

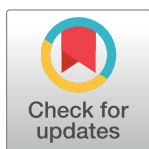
## RESEARCH ARTICLE

# Minimally invasive sampling to identify leprosy patients with a high bacterial burden in the Union of the Comoros

Sofie Marijke Braet<sup>1,2,3\*</sup>, Anouk van Hooij<sup>4</sup>, Epcó Hasker<sup>1</sup>, Erik Fransén<sup>5</sup>, Abdou Wirdane<sup>6,7</sup>, Abdallah Baco<sup>6,7</sup>, Saverio Grillone<sup>6,7</sup>, Nimer Ortuno-Gutierrez<sup>6</sup>, Younoussa Assoumani<sup>6,7</sup>, Aboubacar Mzembaba<sup>6,7</sup>, Paul Corstjens<sup>4</sup>, Leen Rigouts<sup>1,2</sup>, Annemieke Geluk<sup>4</sup>, Bouke Catherine de Jong<sup>1</sup>

**1** Institute of Tropical Medicine, Antwerp, Belgium, **2** University of Antwerp, Antwerp, Belgium, **3** Research Foundation Flanders, Brussels, Belgium, **4** Leiden University Medical Center, Leiden, Netherlands, **5** StatUa Center for statistics University of Antwerp, Antwerp, Belgium, **6** Damien Foundation, Brussels, Belgium, **7** National Tuberculosis and Leprosy control Program, Moroni, Union of the Comoros

\* [sbraet@itg.be](mailto:sbraet@itg.be)



## OPEN ACCESS

**Citation:** Braet SM, van Hooij A, Hasker E, Fransén E, Wirdane A, Baco A, et al. (2021) Minimally invasive sampling to identify leprosy patients with a high bacterial burden in the Union of the Comoros. *PLoS Negl Trop Dis* 15(11): e0009924. <https://doi.org/10.1371/journal.pntd.0009924>

**Editor:** Jessica K. Fairley, Emory University, UNITED STATES

**Received:** March 25, 2021

**Accepted:** October 18, 2021

**Published:** November 10, 2021

**Copyright:** © 2021 Braet et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the manuscript and its [Supporting Information](#) files. The data underlying the findings of this study are retained at the Institute of Tropical Medicine, Antwerp and will not be made openly accessible due to ethical and privacy concerns. Data can however be made available after approval of a motivated and written request to the Institute of Tropical Medicine at [ITMresearchdataaccess@itg.be](mailto:ITMresearchdataaccess@itg.be).

## Abstract

The World Health Organization (WHO) endorsed diagnosis of leprosy (also known as Hansen's disease) entirely based on clinical cardinal signs, without microbiological confirmation, which may lead to late or misdiagnosis. The use of slit skin smears is variable, but lacks sensitivity. In 2017–2018 during the ComLep study, on the island of Anjouan (Union of the Comoros; High priority country according to WHO, 310 patients were diagnosed with leprosy (paucibacillary = 159; multibacillary = 151), of whom 263 were sampled for a skin biopsy and fingerstick blood, and 260 for a minimally-invasive nasal swab. In 74.5% of all skin biopsies and in 15.4% of all nasal swabs, *M. leprae* DNA was detected. In 63.1% of fingerstick blood samples, *M. leprae* specific antibodies were detected with the quantitative αPGL-I test. Results show a strong correlation of αPGL-I IgM levels in fingerstick blood and RLEP-qPCR positivity of nasal swabs, with the *M. leprae* bacterial load measured by RLEP-qPCR of skin biopsies. Patients with a high bacterial load ( $\geq 50,000$  bacilli in a skin biopsy) can be identified with combination of counting lesions and the αPGL-I test. To our knowledge, this is the first study that compared αPGL-I IgM levels in fingerstick blood with the bacterial load determined by RLEP-qPCR in skin biopsies of leprosy patients. The demonstrated potential of minimally invasive sampling such as fingerstick blood samples to identify high bacterial load persons likely to be accountable for the ongoing transmission, merits further evaluation in follow-up studies.

## Author summary

Leprosy is the oldest infectious disease known to humankind. We still do not succeed in curbing its transmission, with more than 200,000 new patients detected worldwide each year. Identifying persons with a high burden of bacteria is key to curb transmission. To identify these persons, bacteria are counted in invasive and painful samples like slit skin

**Funding:** This study was supported by a R2STOP research grant from effect:hope and The Mission To End Leprosy (<https://effecthope.org>, <https://r2stop.org/research/principal-investigators>)(BCdJ), the Q.M. Gastmann-Wichers Stichting (<http://gastmann-wichers.nl>)(AG), the Fonds Wetenschappelijk Onderzoek (FWO, grant 1189219N, <https://www.fwo.be/>) (SMB) and is part of the European and Developing Countries Clinical Trials programme(EDCTP2) supported by the European Union (grant number RIA2017NIM-1847-PEOPLE, <https://www.edctp.org/#>) (SMB, EH, PC, AG, BCdJ). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

smears and skin biopsies. We evaluated whether we can use less invasive samples, like fingerstick blood or nasal swabs, to determine the bacterial load. We found that the level of antibodies against *M. leprae* ( $\alpha$ PGL-I IgM) in fingerstick blood correlates well with the bacterial load determined in skin biopsies from the same leprosy patient. Therefore, a high level of antibodies against *M. leprae* in fingerstick blood might identify persons who pose a potential risk for transmission of leprosy and could be prioritized for contact screening, which is essential for control of the disease.

## Introduction

According to the World Health Organization (WHO) the global leprosy (also known as Hansen's disease) prevalence has decreased to <1 patient per 10,000 population since the year 2000, based on which leprosy is eliminated as a public health problem. However, the annual global incidence has stabilized since 2006, with approximately 200,000 new leprosy patients reported worldwide each year [1], often with heterogeneous distribution in high incidence 'pockets'.

These persistently high incidence areas also occur in settings with solid leprosy control programs, where patients are diagnosed early and treated appropriately with highly effective multidrug therapy. Moreover, in some regions 30% of the leprosy patients occur in children [2], which supports that transmission continues unabatedly. Although transmission pathways of *Mycobacterium leprae* (*M. leprae*) are still not fully understood, evidence shows that the main transmission route appears to be through aerosols/droplets to and from the nasal and oral cavities, also skin-to-skin contact and shedding of bacteria into the environment may play a role [3–5]. An infected contact is thought to be genetically predisposed to progress to either the paucibacillary or multibacillary form of the spectrum, although the majority of infected individuals never develop clinically overt leprosy [6]. Progressing to paucibacillary disease (WHO operational classification:  $\leq 5$  lesions), is associated with a predominant protective Th1 type response, while multibacillary leprosy (WHO operational classification:  $>5$  lesions) infection links with Th2 type response and high levels of anti-*M. leprae* antibodies against phenolic glycolipid I (PGL-I), which are ineffective at controlling this intracellular disease [7]. Untreated multibacillary patients are considered a likely source of transmission, probably even before they develop symptoms [8]. Diagnosis is entirely clinical as stated by the WHO guidelines, relying on the cardinal signs of leprosy. Once a leprosy patient starts multidrug therapy, it is assumed that chances of transmission are drastically reduced since the numbers of viable bacteria are quickly reduced [9].

Since the decreased attention for leprosy in the last century, many clinicians/health care workers lost their acumen to diagnose leprosy, leading to late- and missed diagnoses [10]. Microbiological confirmation, including measurement of the bacterial load, is not standardized nor endorsed by WHO for leprosy diagnosis. The bacterial load can be determined microscopically and by an *M. leprae* specific quantitative real-time PCR (qPCR), typically on slit skin smears or skin biopsies which both represent invasive clinical samples. Unfortunately, access to laboratories facilitating molecular techniques tends to be limited in leprosy endemic countries. Therefore, a low complexity lateral flow assay (LFA) utilizing up-converting reporter particles (UCP) was recently developed to quantitatively detect IgM antibodies against the *M. leprae* specific PGL-I ( $\alpha$ PGL-I) in human serum [11] and fingerstick blood [12], with documented applicability in *M. leprae*- and *M. lepromatosis*-infected squirrels [13,14]. This particular  $\alpha$ PGL-I UCP-LFA on fingerstick blood (further referred to as  $\alpha$ PGL-I test) was found to

correlate with the bacterial index (BI) as determined by slit skin smear microscopy and qPCR on slit skin smear [12,15]. Molecular tests have greater sensitivity for detection of *M. leprae* than microscopy [16]. In 2011, Martinez *et al.* concluded that the qPCR assay targeting a specific repetitive element (RLEP) [17] was more sensitive than qPCR assays using 16rRNA/*sodA*/Ag 85B [18]. In 2018, we resolved that the RLEP-qPCR is also highly specific and that its sensitivity is superior to classical bacillary index (BI) determination on slit skin smear [16]. In 2020,  $\alpha$ PGL-I IgM levels were compared to RLEP-qPCR of slit skin smear [19].

The ComLep study is a cross-sectional study conducted in the Comoros on the island of Anjouan where the average annual incidence rate exceeds 7/10,000. Active case finding is in place since 2008 through Mini Leprosy Elimination Campaigns, which consist of outreach skin clinics at the village level, where anyone with skin problems (including leprosy) is invited for a free dermatological consultation and where free treatment is provided for common minor skin ailments. In the present study, we correlated the RLEP-qPCR based bacterial burden in skin biopsies of leprosy patients as reference, with nasal swab RLEP-qPCR and host-based  $\alpha$ PGL-I test, aiming for less invasive proxy indicators for the total bacterial burden of leprosy disease.

## Material & methods

### Ethics statement

The protocol from the ComLep study (clinicaltrials.gov: NCT03526718) was approved by the institutional Review Board of ITM, by the Ethical Committee of the University of Antwerp (B300201731571) and by the Ethical Committee on the island of Anjouan. Written informed consent was obtained from each participant or from the parent/guardian of each participant under 18 years of age. For minors aged 12–17 years, additional signed assent from the minor was obtained before participation in the study. Participant were allowed to (selectively) refuse sampling.

### Study participants

From January 2017 until January 2018, on the island of Anjouan (Union of The Comoros) leprosy patients were diagnosed within the ComLep study. Diagnosis was based on the so-called cardinal signs, i.e. a typical hypopigmented patch with loss of sensation and/or enlarged peripheral nerves. Patients with  $\leq 5$  lesions were classified as paucibacillary and  $>5$  lesions as multibacillary, according to the WHO operational classification. In addition, whether a patient had  $\geq 25$  lesion was added as a variable, since having  $\geq 25$  lesions, will automatically classify the patient as either borderline borderline, borderline lepromatous or lepromatous according the dermatological aspect of the Ridley-Jopling classification. Only patients who provided both a skin biopsy and a fingerstick blood sample were included in this study (S1 Fig). A contraindication for a skin biopsy was a single lesion occurring in the face. Nasal swabs were collected in addition to these samples. For multibacillary patients who provided a slit skin smear the BI was determined by microscopy.

### Modified Maxwell DNA extraction

The 4 mm skin biopsy and the nasal swab were stored directly after sampling in 1ml of Disolol (ethanol denatured with 1% isopropanol and 1% methyl ethyl ketone) in screw cap vials, which were shipped to ITM. Upon arrival in the laboratory the skin biopsies were manually grinded with mortar and pestle in 1ml PBS. The obtained skin biopsy suspensions and the nasal swab were treated with an inhouse lysis buffer (1.6 M GuHCl, 60 mM Tris pH 7.5, 1%

Triton X-100, 60 mM EDTA, Tween-20 10%) followed by DNA extraction using the Maxwell 16 FFPE Tissue LEV DNA Purification Kit, as described by the manufacturer.

### qPCR assay for *M. leprae* detection

The *M. leprae* bacterial chromosome contains a family of dispersed repeats (RLEP) of variable structure and unknown function. The repetitive RLEP sequence is highly conserved. Thirty-seven copies of RLEP exist in the chromosome, each containing an invariant 545-bp core flanked in some cases by additional segments ranging from 44 to 100 bps. The qPCR detects the *M. leprae*-specific RLEP target. The qPCR assay targets 36 out of the 37 RLEP copies present in the *M. leprae* genome, yielding a highly sensitive test [17]. This RLEP-qPCR assay also has proven high specificity [16]. RLEP-qPCR was done for each sample in analytical triplicate following published protocols, using the StepOnePlus cycler [17]. To monitor for false negative results we included an internal positive control (Universal Exogenous qPCR Positive Control (Eurogentec, Belgium)) labelled with a different fluorescent probe than the probe detecting the RLEP target, which amplifies independently from the main RLEP-qPCR, to rule out qPCR inhibition in the sample.

Quantification was done by adding to each qPCR run a serial dilution of *M. leprae* reference strain NHDP ( $3 \times 10^6$ -30 RLEP copies) (BEI: ref. number 19350). Based on the C<sub>q</sub>-value, slope of the regression line and Y-intercept, the StepOnePlus Software v2.3 provides automatically the RLEP copy number per added template as starting quantity (SQ), to determine the amount of mycobacterial DNA present in a sample. Subsequently, the bacterial load (BL) of the samples was calculated by  $BL = (SQ \times [\text{volume of DNA extract}/\text{volume of template}])/36$  RLEP copy numbers.

### $\alpha$ PGL-I UCP-LFA

The capillary fingerstick blood collected with a disposable 20  $\mu$ l Minivette collection tubes (Heparin coated; Sarstedt), was diluted 1:50 by immediate mixing with 980  $\mu$ l assay buffer. The buffer was supplemented with 1% (v/v) Triton X-100 (100 mM Tris pH 8, 270 mM NaCl, 1% (w/v) BSA) to lyse blood cells. The diluted fingerstick blood sample (50  $\mu$ l) was flowed on lateral flow strips the same day after transportation to the central laboratory at ambient temperature. The lateral flow strips were transported to LUMC. By using an anti-IgM UCP reporter conjugate, the human  $\alpha$ PGL-I IgM antibodies were detected as described in Corstjens et al. (2019) [12]. UCP materials (NaYF<sub>4</sub>:YB3+, Er3+ polyacrylic-acid coated nano-sized particles, 980 nm excitation and 550 nm emission) were obtained from Intelligent Material Solutions, Inc. (Princeton, NJ, USA). A Packard FluoroCount microtiterplate reader compatible with the UCP technology [20], was used to analyse the lateral flow strips. The results were displayed as the ratio value (R) between Test and Flow-Control signal based on relative fluorescence units (RFUs) measured at the respective lines. For this cohort, the  $\alpha$ PGL-I UCP-LFA the R-value threshold was set at 0.29 according to and determined as described in previous studies [12].

### Analytical controls

For molecular analyses, on each sampling day, (negative) environmental control swab samples were collected, to rule out contamination during sampling. A positive (suspension of mouse footpad infected with *M. leprae* Thai 53) and a negative extraction control (water) were extracted in parallel with the clinical samples in each run to check extraction performance and to rule out contamination during the extraction procedure. An internal qPCR control, a positive and negative (water) DNA qPCR control were run simultaneously with the samples to check performance of the qPCR and to rule out DNA contamination during the qPCR procedure.

For serological analyses, at the start of the study non-endemic—and endemic (non-diseased health care staff) control sera were included for the  $\alpha$ PGL-I test.

### Statistical analyses

Data from the assays were log transformed (zero values were replaced by the minimum value for that variable divided by two). Data obtained for the different laboratory assays were evaluated using parametric hypothesis tests. Comparing a quantitative result between the two groups was carried out using the Welch t-test. The difference between months on treatment and the binary outcome of assays, was evaluated with the non-parametric Mann-Whitney U test. The alternative hypothesis stating significant differences between two outcomes was accepted at a significance level of  $\alpha = .05$ .

The multiple regression models modelling bacterial load versus  $\alpha$ PGL-I R-values used the 10-based logarithm of the bacterial load (determined by RLEP-qPCR) as dependent variable, with the 10-based logarithm of the R-values of  $\alpha$ PGL-I test as the independent, continuous variable, and a grouping variable splitting the population into 3 groups: i) patients with a negative nasal swab and less than 25 lesions, ii) patients with either a positive nasal swab or  $\geq 25$  lesions, iii) patients with both a positive nasal swab and  $\geq 25$  lesions. To analyse whether the effect of R-value of the  $\alpha$ PGL-I test on the bacterial load was uniform across the 3 groups, the interaction between  $\alpha$ PGL-I R-values and nasal swab group was tested for significance. The inclusion of the different factors in the multiple regression model was based on a simple linear regression, modelling the effect of each factor on the  $\log_{10}$ (bacterial load in skin biopsy). Significant factors were entered into a multiple linear regression model (S1 Table). Subsequently, this regression model to predict the number of the bacilli was simplified using stepwise-forward selection based on the Akaike information criterion (AIC).

Patients having  $\geq 50,000$  bacilli in a 4mm biopsy were further referred to as high bacterial load (HBL) patients. To evaluate if it was possible to predict the odds of being a HBL-patient, sensitivity and specificity were calculated for the three independent variables (number of lesions,  $\alpha$ PGL-I R-value and nasal swab positivity) separately. For the presence of  $\geq 25$  lesions, for the nasal swab positivity by RLEP qPCR and for the continuous  $\alpha$ PGL-I R-value, a ROC-curve was constructed, and the optimal cut-off value was determined by the Youden index [21], which was 0.81. Subsequently, 2x2 tables were constructed for the predicted versus observed outcome for each binary independent variable. Furthermore, to evaluate whether combining variables would increase the power of predicting the odds of being an HBL-patient, two multiple logistic regressions with presence of  $\geq 25$  lesions (yes or no),  $\alpha$ PGL-I R-value (as a continuous variable) and nasal positivity (yes or no).

All analyses were conducted with R version 3.5.0 for Windows (The R foundation, Vienna, Austria).

### Results

From January 2017 until January 2018, 310 (paucibacillary = 159; multibacillary = 151) leprosy patients were diagnosed. For 263 (84.8%) patients, sampling was complete (fingerstick blood samples and skin biopsies), with 260 providing a nasal swab from both nostrils instead of a nasopharyngeal swab. Based on clinical examination, 117 patients were classified as paucibacillary and 146 as multibacillary leprosy. Of these, 137 multibacillary patients provided a slit skin smear, of which 62 had a BI  $>0$ . At the time of sampling, 53 of the patients (paucibacillary = 18; multibacillary = 35) had received no treatment, versus 210 (paucibacillary = 99; multibacillary = 111) who started their treatment (Table 1). Female patients, patients under 17 years old and patients with no affected nerves are more likely to be paucibacillary (Table 1). All

**Table 1. Representation of the patients included in this study according to different variables.**

	Number of patients				% patients included in the study
Patients included in the study July 2017-January 2018	310				
Patients for whom sampling was complete (skin biopsy*, fingerstick blood)	263/310				84.8%
Patients for whom sampling was complete including nasal swab	260/310				83.9%
<b>Classification</b>					
Paucibacillary	117/263				44.5%
Multibacillary	146/263				55.5%
	<b>Paucibacillary (%)</b>		<b>Multibacillary (%)</b>		<b>OR (95%CI)</b>
<b>Sex</b>					
Female	60/117	(51.3)	54/146	(37.0)	1.79 (1.09, 2.94)
<b>Age (years)</b>					
≤17	75/117	(64.1)	64/146	(43.8)	2.29 (1.39, 3.77)
<b>Number of lesions</b>					
≥25	0/117	(0.0)	57/146	(39.0)	NA
<b>Affected nerves</b>					
0	49/117	(41.9)	21/146	(14.4)	4.29 (2.38, 7.74)
<b>Degree of disability</b>					
0	117/117	(100)	125/146	(85.6)	NA
1			11/146	(7.5)	
2			10/146	(6.8)	
<b>Treatment at sampling timepoint</b>					
Treatment started	99/117	(84.6)	111/146	(76.0)	1.73 (0.92, 3.26)

\* Incomplete sampling was due to either a single lesion occurring in the face, which was a contra-indication for a skin biopsy, or due to (selective) refusal. OR: odds ratio, 95% CI: 95% confidence interval.

<https://doi.org/10.1371/journal.pntd.0009924.t001>

environmental controls tested negative with qPCR, as did the different positive and negative controls included in the DNA extraction and molecular assays, suggesting accurate qPCR results. The endemic and non-endemic controls sera for the αPGL-I test all tested negative.

### Determination of bacterial load with RLEP-qPCR in skin biopsies

Of 263 skin biopsies, 196 (74.5%) tested positive for *M. leprae* DNA with the RLEP-qPCR (Table 2). Of the paucibacillary patients 79/117 (67.5%) tested positive, versus 117/146 (80.1%) of multibacillary patients (Table 2). As expected, bacterial load detected in skin biopsies was significantly higher in multibacillary patients (median: 2601.5 bacilli; mean of log<sub>10</sub>(bacilli): 3.872) than in paucibacillary patients (median: 132.0 bacilli; mean of log<sub>10</sub>(bacilli): 2.12)

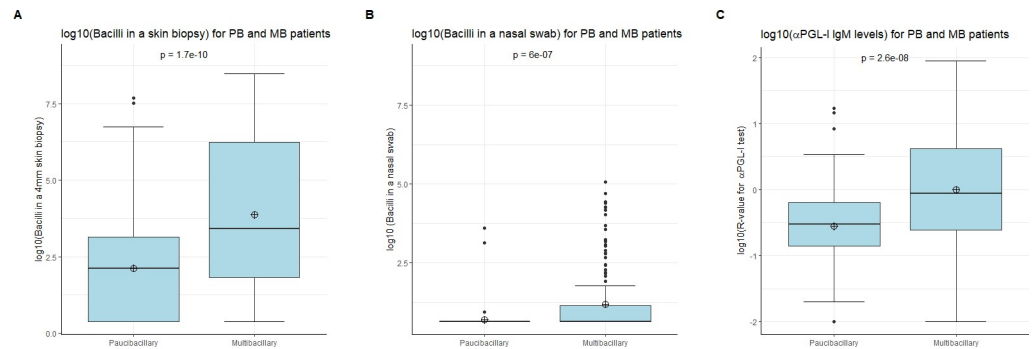
**Table 2. Laboratory assay results.**

	Paucibacillary			Multibacillary						Total (%)			
	N <sub>Pos</sub>	N <sub>Neg</sub>	(%)	BI = 0*			BI > 0*				BI = NA*		
	N <sub>Pos</sub>	N <sub>Neg</sub>	(%)	N <sub>Pos</sub>	N <sub>Neg</sub>	(%)	N <sub>Pos</sub>	N <sub>Neg</sub>	(%)	N <sub>Pos</sub>	N <sub>Neg</sub>	(%)	
Skin biopsy RLEP-qPCR	79	38	67.5%	51	24	68.0%	61	1	98.4%	5	4	55.6%	80.1%
Nasal swab RLEP-qPCR	3	113	2.6%	1	73	1.4%	36	25	59.0%	0	9	0.0%	25.7%
Fingerstick blood αPGL-I UCP-LFA	62	55	53.0%	42	33	56.0%	60	2	96.8%	2	7	22.2%	71.2%

\*BI = bacterial index as determined by microscopy on a skin slit smear; NA = not available, Multibacillary as per WHO clinical definition; Paucibacillary as per WHO clinical definition; N<sub>neg</sub> = number of negatives for the respective assay; N<sub>pos</sub> = number of positives for the respective assay.

<https://doi.org/10.1371/journal.pntd.0009924.t002>





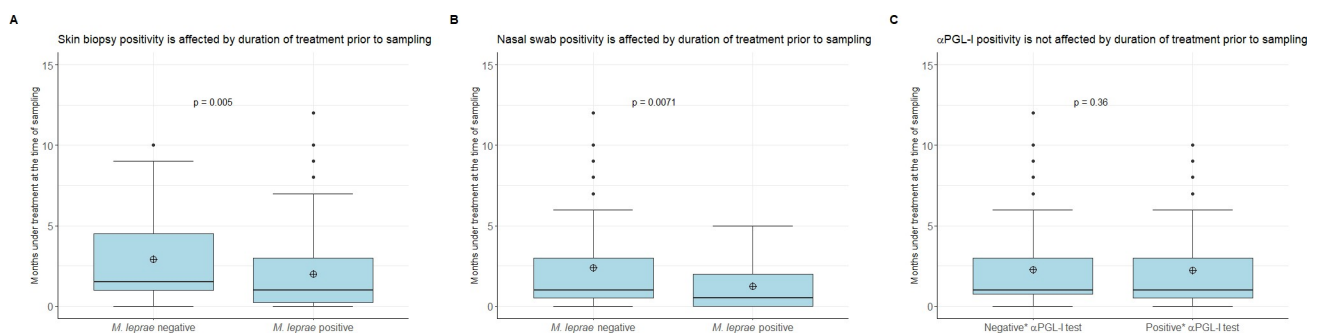
**Fig 1. Difference in outcome measure of the assays between paucibacillary and multibacillary patients as per WHO operational classification.** (A) Outcome measure of RLEP-qPCR on skin biopsies as determined by the bacterial load in a 4mm skin biopsy of paucibacillary and multibacillary patients as per WHO operational classification. (B) Outcome measure of RLEP-qPCR as determined by the bacterial load in nasal swabs of paucibacillary and multibacillary patients. (C) Outcome measure of the  $\alpha$ PGL-I test on fingerstick blood as measured by ratio (R) value, being *relative fluorescence units* measured at test line divided by the signal measured at the flow-control line of paucibacillary and multibacillary patients.

<https://doi.org/10.1371/journal.pntd.0009924.g001>

(Fig 1A;  $p = 1.7e-10$ ). Of the BI-negative multibacillary patients 51 out of 75 (68.0%) tested positive for the presence of *M. leprae* DNA in their skin biopsy (Table 2). In all except one of the 62 BI-positive patients, *M. leprae* DNA was detected (98.4%). For 9 multibacillary patients no slit skin smear, and therefore no BI was available (Table 2). There was no significant difference between the bacterial load detected in skin biopsies of paucibacillary and BI-negative multibacillary patients ( $p = 0.7835$ ). At the time of sampling, patients in whose skin biopsy no *M. leprae* DNA was detected, had on average been treated longer compared to patients with a detectable amount of *M. leprae* DNA in their skin biopsy (Fig 2A;  $p = 0.005$ ).

### Nasal swab positivity determined with RLEP-qPCR

Of 260 nasal swabs, only 40 (15.4%) tested positive for *M. leprae* DNA with the RLEP-qPCR (Table 1). The bacterial load detected in the nasal swabs was significantly higher in multibacillary patients than in paucibacillary patients (Fig 1B;  $p = 6e-07$ ). Only 3 out of the 116 paucibacillary patients (2.6%) tested nasal swab positive, compared to 37/144 (25.7%) multibacillary patients (Table 1). Of the BI-negative multibacillary patients, 1/74 (1.4%) tested positive for *M.*



**Fig 2. Patients with a negative assay result versus patients with a positive assay result with regard to how long they had been taking multidrug therapy prior to sampling.** (A) Patients in whose skin biopsy no *M. leprae* DNA was detected, had on average been treated longer prior to sampling compared to patients with a detectable amount of *M. leprae* DNA in their skin biopsy (B) Patients in whose nasal swab no *M. leprae* DNA was detected, tended to have been treated longer prior to sampling compared to patients with a detectable amount of *M. leprae* DNA in their nasal swab (C) The presence of systemic  $\alpha$ PGL-I is not affected by duration of treatment prior to sampling. R-values for the  $\alpha$ PGL-I test on fingerstick blood. \*Negative/positive means R-value < infection threshold and R-value  $\geq$  infection threshold respectively.

<https://doi.org/10.1371/journal.pntd.0009924.g002>

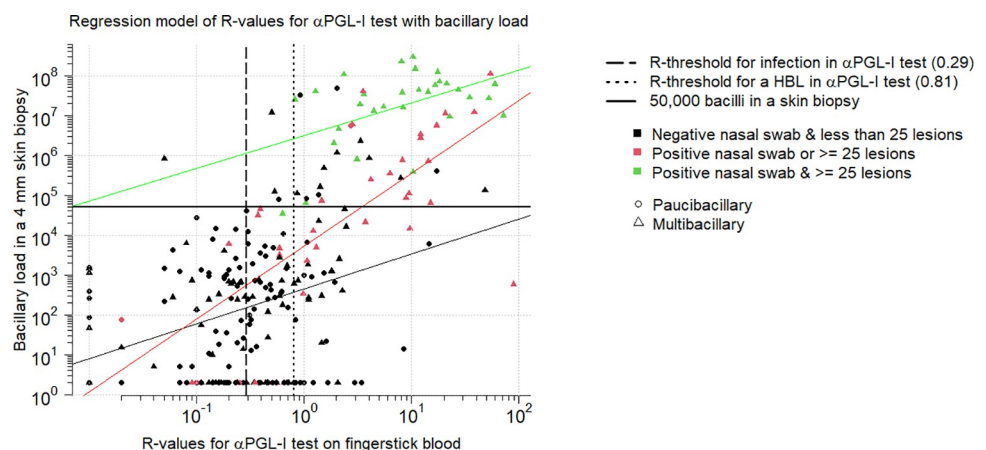
*leprae* DNA in their nasal swab (Table 2), compared to 36/61 (59.0%) of the BI-positive (Table 2). There was no significant difference in bacterial load in nasal swab of paucibacillary and BI-negative multibacillary patients ( $p = 0.9796$ ). However, patients in whose nasal swab no *M. leprae* DNA was detected, tended to have been treated longer prior to sampling compared to patients with a detectable amount of *M. leprae* DNA in their nasal swab (Fig 2B;  $p = 0.0071$ ).

### Level of $\alpha$ PGL-I determined with the $\alpha$ PGL-I test

Fingerstick blood samples of 166/263 (63.1%) patients tested positive for  $\alpha$ PGL-I, including 62/117 (53.0%) paucibacillary patients and 104/146 (71.2%) multibacillary patients (Table 2). The level of  $\alpha$ PGL-I was significantly higher in multibacillary (median Ratio (R)-value: 0.9; mean  $\log_{10}$ (R-value): -0.01) than in paucibacillary (median R-value: 0.3; mean  $\log_{10}$ (R-value): -0.56) patients (Fig 1C;  $p = 2.6e-08$ ). For the BI-negative multibacillary patients 42/75 (56.0%) tested positive for  $\alpha$ PGL-I whereas of the 62 BI-positive multibacillary patients, all but two tested positive for  $\alpha$ PGL-I (96.8%). There was no significant difference between the levels of  $\alpha$ PGL-I R-value detected in fingerstick blood samples of paucibacillary and BI-negative multibacillary patients ( $p = 0.3298$ ). Patients with a negative result for the presence of systemic  $\alpha$ PGL-I had not been treated longer prior to sampling than patients with a positive result (Fig 2C;  $p = 0.36$ ).

### Regression model of the $\alpha$ PGL-I test with the bacterial load determined by RLEP-qPCR

To study the relationship between the  $\alpha$ PGL-I IgM levels (measured as R-value of the  $\alpha$ PGL-I test) and the bacterial load in skin biopsies, we fitted a multiple linear regression model, including 263 patients (Fig 3). The patients were split in three groups: i) patients with a negative nasal swab and less than 25 lesions, ii) patients with either a positive nasal swab or  $\geq 25$  lesions, iii) patients with both a positive nasal swab and  $\geq 25$  lesions. We tested whether the  $\alpha$ PGL-I R-value had an effect on the bacterial load, and if this effect was the same across the 3 groups. We observed a significant relation of the  $\alpha$ PGL-I R-value and the bacterial load in a skin biopsy (S2 Table). This relation varied by group ( $p = 0.01346$  for interaction between



**Fig 3. Multiple regression model to estimate the bacterial load in skin biopsies based on the R-value of  $\alpha$ PGL-I test, the number of lesions and the nasal swab qPCR result.** R-values for the  $\alpha$ PGL-I test on fingerstick blood; HBL = high bacterial load ( $\geq 50,000$  bacilli in a skin biopsy); multibacillary as per WHO operational classification; paucibacillary as per WHO operational classification.

<https://doi.org/10.1371/journal.pntd.0009924.g003>



**Table 3. Linear relationship between log<sub>10</sub>(αPGL-I R-value) and log<sub>10</sub> (bacterial load in a skin biopsy) for each of the three groups.**

	Y-intercept	Slope	Effect Size F-statistic	P-value	Residual standard error	Adjusted R <sup>2</sup>	Pearson's r
<b>Group 1:</b> < 25 lesions and Neg. nasal swab	2.65 (95%CI: 2.51–2.79)	0.88 (95% CI: 0.71–1.05)	24.4	P = 1.696e-06	1.63 (192 df)	0.11	0.34
<b>Group 2:</b> ≥25 lesions or Pos. nasal swab	3.72 (95%CI: 3.46–3.99)	1.83 (95% CI: 1.55–2.10)	40.97	P = 2.609e-07	1.58 (34df)	0.53	0.74
<b>Group 3:</b> ≥25 lesions and Pos. nasal swab	6.50 (95%CI: 6.21–6.78)	0.82 (95% CI: 0.55–1.10)	8.543	P = 0.006792	0.85 (28 df)	0.21	0.46

\* The y-intercept, slope, effect size, p-value, residuals standard error, adjusted R<sup>2</sup> and Pearson's r are given for the linear regression between log<sub>10</sub>(αPGL-I R-value) and log<sub>10</sub> (bacillary load in skin biopsy) for each group

<https://doi.org/10.1371/journal.pntd.0009924.t003>

group and αPGL-I R-values), with the strongest association in the group with either a positive nasal swab or ≥ 25 lesions (Table 3). Exact effect sizes are listed in Table 3. None of the conclusions regarding significance and effect sizes were altered by adding months of treatment prior to sampling to the model.

Out of 263 patients, 64 had a bacterial load in a skin biopsy of ≥50,000 bacilli, further referred to as high bacterial load (HBL) patients. Of all the 64 HBL-patients that provided a fingerstick blood sample, 63 tested positive for αPGL-I test (R-value ≥0.29), and 60 of those had a αPGL-I R-value ≥0.81. All HBL-patients except one provided a nasal swab, of which 34 (54.0%) contained *M. leprae* DNA. The 29 HBL-patients with a negative result for the nasal swab tended to have taken treatment longer at the time of sampling than the nasal swab positive HBL-patients (p = 0.03006). Of the same 64 HBL-patients, 57 provided a slit skin smear, including 52 (91.2%) with a mean BI-value >0 (Table 4). Out of the 64 HBL-patients, 7 (10.9%) were paucibacillary patients, including one with infiltrated lesions (Table 4).

### Identifying HBL-patients

The separate analysis of the three independent variables indicates that based solely on the clinical feature (≥25 lesions) a HBL-patient can be identified with 65.5% sensitivity and 92.4% specificity. The highest sensitivity (93.8%) for identifying a HBL-patient is obtained at αPGL-I R-value ≥0.81, however with a reduced specificity of 80.9%. The nasal swab result as predictor for HBL has the lowest sensitivity (54.0%) with the highest specificity (97.0%) (Table 5). Combining predictors, two models resulted both in an AUC of 0.93; among these two models we chose the simplest one, which includes having ≥25 lesions and the αPGL-I R-value as predictors, which increases sensitivity with 20% in comparison to solely counting lesions. Nasal swab positivity increased the specificity of HBL patient identification at the cost of sensitivity; overall the addition of nasal swab results did not improve the AUC (p-value = 0.99) (Table 6).

### Discussion

Our findings confirm a strong correlation between αPGL-I IgM levels and the *M. leprae* bacterial load as measured by RLEP-qPCR in skin biopsies. To our knowledge this is the first assessment of the correlation of αPGL-I levels with a quantitative molecular measurement in a skin biopsy taken as a proxy for the bacterial load in a leprosy patient. Although a precise determination of the bacterial load by αPGL-I IgM levels is not possible, a range can be estimated. In 2019 Corstjens *et al* [12] demonstrated that the quantitative αPGL-I test results correlated well with the BI of multibacillary patients, and Tio Coma *et al* [19] extended this finding using nasal swab and slit skin smear of contacts and patients in Bangladesh.

Table 4. Characteristics and treatment of the 64 high bacterial load (HBL) patients.

WHO operational classification	Number of HBL-patients	
Paucibacillary	7/64	
Multibacillary	57/64	
	Paucibacillary	Multibacillary
<b>New case /Relapse</b>		
New case	7/7	56/57
Relapse/reinfection*	0/7	1/57
<b><math>\alpha</math>PGL-I test</b>		
R-value <0.81	1/7	3/57
R-value $\geq$ 0.81	6/7	54/57
<b>Slit skin smear</b>		
BI N.A.	7/7	0/57
BI negative	0/7	5/57
BI positive	0/7	52/57
<b>Number of lesions</b>		
<25	7/7	15/57
$\geq$ 25	0/7	42/57
<b>Infiltrated lesions on clinical exam</b>		
no	6/7	37/57
yes	1**/7	20/57
<b>Treatment</b>		
Paucibacillary treatment	6/7	0/57
Multibacillary treatment	1**/7	57/57
<b>Treatment follow-up</b>		
Completed	7/7	50/57
Lost to follow-up	0/7	7/57

R-values for the  $\alpha$ PGL-I test on fingerstick blood; BI = bacterial index as determined by microscopic examination of a skin-slit smear; Multibacillary as per WHO operational classification; Paucibacillary as per WHO operational classification

\*Relapse: the patient was sampled at a second episode of disease, either relapse or reinfection (indistinguishable in this study).

\*\* One PB patient was treated with MB treatment (12 months), due to the presence of infiltrated lesions. The nasal swab positive paucibacillary HBL-patient is not the paucibacillary HBL-patient with infiltrated lesions.

<https://doi.org/10.1371/journal.pntd.0009924.t004>

Table 5. Evaluation of the independent predictors for being an HBL-patients, with 2X2 tables and sensitivity/specificity.

	Number of lesions		$\alpha$ PGL-I R-value		Nasal swab RLEP-qPCR	
	<25	$\geq$ 25	<0.81	$\geq$ 0.81	Negative	Positive
<b>Non HBL-patients</b>	184	15	161	38	191	6
<b>HBL-patients</b>	22	42	4	60	29	34
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
	65.6%	92.4%	93.8%	80.9%	54.0%	97.0%

HBL = high bacterial load ( $\geq$ 50,000 bacilli in a skin biopsy);  $\alpha$ PGL-I R-value = ratio (R) value being the [relative fluorescence units](#) measured at test line divided by the signal measured at the flow-control line of the  $\alpha$ PGL-I test.

<https://doi.org/10.1371/journal.pntd.0009924.t005>

**Table 6. Multiple logistic regression models to predict being an HBL-patient.**

Logistic regression	Variables	AUC	95% Confidence interval	Youden index	Sensitivity Youden index	Specificity Youden index
Multiple logistic regression	$\geq 25$ lesions and $\alpha$ PGL-I R-value	93.1%	89.4% - 96.8%	170.1	93.7%	77.4%
	$\geq 25$ lesions, $\alpha$ PGL-I R-value and nasal swab positivity	93.1%	89.2% - 96.9%	171.6	74.6%	98.0%

$\alpha$ PGL-I R-value = ratio (R) value being the [relative fluorescence units](#) measured at test line divided by the signal measured at the flow-control line of the  $\alpha$ PGL-I test; AUC = area under the curve

<https://doi.org/10.1371/journal.pntd.0009924.t006>

The RLEP-qPCR on skin biopsies was able to confirm 74.5% of all clinically diagnosed leprosy patients, 80.1% of the multibacillary patients, and 98.4% of all BI positive patients. The RLEP-qPCR on nasal swab detected only 15.4% of all leprosy patients, 25.7% of the multibacillary patients, and 60.7% of all BI-positive patients, which is in line with previous data in an Asian cohort [19], yet is 41.2% and 49.6% lower for the paucibacillary and multibacillary patients respectively than in the study performed in non-treated patients conducted by Araujo *et al* [22]. The  $\alpha$ PGL-I test on fingerstick blood of clinically diagnosed patients was positive in 63.1% of all patients included, 71.2% of the multibacillary patients, and increasing to 96.8% in those patients with a positive BI. These findings are in line with the genetic predisposition for T cell driven paucibacillary or B cell driven multibacillary leprosy. While the  $\alpha$ PGL-I test specificity for leprosy could not be estimated in this cohort, given the absence of a large control group of individuals without leprosy, the  $\alpha$ PGL-I IgM is acknowledged to be a marker of *M. leprae* infection rather than disease. The advantage of using the  $\alpha$ PGL-I test on fingerstick blood to support clinical diagnosis, is that this test is minimally invasive, user-friendly and can be performed in remote laboratories such as in the Union of the Comoros, where no specialized facilities are present. Although the RLEP-qPCR on skin biopsies has overall better sensitivity than the  $\alpha$ PGL-I test on fingerstick blood and RLEP-qPCR on nasal swab, the downside of using skin biopsies as a confirmation method, is the invasiveness of the sampling, for which local anaesthesia is necessary and a scar remains. Moreover, to avoid false positives, careful processing of the samples in advanced molecular laboratories needs to be ensured.

### Avoiding misclassification using non-invasive sampling

Untreated leprosy, particularly lepromatous cases, can lead to serious irreversible nerve damage, often resulting in significant disfigurement and disabilities. Treatment is usually based on the spectral phenotype of the disease. Leprosy presents as a spectral disease, which is more complicated than paucibacillary/multibacillary determination, as demonstrated by the Ridley-Jopling classification for which histopathology is crucial [23]. The WHO operational classification, based on counting of lesions as a diagnostic method, has its shortcomings. In this study a negative BI distinguished a group of multibacillary patients whose assay results resembled paucibacillary patients. Also, 7 (10.2%) of the 64 HBL-patients (bacterial load in skin biopsy  $\geq 50,000$ ) would classify as paucibacillary patients following WHO operational classification. The leprosy control team decided at time of clinical diagnosis to categorize one of them as an multibacillary because of the presence of infiltrations. A second paucibacillary patient additionally had a positive nasal swab, which may be transient [24]. Hence, fingerstick blood  $\alpha$ PGL-I testing in addition to counting lesions seem to improve differentiation within the spectrum of multibacillary leprosy patients. Ultimately, improve classification may guide more appropriate treatment.

## Identification of high risk index cases is a critical knowledge gap

For leprosy it is extremely difficult to identify high risk index cases based on secondary cases as the incubation period of leprosy is exceptionally long (on average 5 years and can take up to 20 years or longer). Identification of high risk index cases is key to curb transmission, and therefore this remains an important knowledge gap, as identified during the COR-NTD conference (National Harbor, 2019). That multibacillary patients are primarily responsible for *M. leprae* transmission has been demonstrated several times [25–27] and Sales *et al.* found that patients with a positive BI were four times more likely to transmit the disease to their contact in comparison with multibacillary patients with a negative BI and eight times more likely with a BI > 3 [28]. To obtain a BI score, an invasive sample has to be taken. While  $\alpha$ PGL-I IgM is acknowledged to be a biomarker of *M. leprae* infection rather than disease, our data confirm that in this cohort in the Union of the Comoros high  $\alpha$ PGL-I IgM levels are indicative for a higher bacterial load, which is in line with findings of van Hooij *et al.* in 2017 [15], where they demonstrate that  $\alpha$ PGL-I IgM levels correlate with BI determined by microscopy. By applying arbitrary thresholds, an  $\alpha$ PGL-I R-value of 0.29 could indicate tentative *M. leprae* infection, while a  $\alpha$ PGL-I threshold of 0.81 (in addition to counting lesions) would allow to identify HBL-patients who may pose an increased risk for transmission. Adding the nasal swab, which may also be transiently positive, does not result in extra power for identifying HBL-patients. In the PEOPLE study (clinicaltrials.gov: NCT03662022) ongoing investigation for (close contact) secondary cases will allow to test the hypothesis that high risk index cases of *M. leprae* can be identified with non-invasive sampling. This will help resolve whether they should be prioritized for extensive contact screening beyond the household, to possibly provide prophylactic treatment to these contacts.

## Limitations

Our patients were sampled at variable intervals since the start of treatment limiting our ability to determine diagnostic sensitivity at baseline for the different tests. To control for potential bias, we added this variable to the regression models as a covariate. However, it was nowhere significant, neither in the full model with the interaction between antibodies response and different groups, nor in the linear regression models for the three separate groups (results not shown). None of the conclusions regarding significance and effect sizes were altered by adding months of treatment prior to sampling to the models. Therefore, the month of treatment was not included as a covariate in any of the models, and it is very unlikely that different treatment intervals would bias the results.

In this study we lack a non-exposed population control for the  $\alpha$ PGL-I test, although we incorporated (non-)endemic controls sera. Inclusion of more non-diseased endemic controls may allow to identify titers indicative of (multibacillary) disease versus latent infection.

## Way forward

The extension of the ComLep cohort in the ongoing PEOPLE study, including door-to-door screening for leprosy in highly endemic villages allows to identify the best predictors of transmission to close contacts. Additionally, the genotypic comparison of *M. leprae* can confirm transmission chains. Correlation with our findings on the bacterial burden may help identify high risk index cases and their characteristics, and allow us to test whether leprosy patients with higher outcome in the  $\alpha$ PGL-I test, e.g.  $\alpha$ PGL-I test R-value above 0.81, have significantly more secondary cases than patients with lower  $\alpha$ PGL-I levels in a certain time frame. The ongoing adaptation of the  $\alpha$ PGL-I, full integration of the test in an individually wrapped cassette and improved availability of readers will facilitate actual Point-of-Care testing.

In conclusion, improved approaches for microbiological confirmation of *M. leprae* utilizing less invasive sampling are desired and shown here to be feasible. The bacterial load is not routinely measured for leprosy patients, although it is a known correlate of infectiousness [28]. Counting the number of lesions together with the quantitative  $\alpha$ PGL-I IgM levels can be used as a proxy for the bacterial load in leprosy patients and to better classify patients along the clinical leprosy spectrum. Ongoing studies (i.e. PEOPLE study) are expected to provide further evidence whether counting lesions and  $\alpha$ PGL-I test on fingerstick blood can identify high risk index cases, who can then be prioritized for extensive contact screening beyond the household.

## Supporting information

**S1 Fig. Patient flowchart.** PB = paucibacillary according to the operational WHO classification; \*MB = multibacillary according to the operational WHO classification.  
(DOCX)

**S1 Table. Simple linear regression analysis.** The inclusion of the different factors in the multiple regression model was based on a univariable analysis for each of the factors, estimating the influence of a factor to the bacillar load in the skin biopsy.  
(DOCX)

**S2 Table. Coefficients for the multiple logistic regression, showing no signs of multicollinearity.**  
(DOCX)

**S3 Table. Descriptive statistics for each assay per operational classification.**  
PB = Paucibacillary operational WHO classification; MB = Multibacillary operational WHO classification.  
(DOCX)

## Acknowledgments

We are grateful to Damien Foundation clinical staff for their excellent work in support of leprosy patients and their families.

## Author Contributions

**Conceptualization:** Sofie Marijke Braet, Saverio Grillone.

**Data curation:** Abdallah Baco.

**Formal analysis:** Sofie Marijke Braet, Erik Fransen.

**Investigation:** Sofie Marijke Braet.

**Methodology:** Sofie Marijke Braet, Anouk van Hooij, Abdallah Baco.

**Supervision:** Epco Hasker, Leen Rigouts, Annemieke Geluk, Bouke Catherine de Jong.

**Validation:** Sofie Marijke Braet.

**Visualization:** Sofie Marijke Braet.

**Writing – original draft:** Sofie Marijke Braet.

**Writing – review & editing:** Sofie Marijke Braet, Anouk van Hooij, Epco Hasker, Erik Fransen, Abdou Wirdane, Saverio Grillone, Nimer Ortuno-Gutierrez, Younoussa Assoumani,



Aboubacar Mzembaba, Paul Corstjens, Leen Rigouts, Annemieke Geluk, Bouke Catherine de Jong.

## References

1. Reibel F., Cambau E., & Aubry A. (2015). Update on the epidemiology, diagnosis, and treatment of leprosy. *Med Mal Infect*, 45(9), 383–393. <https://doi.org/10.1016/j.medmal.2015.09.002> PMID: 26428602
2. Hasker E., Baco A., Younoussa A., Mzembaba A., Grillone S., Demeulenaere T., et al. (2017). Leprosy on Anjouan (Comoros): persistent hyper-endemicity despite decades of solid control efforts. *Leprosy review*, 88(3), p 334–342.
3. Martinez T. S., Figueira M. M., Costa A. V., Goncalves M. A., Goulart L. R., & Goulart I. M. (2011). Oral mucosa as a source of Mycobacterium leprae infection and transmission, and implications of bacterial DNA detection and the immunological status. *Clin Microbiol Infect*, 17(11), 1653–1658. <https://doi.org/10.1111/j.1469-0691.2010.03453.x> PMID: 21199152
4. Kotteeswaran G., Chacko C. J., & Job C. K. (1980). Skin adnexa in leprosy and their role in the dissemination of M. leprae. *Lepr India*, 52(4), 475–481. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/7007723> PMID: 7007723
5. Araujo S., Lobato J., Reis Ede M., Souza D. O., Goncalves M. A., Costa A. V., et al. M. (2012). Unveiling healthy carriers and subclinical infections among household contacts of leprosy patients who play potential roles in the disease chain of transmission. *Mem Inst Oswaldo Cruz*, 107 Suppl 1, 55–59. <https://doi.org/10.1590/s0074-02762012000900010> PMID: 23283454
6. Cambri G., & Mira M. T. (2018). Genetic Susceptibility to Leprosy-From Classic Immune-Related Candidate Genes to Hypothesis-Free, Whole Genome Approaches. *Front Immunol*, 9, 1674. <https://doi.org/10.3389/fimmu.2018.01674> PMID: 30079069
7. Bhat R. M., & Prakash C. (2012). Leprosy: an overview of pathophysiology. *Interdiscip Perspect Infect Dis*, 2012, 181089. <https://doi.org/10.1155/2012/181089> PMID: 22988457
8. Ranade M. G., & Joshi G. Y. (1995). Long-term follow-up of families in an endemic area. *Indian J Lepr*, 67(4), 411–425. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8849918> PMID: 8849918
9. Wilder-Smith E. P., & Van Brakel W. H. (2008). Nerve damage in leprosy and its management. *Nat Clin Pract Neurol*, 4(12), 656–663. <https://doi.org/10.1038/ncpneuro0941> PMID: 19002133
10. Geluk A. (2013). Challenges in immunodiagnostic tests for leprosy. *Expert Opin Med Diagn*, 7(3), 265–274. <https://doi.org/10.1517/17530059.2013.786039> PMID: 23537134
11. van Hooij A., Tjon Kon Fat E. M., Richardus R., van den Eeden S. J., Wilson L., de Dood C. J., et al. (2016). Quantitative lateral flow strip assays as User-Friendly Tools To Detect Biomarker Profiles For Leprosy. *Sci Rep*, 6, 34260. <https://doi.org/10.1038/srep34260> PMID: 27682181
12. Corstjens P., van Hooij A., Tjon Kon Fat E. M., Alam K., Vrolijk L. B., Dlamini S., et al. (2019). Finger-stick test quantifying humoral and cellular biomarkers indicative for M. leprae infection. *Clin Biochem*, 66, 76–82. <https://doi.org/10.1016/j.clinbiochem.2019.01.007> PMID: 30695682
13. Schilling A, v. H. A., Corstjens PLAM, Lurz PWW, DelPozo J, Stevenson K, Meredith A, et al. (2019). Detection of humoral immunity to mycobacteria causing leprosy in Eurasian red squirrels (*Sciurus vulgaris*) using a quantitative rapid test. *European Journal of Wildlife Research*, 65.
14. Avanzi C., Del-Pozo J., Benjak A., Stevenson K., Simpson V. R., Busso P., et al. (2016). Red squirrels in the British Isles are infected with leprosy bacilli. *Science*, 354(6313), 744–747. <https://doi.org/10.1126/science.aah3783> PMID: 27846605
15. van Hooij A., Tjon Kon Fat E. M., van den Eeden S. J. F., Wilson L., Batista da Silva M., Salgado C. G., et al. (2017). Field-friendly serological tests for determination of M. leprae-specific antibodies. *Sci Rep*, 7(1), 8868. <https://doi.org/10.1038/s41598-017-07803-7> PMID: 28827673
16. Braet S., Vandelanoot K., Meehan C. J., Brum Fontes A. N., Hasker E., Rosa P. S., et al. (2018). The repetitive element RLEP is a highly specific target for the detection of Mycobacterium leprae. *J Clin Microbiol*, 56(3). <https://doi.org/10.1128/JCM.01924-17> PMID: 29305545
17. Martinez A. N., Lahiri R., Pittman T. L., Scollard D., Truman R., Moraes M. O., et al. (2009). Molecular determination of Mycobacterium leprae viability by use of real-time PCR. *J Clin Microbiol*, 47(7), 2124–2130. <https://doi.org/10.1128/JCM.00512-09> PMID: 19439537
18. Martinez A. N., Ribeiro-Alves M., Sarno E. N., & Moraes M. O. (2011). Evaluation of qPCR-based assays for leprosy diagnosis directly in clinical specimens. *PLoS Negl Trop Dis*, 5(10), e1354. <https://doi.org/10.1371/journal.pntd.0001354> PMID: 22022631
19. Tio-Coma M., Avanzi C., Verhard E. M., Pierneef L., van Hooij A., Benjak A., et al. (2020). Genomic Characterization of Mycobacterium leprae to Explore Transmission Patterns Identifies New Subtype in Bangladesh. *Front Microbiol*, 11, 1220. <https://doi.org/10.3389/fmicb.2020.01220> PMID: 32612587

20. Corstjens P. L., Li S., Zuiderwijk M., Kardos K., Abrams W. R., Niedbala R. S., & Tanke H. J. (2005). Infrared up-converting phosphors for bioassays. *IEE Proc Nanobiotechnol*, 152(2), 64–72. <https://doi.org/10.1049/ip-nbt:20045014> PMID: 16441160
21. Youden W.J., Index for rating diagnostic tests. *Cancer*, 1950. 3(1): p. 32–5. [https://doi.org/10.1002/1097-0142\(1950\)3:1<32::aid-cnrcr2820030106>3.0.co;2-3](https://doi.org/10.1002/1097-0142(1950)3:1<32::aid-cnrcr2820030106>3.0.co;2-3) PMID: 15405679
22. Araujo S., et al., Molecular Evidence for the Aerial Route of Infection of *Mycobacterium leprae* and the Role of Asymptomatic Carriers in the Persistence of *Leprosy*. *Clin Infect Dis*, 2016. 63(11): p. 1412–1420. <https://doi.org/10.1093/cid/ciw570> PMID: 27558564
23. Ridley D. S., & Jopling W. H. (1966). Classification of leprosy according to immunity. A five-group system. *Int J Lepr Other Mycobact Dis*, 34(3), 255–273. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/5950347> PMID: 5950347
24. Klatser P. R., van Beers S., Madjid B., Day R., & de Wit M. Y. (1993). Detection of *Mycobacterium leprae* nasal carriers in populations for which leprosy is endemic. *J Clin Microbiol*, 31(11), 2947–2951. <https://doi.org/10.1128/jcm.31.11.2947-2951.1993> PMID: 8263180
25. Rao P. S., Karat A. B., Kaliaperumal V. G., & Karat S. (1975). Transmission of leprosy within households. *Int J Lepr Other Mycobact Dis*, 43(1), 45–54. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1171831> PMID: 1171831
26. Moet F. J., Pahan D., Schuring R. P., Oskam L., & Richardus J. H. (2006). Physical distance, genetic relationship, age, and leprosy classification are independent risk factors for leprosy in contacts of patients with leprosy. *J Infect Dis*, 193(3), 346–353. <https://doi.org/10.1086/499278> PMID: 16388481
27. Jesudasan K., Bradley D., Smith P. G., & Christian M. (1984). Incidence rates of leprosy among household contacts of "primary cases". *Indian J Lepr*, 56(3), 600–614. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/6549329> PMID: 6549329
28. Sales A. M., Ponce de Leon A., Duppre N. C., Hacker M. A., Nery J. A., Sarno E. N., et al. (2011). Leprosy among patient contacts: a multilevel study of risk factors. *PLoS Negl Trop Dis*, 5(3), e1013. <https://doi.org/10.1371/journal.pntd.0001013> PMID: 21423643