed by HDT Bio Corp.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1177375/full#supplementary-material

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Prediction of the occurrence of leprosy reactions based on Bayesian networks

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Introduction: Leprosy reactions (LR) are severe episodes of intense activation of the host inflammatory response of uncertain etiology, today the leading cause of permanent nerve damage in leprosy patients. Several genetic and non-genetic risk factors for LR have been described; however, there are limited attempts to combine this information to estimate the risk of a leprosy patient developing LR. Here we present an artificial intelligence (AI)-based system that can assess LR risk using clinical, demographic, and genetic data.

Methods: The study includes four datasets from different regions of Brazil, totalizing 1,450 leprosy patients followed prospectively for at least 2 years to assess the occurrence of LR. Data mining using WEKA software was performed following a two-step protocol to select the variables included in the AI system, based on Bayesian Networks, and developed using the NETICA software.

Results: Analysis of the complete database resulted in a system able to estimate LR risk with 82.7% accuracy, 79.3% sensitivity, and 86.2% specificity. When using only databases for which host genetic information associated with LR was included, the performance increased to 87.7% accuracy, 85.7% sensitivity, and 89.4% specificity.

Conclusion: We produced an easy-to-use, online, free-access system that identifies leprosy patients at risk of developing LR. Risk assessment of LR for individual patients may detect candidates for close monitoring, with a potentially

positive impact on the prevention of permanent disabilities, the quality of life of the patients, and upon leprosy control programs.

KEYWORDS

leprosy, leprosy reactions, risk, Bayesian networks, artificial intelligence

1. Introduction

Leprosy is a chronic, disabling infectious disease caused by *Mycobacterium leprae* (*M. leprae*) (1) that affected 141,000 new individuals worldwide in 2021 – a number likely to be underestimated due to potential sub-notification caused by the COVID-19 pandemic – with most cases concentrated in India and Brazil (2). In the classical Ridley & Jopling (R&J) classification system, tuberculoid (TT) and lepromatous (LL) leprosy occupy opposite ends of a continuous disease spectrum that includes three borderline forms (BT, BB, and BL) (3). The TT + BT and BB + BL + LL cases roughly correspond to paucibacillary (PB) and multibacillary (MB) leprosy, according to the treatment-oriented World Health Organization (WHO) classification scheme, respectively (2, 4, 5). Today, it is widely accepted that exposure to *M. leprae* is necessary but not sufficient for the development of leprosy; different sets of host gene variants mediate susceptibility to leprosy in three different stages (6): (i) controlling infection *per se*, that is, the disease regardless of its clinical presentation, (ii) defining the clinical form of disease after the infection is established, and (iii) outlining the risk of developing leprosy reactions (LR) (7, 8).

Leprosy reactions are characterized by an intense and sudden (re) activation of the host inflammatory response that may be diagnosed concomitantly with leprosy, during or even after treatment (9–12). Upon diagnosis, LR requires immediate medical attention to prevent irreversible nerve damage, motor disability, and permanent anatomical deformities. In 2021, 6.04% of newly detected leprosy cases worldwide presented grade-2 disabilities in the diagnosis (2), often due to LR. Cohort studies estimate that, during leprosy, 16 to 56% of the patients will develop irreversible nerve damage, again, mainly due to reactional episodes (13–16). Over the past years, advances in genetic research improved our understanding of the molecular basis of leprosy pathogenesis, and several host genetic variations have been implicated in the control of LR episodes (17–19).

There are two major types of LR of distinct clinical presentation: type-1 (T1R) and type-2 reaction (T2R). T1R affects 10–30% of leprosy patients and occurs primarily within, but not limited to, the first 2 years after leprosy diagnosis (20, 21). Known risk factors for T1R are (i) borderline clinical groups BT-BL (22); (ii) age of leprosy onset, with older individuals being at higher risk (23, 24); (iii) positive bacillary index (25); (iv) an increased number of lesions at leprosy diagnosis (26, 27); (v) detection of *M. leprae* DNA in biopsies of lesions (24); and (vi) genetic/genomic studies have identified an association between T1R and genes *TLR1* (28), *TLR2* (29), *TLR3* (30), *TLR7* (30), *TLR10* (30), *NRAMP1/SCLC11A1* (31), *VRD* (32), *NOD2* (33), *TNFSF15/TNFSF8* (34, 35), lncRNA *ENSG00000235140* (36), *LRRK2* (19), and *PRKN* (19).

Leprosy T2R mainly affects patients classified within the BB-LL range (13, 37). Patients presenting bacterial index higher than 4+ in skin smears are at increased risk for T2R (38, 39). There is a wide variation in the prevalence of T2R in different geographic and endemic settings. In Brazil, approximately 37% of BL and LL cases develop T2R, while in India, Nepal, and Thailand, the proportion is between 19–26% (40). A prospective study involving BL and LL patients from India followed for 11 years, showed that less than 10% of the individuals who developed T2R had a single episode, whereas 62% had chronic T2R (21). In Ethiopia, 63% of leprosy cases had more than one T2R episode, while 37% had a single event (41). Host genetics also seems to play a significant role in controlling the occurrence of T2R, and genes *C4B* (42), *TLR1* (43), *NRAMP1/SCLC11A1* (31), *NOD2* (33, 35), and *IL6* (12, 35) have been implicated as critical molecular players.

One of the challenges of translational medicine is to systematize the analysis of a large amount of patient data to predict a specific outcome. In addition, scientific results from basic research are often difficult to translate into daily medical practice. Artificial Intelligence (AI) methods seek to systematically address often large, complex data sets to provide a base for decision-making. Of particular interest in health care, Bayesian Networks (BN) are among the most successful techniques in processing and unraveling the relationship between a large number of variables, with risk estimation being the outcome (44).

A Bayesian Network (BN) is a graphical model of an outcome variable's posterior conditional probability distribution based on evidence. It contains nodes that represent the random variables and links between pairs of nodes, which represent the causal relationship of these nodes, together with a conditional probability distribution in each node. From the definition, one can deduce that any joint probability distribution may be represented by a Bayesian network, which shows its modeling power: any deterministic model is a particular case of a probabilistic model, and any probabilistic model may be represented as a Bayesian network (45, 46).

Several BN-based systems have been created using medical data, developed for different purposes, and applied to several health conditions such as cardiovascular diseases, liver diseases, cancer and Alzheimer's disease (47–57), including leprosy (44, 58–65). However, few initiatives aim to systematize a large amount of existing information of distinct nature to estimate the risk of the occurrence of a particular event. In the context of leprosy, creating a simple-to-use and flexible platform to predict the risk of LR based on patient data may help minimize the consequences of such aggressive events. Moreover, such a tool could improve leprosy control initiatives and public health systems. Here we present an AI system designed to predict the risk of a leprosy patient to develop LR using a complete or partial dataset of clinical, demographic, and host genetic data.

2. Materials and methods

A flowchart summarizing the three stages of the study and the procedures described next is available in Figure 1.

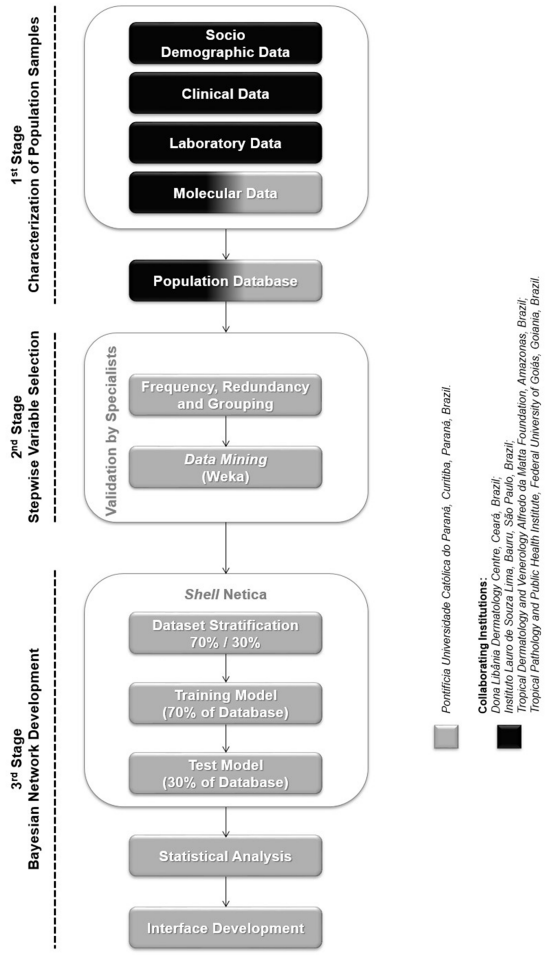

FIGURE 1
Flowchart: study design.

2.1. Population samples

This study used four pre-existing data sets from previous research initiatives of different/independent designs and contexts. The first database included in the study consisted of 409 leprosy patients diagnosed at the Reference Center for Diagnosis and Therapy located in Goiania, central-western Brazil, between February 2006 and March 2008, originally used for the genetic study that identified an association between T2R and variants of the *IL6* gene. A complete description of the Goiania population has been published elsewhere (12). Later, the Goiania population was used for an expanded investigation involving a larger number of candidate genes that detected an association between T1R and variants of the gene *TNFSF8* (34). Finally, an association between T1R and lncRNA *ENSG00000235140* (36) and *LRRK2* (unpublished data) was also detected in the Goiania sample. Two additional databases comprised 533 patients recruited at the Dermatological Center Dona Libânia, Fortaleza, northeast Brazil, and 137 patients diagnosed with leprosy at the Fundação Alfredo da Matta, Manaus, north Brazil. Enrolment of these two population samples was performed under a single protocol of a clinical study described previously (66) and conducted by the Tropical Medicine Center of the University of Brasília between March 2007 and February 2012. Finally, a fourth database consisted of 371 patients diagnosed with leprosy at the Instituto Lauro de Souza Lima, Bauru, southeast Brazil, between March 2008 and January 2013, originally for a genetic study that detected an association between leprosy and variants of the *TLR1* (67) and *NOD2* (68) genes. For all databases, leprosy diagnosis/classification was defined after detailed dermatological and neurological examination by specialized leprologists, complemented by bacilloscopy and histopathology of skin lesions. All cases were classified following the R&J scheme (3). Patients were followed up for at least 2 years since diagnosis to monitor LR occurrence. Individuals who did not present LR at the initial diagnosis or during follow-up, were defined as non-reactional leprosy patients.

All patients were treated for leprosy according to WHO/MDT guidelines and for LR with the appropriate therapy. All subjects were evaluated for an extensive clinical, socioeconomic, and demographic information list.

2.2. Variable selection

The four databases included in this study were composed of clinical and laboratory parameters, most of them obtained for descriptive, epidemiological purposes unrelated to the occurrence of LR. Each one of the databases was subjected individually to a two-step, unbiased process aiming to identify those variables exerting the highest impact upon the risk of LR, thus, to be included in the system, as follows:

2.2.1. Frequency, redundancy, and grouping

The first selection step consisted of removing variables with low frequency (less than 15%) of occurrence and/or mutually correlated (redundant), consequently capturing the same information. In the case of redundant variables, the most frequent was selected to capture the information of the set.

2.2.2. Data mining

Data mining is one of the main stages of the knowledge extraction process from large databases, also known as KDD – Knowledge Discovery in the Databases (69). This AI method is defined as the process of discovering patterns in data to generate helpful information for the decision-making (70). WEKA (Waikato Environment for Knowledge Analysis) is an open-source program with a collection of algorithm implementations of various data mining techniques, such as pre-processing, classification, clustering, and visualization (71). This study used WEKA in the second variable selection step to identify those hierarchically important for LR occurrence in the population samples. The variables were selected using the C4.5 algorithm, which creates a decision tree and identifies the most relevant and non-redundant variants, thus reducing the number of attributes. The C4.5 selection is made according to the gain ratio, which is a normalization of the information gain, a parameter based on the entropy measure (originating from information theory) closely related to the maximum likelihood estimations (MLE) and usually used to make inferences about parameters of the underlying probability distribution from a given dataset (45, 72–75).

Four dermatologists/hansenologists with extensive experience in the area continuously validated the two-step variable selection

through a qualitative process based on their experience in the field of leprosy diagnosis. These specialists were also involved in conducting system performance assessments, evaluating usability, and organizing the workflow for integrating data from the four databases. By leveraging the knowledge and expertise of specialists, clinical decision systems can be effectively validated and optimized for real-world clinical use (76). Criteria for selecting the specialists were; (i) holding MD/Ph.D. degrees in dermatology/hansenology; (ii) having more than 10 years of experience in leprosy diagnosis; (iii) being representative of regions of Brazil with different levels of leprosy endemicity.

Finally, two datasets contributed with genotypic information: Goiania for genes *IL6, TNFSF8*, *LRRK2*, and *ENSG00000235140* and Bauru for *TLR1* and *NOD2*, all previously studied in these population samples.

2.3. System development

The system was created as a BN using Shell NETICA (Norsys Software Corporation) (77) with a customized dynamic interface considering the number of variables in the database. The system was designed to operate with complete or partial information, which is of critical importance considering the translational bias of the proposal and the fact that several leprosy centers may not have access to all the information included, particularly the molecular genetic data. The system loads a spreadsheet in which columns and lines refer to the variables and records, respectively. Each variable (column) is related to one node of the BN. The variables comprise demographic, clinical, laboratory, and genetic data (markers). For each one of the databases, two groups were formed randomly to create the network: the test file, with 30% of patients, and the training file, with 70% of patients, both stored in an Excel file format.

The system's performance was assessed by its accuracy, sensitivity, specificity, and negative and positive predictive values. The patient's predicted outcome was defined by the class with higher risk, as estimated by the system. Predictive values were calculated using the prevalence of occurrence of reversal reactions observed for the studied population samples. The feature "importance" was also measured using the F_1 score, which is the harmonic mean between positive predictive value (PPV) and sensitivity. The F_1 score was calculated accordingly to the equation $F_1\ score = 2 * \frac{PPV * sensitivity}{PPV + sensitivity}$ using Python 3.7.9.

3. Results

Table 1 summarizes information on age, gender, and clinical form of leprosy according to the R&J classification system for T1R, T2R and non-reactional leprosy patients groups of all population samples. The mean age at diagnosis ranged from 40 to 59 years old, and males were consistently more frequent than females across all four population samples. Leprosy clinical form most frequently observed was BT (479, 33%) followed by BL (379, 26.1%), LL (346, 23.8%), BB (134, 9.2%), and TT (100, 6.9%). For the combined sample, 51% were non-reactional leprosy patients, 25.9 and 23.1% developed T1R or T2R, respectively. As expected, T1R was observed more often in BT + BB + BL cases, and T2R occurred more often in BL and LL individuals (Table 1).

TABLE 1 Distribution of sex, age at diagnosis, and clinical type of disease of leprosy-affected individuals with T1R, T2R, and non-reactional leprosy patients in each population sample.

	Patients, No. (%)														
	Goiania			Fortaleza			Manaus			Bauru			Combined		
Age, Years (Mean ± SD)	44.63 ± 16.67			45.15 ± 14.25			40.00 ± 15.39			59.00 ± 18.04			48.00 ± 17.29		
Sex															
Male	234 (57.1)			352 (66.0)			100 (72.9)			258 (69.5)			944 (65.1)		
Female	175 (42.9)			181 (34.0)			37 (27.1)			113 (30.5)			506 (34.9)		
Ridley&Jopling Classification	NRLP	T1R	T2R	NRLP	T1R	T2R	NRLP	T1R	T2R	NRLP	T1R	T2R	NRLP	T1R	T2R
TT	22	0	0	28	0	0	16	0	0	34	0	0	100	0	0
BT	124	79	0	164	24	0	36	4	0	18	30	0	342	137	0
BB	16	29	3	12	14	0	2	3	0	27	27	1	57	73	4
BL	26	46	8	47	71	66	12	28	10	12	20	33	97	165	117
LL	28	0	28	33	0	68	5	0	16	66	0	102	132	0	214
I	0	0	0	6	0	0	5	0	0	1	0	0	12	0	0
HI (Mean)	–	–	–	–	–	–	–	–	–	1.73	2.69	3.84	1.73	2.69	3.84
Proportion per Group	52.9	37.6	9.5	54.4	20.5	25.1	55.5	25.5	19.0	42.6	20.8	36.6	51.0	25.9	23.1
Total	409			533			137			371			1,450		

BB, borderline borderline; BL, borderline lepromatous; BT, borderline tuberculoid; HI, histological index; I, indeterminate leprosy; LL, lepromatous leprosy; NRLP, non-reactional leprosy patients; SD, standard deviation; TT, tuberculoid leprosy; T1R, type-1 reaction; T2R, type-2 reaction.

Our strategy for variable selection led to the inclusion of 34 demographic, clinical, laboratory, and genetic parameters (Supplementary Table S1) related to the occurrence of LR in the population samples (Table 2). Since the initial set of variables was not the same across the four datasets – thus, the variables selected by the two-step process and validated by the specialists were not necessarily the same – the prediction system was designed to include all variables selected in each population sample. Detailed information about the distribution of the included variables across the four different datasets is available in Supplementary Table S2.

The risk-prediction system was developed to allow the use of each of the four databases individually as a reference, as well as to use a single, combined dataset, thus favoring customization and facilitating the inclusion of new data sets. The system – named SEPAREH (from Portuguese: *Sistema Especialista Para Avaliação de Risco de Estado Reacional em Hanseníase*; in English: Specialist System for Evaluation of Risk of Occurrence of Reactional States in Leprosy) is designed to present a friendly graphical user interface (Figure 2), which allows the primary care professional to use it intuitively. Variation of the patient's risk of developing one of the two types of LR is shown in real time, as each available clinical and/or genetic information is included in the interface. The platform can be accessed for free at https://orfeu.ppgia.pucpr.br/separeh.[1]

The overall sensitivity and specificity of the system, as estimated using the combined dataset of 1,450 patients, was 79.3% (95% CI 73.9–84.7) and 86.2% (95% CI 81.6–90.8), respectively. Accuracy reached 82.7% (95% CI 79.2–86.3), and positive and negative predicted values were 85.1% (95% CI 80.2–90.1) and 80.6% (95% CI 75.5–85.7), respectively.

To assess the importance of each of the variables individually, modeling was carried out after removing one at a time, and the impact on system performance was measured through changes in sensitivity, specificity, and F1. As summarized in Figure 3, the three attributes exerting the highest impact were R&J classification, combined genetic markers, and histological index. Interestingly, the highest estimates of accuracy, sensitivity, specificity, and both negative and positive predictive values were observed for the Bauru and the Goiania datasets, for which genotypic data was available, even higher than what was observed for the combined dataset of much larger sample size (the only exception being the positive predictive value for Bauru: 82.7% vs. 85.1% for the combined dataset) (Table 3).

TABLE 2 Demographical, clinical, laboratory, and genetic variables selected in the study.

Data	Variable information[a]
Socio-demographic	Sex
	Age group
	Ethnicity
Clinical	Multidrug therapy
	First signs and symptoms
	Ridley-Jopling classification
	Number of skin lesions
	Type of lesion
	Color of lesion
	Sensibility testing
Laboratory	Bacilloscopic index
	Histological index
	PGL-1
Genetic	*IL6* markers (4)
	NOD2 marker (1)
	TLR1 markers (2)
	TNFSF8 markers (4)
	*ENSG00000235140*markers (4)
	LRRK2 markers (3)
Family History	First degree[b]
	Second degree[c]
	Contact[d]

[a]Self-report in years since noticing the early signs and symptoms of leprosy.
[b]Father, mother, child, and sibs affected by leprosy.
[c]Cousins, nephews, uncles/aunts, grandparents, and grandchildren affected by leprosy.
[d]Close household contact affected by leprosy.

4. Discussion

As an outcome of contact with its causative agent, leprosy is controlled by multiple environmental and socioeconomic factors and innate characteristics of both the host and pathogen. The specific contribution of each of these factors to the risk of developing leprosy and its endophenotypes is widely unknown. Today, LRs constitute a significant cause of disabilities associated with leprosy; thus, predicting patients at higher risk of developing LR at the time of leprosy diagnosis may help prevent permanent neural impairment. However, an accurate estimate of this risk demands analyzing a very complex set of variables, which is difficult – if not impossible – to perform by an unassisted primary healthcare professional. Here we present an easy-to-use, flexible, and automated system that identifies leprosy patients at increased risk of developing LR based on clinical, socio-economical, laboratory, and genetic data. Patients at high risk are candidates for close monitoring during and after treatment, aiming to prompt the management of these aggressive events, minimizing the likelihood of permanent disabilities. Our platform translates basic scientific data into a direct application that may immediately impact leprosy patients' quality of life and leprosy control programs' effectiveness.

The three features that exerted the highest impact on the system's performance were the R&J classification, the histological index, and the combined effect of the genetic markers (Figure 3). The R&J class is a well-accepted major risk factor for reversal reactions (7, 13, 21, 22, 37, 40, 41). As expected, simulations confirm that patients in the tuberculoid pole of the spectrum tend to have a higher chance of developing no reversal reaction (98% ~ when the classification is TT). As clinical form moves towards borderline, the probability of a T1R rises from <1 to 53%~ when the category is BB and, finally, patients

1 The access to the platform is limited to HTTPS protocol. In case of difficulty accessing the platform, please certify whether HTTPS is being used.

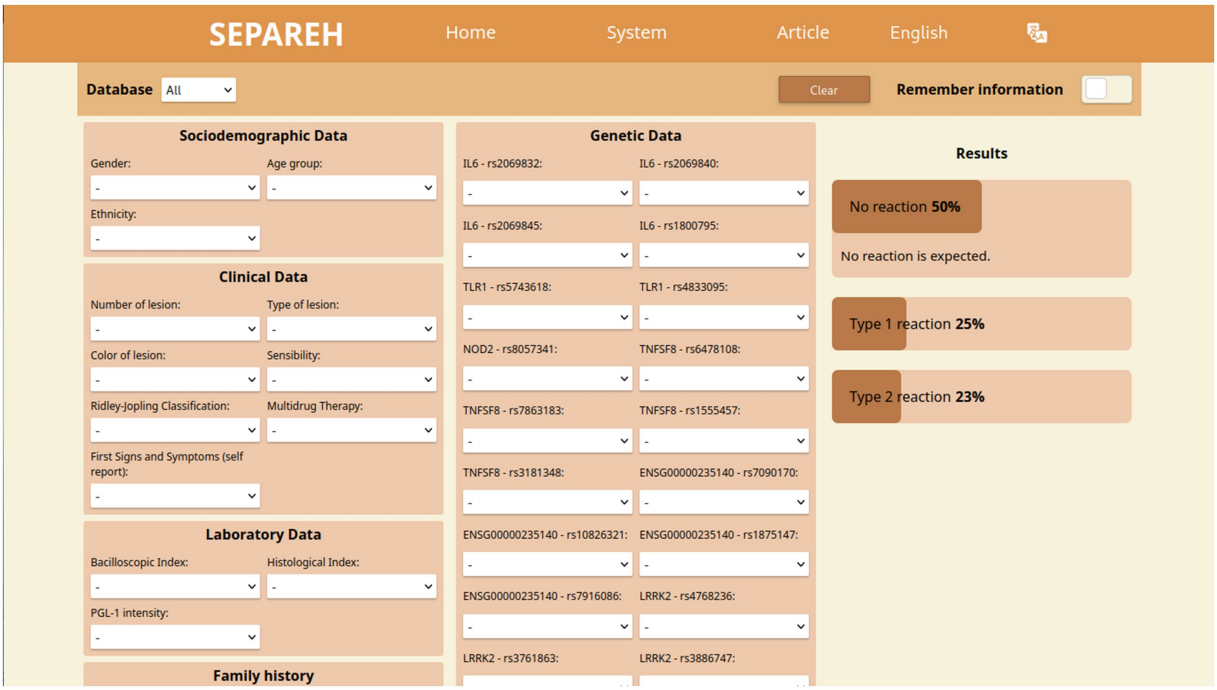

FIGURE 2
SEPAREH interface.

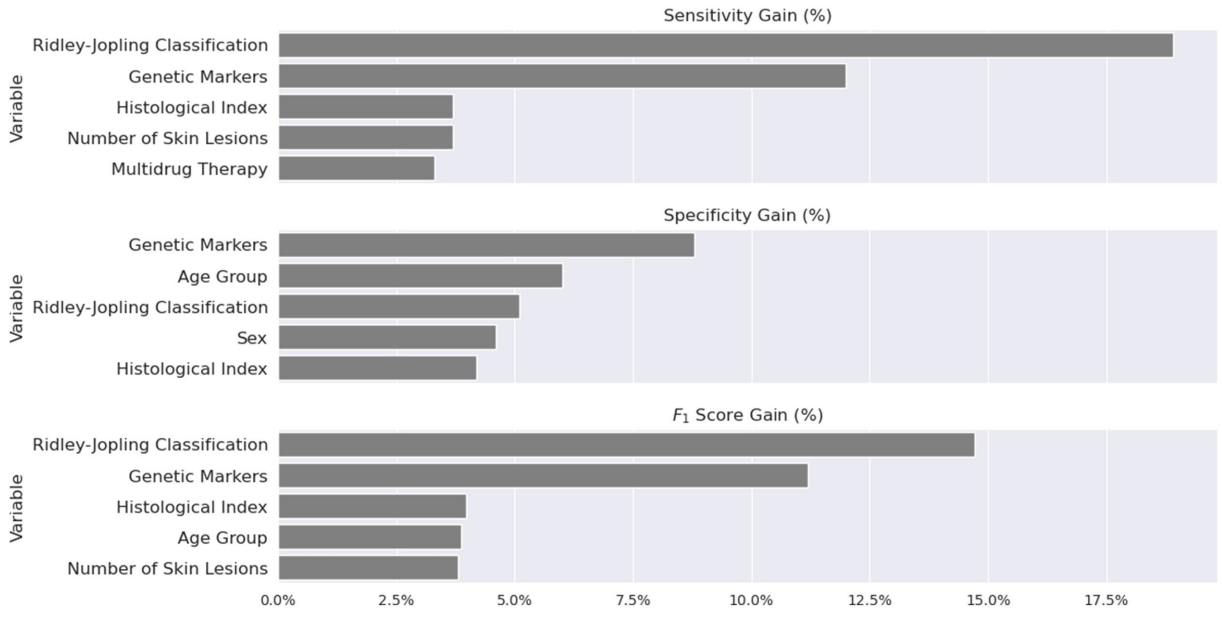

FIGURE 3
Top 5 most essential features measured in relative gain using sensitivity, specificity, and the F1 score.

at the lepromatous pole have a higher risk of developing T2R – more specifically, 61%~ when the type is LL. The second top-three parameter impacting the system is the histological index. An index equal to 2+ increases the risk of T1R to 56%~; values higher than 5+ shift the risk towards T2R – 45%~ when the histological index is 6+. This behavior is expected since an increase in the histological index is highly correlated with a higher bacterial load and, consequently, a move toward the lepromatous pole of the disease. A histological index higher than 5+ is also a well-known risk factor for developing T2R (38, 39). Finally, genetic data seems critical to improving the system's performance, which suggests that understanding the true, exact nature of LR depends on the description of the underlying genetic mechanisms.

We are aware of the study's limitations: we have had limited access to genetic information across the population samples; including genotypic data for additional, known LR susceptibility genes would likely positively impact the system's performance. In addition, the heterogeneity of the databases, originally obtained for independent studies of distinct designs, prevented a comprehensive analysis of the performance of the system, which we understand was yet quite remarkable, likely due to the ability of Bayesian methods to estimate risk using all available – even if partial – information. This is important considering that not all leprosy centers across the globe will have access to molecular data of all the patients; in these cases, the platform can still help estimate the risk of LR using only the clinical/laboratory and demographic data with fair sensitivity and specificity, as observed for the Fortaleza and Manaus datasets (Table 3). Of note: the heterogeneity of the dataset is known to enhance the quality of a trained model, since it tends to improve the generalization capturing a more comprehensive understanding of the problem and its nuances. Thus, the inclusion of diverse datasets is a known strategy to improve the performance of machine learning models. For example, in the field of Random Forests, the use of diverse datasets has been explored as a method to enhance the model's accuracy and robustness (78). This principle extends to various domains, including computer vision (79), and conversational AI (80). For a comprehensive evaluation and refining of the system, datasets enrolled prospectively with these specific purposes will be necessary.

5. Conclusion

We produced SEPAREH as an easy-to-use, online, free-access system that identifies leprosy patients at higher risk of developing LR. We believe that SEPAREH can be useful to help primary healthcare services to establish a protocol for patient follow-up dedicated to improving early diagnosis and prevention of the devastating consequences of untreated LR. Ultimately, risk assessment of LR for individual patients may be of potential positive impact on the prevention of permanent disabilities, the quality of life of the patients, and upon leprosy control programs.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

TABLE 3 Results obtained for each population sample.

Population sample	Two-by-two contingency				Results	95% CI
Combined		NRLP	LR	Total	Sensitivity = 79.3%	73.9–84.7%
	NRLP	187	45	232	Specificity = 86.2%	81.6–90.8%
	LR	30	172	202	PVP = 85.1%	80.2–90.1%
	Total	217	217	434	PVN = 80.6%	75.5–85.7%
					Accuracy = 82.7%	79.2–86.3%
Goiania		NRLP	LR	Total	Sensitivity = 85.7%	76.5–94.9%
	NRLP	59	8	67	Specificity = 89.4%	82.0–96.8%
	LR	7	48	55	PVP = 87.3%	78.5–96.1%
	Total	66	56	122	PVN = 88.0%	80.3–95.8%
					Accuracy = 87.7%	81.9–93.5%
Bauru		NRLP	LR	Total	Sensitivity = 82.7%	72.4–93.0%
	NRLP	51	9	60	Specificity = 85.0%	76.0–94.0%
	LR	9	43	52	PVP = 82.7%	72.4–93.0%
	Total	60	52	112	PVN = 85.0%	76.0–94.0%
					Accuracy = 83.9%	77.1–90.7%
Fortaleza		NRLP	LR	Total	Sensitivity = 78.1%	68.6–87.6%
	NRLP	62	16	78	Specificity = 71.3%	61.8–80.8%
	LR	25	57	82	PVP = 69.5%	59.5–79.5%
	Total	87	73	160	PVN = 79.4%	70.5–88.4%
					Accuracy = 74.3%	67.6–81.1%
Manaus		NRLP	LR	Total	Sensitivity = 77.8%	58.6–97.0%
	NRLP	18	4	22	Specificity = 78.3%	61.4–95.1%
	LR	5	14	19	PVP = 73.7%	53.9–93.5%
	Total	23	18	41	PVN = 81.8%	65.7–97.9%
					Accuracy = 78.0%	65.4–90.7%

LR, leprosy reactions; NRLP, non-reactional leprosy patients; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

Ethics statement

This study was approved by the Brazilian Committee for Ethics in Research (CONEP) (protocol 1.722.447). All patients signed an informed consent to participate in the study; for patients <18 years old, the informed consent was signed by one of the parents or the legal guardian.

Author contributions

RA, EH, DC, CM, and JN contributed to defining the AI-based protocol and data modeling. LH, GR, and EH developed the online platform. MA contributed to the recruitment and clinical characterization of the Tropical Pathology and Public Health Institute, Goiania, Goiás patients. AL, CS, AB, and PR contributed to the recruitment and clinical description of the Lauro de Souza Lima Institute, Bauru, São Paulo patients. MP and HS contributed to the recruitment and clinical characterization of the Dona Libânia Dermatology Centre patients, Fortaleza, Ceará. RC contributed to the recruitment and clinical description of the Alfredo da Matta Foundation patients, Manaus, Amazonas. MO contributed to the statistical analysis. VF contributed to the generation of the original genetic data. SB-S, GP, and MP contributed to coordinating the original study under which the population samples were recruited and characterized. RA, EH, CM, JN, and MM helped to draft the manuscript. MM is the principal investigator, the main responsible for the study design and execution, and provided senior supervision throughout the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1233220/full#supplementary-material

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Specialized active leprosy search strategies in an endemic area of the Brazilian Amazon identifies a hypermutated *Mycobacterium leprae* strain causing primary drug resistance

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Introduction: Leprosy, an infectious disease caused by *Mycobacterium leprae*, remains a public health concern in endemic countries, particularly in Brazil. In this study, we conducted an active surveillance campaign in the hyperendemic city of Castanhal in the northeastern part of the state of Pará using clinical signs and symptoms combined with serological and molecular tools to diagnose new cases and to identify drug resistance of circulating *M. leprae* strains and their distribution in the community.

Methods: During an active surveillance of one week, we enrolled 318 individuals using three different strategies to enroll subjects for this study: (i) an active survey of previously treated cases from 2006 to 2016 found in the Brazil National Notifiable Disease Information System database ($n = 23$) and their healthy household contacts (HHC) ($n = 57$); (ii) an active survey of school children (SC) from two primary public schools in low-income neighborhoods ($n = 178$), followed by visits to the houses of these newly diagnosed SC ($n = 7$) to examine their HHC ($n = 34$) where we diagnosed additional new cases ($n = 6$); (iii) and those people who spontaneously presented themselves to our team or the local health center with clinical signs and/or symptoms of leprosy ($n = 6$) with subsequent follow-up of their HHC when the case was confirmed ($n = 20$) where we diagnosed two additional cases ($n = 2$). Individuals received a dermato-neurological examination,

5 ml of peripheral blood was collected to assess the anti-PGL-I titer by ELISA and intradermal earlobe skin scrapings were taken from HHC and cases for amplification of the *M. leprae* RLEP region by qPCR.

Results: Anti-PGL-I positivity was highest in the new leprosy case group (52%) followed by the treated group (40.9%), HHC (40%) and lowest in SC (24.6%). RLEP qPCR from SSS was performed on 124 individuals, 22 in treated cases, 24 in newly diagnosed leprosy cases, and 78 in HHC. We detected 29.0% (36/124) positivity overall in this sample set. The positivity in treated cases was 31.8% (7/22), while in newly diagnosed leprosy cases the number of positives were higher, 45.8% (11/23) and lower in HHC at 23.7% (18/76). Whole genome sequencing of *M. leprae* from biopsies of three infected individuals from one extended family revealed a hypermutated *M. leprae* strain in an unusual case of primary drug resistance while the other two strains were drug sensitive.

Discussion: This study represents the extent of leprosy in an active surveillance campaign during a single week in the city of Castanhal, a city that we have previously surveyed several times during the past ten years. Our results indicate the continuing high transmission of leprosy that includes fairly high rates of new cases detected in children indicating recent spread by multiple foci of infection in the community. An unusual case of a hypermutated *M. leprae* strain in a case of primary drug resistance was discovered. It also revealed a high hidden prevalence of overt disease and subclinical infection that remains a challenge for correct clinical diagnosis by signs and symptoms that may be aided using adjunct laboratory tests, such as RLEP qPCR and anti-PGL-I serology.

KEYWORDS

leprosy, *Mycobacterium leprae*, household contacts, school children, drug resistance

1. Introduction

Leprosy, caused by the human pathogen *Mycobacterium leprae*, is a chronic, slowly evolving disease that causes damage to skin and nerves resulting in a wide array of skin lesions, nerve inflammation and pain leading to nerve impairment, loss of sensation, muscle weakening, atrophy and bone loss leading to disfigurement and disability with resulting social stigma. It remains a public health problem, especially in middle and low-income countries, such as India, Brazil, and Indonesia, where 79.6% of all global new cases were reported in 2019, when 202,185 new cases were detected globally. Brazil detected the second largest number of cases worldwide after India, with 27,863 new cases (1). The Brazil Amazon region, besides being highly endemic, has been depicted as having a very high hidden prevalence of leprosy (2). *M. leprae* primarily infects the peripheral nerves and later the skin (3, 4). Transmission from person to person is thought to be through the aerosol route, mainly in persons living in close contact for extended periods of time (5). Therefore, daily and continuous exposure with untreated patients make household contacts (HHC) a high-risk group in disease control strategies (6). Leprosy in children below 15 years old indicates recent infection to the bacillus during the early years of life and active circulation of bacilli in the community (7). This group was included as a target in the strategy for early detection and disrupting the transmission chain, aiming for the elimination of leprosy as a public health problem by the World Health Organization (8).

Early detection through contact tracing and active surveillance is essential to break the chain of transmission, to prevent severe neural involvement and physical disabilities due to disease progression. The diagnosis still relies on identifying well-characterized clinical signs and symptoms, with the detection of peripheral nerve damage, loss of sensation, and skin lesions. Laboratory tools, such as bacilloscopy in slit skin smears (SSS) (9), histopathology of skin lesions and molecular biology for detecting the *M. leprae*-specific repetitive element RLEP in SSS and skin biopsy (10, 11) as well as anti-PGL-I serology titer (2, 12, 13) support case elucidation, patient and HHC follow-up, and evaluation of subclinical infection in the community.

Together with the difficulties in the clinical diagnosis of leprosy and the absence of laboratory tools, drug resistance is an aggravating factor in controlling leprosy. The emergence of drug resistance has been reported since 1960 (14), and the presence of point mutations within genes in the drug resistance determining region (DRDR) is widely considered an important molecular signature for drug resistance in leprosy (15). Mutations in the *folP1* and *rpoB* genes confer resistance to the first line drugs used in the multidrug therapy (MDT) regimen, dapsone and rifampicin respectively, while mutations in *gyrA* and *gyrB* confer resistance to quinolones, second-line drugs of choice for leprosy treatment (16, 17).

Drug-resistant strains from 2009–2015 were recently described worldwide from MB leprosy cases from 19 sentinel countries for resistance to rifampicin, dapsone and ofloxacin showing around 2.3% in new cases and 4.5% in relapsed cases with 154 out of 1,932 (8%) *M. leprae* strains found overall with drug resistant mutations (18). In

Brazil, a study with relapsed leprosy patients from the states of Rio de Janeiro, Espírito Santo, Amazonas, Pará and Ceará showed mutations associated with drug-resistance in *folP1* (5.3%), *rpoB* (7%), and *gyrA* (2.6%) (19). In the Brazilian Amazon region, the detection of drug resistance variants reached 43.2% among leprosy patients in a former leprosy colony, Prata Village (20), that is located less than 40 Km from Castanhal, the city of our study.

In this study, we conducted an active surveillance campaign in the hyperendemic city of Castanhal using clinical signs and symptoms combined with serological and molecular tools to diagnose new cases and to identify drug resistance of circulating *M. leprae* strains and their distribution in the community.

2. Methods

2.1. Study area

Castanhal is a municipality located 68 Km from Belém, the capital city of the state of Pará, Northern Brazil Amazon region, with an estimated population of 198,000 inhabitants in 2018 (21). The municipality has 76.4% of the urban population covered by the Family Health Strategy (SUS, the primary health service in charge of leprosy diagnosis), which is much higher than Belém that had only 22% coverage in 2016 (22). The average leprosy new case detection rate in the last ten years in Castanhal was 42.7/100,000 inhabitants, considered hyperendemic according to the WHO and Brazilian Ministry of Health (23).

2.2. Fieldwork

Fieldwork was carried out using three distinct strategies: (i) active survey of reported and multidrug therapy (MDT) treated cases from 2006 to 2016 at the Brazil National Notifiable Disease Information System and their HHC (Figure 1A), (ii) active survey of school children (SC) from two primary public schools in peripheral and low-income neighborhoods, followed by a visit of the houses of the SC diagnosed with leprosy to examine their HHC (Figure 1B), and (iii) people who spontaneously presented themselves to our team, or the local health center, with signs and/or symptoms of leprosy as well as a visit to their HHC when the case was confirmed (Figure 1C).

The active survey was conducted according to the following scheme. All subjects were clinically evaluated by our team of health professionals (including a leprosy specialist, nurse and physiotherapist) and had peripheral blood and earlobe SSS collected according to established protocols. Biopsy of skin lesions was performed for pathological analyses by hematoxylin and eosin to detect cellular infiltrates and Fite-Faraco staining for quantifying acid-fast bacilli (AFB) (24) in a logarithmic index, resulting in the bacillary index (BI) registered from 0 to 6+ (25) depending on the number of AFB detected in the sample. The sample's BI is related to the number of

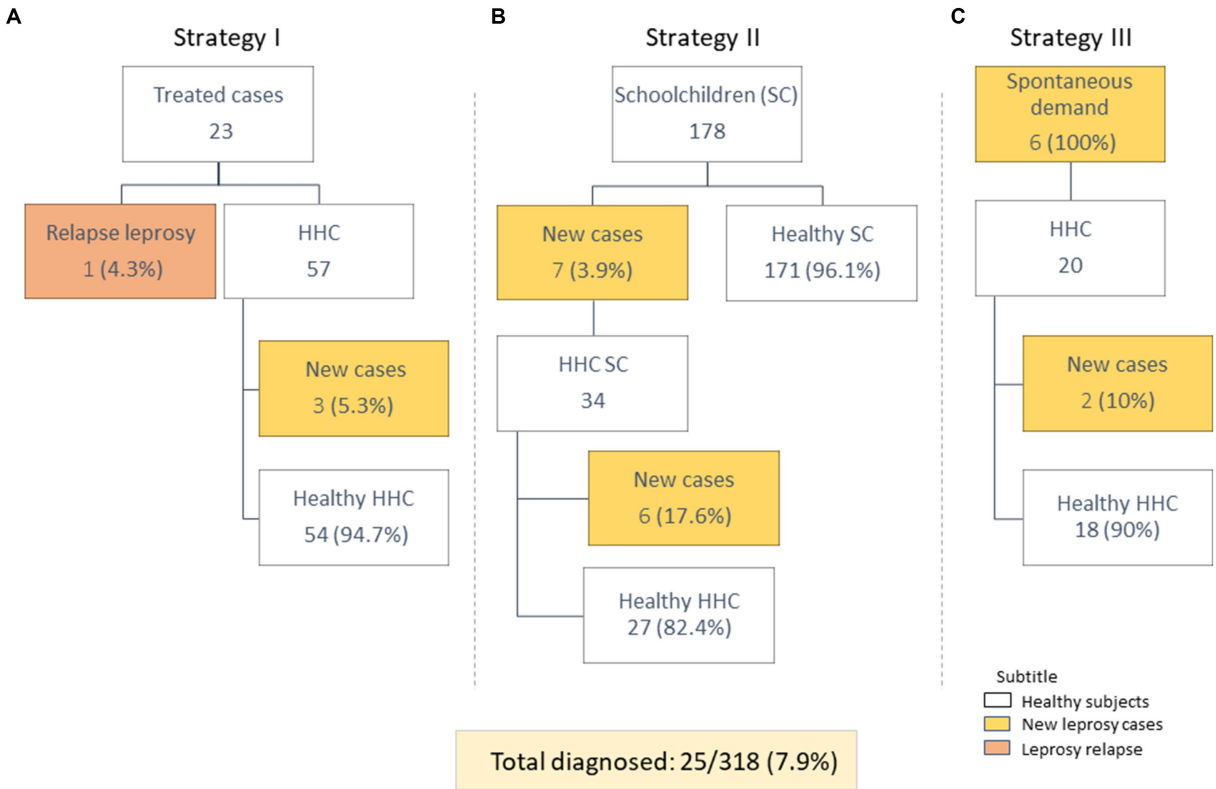

FIGURE 1
Distribution of study participants, according to each of the three active search strategies. **(A)** Strategy I: identified previously treated leprosy patients with follow-up with their HHC. **(B)** Strategy II: examined schoolchildren with follow-up of their HHC. **(C)** Strategy III: identified individuals who came to the clinic with suspected symptoms of leprosy with follow-up of their HHC.

M. leprae genome copies in the sample collected which is a determining factor for predicting the success rate for *M. leprae* whole genome sequencing (WGS) (26).

2.3. Clinical evaluation

In the clinical evaluation, the leprosy physician examines the skin of each individual and when suspected characteristic skin lesions were detected, a sensitivity test was performed using the Semmes-Weinstein monofilament (27). Based on the Simplified Neurological Evaluation protocol proposed by the Brazil leprosy control program (28), peripheral nerves were examined by palpation as well as determining sensitivity, motor and autonomic functions for all nerves, including trigeminal, facial and auricular on face and neck; radial, radial cutaneous, median and ulnar nerves in the upper limbs; and fibular, superficial fibular and tibial nerves in the lower limbs. The assessment of neural impairment and grade of disability varied from 0 to 2, where grade 0 represents an absence of physical disability, grade 1 those individuals with decrease or loss of sensitivity on hands and/or feet, and grade 2 those with visible physical disabilities in eyes and/or limbs (8).

2.4. Laboratory analyses

Five milliliters of peripheral blood were collected from all individuals for the serological assay for the detection of anti-PGL-I antibodies by the ELISA technique, using the ND-O-HSA antigen, through a protocol described previously (29).

SSS were collected from both earlobes in one eppendorf tube containing 70% ethanol (13). After rehydration of the pelleted material in phosphate buffer saline (PBS), DNA extraction was performed using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) following the manufacturer's recommendations. Amplification of the specific *M. leprae* RLEP region was performed by quantitative PCR (qPCR) using forward LP1 (5′-GTGAGGGTAGTTGTT-3′) and reverse LP2 (5′-GGTGCGAATAGTT-3′) primers (30). The qPCR amplification mixture contained 5 µL of PCR grade water, 10 µL of SYBR green fluorescent DNA binding dye, 1 µL of primers and 10 ng of total DNA or 10 ng of positive control *M. leprae* DNA, or 4 µL of PCR grade water as a negative control, in a total volume of 25 µL per reaction. Each reaction was conducted in duplicate and the contents were processed and read by an Applied Biosystems® 7,500 Real-Time PCR System. The reaction occurred with the following specifications: Uracil-DNA glycosylase (UDG) at 50°C for 2 min, prior 95°C for 2 min for initial denaturation followed by 45 cycles, each cycle consisting of denaturation at 95°C for 15 s, annealing at 58°C for 15 s and extension at 72°C for 1 min. A melting curve was performed in each experiment. A standard amplification curve was prepared with purified *M. leprae* starting at 10^9 bacilli genome copies/µL. The standard curve was composed of five points and was performed by serial dilution (1,100 to 1,5,000). The melting curve was used to analyze the specificity of the amplification. The results were obtained according to the first fluorescence signal detection cycle threshold (Ct). The sample was considered positive when duplicate samples showed a Ct less than 45 cycles. The standard curve was performed on each plate and included three negative control samples for each experiment.

Two skin biopsies were collected from each patient showing altered sensitivity skin lesions by a dermatologist using a 4 mm disposable punch (25). One fragment was stored in 10% formalin for histopathological examination and the other fragment was placed in 70% alcohol for WGS. Formalin fixed samples were dehydrated, clarified, and embedded in paraffin. Slides of 5 µm thickness were obtained from blocks sectioned with a microtome and subsequently deparaffinized. Sections were stained with hematoxylin–eosin to evaluate cellular infiltration and with Fite-Faraco for AFB detection (31).

For WGS of skin biopsy material, DNA was extracted using a pre-established protocol combining host tissue digestion and the QIAmp microbiome kit for host DNA depletion, strong bacterial cell lysis, and silica-based purification (26). Libraries with low *M. leprae* content underwent enrichment using whole-genome tiling arrays as described previously (32).

2.5. Statistical analysis

To compare the medians of the test results, the Mann–Whitney test was performed for two independent non-parametric samples. The statistical test and the plotting of results on graphs were performed using the GraphPad prism® program (version 6.1), the significance level of 0.5 ($p \leq 0.05$) was used.

3. Results

During the fieldwork week, we evaluated a total of 318 individuals and diagnosed 25 cases (7.9%) using the three different strategies (Figure 1). In the previously treated case group, we evaluated 23 individuals and diagnosed one relapse (1/23; 4.3%). Among their HHC, three new cases were diagnosed (3/57; 5.3%) (Figure 1A). In the SC survey, 178 students were examined and 7 were diagnosed as new leprosy cases (7/178, 3.9%). The HHC of newly diagnosed SC were examined and six of 34 of these (17.6%) were diagnosed (Figure 1B). Six individuals with spontaneous demand (those who visited the clinic with symptoms of leprosy) were diagnosed (6/6; 100.0%) and two of the 20 HHC from these new cases (2/20; 10.0%) were diagnosed (Figure 1C).

The newly diagnosed leprosy cases (n = 25) ranged in age from 4 to 64 years old. Of these, nine (9/25; 36%) were children under 15 years old. The clinical forms were classified as: Primary neural (2/25; 8%), Indeterminate (3/25; 12%), Tuberculoid (1/25; 4%), Borderline (17/25; 60%), and Lepromatous leprosy (2/25; 8%). The disability grade of new cases was categorized as: Grade 0 (17/25; 68%), Grade 1 (6/25; 24%), and Grade 2 (2/25; 8%).

The anti-PGL-I IgM antibody titer was positive in 32.7% of all individuals (104/318). Among newly diagnosed leprosy cases, the positivity was 52% (13/25), the O.D. median was 0.31 while for treated cases the positivity was 40.9% (9/22) with an O.D. median of 0.21. HHC were positive in 40.0% (40/100) with an O.D. median of 0.24 and 24.6% (42/171) of SC were positive with an O.D. median of 0.18 (Table 1 and Supplementary Table S1). The statistical test showed a significant difference between SC and HHC ($p = 0.003$, 95% CI −0.09 to −0.019) and between SC and new leprosy cases ($p = 0.018$, 95% CI −0.017 to −0.198) (Figure 2).

RLEP qPCR from SSS was performed on 124 individuals, 22 in treated cases, 24 in newly diagnosed leprosy cases, and 78 in HHC. We detected 29.0% (36/124) positivity overall in this sample set. The positivity in treated cases was 31.8% (7/22), while in newly diagnosed

leprosy cases the number of positives were higher, 45.8% (11/23) and lower in HHC at 23.7% (18/76). The percentage of double-positives overall (anti-PGL-I IgM+/RLEP qPCR+) was 5.6% (7/124). In the individual groups double positivity was 16.7% (4/24) for new leprosy cases, 4.5% (1/22) for treated cases and 2.6% (2/78) for HHC (Table 1).

A total of 22 skin biopsies were sampled from newly diagnosed leprosy patients. Three samples (3/22, 13.6%) were confirmed as leprosy by histopathology due to the presence of AFB. Three samples (3/22, 13.6%) were classified as superficial spongious dermatitis; three samples (3/22, 13.6%) were classified as granulomatous dermatitis and 13 (13/22, 59.2%) were classified as superficial perivascular dermatitis. RLEP qPCR was performed for 17 biopsies and was positive in seven of these (7/17, 41.2%), among which only three (42.8%) were positive for AFB and confirmed as leprosy by histopathology. Of the remaining samples, 2/7 (28.6%) were characterized as superficial perivascular dermatitis while the other 2/7 (28.6%) were characterized as granulomatous dermatitis.

Only five of the RLEP positive samples had enough bacillary DNA for WGS (n = 2) or to fully sequence the drug resistance determining region (DRDR) by PCR sequencing (n = 3). The two strains fully sequenced were covered 111 (patient 3702) and 57 times (patient 51447), respectively. 51447 was wild type (WT) for *rpoB*, *folp1*, *gyrA*, and *gyrB* while another was found to be a hypermutated *M. leprae* strain (3702), with multiple mutations in the DRDR genes *folp1* (P55L), *gyrA* (V731I) and *gyrB* (T503I). There were additional mutations found in a number of other genes, including *fadD9* (G796S), *ribD* (A63T), *pks4* (M14I) and *nth* (N142fs) (26). Raw genome sequences were deposited into the NCBI Sequence Read Archive (SRA) with biosample numbers SAMN07514430 (3702 or Br2016-15) and SAMN36810538 (Br51447). Both of these isolates were SNP type 4N which predominates in this region (Supplementary Table S1). The remaining three samples were WT in *rpoB* and *folp1* and two were WT for *gyrA*. None of the three amplified the *gyrB* gene, so this gene could not be characterized.

TABLE 1 Positivity of anti-PGL-I IgM, molecular detection of RLEP and association of the two tests in the groups of the study.

Groups	Anti-PGL-I					RLEP (qPCR)				Double			
	Median	Positive		Negative		Positive		Negative		Positive		Negative	
	(O.D.)	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)
New leprosy cases	0.31	13	52.0	12	48.0	11	45.8	13	54.2	4	16.7	5	20.8
Leprosy treated cases	0.21	9	40.9	13	59.1	7	31.8	15	68.2	1	4.5	7	31.8
HHC[a]	0.24	40	40.0	60	60.0	18	23.1	60	76.9	2	2.0	32	34.0
SC[b]	0.18	42	24.6	129	75.4	NA[c]	NA	NA	NA	NA	NA	NA	NA
Total		104	32.7	214	67.3	36	29.0	88	71.0	7	5.0	44	31.4

[a]HHC: Household contacts.
[b]SC: School children.
[c]NA: Not available.

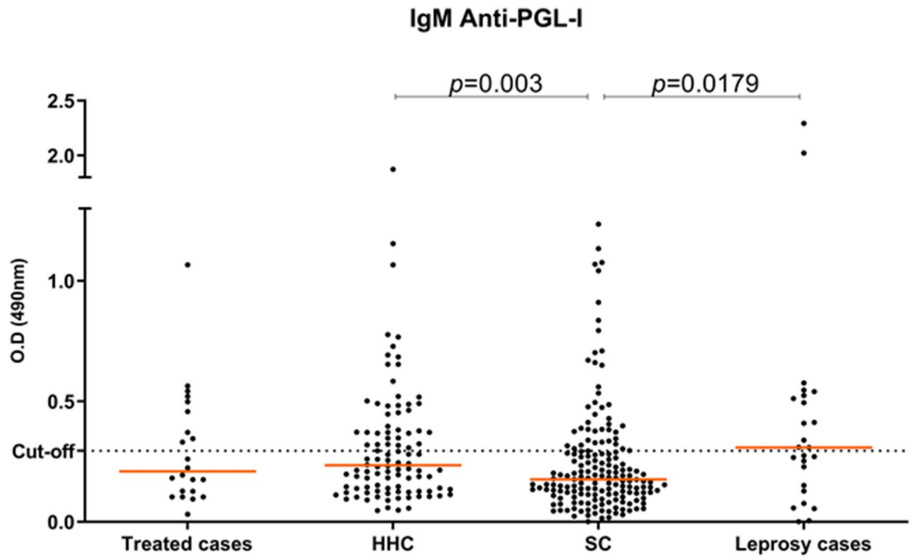

FIGURE 2
Titer of anti-PGL-I antibodies for all individuals according to study groups: treated cases (n = 22); HHC (n = 100); SC (n = 171); new leprosy cases (n = 25).

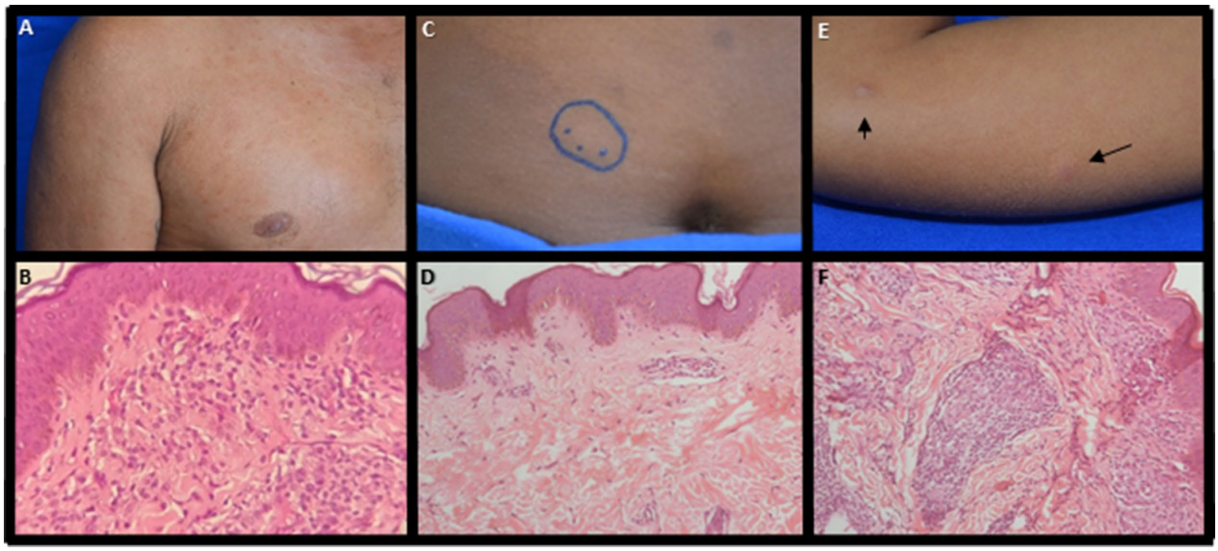

FIGURE 3
Clinical and pathological examination evaluation. Primary drug-resistant leprosy case **(A)** presence of infiltrative lesions and nodules disseminated through the integument; **(B)** dense granulomatous inflammatory infiltrate composed of lymphocytes, epithelioid histiocytes of foamy cytoplasm and plasmocytes, involving vessels, nerve filaments and superficial and deep plexus attachments; **(C)** spouse presented hypochromic plaque in the abdomen; **(D)** epidermis with a mild acanthosis and dermis with minimal perivascular lymphocytic infiltrate in the upper dermis and negative AFB. **(E)** son with hypochromic maculae with the presence of tubers in the right arm and elbow; **(F)** dense granulomatous inflammatory infiltrate of nodular architecture, composed of lymphocytes, plasmocytes and cytoplasmic epithelioid histiocytes with few positive AFB.

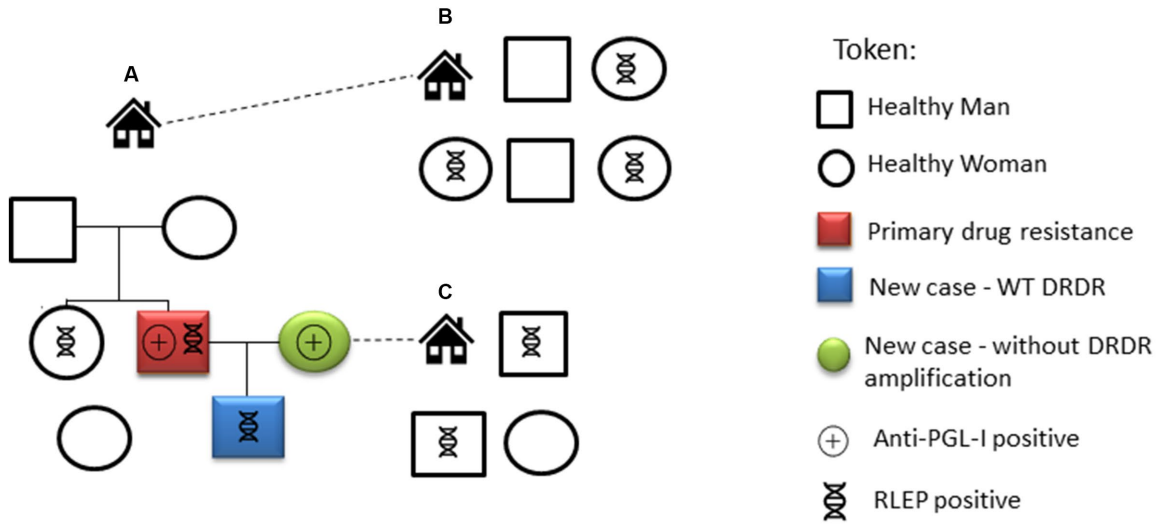

FIGURE 4
Evaluation and laboratory exams of the primary drug-resistant leprosy case and his contacts. House A residents: the individual primary drug-resistant case (red square), his spouse (green circle), their son (blue square) and other relatives. Residents of house B, located close to house A, are life-long contacts. Relatives of the spouse live in house C. The positive results for anti-PGL-I serology and detection of RLEP for each individual are shown.

3.1. Case findings of three diagnosed leprosy patients in a single extended family

3.1.1. The primary multidrug-resistant leprosy case

A 31-year-old male with no prior history of leprosy presented infiltrative and nodular lesions disseminated throughout the skin, including face and ears, for at least one-year. After clinical evaluation, he was diagnosed with lepromatous leprosy (Figure 3A). The neurological evaluation showed three affected nerves with no disability (DG0). Adjunct laboratory tests demonstrated positive results for: SSS (BI 3.5), anti-PGL-I IgM antibody (O.D. = 2.02), positive RLEP qPCR in SSS (Ct = 32) and histopathological examination showing a dense superficial and deep granulomatous inflammatory infiltrate with a nodular architecture composed of lymphocytes, epithelioid histiocytes of foamy cytoplasm and

plasmocytes involving vessels and nerve filaments (Figure 3B). The Fite-Faraco staining was also positive, with AFB either isolated or forming globi classified histopathologically as borderline lepromatous (BL) according to Ridley and Jopling classification. Molecular evaluation of the skin lesion was RLEP positive. Whole genome sequencing identified the strain as SNP subtype 4 N and as a hypermutated *M. leprae* strain with multiple mutations in the DRDR genes *folp1* (P55L), *gyrA* (V731I), *gyrB* (T503I), and in several other genes, including *fadD9* (G796S), *ribD* (A63T), *pks4* (M14I) and *nth* (N142fs). Regarding the treatment of this patient, after 11 doses of standard MDT, new nodular lesions in the lower limbs continued to appear at which time the WGS results confirmed dapsone resistance. The treatment regimen substituted daily minocycline 100 mg for dapsone and after 12 additional doses with this modified regimen the patient showed improvement in the clinical and laboratory parameters including an absence of active lesions, a decrease in the BI to 2.5 and a lower anti-PGL-I titer to 0.42. These laboratory parameters continued to decrease 6 months after medical discharge with a BI of 2.0 and a negative anti-PGL-I titer of 0.27. Fourteen of the HHC of this individual were evaluated, and 2 (14.3%), a spouse and son, were diagnosed with clinical signs and symptoms of leprosy. The IgM anti-PGL-I titers were positive only in 2 samples (14.3%) and negative in the remaining ten HHCs, varying from O.D. 0.11 to 0.28. The amplification of the RLEP of SSS samples by qPCR was positive in 6/12 HHC (50%) considered clinically healthy (Figure 4).

The spouse, 21-years-old, presented a hypochromic skin plaque larger than 10 cm diameter with imprecise edges (pseudopods) on the abdomen (Figure 3C) with loss of sensation, and DG0. She was classified as borderline leprosy (BT) with a positive anti-PGL-I titer (O.D. = 0.41) and negative for RLEP by qPCR in SSS. The histopathological examination of the skin biopsy showed an epidermis with mild acanthosis and minimal perivascular lymphocytic infiltrate in the upper dermis. AFB were absent by the Fite-Faraco stain, and the lesion was diagnosed as minimal superficial perivascular dermatitis (Figure 3D).

The son, 4-years-old, had hypochromic lesions with the presence of tubers in the left forearm, left forehead, and right forearm (Figure 3E) associated with thickening of the left ulnar nerve, DG0 and negative serology (O.D. = 0.28). He was also clinically classified as borderline leprosy. Histopathological examination demonstrated a dense granulomatous inflammatory infiltrate of nodular architecture composed of lymphocytes, plasmocytes and cytoplasmic epithelioid histiocytes (Figure 3F), with AFB (1+) on the sections examined by Fite-Faraco stain with the diagnostic definition of borderline tuberculoid (BT) leprosy by histopathology according to Ridley and Jopling classification. The qPCR performed on the SSS sample was positive (Ct = 41.4) for RLEP of *M. leprae*. The molecular analysis of the skin biopsy showed WT alleles in *rpoB*, *folp1*, *gyrA* and *gyrB*.

4. Discussion

Castanhal is a city in the northeastern part of Pará state, an endemic area that has been monitored by our leprosy surveillance team since 2010 (12). The municipality presents structural challenges in terms of public health, including the capacity to diagnose leprosy cases early and perform contact tracing and follow-up. In only 1 week of fieldwork, our group detected 25 new cases, which represents 71.4% of the number of cases detected in a year before the study (35 new cases) (29). The delay in diagnosis was supported by the presence of grade 2 physical disability (DG2) in 8% of cases and the number of new cases of leprosy in children under 15 (9/25, 36%) indicates ongoing recent infection from multiple foci of spread within the community corresponding to 4.5-fold more than was diagnosed by the local health team in 2015 (23). Leprosy diagnosis is primarily based on clinical signs and symptoms identified by well-trained leprologists. Laboratory tests with high sensitivity and specificity are not able to diagnose those with leprosy in all clinical forms and cannot even predict which at-risk HHC with positive anti-PGL-I titers will eventually progress to disease (33). However, laboratory tools may help identify biomarkers of subclinical infection, supported by the fact that individuals who do not show obvious clinical signs and symptoms of leprosy, considered healthy contacts, can be identified as having been infected if they have a positive anti-PGL-I titer and/or confirmed acid-fast bacilli or RLEP PCR positivity in SSS or skin lesion biopsy (13). We have previously shown that HHC with a positive anti-PGL-I titer have an 8.6-fold higher risk of progressing to disease than those with negative serology within 4 years (12). In this current study, almost 10% of the HHC had a confirmed leprosy diagnosis and 40.0% of clinically healthy HHC were seropositive. This means that 4 out of 10 HHC have this higher risk of developing leprosy.

Another important tool is the detection of *M. leprae* DNA, which may assist in the monitoring of asymptomatic HHC in an endemic area (34). In our study, we used RLEP, a repetitive region with up to 37 copies in the *M. leprae* genome (35). Therefore, its detection is efficient even when there are low levels of *M. leprae* DNA in different samples (10) and correlates with the bacilloscopy index and the clinical form (11). In our study, 23.1% of HHC had a positive RLEP qPCR result in SSS. In addition, we found that 2% of HHC were double-positive (anti-PGL-I+/RLEP qPCR+), results that we have previously established as likely representing latent leprosy disease (13). These individuals live in an endemic area, have leprosy cases in their household, are positive for *M. leprae* DNA in the ear lobe and show a non-protective immune response against the bacillus allowing its ability to grow and spread. Despite not showing clinical signs and symptoms of leprosy, individuals positive for both biomarkers of infection likely are subclinical with latent disease and need continuous monitoring by the local health team. Moreover, the presence of *M. leprae* confirmed by intradermal smear microscopy or skin biopsy is one of the cardinal signs for leprosy case definition by the WHO (36). In fact, RLEP qPCR is just a more sensitive method to detect *M. leprae* through the presence of DNA in either SSS or skin biopsy, and this alone should be considered sufficient to diagnose such individuals and to subsequently treat them early with MDT to effectively break the transmission chain and to avoid a delayed diagnosis with severe nerve damage and disability.

Our strategies of active surveillance for new cases among contacts of former patients that had already been treated and among school children allows many of these cases to be diagnosed in their earliest clinical manifestations, with light clinical signs and symptoms without significant nerve damage or disability, which are often poorly understood by the patient, their family and even for many untrained professionals. Thus, early diagnosis and treatment of cases prior to the development of nerve damage are extremely important to break the

transmission chain and to avoid disfigurement and disabilities that can lead to stigma and social isolation.

The patient found with drug resistant *M. leprae* was apparently a case of primary drug resistance with no previous history of the patient being treated for leprosy. Luckily, our study showed that the son of this patient was not infected by this hypermutated strain, his strain was WT and drug sensitive. A limitation of this study was that although six of the 12 individuals in this extended family were RLEP+, none of these individuals had enough DNA to allow for sequencing and the spouse, who was diagnosed with leprosy, was qPCR negative for RLEP. Nevertheless, the finding of a patient with a strain resistant to dapsone, one of the main drugs used in the MDT regimen to treat most patients, in addition to mutations in *gyrA* and *gyrB* indicating possible resistance to fluoroquinolones, important second-line drugs used for the treatment of leprosy, should draw attention to the increased danger and prevalence of multidrug resistant strains and provide an incentive for increased funding for testing more clinical strains for drug resistance, especially in endemic areas. There is also a need to seek new alternative drug regimens that can be substituted in cases of resistance to the three main drugs used in MDT as was eventually used to treat the patient with the hypermutated strain and to identify new and more effective antimycobacterial drugs to facilitate a real break in the transmission chain of these strains in the community (37).

5. Conclusion

Our surveillance activities in just 1 week in an area hyperendemic for leprosy in the Amazon region of Brazil (Castanhal, Pará State) showed high transmission rates of leprosy. It also revealed a high hidden prevalence of overt disease and subclinical infection that remains a challenge for correct clinical diagnosis by signs and symptoms that may be aided using adjunct laboratory tests, such as RLEP qPCR and anti-PGL-I serology. The spread of leprosy can be worsened by the presence of drug resistant *M. leprae* strains that are potentially circulating in this population, which should be monitored more closely.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at (NCBI Sequence Read Archive, SRR6241736).

Ethics statement

The studies involving human participants were reviewed and approved by Institute of Health Sciences Research Ethics Committee from Pará Federal University (CAAE 26765414.0.0000.0018 CEP-ICS/UFPA). Written informed consent to participate in this study was provided by the participant or participants' legal guardian/next of kin. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from all individuals and/or minors' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article, including data in Supplementary Table S1.

Author contributions

RB, JB, JS, MS, and CS: contributed to the research design and writing the draft manuscript. RB, AG, MB, PCa, MB, MF, AN, SB, PCo, GC, ÂR-d-S, and CS: collected data and organized the database. RB, GC, CA, JS, MS, and CS: performed statistical and data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1243571/full#supplementary-material

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Field-friendly anti-PGL-I serosurvey in children to monitor *Mycobacterium leprae* transmission in Bihar, India

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Background: It has been amply described that levels of IgM antibodies against *Mycobacterium leprae* (*M. leprae*) phenolic glycolipid I (PGL-I) correlate strongly with the bacterial load in an infected individual. These findings have generated the concept of using seropositivity for antibodies against *M. leprae* PGL-I as an indicator of the proportion of the population that has been infected. Although anti-PGL-I IgM levels provide information on whether an individual has ever been infected, their presence cannot discriminate between recent and past infections. Since infection in (young) children by definition indicates recent transmission, we piloted the feasibility of assessment of anti-PGL-I IgM seroprevalence among children in a leprosy endemic area in India as a proxy for recent *M. leprae* transmission.

Material and methods: A serosurvey for anti-PGL-I IgM antibodies among children in highly leprosy endemic villages in Bihar, India, was performed, applying the quantitative anti-PGL-I UCP-LFA cassette combined with low-invasive, small-volume fingerstick blood (FSB).

Results: Local staff obtained FSB of 1,857 children (age 3–11 years) living in 12 leprosy endemic villages in Bihar; of these, 215 children (11.58%) were seropositive for anti-PGL-I IgM.

Conclusion: The anti-PGL-I seroprevalence level of 11.58% among children corresponds with the seroprevalence levels described in studies in other leprosy endemic areas over the past decades where no prophylactic interventions have taken place. The anti-PGL-I UCP-LFA was found to be a low-complexity tool that could be practically combined with serosurveys and was well-accepted by both healthcare staff and the population. On route to leprosy elimination, quantitative anti-PGL-I serology in young children holds promise as a strategy to monitor recent *M. leprae* transmission in an area.

KEYWORDS

children, leprosy, anti-*M. leprae* PGL-I antibodies, infection, diagnostics, serosurvey, transmission, upconversion

Introduction

Leprosy, caused by *Mycobacterium leprae (M. leprae)* or *M. lepromatosis*, is a debilitating neglected tropical disease (NTD) still predominantly forming a health threat for poor and marginalized populations from over 120 countries (1–3). It is a chronic infectious disease that can cause long-term nerve damage and often results in both physical and social disabilities (4, 5). *M. leprae* is believed to be transmitted via aerosol droplets from the respiratory system, during repeated and close contact with untreated patients (6). Only approximately 5% of persons infected with *M. leprae* develop disease symptoms (4). However, it is assumed that infected, asymptomatic individuals carrying sufficient amounts of the mycobacterium contribute to its transmission (7, 8).

Multidrug therapy (MDT) effectively kills *M. leprae* and provides an effective cure if treatment is initiated timely. Following MDT's introduction in 1981, leprosy prevalence has significantly dropped. Yet, transmission of *M. leprae* remains, reflected by over 9,000 new child cases detected in 2022 worldwide (9). It is also believed that large numbers go undetected as a result of the drop in leprosy-focused healthcare following the declaration of leprosy elimination on the global level (10).

The WHO's Global Leprosy Strategy 2021–2030 aims to significantly reduce the number of new cases with grade 2 disability and new child cases by focusing on early detection of disease and interruption of transmission. To achieve the latter, it is vital to identify and treat sources of infection, e.g., multibacillary (MB) cases with high bacillary loads that are highly likely to transmit bacteria (11, 12). Detecting *M. leprae*-infected individuals lacking clinical symptoms who can transmit the bacterium or develop leprosy themselves remains a major challenge for dedicated control programs. Monitoring leprosy elimination is currently conducted by evaluating the proportion of new child cases (below age 15) among the total number of new cases detected (9, 13, 14). As only a small percentage of *M. leprae*-infected individuals progress to disease and it can take many years before symptoms of leprosy manifest (15), using new cases to monitor elimination does not provide sufficiently accurate and up-to-date information with respect to (elimination of) transmission.

Household contacts of MB leprosy cases have been reported to be most vulnerable to contracting disease (16, 17). Levels of IgM antibodies against phenolic glycolipid I (PGL-I), a specific cell wall component of *M. leprae,* correlate strongly with the bacterial index (BI) of *M. leprae*-infected individuals (8, 18, 19). Moreover, based on a literature review covering reports on serology for leprosy from 1987 to 2020 worldwide, we showed that quantitative anti-PGL-I serology in young children as a measure for *M. leprae* infection provides the potential for assessing recent transmission rates in a community (20, 21). These findings, on top of the observation that MB cases are more likely to transmit bacteria, provide a rationale for identifying seropositive individuals to study transmission. Although the presence of antibodies cannot discriminate between recent and past infection, infection in young children by definition indicates recent transmission. Therefore, the assessment of seropositivity in children is recommended by the WHO "Task Force on *definitions, criteria and indicators for interruption of transmission and elimination of leprosy*" as an indicator to monitor elimination (9) and offers a tool to study the effects of interventions including post-exposure prophylaxis (PEP).

Over the past years, we have developed a robust, user-friendly test that detects anti-PGL-I IgM antibodies using the unique upconverting reporter particle (UCP) technology in a low-cost lateral flow-based assay (LFA) format [(18, 22–26) and Pierneef et al. (*manuscript in preparation*)]. The anti-PGL-I UCP-LFA offers a sensitive, low-complexity rapid test to quantitatively determine anti-PGL-I IgM levels in capillary and venous blood (24, 27).

In India, the new leprosy child case detection rate was approximately 10 per 1,000,000 child population in 2020 (28). Of the new cases of all ages registered in India, 15 to 20% (16,000–20,000 individuals each year) are located in Bihar (29), a socio-economically poor state in the eastern part of the country with an estimated population of over 100 million people (30). In this study, we report for the first time the application of the fully integrated anti-PGL-I UCP-LFA cassette in a larger serosurvey using fingerstick blood (FSB). We aimed to assess the use of the anti-PGL-I UCP-LFA as a tool for measuring *M. leprae* infection among children as a proxy for transmission in Bihar, India.

Methods

Study participants

Study participants were consenting individuals living in a Health and Demographic Surveillance System (HDSS) site in Muzaffarpur district, Bihar, India.

Children cohort

As part of the Tropical Medicine Research Center (TMRC) study on leishmaniasis in the already-existing Muzaffarpur HDSS, a door-to-door screening was performed by the staff of the Banaras Hindu University (BHU; Varanasi, India) (31, 32). The screening started in August 2020 and was completed in January 2021. FSB from children ($n = 1,857$; 987 male/870 female) between 3 and 11 years of age living in Bihar was collected (Table 1). The inclusion criteria were all children without any signs of leprosy or other diseases residing permanently in one of the following villages in the old HDSS area (32): Singar Phulkahan, Madhopur Chhapra, Godai Phulkahan, Godai Jamal, Vishwanathpur, Raksha North, Raksha North Chauk, Raksha South West, Raksha South, Raksha Deah, Nariyar Nawada, and Arizpur Kothi. Excluded were children below 2 years of age. BCG status was not recorded in this study but has been above 84% and almost uniform across India since the time of the study (2020) (33).

Leishmaniasis

In addition, the following biobank samples collected during the TMRC study in Bihar on leishmaniasis were included.

Serum samples from leishmaniasis patients from the same area were collected, including visceral leishmaniasis (VL; age 12–52; *n*

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TABLE 1 Age and gender of the 1,857 children per village of residence.

Village	Village name	# Children	Age mean (range)	Gender (% female)
A	Singar Phulkahan	114	7 (3–9)	43.86
B	Madhopur Chhapra	126	7 (5–9)	45.24
C	Godai Phulkahan	216	7 (5–9)	50.46
D	Godai Jamal	110	7 (5–9)	45.45
E	Vishwanathpur	89	7 (5–9)	49.44
F	Raksha North	164	7 (5–11)	49.39
G	Raksha North Chauk	204	7 (5–9)	48.04
H	Raksha South West	97	7 (5–9)	50.52
I	Raksha South	411	7 (5–9)	47.69
J	Raksha Deah	250	7 (3–9)	40.80
K	Nariyar Nawada	70	7 (5–9)	48.57
L	Arizpur Kothi	6	7 (5–9)	0
	Total	1,857	7 (3–9)	46.85

The number (#) of children, mean age (range), and percentage of females per village of residence are provided.

= 20) and post-kala-azar dermal leishmaniasis (PKDL; age 12–62; $n = 20$). VL patients were rK39 antibody/splenic aspirate positive and displayed clinical symptoms of active VL. PKDL patients were rK39 antibody or skin slit smear/PCR positive and had a history of VL (34).

Asymptomatic individuals

Serum samples from asymptomatic individuals (ASY; age 10–70) were collected. ASY were individuals without any clinical symptoms of leishmaniasis, who were seropositive for rK39 antibodies in the Direct Agglutination Test (DAT; cutoff titer for positivity ≥1:1,600) (34).

Endemic controls

Serum samples from endemic controls (ECs; age 8–50; $n = 20$) were collected as part of the NIH-TMRC project. ECs were individuals who tested seronegative for rK39 antibodies/DAT (34).

Fingerstick blood collection

FSB was collected using disposable 20 µl Minivette® collection tubes (Heparin coated; Sarstedt) and directly mixed with 980 µl high salt finger stick (HSFS) buffer: 100 mM Tris pH 8.0, 270 mM NaCl, 1% (v/v) Triton X-100, and 1% (w/v) BSA. FSB was applied to the anti-PGL-I UCP-LFA cassette on the spot immediately after collection.

Serum collection

For samples that were used as positive and negative controls throughout the study, venous blood samples were collected, in 4 ml plain BD vacutainer serum tubes (BD, Franklin Lakes, NJ, USA). Tubes were centrifuged at an RCF of 500 xg for 10 min and sera were subsequently aliquoted and frozen (−80°C) until use.

Anti-PGL-I UCP-LFA cassette

Fully integrated and individually packaged UCP-LFA cassettes for the detection of human anti-PGL-I IgM antibodies were produced by MaximBio (Rockville, MD, USA; schematic overview shown in Figure 1). The air-tight pouches with test cassettes contained silica dry packs allowing extended shelf life and protection against humidity. The Test (T) line on the LF strip comprised 100 ng of synthetic PGL-I, phenolic trisaccharide functionalized with a hexanoic acid linker for conjugation to BSA [NPT1-H-BSA; Leiden, the Netherlands (35)]. The Flow-Control (FC) line comprised 100 ng rabbit anti-goat IgG [G4018; Sigma-Aldrich, Inc., St. Louis, MO, USA). Goat IgG specific for anti-human IgM (I0759; Sigma-Aldrich, Inc., St. Louis, MO, USA] was conjugated to polyacrylic acid functionalized UCPs [200 nm, $NaYF_4$:Yb^{3+}, Er $^{3+}$; Intelligent Material Solutions Inc. (IMS); Princeton, NJ, USA MS] according to previously described protocols at a concentration of 50 µg antibody per mg UCP (23). Stock solutions were kept at 4°C until use. To dry the UCPs onto the glass fiber conjugate-release pad, the material was diluted in a buffer containing 100 mM Tris pH 8.0, 270 mM NaCl, 10% (w/v) sucrose, 1% (w/v) BSA, 0.5% Tween-20, and striped at a density of 100 ng/mm. Components were mounted on plastic backing cards which were cut into LF strips of 4.8 mm width by 6 cm length, added to an appropriate cassette, and individually sealed in a pouch together with a silica dry pack.

UCP-LFA

To initiate LF, 50 µl of the 50-fold diluted FSB or serum sample was added to the test cassette. Cassettes were analyzed using a UCP-adapted portable reader (LFR; Qiagen, Hilden, Germany; Figure 1). The results were calculated as the ratio value (R) between T and FC signals based on relative fluorescence units (RFUs) measured at the respective lines. The cutoff for positivity ($R \geq 0.12$) for the UCP-LFA batch used in this study was based on the median of a sextuple test of a standard control serum sample (+) performed in India plus the standard deviation (SD). Measurements in India yielded an identical cutoff level as in Leiden using aliquots of the same control serum sample (+). In addition, one highly anti-PGL-I IgM-positive serum sample (++) and one negative serum sample (–) were tested as control samples in sextuple to monitor the reproducibility of the test.

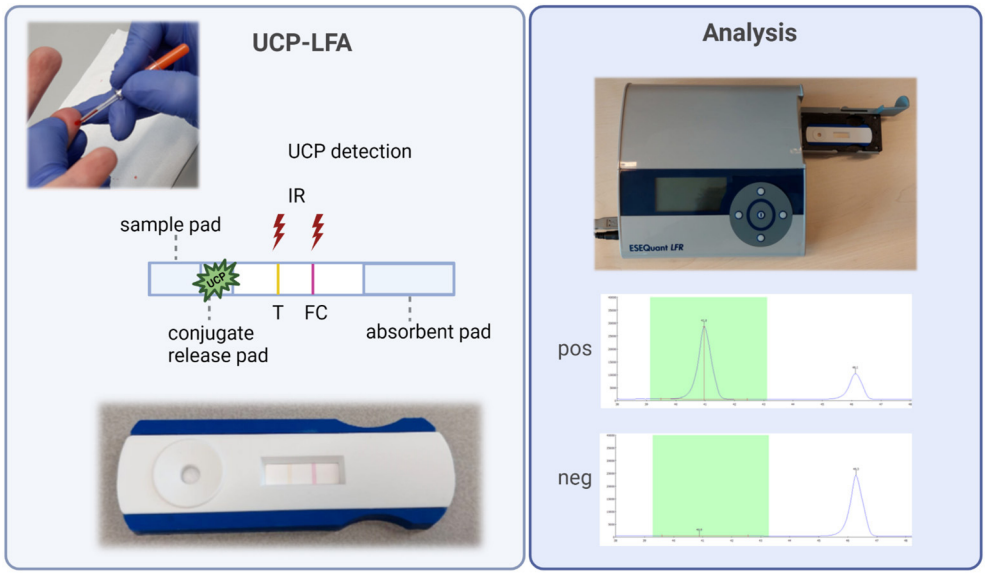

FIGURE 1
Fully integrated anti-PGL-I UCP-LFA cassette and analysis. **Left:** FSB is collected with a minivette (20 μl). To initiate LF, a diluted FSB sample is added to the cassette onto the sample pad. Hydration of the anti-IgM UCP-conjugate will allow the binding of anti-PGL-I IgM antibodies from the sample to the conjugate and sequential binding to the T line with synthetic PGL-I. The FC line can bind UCP conjugate not bound to the T line. Color-coded T and FC lines visible by the eye disappear upon flow. **Right:** UCP signals are detected with a reader upon excitation with 980 IR light generating 540 nm emission. Results for positive and negative samples are shown. Results are calculated as R-value, with the T signal divided by the FC signal. This figure was created with BioRender.com. FC, flow control line; FSB, fingerstick blood; IgM, immunoglobulin M; IR, infrared excitation; PGL-I, phenolic glycolipid I; R, ratio value, result of the UCP-LFA; T, test line; UCP-LFA, upconverting reporter particle lateral flow assay.

Ethics

This study was performed in accordance with the Helsinki Declaration (7th revision, 64th Meeting, 2013, Fortaleza). This study was a component of Muzaffarpur NIH-TMRC HDSS for which ethics approval was received from the institutional review boards of the Institute of Medical Sciences, Banaras Hindu University (BHU; reference number Dean/2017/EC/185; Dated 24/10/2017), Varanasi, India, and the review committee of the U.S. National Institutes of Health (NIH). The institutional review board of BHU has received accreditation from the National Institutes of Health in the United States. Ethics approval was also obtained by the institutional review board of the Institute of Tropical Medicine, Antwerp (reference number 1305/19), and the Ethics Committee of the University Hospital of Antwerp (reference number 19/28/342), Belgium. All data were anonymized. Participants were informed about the study objectives, sampling protocol, and their right to refuse to take part or withdraw from the study without consequences for their treatment at any point in time. The refusal rate was lower than 5%. Written informed consent was obtained from a parent, guardian, or village head before enrollment. Consent forms were kept in a secure file cabinet at Kala-azar Medical Research Center (KAMRC), Muzaffarpur, Bihar, India.

Statistical analysis

GraphPad Prism version 9.0.1 for Windows (GraphPad Software, San Diego, CA, USA) and RStudio version 4.2.1 (Boston, MA, USA) were used to perform statistical analysis. The distribution of anti-PGL-I data was checked for normality by plotting a histogram. Data were then log-transformed based on the natural logarithm of the anti-PGL-I R-value plus 1. Mann–Whitney U-tests and Kruskal–Wallis tests were performed to determine the statistical significance between two and three independent groups, respectively. A logistic regression analysis was performed to assess the association between age and anti-PGL-I positivity.

Results

This was the first time applying the anti-PGL-I UCP-LFA cassette format at a larger scale in a field setting. A quality control protocol (as described in the Methods) was performed to monitor reproducibility (data not shown). The anti-PGL-I UCP-LFA was considered feasible and accepted by both healthcare staff and the population.

Serosurvey for anti-PGL-I IgM among children in India

To assess seroprevalence for anti-PGL-I IgM in a leprosy endemic area, FSB samples of 1,857 children living in the state of Bihar, India, were obtained during a field serosurvey and screened using the anti-PGL-I UCP-LFA cassette. Among these children, 215 (11.58%) tested positive for anti-PGL-I IgM (Figure 2A; Supplementary Table 1). Anti-PGL-I R values ranged from 0 to 0.84 with a median of 0.04 (IQR 0.02–0.07). The distribution appeared

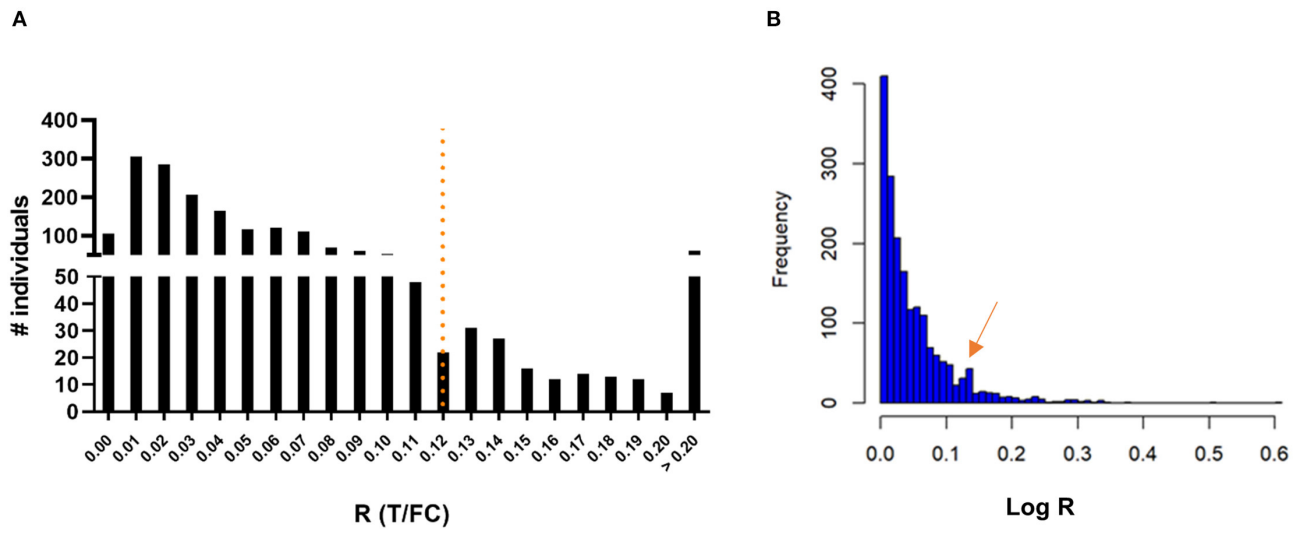

FIGURE 2
Presence of anti-PGL-I IgM antibodies in FSB of 1,857 children 3–11 years old in Bihar, India. UCP-LFA cassettes were used to obtain a quantitative R-value (T/FC) indicating the presence of anti-PGL-I IgM antibodies using a UCP reader. **(A)** Histogram with a mode of 0.01 for R: the highest R-value detected was 0.84 ($n = 1$). The cutoff for positivity ($R \geq 0.12$) for the UCP-LFA batch used in this study is indicated by the dotted line and was based on the median of a sextuple test performed in India of a standard control serum sample (+) plus its standard deviation (SD). **(B)** Histogram of log-transformed R-values. FC, flow control line; IgM, immunoglobulin M; PGL-I, phenolic glycolipid I; R, ratio value, result of the UCP-LFA; T, test line.

unimodal with a mode of 0.01 and strongly skewed to the right. Upon log transformation (based on the natural logarithm of the anti-PGL-I R-value plus 1), the distribution was still very skewed with a small dip around 0.11, followed by a much lower second mode. The log-transformed value of 0.11 corresponds with 0.12 on the original scale, and this confirms the cutoff value of $R \geq 0.12$ used, as shown by the second peak in the histogram (Figure 2B). From the seropositive children with R-values >0.2 (median of seropositive children plus SD of seronegative children; $n = 61$), 51 could be followed up in October 2021 and examined for signs and symptoms of leprosy. One child (male, 9 years old) was diagnosed at follow-up at the primary healthcare center as a new paucibacillary (PB) leprosy case 1 year after the serosurvey (test performed in October 2020; R-value 0.21), showing a lesion on his left upper arm.

For all participants, information on age, gender, and place of origin was recorded at the time of sampling FSB. The ages of the children in this cohort ranged from 3 to 11, with the majority of the children being 5 ($n = 410$), 6 ($n = 353$), 7 ($n = 392$), 8 ($n = 430$), or 9 ($n = 268$) years of age. For children aged 5–9 years, the percentage of seropositive children showed an increasing trend with age: from 7.32% at age 5 to 14.55% in age group 9 (Figures 3A, B; Supplementary Table 2). The odds of testing positive for anti-PGL-I IgM in this age range (5–9) increased by 18% per year (logistic regression; $p = 0.002$).

Ages were represented equally among the 987 male and 870 female participants (Table 2). No correlation was found between gender and the presence of anti-PGL-I IgM antibodies (Mann–Whitney U-test; $p = 0.2314$; Figure 3C). For female and male participants, almost identical seropositivity percentages of 11.61 and 11.55% were found, respectively.

Children from 12 different villages were included (Supplementary Figure 1). The majority of the children were resident in Raksha South ($n = 411$). In general, seropositivity rates among the villages were similar (Figure 3D; Supplementary Table 3). However, a slight increase in levels of antibodies (R-values) among Raksha South with villages Madhopur Chhapra, Godai Phulkahan, Godai Jamal, Raksha North, and Raksha Deah was observed (Kruskal–Wallis; $p = 0.0003$ to $p = 0.0154$).

Screening sera of leishmaniasis patients and controls

In addition to being endemic for leprosy, the area of Bihar is also endemic for leishmaniasis (32, 36). As leishmaniasis is a differential diagnosis of leprosy (37), although not the main purpose of this study, biobanked serum samples from individuals from exactly the same area with VL ($n = 20$), PKDL ($n = 20$), asymptomatic *Leishmania donovani (L. donovani)* infection ($n = 20$), and endemic controls ($n = 20$) were tested with the anti-PGL-I UCP-LFA cassette as well. Two of the 60 individuals with confirmed *L. donovani* infection tested positive (3.33%) for anti-PGL-I IgM (Table 3): one ($R = 0.12$) PKDL case and one person with an asymptomatic *L. donovani* infection ($R = 0.26$). None of the endemic controls (age 8–50) from the same area tested positive for anti-PGL-I IgM (Table 4). Moreover, plasma samples of young children (age 1–6; $n = 37$) from a non-endemic area (the Netherlands) all tested negative using the anti-PGL-I UCP-LFA cassette (Pierneef et al., *unpublished data*).

Discussion

Measuring seroprevalence among young children represents a potential tool to monitor the intensity of recent transmission,

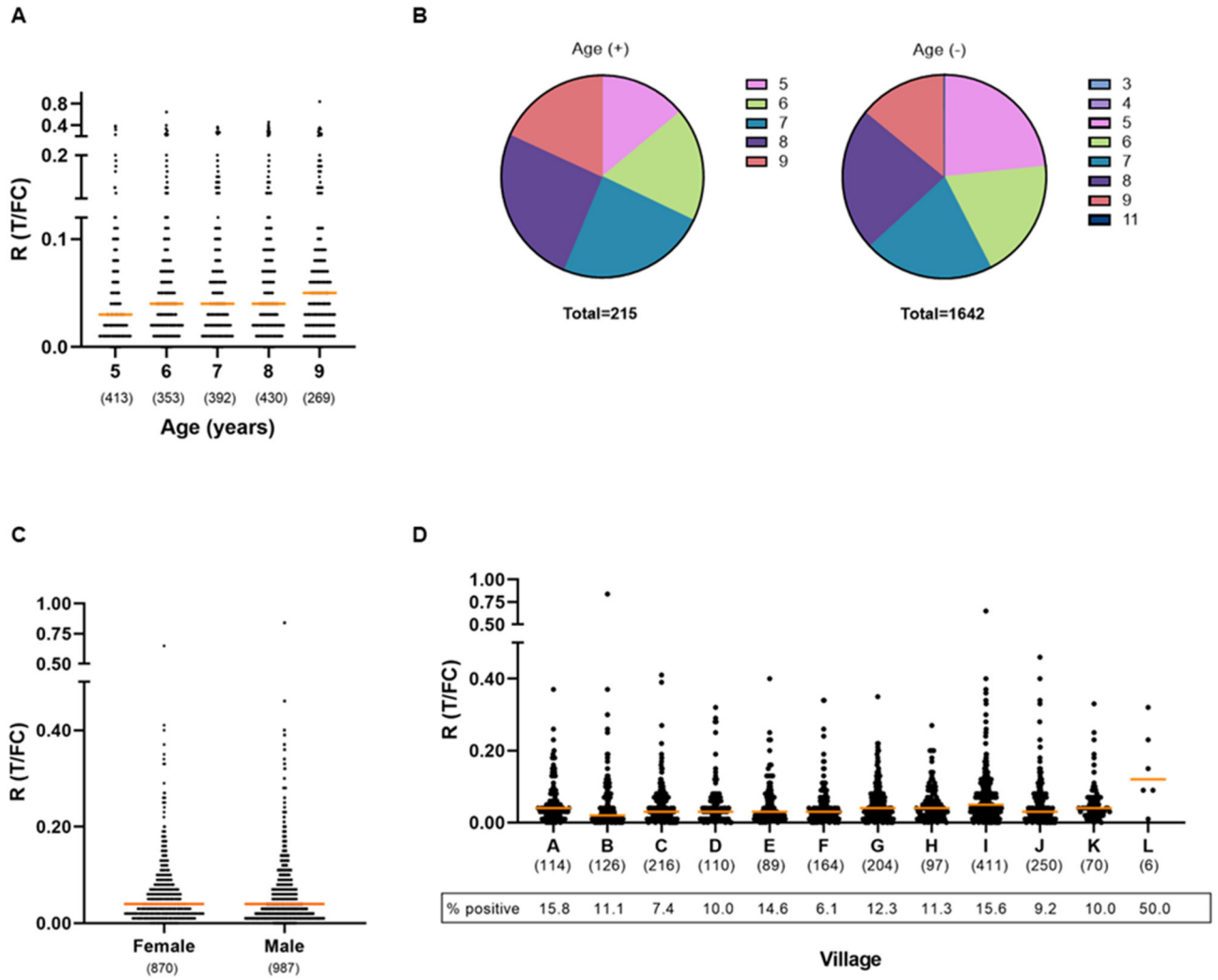

FIGURE 3
Presence of anti-PGL-I IgM antibodies in FSB of children in relation to age, gender, and place of residence. UCP-LFA cassettes were used to obtain a quantitative ratio (R) value (T/FC) indicating the presence of anti-PGL-I IgM using a UCP reader. Median values for each group are indicated by horizontal bars. The cutoff for positivity ($R \geq 0.12$) for the UCP-LFA batch used in this study was based on the median of a sextuple test performed in India of a standard control serum sample (+) plus its standard deviation (SD). The numbers of individuals per group are given in parentheses. **(A)** Anti-PGL-I IgM R values per age group. Two children aged 3 years and one child aged 4 years were included in the 5-year-old age group and one child aged 11 years was included in the 9-year-old age group. **(B)** Venn diagrams displaying the proportion of children per age category for anti-PGL-I IgM-positive (+) and -negative (−) children. Colors indicate the age groups. **(C)** Anti-PGL-I IgM R values per gender. **(D)** Anti-PGL-I IgM R values per village. A: Singar Phulkahan, B: Madhopur Chhapra, C: Godai Phulkahan, D: Godai Jamal, E: Vishwanathpur, F: Raksha North, G: Raksha North Chauk, H: Raksha South West, I: Raksha South, J: Raksha Deah, K: Nariyar Nawada, L: Arizpur Kothi. FC, flow control line; IgM, immunoglobulin M; PGL-I, phenolic glycolipid I; R, ratio value, result of the UCP-LFA; T, test line.

particularly when antibody levels are assessed quantitatively. Changes in seroprevalence measured in repeated cross-sectional surveys among young children of a certain age group in an area can indicate the rate of transmission as well as the effect of control measures or interventions in an area. On route to leprosy elimination, an indicator for the intensity of transmission would be highly valuable, as the proportion of child cases, which is currently applied by the WHO, does not provide information swiftly as it can take years for an infected individual to develop leprosy. Moreover, it is not an accurate representation of infection as only a minority of the infected individuals develop the disease (9).

In this study, using the anti-PGL-I UCP-LFA, we found seropositivity of 11.58% among children in Bihar, a leprosy endemic state in India, with a prevalence rate of 17.1 per 10,000 population in 2019 (31), which is above the elimination threshold of 1 per 10,000 population defined by the WHO (38). In agreement with previous research, this percentage falls in line with the seroprevalence (median 14.9%) found in studies from endemic areas—mostly Brazil, India, and Indonesia (20). Seropositivity slightly increased with age (between 5 and 9 years of age). Previous reports describe a similar increase with age, followed by a decrease in antibodies after the age of 20 years (20, 39). Children of school-going age would thus be a suitable target group for sensitive assessment of recent *M. leprae* infection.

The slightly higher R-values (individual anti-PGL-I IgM levels) found in Raksha South compared to five other villages could not be

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TABLE 2 Age distribution among female and male children aged 3–11 in Bihar, India.

Gender	Age	# Children	Cumulative # children	Percentage (%)	Cumulative percentage (%)
Female	3	2	2	0.23	0.23
	4	0	2	0	0.23
	5	192	194	22.07	22.30
	6	148	342	17.01	39.31
	7	206	548	23.68	62.99
	8	203	751	23.33	86.32
	9	119	870	13.68	100
	11	0	870	0	100
Male	3	0	0	0	0
	4	1	1	0.10	0.10
	5	218	219	22.09	22.19
	6	205	424	20.77	42.96
	7	186	610	18.84	61.80
	8	227	837	23.00	84.80
	9	149	986	15.10	99.90
	11	1	987	0.10	100

The number (#) of children per age and gender are provided with the corresponding percentage of that group in relation to total male/female participants.

TABLE 3 Ratio values for anti-PGL-I IgM measured in individuals infected with L. donovani in Bihar, India.

R	# Individuals	Cumulative # individuals	Percentage (%)	Cumulative percentage (%)
0.00	15	15	25.00	25.00
0.01	16	31	26.67	51.67
0.02	12	43	20.00	71.67
0.03	7	50	11.67	83.33
0.04	2	52	3.33	86.67
0.05	2	54	3.33	90.00
0.06	2	56	3.33	93.33
0.07	1	57	1.67	95.00
0.09	1	58	1.67	96.67
0.12	1	59	1.67	98.33
0.26	1	60	1.67	100

The number (#) of individuals per ratio (R) value is provided with the corresponding percentage of that group in relation to the whole cohort. The cutoff for positivity ($R \geq 0.12$) for the UCP-LFA batch used in this study was based on the median of a sextuple test performed in India of a standard control serum sample (+) plus its standard deviation (SD).

explained by differences in the age and/or gender of the children as compared to the other villages. However, group sizes were not equal and Raksha South had a notably higher number of participants ($n = 411$), possibly affecting the analysis.

Previous studies reported that seroprevalence was stable over time if leprosy incidence in an area remained unchanged (20). A significant limitation hindering direct comparison of previous seroprevalence results from endemic areas (mostly from India, Brazil, and Indonesia) was the use of different assays measuring anti-*M. leprae*-specific antibodies. Measurements were performed using either FSB or serum with variable dilutions, and target antigens varied from native to synthetic PGL-I recognized by various IgM, IgG, or IgA isotypes. In addition, cutoff values were often chosen arbitrarily. However, analysis of data available from China showed that if the same method was used, a decrease in disease prevalence or new case detection rate corresponded to a decrease in anti-*M. leprae* antibody seroprevalence in children (21), adding the potential of measuring antibodies in children as a tool for recent transmission.

Timely diagnosis and treatment can prevent disabilities from developing and the mycobacterium from spreading (40). Active case-finding approaches by healthcare workers are a proven

TABLE 4 Ratio values for anti-PGL-I IgM measured in endemic controls in Bihar, India.

R	# Individuals	Cumulative # individuals	Percentage (%)	Cumulative percentage (%)
0.00	2	2	10.00	10.00
0.01	3	5	15.00	25.00
0.02	8	13	40.00	65.00
0.03	2	15	10.00	75.00
0.04	1	16	5.00	80.00
0.05	2	18	10.00	90.00
0.09	1	19	5.00	95.00
0.10	1	20	5.00	100

The number (#) of individuals per ratio (R) value is provided with the corresponding percentage of that group in relation to the whole cohort. The cutoff for positivity ($R \geq 0.12$) for the UCP-LFA batch used in this study was based on the median of a sextuple test performed in India of a standard control serum sample (+) plus its standard deviation (SD).

method to identify cases at early stages (41). In addition to the use of serology to monitor transmission, large-scale screenings have the potential to early identify new cases. Although the presence of anti-PGL-I IgM does not predict disease, seropositive individuals are at an increased risk of developing leprosy (42). In this study, 51 children were followed up and screened for clinical symptoms. One boy aged nine (anti-PGL-I IgM R-value 0.21) was diagnosed at follow-up with PB leprosy indicating the additional benefit of community seroscreening in children even for PB leprosy. As we took a cost-efficient approach, it was outside the scope of this project to follow-up all seropositive children for clinical examination which limits the additional impact of the study. In future studies, follow-up of all seropositive children as well as a subgroup of randomly selected seronegative children should be included besides additional screening of contacts of seropositive children to identify the source of transmission.

Single-dose rifampicin (SDR) PEP is a preventive treatment for leprosy which can decrease the risk of developing disease among (household) contacts of leprosy cases (43). Large-scale, international research proves that SDR-PEP is safe and the WHO recommends its use in the combat against leprosy (44). As many activities including PEP administration are ongoing worldwide and over 175,000 individuals have already received this regimen (44), it is of interest to study the effect over time on *M. leprae* transmission (measured by seroprevalence rates in children) of such prophylactic interventions and to compare the effect of PEP on transmission between countries. Crucial would be the use of one standardized, quantitative assay, which is preferentially field-friendly and easy to use at a large scale. Therefore, the low-complexity UCP-LFA cassette—which is more robust and easier to perform than the ELISA—quantitatively detecting anti-PGL-I IgM in FSB that was field-tested in this study offers a particularly suitable format for this purpose.

Since PKDL is a differential diagnosis of leprosy (37), although not the main aim of this study, we assessed the potential of the anti-PGL-I UCP-LFA to discriminate between the two infections. We found two out of 60 (3.33%) individuals infected with *L. donovani* to test positive for anti-PGL-I IgM. However, while those individuals are living in an area where both diseases are endemic, previous exposure to *M. leprae* cannot be excluded. Furthermore, in an area not endemic for leprosy, young children all tested negative in the anti-PGL-I UCP-LFA cassette, arguing for the specificity of the test. The HDSS team is experienced in conducting studies for leishmaniasis in the field, and this provides opportunities for combining leishmaniasis with leprosy research (32). Performing serosurveillance to monitor the transmission of multiple NTDs simultaneously by pooling expertise into one joint operation could save cost as well as time. To this end, the design of a (combined) rapid test for the detection of antibodies against *L. donovani* (and *M. leprae*) could also be valuable and is considered for future research (45).

Conclusion

Screening for quantitative assessment of anti-PGL-I IgM levels in children identified 11.58% seropositivity in 12 villages in Bihar, India. These data are in line with seroprevalence data reported (20, 21) for other endemic areas without PEP in the past decades. There was a slight, significant increase with age, but no difference in seropositivity between genders was observed. Anti-PGL-I IgM antibodies in young children are a useful indicator for *M. leprae* infection, thereby serving as a proxy for recent transmission in an area and thus can be used as a tool for monitoring the reduction of transmission when new cases are scarce. A follow-up study 5 to 10 years later, again assessing the anti-PGL-I IgM antibodies in these villages in Bihar in the same age groups, would provide insight into the changes in transmission in this area. In addition, similar studies should be conducted in areas where leprosy is less endemic or not endemic anymore to further validate this tool for monitoring the elimination of transmission.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Institutional Review Boards of the Institute of Medical Sciences, Banaras Hindu University (BHU; reference number Dean/2017/EC/185; Dated 24/10/2017), Varanasi, India, and the review committee of the U.S. National Institutes of Health (NIH). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

Conceptualization: AG. Data curation: LP, AH, MM, and ZZ. Formal analysis: LP, AH, EH, and AG. Funding acquisition: PM, SS, EH, and AG. Investigation: LP, PM, AH, SS, AS, RK, DJ, MM, AK, and ZZ. Methodology: EH and AG. Supervision: PC and AG. Visualization: LP, PM, AH, PC, EH, and AG. Writing—original draft: LP and AG. Writing—review and editing: LP, PM, AH, KC, PC, EH, and AG.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1260375/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Map of the research area indicating the included villages in Bihar, India, and the seropositivity percentages measured in children. The locations of 10 villages (A–J) in Bihar, India, are provided with the corresponding percentage of children testing positive for anti-PGL-I IgM. Note that two villages (K, L) were not shown on this map, as they were located too far away. UCP-LFA cassettes were used to obtain a quantitative ratio (R) value (T/FC) indicating the presence of anti-PGL-I IgM using a UCP reader. The cutoff for positivity ($R \geq 0.12$) for the UCP-LFA batch used in this study was based on the median of a sextuple test performed in India of a standard control serum sample (+) plus its standard deviation (SD). A, Singar Phulkahan; B, Madhopur Chhapra; C, Godai Phulkahan; D, Godai Jamal; E, Vishwanathpur; F, Raksha North; G, Raksha North Chauk; H, Raksha South West; I, Raksha South; J, Raksha Deah; K, Nariyar Nawada; L, Arizpur Kothi. Anti-PGL-I, anti-phenolic glycolipid I; FC, flow control line; IgM, immunoglobulin M; R, ratio value, result of the UCP-LFA; T, test line.

SUPPLEMENTARY TABLE 1
Ratio values for anti-PGL-I IgM measured in children aged 3–11 in Bihar, India.

SUPPLEMENTARY TABLE 2
Anti-PGL-I IgM positivity in children aged 5–9 in Bihar.

SUPPLEMENTARY TABLE 3
Place of residence of the children aged 3–11 in Bihar and corresponding percentage of anti-PGL-I IgM positive children.

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Ticks are unlikely to play a role in leprosy transmission in the Comoros (East Africa) as they do not harbour *M. leprae* DNA

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Introduction: Leprosy, one of the oldest known human diseases, continues to pose a global challenge for disease control due to an incomplete understanding of its transmission pathways. Ticks have been proposed as a potential contributor in leprosy transmission due to their importance as vectors for other infectious diseases.

Methods: In 2010, a sampling of ticks residing on cattle was conducted on the islands Grande Comore, Anjouan, and Mohéli which constitute the Union of the Comoros where leprosy remains endemic. To investigate the potential role of ticks as a vector in transmission of leprosy disease, molecular analyses were conducted.

Results: Out of the 526 ticks analysed, none were found to harbour *Mycobacterium leprae* DNA, as determined by a quantitative polymerase chain reaction (qPCR) assay targeting a family of dispersed repeats (RLEP) specific to *M. leprae*.

Discussion: Therefore, our results suggest that in the Union of the Comoros, ticks are an unlikely vector for *M. leprae*.

KEYWORDS

leprosy, *Mycobacterium leprae*, ticks, transmission, vector, reservoir, cattle

Introduction

Leprosy is a mutilating disease caused by the intracellular bacilli *Mycobacteria leprae* (*M. leprae*) and/or *lepromatosis* (1). Despite the World Health Organization (WHO) removing leprosy from its list of public health concerns in 2001, the lack of significant reduction in new cases and the detection of leprosy in children indicate that transmission of the disease is still ongoing (2). This is evident in regions where active measures are taken to identify cases, such as door-to-door screenings, which consistently uncover new leprosy patients. Additionally, the prevalence of severe disabilities at the time of diagnosis in many countries suggests delayed detection and diagnosis (3). As a result, it is becoming increasingly apparent that we only see the tip of the iceberg of the global leprosy burden.

The exact transmission route of leprosy has not been fully elucidated yet. Different sites have been identified as potential entry and exit for *M. leprae* bacilli to the human body, namely the nose, mouth and skin (4). The highest bacillary burden is found in the epidermis of leprosy patients (5). The most probable transmission route of leprosy is via the aerial route (6), caused by the prolonged close contact to leprosy patients. Especially multibacillary patients are considered to drive leprosy transmission, given the high bacterial load. However, the nine-banded armadillo (7, 8), red squirrels (9), and chimps (10) have been confirmed as animal reservoirs and zoonotic transmission of *M. leprae* has been confirmed by genotyping (8) infected armadillos and leprosy patients in the US. Thus, the question as to whether the transmission pathway is direct or (partially) vector-driven remains unresolved (4).

The vector competence of *Amblyomma sculptum* from the family of hard ticks (Ixodidae) for *M. leprae* was demonstrated by Ferreira et al. (11) by artificially feeding adult females with *M. leprae* Thai-53 infected rabbit blood. Transovarial transmission of *M. leprae* was confirmed by the *M. leprae* specific RLEP qPCR. These findings are supported by results of Tongluan et al. (12) who injected *Amblyomma maculatum* ticks at adult and nymph stage with an *M. leprae* Thai-53 suspension derived from infected nude mice footpads. They confirmed the presence of *M. leprae* DNA in F_1 larvae and F_1 nymphs via RLEP qPCR. Both studies were able to grow *M. leprae* in cell lines derived from ticks, viability was confirmed by examination of the normalized expression levels of the *M. leprae esxA* gene (12) or 16S rRNA RT-qPCR (11). Transmission of *M. leprae* to a vertebrate host followed by an infection was only shown for *M. leprae* cultivated in and isolated from tick-derived cell lines. When inoculated directly into the footpad, these bacilli are able to establish a prolonged infection in mice (11, 12). Blood-feeding tick larvae were able to transfer viable *M. leprae* to a rabbit model. However, rabbit skin was analysed already 5 days after inoculation, a time frame too short to confirm a stable infection of the vertebrate host (11). Even though these studies were mainly aiming towards being able to grow *M. leprae* bacilli *in vitro* in a cell line, the experimental data suggests that there is a possibility for a transmission route of leprosy via ticks after taking their blood meal on a person with leprosy.

The Union of the Comoros has the highest *per capita* incidence of leprosy in Africa [as high as seven cases per 10,000 individuals on Anjouan (13, 14)], making it the only country of the African continent that did not reach the elimination target of less than 1 patient/10,000 population postulated by the WHO (3, 15). Despite the persistent efforts of the National Tuberculosis and Leprosy Control Programme, including intensified screenings since 2008 and the administration of post-exposure prophylaxis within the framework of the PEOPLE and BE-PEOPLE studies (Clinicaltrials.gov: NCT03662022 and NCT05597280), leprosy, a poverty-related disease, remains endemic on the islands Anjouan and Mohéli. In contrast, the wealthiest of the three islands, Grande Comore, is not considered a leprosy endemic region. Leprosy has a long incubation period of several months to decades, with an average of 2–4 years (16), which implies ongoing transmission of the disease by the high proportion of affected children on Anjouan and Mohéli (2). The potential contribution of non-human animal and environmental reservoirs to the transmission of leprosy represents a knowledge gap towards interrupting leprosy transmission. Further, the role of ticks as biological or mechanical vector has not been confirmed by epidemiological studies yet. Therefore, this study sought to investigate the presence of *M. leprae* DNA in a tick collection obtained from the Union of the Comoros as a means of further elucidating the potential involvement of ticks as a vector in leprosy transmission.

Materials and methods

Samples

A total of 526 ticks from a previously described collection (17) from the Union of the Comoros were selected for screening for the presence of *M. leprae* DNA. Specimens were shipped and stored in molecular grade pure ethanol (Avantor, United States) at −20°C to the Institute of Tropical Medicine in Antwerp, Belgium. From the leprosy-endemic islands Anjouan ($n = 134$) and Mohéli ($n = 129$) 263 ticks were available. The prevalence of leprosy on Anjouan and Mohéli by the end of 2017 was 4.57/10,000 (18). A summary of leprosy prevalence per sampled district on Anjouan and Mohéli can be found in Supplementary Table S1. As a comparator, $n = 263$ ticks were selected from Grande Comore where leprosy is not endemic. All ticks were morphologically inspected and classified according to the guide by Walker et al. (19) before they were molecularly examined for the presence of *M. leprae* DNA.

DNA extraction

One half of each tick was used for DNA extraction. The ticks were ground with a mortar and pestle in 1 mL phosphate-buffered saline. To avoid DNA contamination mortars and pestles were autoclaved, treated with bleach, and rinsed prior to use and a new set was used for each sample. Subsequently, 200 µL of the resulting suspension were incubated with 200 µL in-house lysis buffer (Tris-HCl – pH 7.5, EDTA 0.5 M pH 8, 6 M GuHCl, Tween 20, Triton X-100, diatomaceous earth) and 20 µL proteinase K solution (Promega, United States) in a shaking incubator for 1 h at 60°C and 200 rpm. The lysed suspension was further extracted with the Maxwell® 16 FFPE PLUS Tissue LEV DNA purification Kit (Promega, United States), following the manufacturers' protocol. To control for contamination throughout the extraction procedure, each run included a negative (molecular grade water) and a positive extraction control (suspension of mouse footpad infected with *M. leprae* Thai-53, BEI reference number: 19352).

qPCR assay

To quantify *M. leprae* DNA in the tick extracts, a qPCR assay targeting a family of dispersed repeats (RLEP) (20) was used as described previously (21) for 45 cycles (positivity cut-off <40 C_q), using the StepOnePlus™ qPCR cycler and StepOne software v2.3 (Applied Biosystems, United States), the primer and probe sequences and cycling conditions can be found in Supplementary Table S2. With this assay 36 out of 37 RLEP copies in the *M. leprae* genome are detected. Samples were tested in triplicate and considered positive when two of the three replicates were under the positivity cut-off. Non-template controls (molecular grade water) to control for contamination during the qPCR procedure and a gDNA (*M. leprae* NHDP, BEI reference number: 19350) standard curve for

quantification with 1:10 dilutions from 3×10^5 to 3×10^1 RLEP copies were included in each run. An internal positive control (IPC, Eurogentec, Belgium) was spiked into each well to detect inhibition during the qPCR run.

Statistical analysis

To determine the significance of the difference between ticks selected from the leprosy endemic (Anjouan and Mohéli) and non-endemic (Grande Comore) islands, the one-proportion z-test was applied. The significance of the sample rate ratio of ticks investigated in this study compared to the complete tick collection by Yssouf et al. (17) was calculated with the Fisher's exact test. All statistical analyses were performed with R, version 4.3.0 for macOS (The R foundation, Vienna, Austria), the alternative hypothesis, stating significant differences between variables, was accepted at a significance level of alpha = 0.05.

Results

Morphological classification of ticks

Of the 263 ticks from the endemic islands of Anjouan and Mohéli, 253 (96.2%) were identified as *Rhipicephalus microplus* and 10 (3.8%) as *Amblyomma variegatum* (Table 1). The sample rate ratio analysis of species classification showed that *A. variegatum* was slightly underrepresented in the subset examined in our study with a proportion of 3.8% compared to 9.8% in the complete original collection by Yssouf et al. (17) (Supplementary Table S3).

In our study an additional classification of the ticks by developmental stage and sex was conducted. Most of the ticks from the endemic islands were adults ($n = 184$, 70.0%), followed by ticks in the nymph stage ($n = 77$, 29.3%). Only $n = 2$ larvae (0.8%) were available for analysis (Table 2). The majority of collected ticks was identified as female ($n = 109$, 81.3% from Anjouan; $n = 102$, 79.1% from Mohéli; $n = 167$, 63.5% from Grande Comore). For a small proportion of ticks (4.6%) the sex could not be identified in our study because the determining features in some nymphs and larvae were inconclusive (Table 2).

Detection of *M. leprae* DNA by RLEP qPCR

None of the 526 tested DNA extracts from ticks resulted in a positive result in the RLEP qPCR. For none of the triplicates an amplification curve showed during the qPCR assay and therefore the positivity cut-off of 40 C_q was fulfilled. The limit of detection of the RLEP qPCR assay is as low as 30 RLEP copies per 2 µL added to each qPCR reaction, which correlates with approximately one *M. leprae* bacillus. All positive extraction controls resulted in a positive qPCR result. Negative extraction controls and non-template controls were negative on qPCR, indicating the absence of DNA contamination during the extractions and qPCR assays. IPC was spiked into the DNA extracts before qPCR quantification. Results were consistent within each qPCR run which confirms the absence of qPCR inhibition. A summary of the qPCR results of RLEP and IPC can be found in Supplemental File 1.

Discussion

This study is the first to use molecular tools to screen wild, animal-derived ticks from a leprosy endemic country for the presence of *M. leprae*. The absence of *M. leprae* DNA was confirmed in all tested specimens from the Comoros. Next to *M. leprae*, *M. lepromatosis* can also cause leprosy disease in humans (1). We have tested the leprosy patient cohort in the Comoros for the presence of *M. lepromatosis* DNA by qPCR assay, with results suggesting that *M. leprae* is the only causative agent for leprosy on the Comoros (manuscript in preparation). Therefore, in this study ticks were only screened for the presence of *M. leprae* DNA.

In the search for drivers for leprosy transmission, two previous studies (11, 12) identified ticks from the genus *Amblyomma* as potential competent vectors for *M. leprae*. More specifically, under experimental conditions the transovarial transmission and the survival of *M. leprae* in female ticks and tick-derived cells was confirmed. The majority of the wild tick collection analysed in our study were adult females, which are able to harbour and transmit *M. leprae* under experimental conditions. The small proportion of nymphs, which is the developmental stage most likely to parasitize humans and transmit other tick-borne diseases such as lyme disease (22) and ehrlichiosis (23), could explain our inability to detect *M. leprae* DNA in the tick collection that was studied.

Further, the tick collection consisted of a small ratio of *Amblyomma* ticks, the species with proven capacity to harbour *M. leprae* (11, 12), compared to *R. microplus*. Only 10 out of 263 (3.8%) ticks from the endemic islands Anjouan and Mohéli were *A. variegatum* while Yssouf et al. (17) classified 73 out of 742 (9.8%) ticks as *A. variegatum*. The reason for the different species distribution is that only a subset of the original collection was available for analyses at ITM, Antwerp. The selected number of ticks from Grande Comore, used as non-endemic controls, was matched to the species distribution found for the endemic islands in this study. Accordingly, the percentage of *A. variegatum* was

TABLE 1 Species distribution of ticks over the three islands of the Union of the Comoros classified according to Walker et al. (19).

Group	Island	*R. microplus*		*A. variegatum*		Total	
Islands endemic for *M. leprae* transmission	Anjouan	131/134	(97.8%)	3/134	(2.2%)	134	263
	Mohéli	122/129	(94.6%)	7/129	(5.4%)	129	
Island non-endemic for *M. leprae* transmission	Grande Comore	254/263	(96.6%)	9/263	(3.4%)	263	
Total		507/526	(96.4%)	19/526	(3.6%)	526	

TABLE 2 Distribution of developmental stages and sex of ticks classified and investigated in this study.

	Endemic (Anjouan + Mohéli)		Non-endemic (Grande Comore)	
Developmental stage				
Adult	184	(70.0%)	243	(92.4%)*
Nymph	77	(29.3%)	20	(7.6%)*
Larva	2	(0.8%)	0	(0.0%)
Total	263	(100%)	263	(100%)
Sex				
Female	211	(80.2%)	167	(63.5%)*
Male	43	(16.3%)	81	(30.8%)*
Undetermined	9	(3.4%)	15	(5.7%)
Total	263	(100%)	263	(100%)

*Proportions that are significantly different ($p < 0.05$) in the sample proportion from the non-endemic island compared to the endemic islands.

smaller than the one found by Yssouf et al. on this island. However, both *Rhipicephalus* and *Amblyomma* ticks belong to the family of Ixodidae (or hard ticks). In their previous studies Tongluan et al. and Ferreira et al. were able to maintain *M. leprae* in *Ixodes*-derived cell lines which suggests a similar potential of all members of the Ixodidae family as a vector for *M. leprae*.

Even though the ticks analysed in our study were collected from cattle and goats and not from humans, feeding of cattle ticks on humans seems probable in situations where humans and livestock live closely together. For both *R. microplus* and *A. variegatum* which mainly feed on cattle and other large animals (24), such cross-over events have been reported (25–27). A recent publication by Faber et al. (28) is raising the hypothesis that a skin disease in water buffaloes described as lepra bubalorum could be caused by *M. leprae* and therefore act as animal reservoir. However, evidence for cases in Indonesia is only historical as there were no further reports for lepra bubalorum in cattle since 1961 (29) and there is no water buffalo population described in the Union of the Comoros (30).

Different other vectors have been suggested for the transmission of *M. leprae*, e.g., arthropods such as mosquitos (Aedes, Culex, Rhodnius) (31–33), flies (*Musea, Calliphora and Stomoxys*) (34), and sand flies (*Phlebotomus, Sergentomyia*). The latter are unlikely vectors as they cannot maintain viable *M. leprae* bacilli (35). Early studies on mosquitos confirmed the presence of acid-fast bacilli in the proboscis of mosquitos (*A. aegypti* and *C. fatigans*) after experimentally feeding on untreated leprosy patients (31, 32). However, viability determined by fluorescence microscopy reduced within seven days after feeding (32). Da Silva Neumann et al. have investigated *R. prolixus*, *A. aegypti*, and *C. quinquefasciatus* as possible vector, with the result that only *R. prolixus* has the ability to defecate infective *M. leprae* up to 20 days after infection with *M. leprae* Thai-53 infected rabbit blood (33). Additionally, amoeba have been found to have vector potential as they can phagocytose *M. leprae*. *In vitro* experiments showed that *M. leprae* can survive up to 72 h within the *Acanthamoeba* and up to 8 months in amoebal cysts while retaining infectivity for a nude mouse model (36, 37). However, for none of these vector candidates a clear correlation with leprosy infections in humans was identified.

Even though Ixodes ticks are potential competent vectors for *M. leprae in vitro* and pathogen transmission from livestock to humans via ticks is probable, all ticks from Anjouan, Mohéli, and Grande Comore that were investigated tested negative for *M. leprae* DNA. This finding lessens the chance that leprosy is a tick-borne zoonosis in the Union of the Comoros, rather than spread by human-to-human transmission.

Our results support the hypothesis that most leprosy infections are caused by human-to-human interactions rather than by a non-human animal or environmental reservoir of *M. leprae* and that close contact to a leprosy patient is the driving force of transmission. For the definitive exclusion of the role of ticks in the transmission of leprosy disease, a larger number of ticks also from other leprosy endemic regions should be analysed. The exploration of human-derived ticks and particularly ticks parasitising leprosy patients should be the focus of such studies. Further, qualitative case control studies investigating daily activities of leprosy patients and healthy controls will be useful for the generation of new hypotheses on the driving factors of leprosy transmission.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

All ticks were manually sampled in the presence of cattle owners. No suffering was imposed to sampled animals and the disturbance associated with sampling was underneath the regulatory threshold requiring the submission of the protocol to an institutional ethics committee, as described on European Union Directive 2010/63/UE. For the study of ticks, ethical approval was not required in accordance with the national legislation and the institutional requirements.

Author contributions

LK, EC, BJ, and SB contributed to conception and design of the study. AY and PT were involved with the tick sampling and provided the tick collection. EC and MD-L were involved in the methodology, investigation, and validation. LK, EC, and SB performed the formal analysis and statistics. SB was the project administrator and together with BJ supervised the study. LK wrote the original manuscript draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1238914/full#supplementary-material

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Case report: Cyclophosphamide pulse therapy for chronic recalcitrant erythema nodosum leprosum

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Chronic recalcitrant erythema nodosum leprosum (ENL) or type 2 reaction (T2R) is a severe condition found in approximately 50% of multibacillary leprosy subjects. T2R is associated with important morbidities and may lead to several disabilities, not only due to nerve damage but also due to the prolonged use of corticosteroids, thalidomide, or immunosuppressors. We describe here four leprosy patients with chronic recalcitrant ENL treated with cyclophosphamide pulse therapy. All subjects had been on prednisone and thalidomide therapy for at least 30 months but showed inflammatory activity when doses were reduced. Pulse therapy with 1.0 g of cyclophosphamide was used every 4–6 weeks for a minimum of three applications. After pulse therapy, all cases presented total or partial regression of symptoms, and we were able to taper thalidomide and prednisone doses, with better control of ENL, avoiding further hospital admissions and disabilities. No side effects were observed during or after infusion therapy. Cyclophosphamide pulse therapy may be useful and safe to control chronic recalcitrant ENL.

KEYWORDS

leprosy, pulse therapy, cyclophosphamide, erythema nodosum leprosum (ENL), type 2 reaction

Introduction

Erythema nodosum leprosum (ENL) or type 2 reaction (T2R) is a common and severe immune-inflammatory complication of multibacillary leprosy. T2R is associated with increased levels of inflammatory cytokines and chemokines, such as TNF and IL-6, among others, not only in cutaneous lesions but also in several internal organs, leading to systemic involvement. ENL is characterized by the presence of subcutaneous acute and painful nodules associated with fever, myalgia, asthenia, arthritis, neuritis, generalized lymphadenopathy, and many other symptoms, sometimes requiring hospitalization (1, 2).

Chronic recalcitrant ENL is a hard-to-treat condition that imposes long-term use of corticosteroids, thalidomide, or immunosuppressors, resulting in significant morbidity and increasing the risk of disability in those patients (1, 2). Other drugs, such as pentoxifylline and clofazimine, have been used but seem to be effective in less severe cases. More recently, other therapeutic strategies have been used in an attempt to control ENL, such as anti-TNFα agents and apremilast (3–5).

Pulse therapy with cyclophosphamide has been advocated for the treatment of connective tissue diseases, neutrophilic dermatosis, bullous diseases, and other autoimmune and inflammatory conditions (6).

In this study, we describe four leprosy patients with chronic recalcitrant ENL who were under prednisone and thalidomide therapy for at least 30 months. Pulse therapy with 1.0 g of cyclophosphamide was used every 4–6 weeks for a minimum of three applications.

Case description

From January 2018 to July 2023, four ENL patients were evaluated after inclusion in the cyclophosphamide pulse therapy protocol due to chronic, relapsing, and difficult-to-control episodes of severe ENL after at least three attempts to lower the dose after taking prednisone or thalidomide. Table 1 shows demographic, clinical, and therapeutic characteristics. The females predominate males at a 3:1 ratio, with ages ranging from 24 to 41 years. Patients were diagnosed using the Ridley–Jopling criteria (7) upon clinical evaluation, histopathology, and a positive bacillary index. Serology tests for HIV, HTLV-1, B, and C hepatitis viruses were negative (8). All cases had long-term use (36–61 months) of prednisone or thalidomide. Prednisone daily doses ranged from 80 to 2.5 mg, and thalidomide daily use varied from 400 to 50 mg.

The duration of ENL ranged from 48 to 62 months, and all cases were classified as presenting a severe reaction (9) with several episodes of reactivation during the observation period, especially upon any tentative lower prednisone or thalidomide doses. During the clinical activity of T2R, the patients presented more than 20 subcutaneous nodules associated with systemic symptoms such as fever, myalgia, arthritis, neuritis, lymphadenopathy, and edema of the extremities (Table 1). Three out of four subjects were presented with ENL before multidrug therapy (MDT), whereas one patient (number 2) developed ENL during MDT. However, this patient was diagnosed with a reversal reaction (RR) before MDT. She presented BL, and after this first RR and MDT initiation, she developed a series of recurrent ENL episodes, and no further RR was detected.

Cyclophosphamide pulse therapy was initiated during hospitalization for 1 or 2 days with 1.0 g diluted in saline 0.9% by intravenous infusion in 4 h. Before pulse therapy, all subjects performed the following laboratory evaluations: blood count, liver enzymes, blood glucose, BUN, creatinine, chest x-ray, and urinalysis. No adverse events (AEs) were associated with pulse therapy, in contrast with several AEs presented due to prednisone and thalidomide chronic use, such as Cushing syndrome, acne, diabetes, and deep venous thrombosis. After more than 3 years of prednisone and thalidomide use, one patient (number 4) was diagnosed with latent tuberculosis. She was treated with isoniazid and rifampicin and considered cured.

A positive effect of cyclophosphamide pulse therapy in all patients was confirmed by a better control of ENL symptoms, allowing the use of a lower dosage of prednisone or thalidomide (Table 1). The daily average dosage of thalidomide dropped in only one patient, from 400 to 100 mg. However, the prednisone daily average dose was lower in three out of four subjects (50–74% reduction) after pulse therapy.

In the three subjects presenting no disabilities at diagnosis and before ENL treatment, despite the chronicity and severity of reaction episodes, it was possible to avoid any development of disabilities. Patients 2, 3, and 4 needed to be hospitalized due to the intensity of their reaction before using pulse therapy. No one needs hospitalization due to ENL symptoms after the first cyclophosphamide cycle. One subject (number 2) was discharged from the outpatient clinics after no signs or symptoms of ENL without using prednisone, thalidomide, or any other immunosuppressive drug for at least 5 months. However, the other three patients remain on low-dose prednisone and thalidomide therapy.

Discussion

Reactions are the main source of disabilities in leprosy, not only associated with neuritis but also with systemic involvement. ENL may be a longstanding complication of leprosy in approximately 50% of multibacillary cases, leading to the use of high dosages of thalidomide, prednisone, or immunosuppressors for a long period of time, in most cases, for many years (1, 2). Unfortunately, most patients develop severe morbidities and even death associated with prolonged use of corticosteroids (2). In addition to all the physical consequences, the impact of reactions in social, economic, and psychological domains may be underestimated (10–12). Leprosy remains a burden in more than 120 countries and is considered by the WHO to be the most common infectious cause of disability in the world (13). Nevertheless, the incredible negligence toward the disease is reflected in the very few alternatives and trials for the development of new drugs that are more effective and safer for managing reactions (14).

ENL is mediated by increased peripheral production of chemokines and cytokines like IL-6, IFN, IL-17, and TNFα, immune complex deposits, and neutrophil infiltration in the skin and internal organs. There is also the participation of T-cells and the activation of intermediate monocytes, which contribute to the development of tissue damage (15–17).

In addition to corticosteroids and thalidomide, which prolonged use is associated with several side effects, other options such as pentoxifylline, clofazimine, and immunosuppressors may also require a long period of use, with variable effectiveness along with toxicity. More recently, anti-TNFα drugs and apremilast have been used for treating chronic and difficult-to-control ENL with favorable results. Etanercept (6 cases), infliximab (2 cases), and adalimumab (1 case) were employed in variable dosages, and rapid response (hours) was observed with infliximab use (3, 4, 18). However, no prospective controlled trial has been published yet, and besides the high costs, anti-TNFα agents may be associated with the reactivation of tuberculosis. Additionally, anti-TNFα therapy has been associated with leprosy relapse or the efficacy of MDT drugs in leprosy patients under treatment (4). In a pilot study, apremilast—an oral phosphodiesterase-4 inhibitor that decreases the Il-17 pathway and multiple inflammatory cytokines—was used in 12 patients with chronic or recurrent ENL for 6 months with promising results (5). Unfortunately, apremilast has a high cost and may require prolonged use, limiting its indication.

Due to its immunosuppressive effects, cyclophosphamide is used as a treatment for various autoimmune diseases. It suppresses T and B cells and decreases antibodies, adhesion molecules, and cytokine production (6, 19). Cyclophosphamide has shown a role in corticosteroid-sparing in pemphigus and lupus disease (20–22). In the

TABLE 1 Demographic, clinical and therapeutic characteristics of chronic relapsing ENL patients.

Patient	Age	Sex	R&J	Duration of ENL (months)	ENL characteristics	Pulse therapy cycles	Thalidomide before pulse therapy*	Thalidomide after pulse therapy**	Prednisone before pulse therapy*	Prednisone after pulse therapy**	Degree of disability before	Degree of disability after	Clinical outcome
1	41	F	LL	62	Subcutaneous nodules, acroedema, arthralgia, fever, lymphadenopathy, neuritis	6	200 mg	200 mg	60 mg	10 mg	2	2	Few and small nodules; no other symptoms; using thalidomide 200 mg and prednisone 10 mg
2	36	F	BL	58	Subcutaneous nodules, fever, myalgia	5	400 mg	100 mg	50 mg	18 mg	0	0	Discharge after 5 months without drugs and no clinical activity
3	28	M	LL	48	Subcutaneous nodules, acroedema, arthritis[¶], fever, myalgia	4	400 mg	400 mg	60 mg	10 mg	0	0	Polyarthralgia, no nodules or other symptoms; thalidomide 400 mg and prednisone 5 mg; methotrexate 20 mg weekly (using before pulse therapy)
4	24	F	LL	55	Subcutaneous nodules, acroedema, arthralgia, astenia, fever, headache	3	400 mg	400 mg	20 mg	20 mg	0	0	No nodules or other symptoms; thalidomide 400 mg; prednisone 15 mg

*Daily dosage in average from the first ENL episode until the first pulse therapy infusion. **Daily dosage in average from the last pulse therapy until the last consultation. [¶]A seronegative polyarthritis since the first ENL episode, requiring methotrexate use (20 mg/week) associated with prednisone and thalidomide before pulse therapy.

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treatment of autoimmune diseases, intravenous cyclophosphamide pulse therapy has been administered at 500–1,000 mg/m^2, at 3–4 weeks for 3–6 months, alone or in association with methylprednisolone (6, 20, 21). Although daily oral administration is possible, pulse therapy has been shown to be safer (less leukopenia, amenorrhea, and teratogenicity) without difference in reactivation rates for vasculitic diseases (22, 23).

Our data suggest that pulse therapy with cyclophosphamide may be useful to avoid high dosages of prednisone, hospitalizations due to severe ENL relapses, and the development of disabilities. All these advantages may also provide a potential cost-effectiveness advantage in favor of pulse therapy use. The limitations of our case series are the retrospective design and the absence of a control group. Additionally, the cyclophosphamide pulse therapy schedule was used for variable periods of time ranging from 4 to more than 6 weeks, which could not be enough to achieve the necessary immune-inflammatory modulation required for a better therapeutic outcome. However, pulse therapy with cyclophosphamide should be considered in steroid-dependent patients with severe and recrudescent ENL. To our knowledge, there is no previous data about the use of cyclophosphamide pulse therapy in the management of ENL. Future prospective and controlled studies in a larger number of patients should be conducted to evaluate the efficacy of cyclophosphamide pulse therapy in the treatment of chronic recalcitrant ENL.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Fameb, Federal University of Bahia. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

GM: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Validation. TA: Data curation, Investigation, Methodology, Writing – original draft, Validation. FB: Data curation, Methodology, Writing – original draft. PM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing, Validation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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