Prototype multi-biomarker test for point-of-care leprosy diagnostics

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HIGHLIGHTS

Prototype MBT that quantitatively detects six host-derived biomarkers is developed

The immunopathological spectrum of leprosy is ideally suited to evaluate the MBT

MBT discriminated patients with leprosy from controls in a high and non-endemic area

Application of the MBT using low invasive fingerstick blood is technically feasible
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SUMMARY
To end the decade-long, obstinately stagnant number of new leprosy cases, there is an urgent need for field-applicable diagnostic tools that detect infection with Mycobacterium leprae, leprosy’s etiologic agent. Since immunity against M. leprae is characterized by humoral and cellular markers, we developed a lateral flow test measuring multiple host proteins based on six previously identified biomarkers for various leprosy phenotypes. This multi-biomarker test (MBT) demonstrated feasibility of quantitative detection of six host serum proteins simultaneously, jointly allowing discrimination of patients with multibacillary and paucibacillary leprosy from control individuals in high and low leprosy endemic areas. Pilot testing of fingerstick blood showed similar MBT performance in point-of-care (POC) settings as observed for plasma and serum. Thus, this newly developed prototype MBT measures six biomarkers covering immunity against M. leprae across the leprosy spectrum. The MBT thereby provides the basis for immunodiagnostic POC tests for leprosy with potential for other (infectious) diseases as well.

INTRODUCTION
For over a decade, the annual number of newly detected leprosy cases has stagnated around 200,000 including children (World Health Organisation, 2019). This indicates that transmission of the causative agent of leprosy, Mycobacterium leprae (M. leprae), is still ongoing. Leprosy can be effectively cured by multidrug therapy (MDT), and early identification and treatment of patients with leprosy prevents irreversible nerve damage correlated with advanced stages of the disease (Thangaraju and Venkatesan, 2020). Prevention of disability reduces health as well as socioeconomic burden on leprosy-affected individuals, as their visible handicaps can lead to loss of income or unemployment due to social stigma and exclusion. Currently, leprosy diagnosis is based on clinical symptoms requiring well-trained clinicians. As a result of the declaration by the World Health Organization (WHO) in 2000 that the global target of leprosy elimination had been reached (World Health Organisation, 2002), leprosy control activities received considerably less attention and leprosy care was integrated in general healthcare programs. This leads to diminished leprosy expertise among clinicians which currently results in frequent missed or delayed diagnosis (Smith et al., 2015). Undiagnosed patients and M. leprae-infected individuals (yet) without clinical symptoms are likely to contribute significantly to the ongoing transmission (Pedrosa et al., 2018), which is emphasized by the fact that 75% of the new leprosy cases in high endemic areas cannot be directly attributed to known index cases (Richardus et al., 2005; Ortuno-Gutierrez et al., 2019a). Implementation of diagnostic tests specific for M. leprae infection in contact and population surveys will allow the identification of M. leprae-infected individuals as target for post-exposure prophylaxis (PEP), as well as detection of early stage leprosy for timely treatment (Blok et al., 2018; GPZL, 2019). Such diagnostic tests are not yet available (GPZL, 2019; Smith et al., 2016). Moreover, in order to implement novel tools in leprosy endemic areas, which are often resource-limited settings, diagnostic tests need to be available in a user- and field-friendly, rapid test format.

Leprosy has a wide spectrum of clinical manifestations which are closely related to the host immune response against M. leprae. In patients with multibacillary (MB) leprosy (individuals with high bacillary
loads), IgM antibody responses to phenolic glycolipid-I (PGL-I), a cell wall component of *M. leprae*, are frequently detected (van Hooij et al., 2017). In paucibacillary (PB) leprosy, this antibody response is generally absent, but instead, biomarkers of (Th1-)cell-mediated immunity are observed (van Hooij et al., 2018, 2019). Examining the anti-*M. leprae* antibody response only is therefore not sufficient to identify patients at both sides of the leprosy spectrum but requires detection of multiple biomarkers specific for humoral as well as cellular immunity (Geluk, 2013a). Recently, we identified host biomarkers associated with leprosy in *M. leprae* antigen-stimulated whole blood assays (WBAs) and plasma from a leprosy endemic population in Bangladesh. A host biomarker signature of aPGL-I IgM, IP-10, CRP, ApoA1, and S100A12 was identified, covering both the humoral and cellular pole of the immunopathologic leprosy spectrum (van Hooij et al., 2019; Nausch and Jacobsen, 2019). High aPGL-I IgM, IP-10, and CRP levels, relative to controls, were associated with MB leprosy, whereas ApoA1 and S100A12 levels were critical for identification of both patients groups. For patients with PB leprosy, ApoA1 was identified as the most important biomarker (van Hooij et al., 2019). ApoA1 and S100A12 levels also differentiated highly exposed contacts from endemic controls (ECs), identifying potentially *M. leprae*-infected individuals (van Hooij et al., 2020). In addition, CCL4 showed added diagnostic value in overnight stimulated WBA samples, particularly for patients with PB leprosy (van Hooij et al., 2019) and was also associated with *M. leprae* infection among household contacts (van Hooij et al., 2020).

Utilizing the unique up-converting reporter particles (UCPs), individual lateral flow (LF) test strips for separate detection of each of the five identified biomarkers were previously developed and applied to several cohorts from different geographic regions (van Hooij et al., 2016, 2018, 2019). UCP-LF is virtually background free as the up-conversion upon excitation with infrared light does not occur in nature. This prevents autofluorescence with other assay components, providing a rapid and highly sensitive point-of-care (POC) test format (Corstjens et al., 2005; Sedlmeier et al., 2016; Tanke et al., 2014). In contrast to most POC tests (Buhrer-Sekula et al., 2003), the results generated by UCP-LF tests are quantitative. This allows cross-sectional comparison of test groups, as well as intra-individual longitudinal monitoring, at the POC level.

Aiming at user- and field-friendly test applications, we developed a multi-biomarker test (MBT) strip that allows simultaneous detection of these six biomarkers on one strip rather than separate strips for each biomarker. To demonstrate feasibility of this MBT to identify patients with leprosy and *M. leprae*-infected individuals, we analyzed banked plasma and serum samples of patients with leprosy from a highly endemic area in Bangladesh (Richards et al., 2013), as well as an area in South Korea, that has reached the WHO elimination target (registered prevalence of less than 1 case per 10,000 population) in 1984 (Lee et al., 2015) but still reports new (import) leprosy cases annually (World Health Organisation, 2019). Finally, we pilot tested the MBT in Bangladesh collecting and directly testing fingerstick blood (FSB) samples from patients with leprosy and their contacts in the field to assess POC application of the MBT.

**RESULTS**

**The MBT format**

MBT strips comprising six test lines (biomarkers) with their respective flow control lines were produced using the sequence of biomarkers as indicated in Figure 1A. For this study, a predefined five-biomarker signature for leprosy (aPGL-I IgM, IP-10, CRP, S100A12, and ApoA1 (van Hooij et al., 2019)) was incorporated in the MBT strip format. As we envisage use of MBT strips both as POC tests and as user-friendly rapid tests for overnight stimulated whole blood samples, CCL4 as a biomarker for PB leprosy (van Hooij et al., 2016, 2018, 2019) was included as the sixth biomarker. The readout provided by the luminescent reporter technology (UCP) is indicated as the R value, for each biomarker individually. The R value is a relative value that quantifies the difference between the signal intensity of the test line and flow control. For each biomarker, this relative value can be converted to concentrations by generating a standard curve using known (recombinant) biomarker concentrations.

Application of the MBT to representative samples of EC and clinically diagnosed patients with MB and PB leprosy clearly showed the difference in peak height and concomitant R values between the plasma samples of the patient with MB leprosy and the EC, as well as the WBA sample of the patient with PB leprosy (Figure 1A). Importantly, overnight stimulation with *M. leprae* antigens, as demonstrated for the patient with PB leprosy, increased CCL4 levels which were undetectable in unstimulated samples/sera (van Hooij et al., 2019). Moreover, the dichotomy in aPGL-I IgM between MB and PB unambiguously confirmed the presence of antibodies in the former leprosy type and absence in the latter.
The MBT format thus enabled simultaneous, quantitative detection of six biomarkers in one test (Figure 1A), thereby representing a unique feature for user-friendly lateral flow assays. To explore scoring procedures for the readout, we defined and evaluated the NUM score and ALGO score. The NUM score is based on the sum of the number of positive biomarkers detected in the MBT providing a quick and easy-to-interpret readout. This required determination of a cutoff R value to discriminate patients with leprosy from controls, which was done using the Youden’s index (Fluss et al., 2005) for each individual biomarker. The ALGO score was based on an algorithm that contributes higher weights to R values associated with disease and was calculated using the median R values of the individual biomarkers for the patients with leprosy.

MBT evaluation in two cohorts with varying leprosy endemicity

To further explore the MBT performance, MBT strips were applied to banked plasma samples from Bangladesh and banked sera from South Korea.

Bangladesh (high endemic area)

Since the biomarkers studied here are generally not present in the same concentration range in blood, the optimal sample dilution per biomarker (10-fold and 1000-fold) was first determined. Results indicated that the biomarkers present in high concentrations (ApoA1, CRP, and αPGL-I IgM) based on previously obtained ELISA data (van Hooij et al., 2019) distinguished patients with leprosy from controls effectively using 1000-fold dilutions, whereas for detection of IP-10 and S100A12, 10-fold dilutions were required (Figure S1). CCL4 could not be detected in unstimulated plasma samples, in line with what we have observed.

Figure 1. MBT schematic overview and scoring

(A) The MBT strip consists of 12 parallel lines, of which six are test lines (Tn) and six are flow controls (FCn). Each Tn and FCn pair measures a single biomarker: αPGL-I IgM, IP-10, CRP, CCL4, S100A12, ApoA1. The up-converting reporter particles (UCP) are incorporated in the sample pad. The strip is read using a portable reader perpendicular to the sample flow. The test readout is a pattern of peaks, showing the signal of each of the 12 lines resulting in a ratio value (R) per biomarker (Tn/FCn). R values for each biomarker are displayed for plasma samples of a patient with multibacillary leprosy (MB, orange) and an endemic control (EC, green) and an M. leprae antigen-stimulated whole blood assay sample of a patient with paucibacillary leprosy (PB; blue).

(B) Two scores were calculated using the R values, the NUM score and the ALGO score. The NUM score is the sum of positive biomarkers per individual (green; R value above the cutoff). The ALGO score is based on an algorithm that contributes higher weights to R values associated with disease and was calculated using the median R values of the individual biomarkers for the patients with leprosy.
previously (van Hooij et al., 2019). For the other five individual biomarkers, areas under the curve (AUCs) observed in this cohort were comparable to results from an earlier study (van Hooij et al., 2019) using the same plasma samples but with multiple singleplex UCP-LF strips (Figure S2).

The NUM score as determined previously with singleplex UCP-LF strips accurately distinguished patients with leprosy from healthy endemic controls (ECs). Application of the NUM score to the MBT results showed a similar AUC (AUC: 0.9: p < 0.0001; Figure 2A). This score thus performed equally well for the MBT as for singleplex strips, signifying the potential of this MBT readout to identify patients with leprosy in endemic areas.

South Korea (non-endemic)

The MBT was also evaluated using serum samples from a South Korean cohort (Figure S3). Application of the NUM score to the MBT data significantly discriminated patients with leprosy from healthy controls living in that area (AUC: 0.88; p < 0.0001) (Figure 2A). Furthermore, patients with leprosy showed significantly higher NUM scores than contacts of patients with leprosy or patients with other dermatological diseases (ODDs) (household contact [HC]: p = 0.0079; ODD: p = 0.003; Figure 2B). These data indicate the applicability of the MBT to identify patients with leprosy also in a non-endemic area.

Although the NUM score performed equally well in both cohorts to differentiate patients with leprosy from healthy controls, it transformed the quantified MBT readout to a qualitative result (positive or negative) per biomarker. Apart from αPGL-I IgM, the other five MBT biomarkers are also present in unexposed, healthy individuals but the R values observed differed between patients and controls. Thus, to evaluate the difference in biomarkers between test groups, we stratified R values as strongly, intermediately, or not associated with disease, based on the median R value per biomarker determined for the group of patients with leprosy in each country (Table S1). This showed that the earlier observed pattern for patients with MB leprosy of high αPGL-I IgM, CRP, and IP-10 R values (van Hooij et al., 2019) was confirmed in both the Bangladeshi and South Korean cohorts. Similarly, MBT data of both cohorts showed that the ApoA1 R values in patients with PB leprosy differed from those in healthy controls (Figure 3). Interestingly, contacts of patients with leprosy in South Korea showed ApoA1 R values similar to those of patients with PB leprosy. This indicates not only the potential of this biomarker for discriminating PB from ODDs but also to detect M. leprae exposure/infection. An overview of the biomarkers differentiating controls from patients

![Figure 2. The MBT NUM score identifies patients with leprosy](image-url)
with MB and PB leprosy, HCs, and patients with ODDs indicates that assessing a combination of biomarkers is essential to allow proper interpretation of the MBT outcome (Figure 3C).

To reflect the effect of the observed patterns in individual biomarker R values in the MBT results, we assessed the second scoring method. This ALGO score showed a clear gradient from patients with MB leprosy to patients with PB leprosy and healthy controls in Bangladesh (Figure 4). For the South Korean cohort, however, the ALGO score of patients with PB leprosy did not differ from the scores observed in controls and contacts. Interestingly, patients with MB leprosy clearly showed the highest ALGO scores, and scores \( R \geq 5 \) were uniquely observed in this patient group (Figure 4B). This observation implicates that the ALGO score is associated with bacterial load in patients with leprosy.

In summary, the MBT accurately detected multiple biomarkers using a single test strip and allowed detailed assessment of biomarkers in blood samples. Two scoring methods were explored to interpret the MBT results, the easy-to-use NUM score to indicate the number of positive biomarkers and the more quantitative ALGO score reflecting the number of biomarkers per individual displaying R values strongly, intermediate, or not associated with disease. Application of these scoring methods facilitates interpretation of the quantitative MBT readout to identify patients with leprosy and \( M. \text{leprae} \)-infected individuals.
MBT evaluation in fingerstick blood

A pilot test to evaluate the use of FSB combined with the MBT was performed in Bangladesh aiming at future POC application. All 42 FSB samples were collected from patients and contacts visiting the field hospital on the same day. Analysis of the MBT strips by a portable UCP reader showed that all biomarkers could be clearly detected in FSB enabling the determination of R values. R values similar to those in plasma/sera samples were obtained (Figure S4), demonstrating that hemoglobin formed by hemolysis did not hamper the UCP signal. Importantly, like in sera and plasma samples, higher NUM scores were more frequently observed in FSB of patients with leprosy compared to HCs (Figure S4). Hence, application of the MBT using low invasive FSB samples is technically feasible at low-resource settings providing potential for MBT use at POC.

**DISCUSSION**

This study provides proof of concept for the use of the MBT platform, thereby representing, to the best of our knowledge, the first demonstration of a diagnostic tool simultaneously and quantitatively detecting multiple host biomarkers with a user-friendly test easily applicable with FSB in the field.

Leprosy is ideally suited as a model disease to test this platform due to the close parallel between the ability of the host to establish effective immunity to *M. leprae* and the inter-individual variability in clinical manifestations, ranging from self-limited (PB) disease with a predominant Th1 response to disseminated (MB) disease characterized by extensive anti-*M. leprae* antibody titers (Geluk, 2013a, 2013b, 2018). This study showed POC testing of a biomarker signature covering humoral and cellular immune responses...
against *M. leprae* (van Hooij et al., 2019). The combination of six biomarkers in this new strip format in a single MBT device avoids running six individual tests and as such is a major step forward toward POC near-patient applications. Moreover, the procedure is less prone to error as the automated reader will immediately provide the MBT result; this would be much more complicated when running the six individual tests in sequence. The six-marker MBT strip provided similar test results as previously obtained with individual UCP-LF strips for each of the biomarkers separately (van Hooij et al., 2019). This clearly demonstrated technical feasibility of this new diagnostic platform.

Besides, enabling detailed evaluation of six biomarkers individually, the MBT allows combined analysis of multiple biomarkers as part of a biomarker signature. To allow for scoring, two methods were explored which are independent of each other. The NUM score, indicating the number of biomarkers with a value above the biomarker-specific cutoff, allowed discrimination of patients with leprosy from their contacts and healthy individuals. The ALGO score represents a more direct quantitative score linking the relative biomarker R values with leprosy disease. Irrespective of leprosy endemicity, patients with MB leprosy showed the highest ALGO scores, confirming the association of these MBT-implemented biomarkers with disease severity and bacterial load (van Hooij et al., 2019). Both scores are an example of the ample possibilities to analyze the MBT readout. As described previously, standard curves can also be generated to convert the quantifiable R values to absolute concentrations (Corstjens et al., 2011, 2016).

Selection of a suitable scoring method depends on the aim of the study. The NUM score provides a quick interpretation of the test result suitable for large-scale screening studies, for instance, to identify *M. leprae*-infected individuals that contribute to the perpetuating transmission. HCs of patients with MB leprosy are at the highest risk of acquiring *M. leprae* infection (Bakker et al., 2006; Goulart et al., 2008; Sales et al., 2011) and thus represent candidates for preventive drug administration in multiple studies (Barth-Jaeggi et al., 2016; Mieras et al., 2018; Ortuno-Gutierrez et al., 2019b; Richardus et al., 2020; Tiwari et al., 2020) to prevent progress to leprosy disease, as well as decrease transmission. Since June 2018, the WHO guidelines for leprosy control have included single-dose rifampicin as PEP for leprosy prevention (World Health Organization, 2010). The MBT could aid in the identification of *M. leprae*-infected individuals eligible for PEP to allow a more efficient and better targeted drug administration approach.

For personalized diagnostics and monitoring of the treatment response, the more detailed evaluation by the ALGO score could be informative. On the other hand, the MBT can be useful as adjunct diagnostic for patients presenting with symptoms suggestive of leprosy in both leprosy endemic and non-endemic countries. Identification of patients with PB leprosy lacking anti-*M. leprae* antibodies is challenging using the currently available diagnostic methods leading to delayed or misdiagnosis. In Bangladesh, the MBT result separated patients with PB leprosy clearly from ECs, although in the low endemicity setting in South Korea, the current biomarker signature could not distinguish the small-sized cohort of patients with PB leprosy (*n* = 6). Separate evaluation of biomarkers, however, indicated that ApoA1 differed significantly in these patients with PB leprosy from controls, corroborating the potential of ApoA1 as a biomarker for PB leprosy (van Hooij et al., 2019). *M. leprae*-exposed HCs in South Korea showed a similar ApoA1 response as the patients with PB leprosy, as observed previously in Bangladesh (van Hooij et al., 2020). In contrast to our findings in Bangladesh (van Hooij et al., 2020), R values of S100A12 in contacts and patients with PB leprosy were similar to those of healthy controls in South Korea. Leprosy is no longer endemic in this country; it has to be taken into account that the frequency of exposure to *M. leprae*, as well as other environmental pathogens, can definitely influence biomarker levels, stressing the importance of quantitative measurements.

For global application including identification of patients with PB leprosy, the currently implemented biomarker signature will need fine-tuning and evaluation in large cohorts is warranted. The flexible MBT format allows replacement of biomarkers upon identification of additional candidate markers. To identify new biomarkers, especially for PB leprosy, the scope can be widened from the broadly studied immune markers to metabolic markers, which contribute to leprosy pathogenesis as well (Mayboroda et al., 2016; Cruz et al., 2008; Silva et al., 2017). New techniques (Koeken et al., 2020; Mourits et al., 2020) to identify disease markers in HCs developing PB leprosy in high throughput fashion are currently explored by us.

An important advantage of the MBT is its field applicability, ensuring implementation in low-resource settings. Furthermore, its flexible format also enables the application of the MBT to other diseases for which diagnosis will benefit from the quantitative detection of multiple biomarkers simultaneously. Serum
Biomarker signatures have been described, for example, for tuberculosis (Chegou et al., 2016; Jacobs et al., 2016), rheumatoid arthritis (Li et al., 2018), and inflammatory bowel disease (Knutson et al., 2013). More recently, it was also described for patients with COVID-19 that cytokines play an important role in determining the outcome of infection besides SARS CoV-2-specific antibodies (Grifoni et al., 2020; Sekine et al., 2020).

In this study, we demonstrated the technical feasibility and applications of the MBT platform for leprosy diagnostics by successfully implementing host biomarkers covering a well-defined biomarker signature for leprosy, on one MBT strip. Moreover, the MBT was not only compatible with plasma and serum but allowed POC testing with FSB samples. Thus, the MBT format represents a step forward in the development of the urgently needed immunodiagnostic POC test for detection of *M. leprae* infection and early stage leprosy.

**Limitations of the study**

We acknowledge several limitations to the findings in this study: of the patients with leprosy studied for the fingerstick blood assay we some were already treated with MDT. This cohort was therefore less homogeneous and showed variable associations with disease depending on the duration of treatment. As this study aimed at developing a test platform rather than evaluating a biomarker signature, relatively small sample sizes were tested. For the same reason, direct comparison of FSB and plasma/serum was not included, and intra-individual differences in biomarker R values in these different samples could therefore not be determined.

**Resource availability**

**Lead contact**

Further information and requests for resources and reagents should be directed to A.geluk@lumc.nl.

**Material availability**

There are restrictions to the availability of MBT due to the fact that the MBT currently is an in house produced test.

**Data and code availability**

The published article includes all data generated or analyzed during this study.

**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.102006.

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**AUTHOR CONTRIBUTIONS**

A.G. and A.H. designed research of the study. A.S.C., J.R., K.A., and J.P.K. enrolled patients and performed and registered clinical diagnosis. A.H., D.J., E.T.K.F., M.K., and S.S. performed research. A.G. and A.H. analyzed the data. A.G., A.H., and P.C. wrote the paper. All authors agreed with manuscript results and conclusions.
DECLARATION OF INTERESTS

The authors declare to have no conflicts of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supplemental Information

Prototype multi-biomarker test for point-of-care leprosy diagnostics

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SUPPLEMENTAL INFORMATION

PROTOTYPE MULTI-BIOMARKER TEST STRIP FOR POINT-OF-CARE LEPROSY DIAGNOSTICS

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The percentages of males and females, individuals receiving multidrug therapy (MDT), the mean bacteriological index (BI) and the age range of the multibacillary (MB) and paucibacillary (PB) patients, household contacts (HC), healthy (endemic) controls ((E)C) and patients with other dermatological diseases (ODD) in the study cohorts from Bangladesh (plasma and fingerstick blood (FSB)) and South Korea (serum). Ratio (R) values for αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 determined by the MBT strip in these samples were used to calculate the optimal cut-off (as determined by Youden’s index) to discriminate leprosy patients from controls applying the indicated dilution.
The MBT readout (R-value) was also stratified based on the association with disease as strongly, intermediately or not associated. These categories are based on the patient median (Median).

Strong association: \( R \geq 2 \times \text{median of patient group} \), intermediate association: median of patient group \( \leq R < 2 \times \text{median of patient group} \), no association: \( R < \text{median of patient group} \).

AUC: Area under the curve (as determined by computing receiver operating characteristic curves for leprosy patients compared to controls). NA = not applicable
Supplementary Figures

**Supplementary Figure S1:** Levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 measured by multibiomarker test (MBT) strips (Bangladesh cohort) related to Figures 2 -4.

The levels of αPGL-I IgM, IP-10, CCL4, CRP, S100A12 and ApoA1 were assessed by applying 10- and 1000-fold diluted plasma samples of leprosy patients (n=36) and endemic controls (EC; n=20) to MBT strips. CCL4 was not detected in these plasma samples, a graph was therefore not included. Multibacillary patients (MB; n=21) are indicated with orange dots and paucibacillary patients (PB; n=15) with blue dots. Ratio values (R) for each biomarker were calculated by dividing the peak area of the test line by the peak area of the flow control line (y-axis). The dashed line indicates the optimal study cut-off value to discriminate leprosy patients from controls, determined by the Youden’s index for the optimal dilution (10-fold: IP-10, S100A12; 1000-fold αPGL-I IgM, CRP, ApoA1).
Supplementary Figure S2: Areas under the curve (AUCs) for αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 related to Figure 2.

Receiver operating characteristic (ROC) curves were computed and the respective AUC was calculated. AUCs obtained using either UCP-LFA strips specific for a single biomarker (singleplex) or multibiomarker test (MBT) strips were determined as a measure for discrimination between leprosy patients and endemic controls (EC). αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 were assessed in plasma samples of leprosy patients (MB=21; PB=15) and EC (n=20). The AUCs (y-axis) were calculated based on the Ratio values for each marker for singleplex strips (blue) or MBT strips in the 10-fold (dark green) and 1000-fold (mint green) dilution. Singleplex data was described previously, samples were diluted 10-fold (IP-10), 100-fold (αPGL-I IgM and S100A12), 1000-fold (CRP) and 10000-fold (ApoA1). The dashed line at 0.5 indicates a non-discriminatory AUC.
Supplementary Figure S3: Levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 measured by multibiomarker test (MBT) strips (South Korea cohort) related to Figures 2-4.

The levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 were assessed in serum samples of leprosy patients (n=25), household contacts (HC; n=25), healthy controls (C; n=24) and patients with other dermatological diseases (ODD; n=24) from South Korea using MBT strips. Results of the optimal dilution per biomarker are shown, 10-fold for IP-10 and S100A12 and 1000-fold for CRP, ApoA1 and αPGL-I IgM. Multibacillary patients (MB; n=19) are indicated with orange dots and paucibacillary patients (PB; n=6) with blue dots. Ratio values (R) (y-axis) were calculated by dividing the peak area of the test line by the peak area of the flow control line. The dashed line indicates the optimal study cut-off value to discriminate leprosy patients from controls, determined by the Youden’s index.
Supplementary Figure S4: Fingerstick blood (FSB) levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 measured by MBT strips related to Figure 2.

The levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 were assessed in FSB samples (two dilutions) of leprosy patients from Bangladesh (Leprosy; n=27) and household contacts (HC; n=15) using MBT strips. Patients treated for several months when FSB samples were taken (91% MB (n=10), 12,5% PB (n=2)) were included. Results of the optimal dilution per biomarker are shown, 10-fold for IP-10 and S100A12 and 1000-fold for CRP, ApoA1 and αPGL-I IgM. Ratio values (R) (y-axis) were calculated by dividing the peak area of the test line by the peak area of the flow control line. MBT NUM-scores were determined for HC, PB and MB patients. The dashed line indicates the optimal study cut-off value to discriminate leprosy patients from controls, determined by the Youden’s index.
Transparent Methods

Study participants: Leprosy was diagnosed based on clinical, histological and bacteriological observations and classified as MB or PB as described by the WHO (World Health Organisation, 2019) and the bacteriological index (BI) was determined (Richardus et al., 2013). In Bangladesh 63 leprosy patients, 15 household contacts (HC) and 20 endemic controls (EC) were recruited between January 2013 and 2019 in leprosy endemic areas in Bangladesh as part of the MALTALEP/IDEAL trial (Richardus et al., 2013, van Hooij et al., 2020). Staff of leprosy- or TB clinics were excluded as EC (Supplementary Table S1). In South Korea participants included 25 leprosy patients, 25 frequent contacts of the patients (HC: 88% family contact, 12% office contact), 24 individuals with a history of other dermatological diseases (ODD) and 24 healthy controls from the same area (C) (Supplementary Table S1). ODD showed symptoms similar to leprosy, including patients with psoriasis vulgaris, eczema, fungal infections and sarcoidosis.

Ethics: This study was performed according to the Helsinki Declaration (2008 revision) and the study protocol was approved by the National Research Ethics Committee (Bangladesh Medical Research Council) (Ref no. BMRC/NREC/2010-2013/1534). Participants were informed about the study objectives, the samples and their right to refuse to take part or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. All patients received treatment according to national guidelines.

Leprosy prevalence: The prevalence in the four Bangladeshi districts (Nilphamari, Rangpur, Panchagar and Thakurgaon) was 0.9 per 10,000 and the new case detection rate 1.18 per 10,000 (Rural health program, the leprosy mission Bangladesh, yearly district activity report 2018). The leprosy prevalence in South Korea was 0.025 per 10,000 (2018 (World Health Organisation, 2019)).
**Samples:** Plasma and whole blood assay samples were collected in Bangladesh, shipped to the LUMC on dry ice and stored at -80 °C until further testing (Khadge et al., 2015). For the WBA, 4ml venous blood was drawn and 1ml was applied directly to a microtube precoated with 10 μg *M. leprae* whole cell sonicate (WCS) or without stimulus (Med). After 24 h incubation at 37°C the microtube was frozen at −20°C. Serum samples from South Korea were collected and stored at -80 °C until testing by local health care workers. An extensive standard operating procedure and a quality control sample were provided to limit procedural differences. FSB was collected using disposable 50 μl Minivette® collection tubes (Heparin coated; Sarstedt) and directly mixed with 455 μl high salt finger stick (HSFS) buffer supplemented with 1% (v/v) Triton X-100 (HSFS;100mM Tris pH 8, 270mM NaCl, 1% (w/v) BSA). FSB was tested directly after collection in a reference center for leprosy patients in Bangladesh (The Leprosy Mission International, Bangladesh, Nilphamari Hospital).

**Multi-biomarker test (MBT) production:** MBT strips were assembled by mounting 10 mm glass fiber sample/conjugate pad (Glass Fiber Conjugate Pad #8964, Ahlstrom), 25 mm laminated nitrocellulose membrane (Sartorius UniSart CN95) and 10 mm absorbent pad (High Purity Cotton Grade #320, Ahlstrom) on a 36 mm plastic backing card; all strip materials were obtained from Kenosha (Amstelveen, the Netherlands). Sample/conjugate and absorbent pad overlap respectively 3 and 6 mm with the nitrocellulose. Nitrocellulose was pre-striped such that each MBT strip contained 6 Test (T) lines with respective Flow Control (FC) lines, providing capture zones for the six biomarkers. The six pairs of capture lines (T and FC) were distributed evenly over the 50 mm wide MBT strip starting at 14 mm from the base of the MBT strip. Capture lines were located in a linear array of slanted lines, such that liquid only passed single capture lines, with T and FC lines separated by 3 mm and a 5mm distance between individual T and FC pairs. Sample flow direction and scanning direction of the reader are perpendicular. Each Test (Tₙ) line comprised 300 ng capture molecules (Figure 1): T₁ = ND-
O-HSA, obtained through the Biodefense and Emerging Infections Research Resources Repository (https://www.beiresources.org/) (Cho et al., 1983); T_2 = mouse-anti-IP-10 mAb (BC-55, Diaclone Research, Besancon, France); T_3 = mouse-anti-CRP mAb (C5, Labned.com, Amstelveen, Netherlands); T_4 = mouse-anti-CCL4 mAb (MAB271, R&D systems, Minneapolis, USA); T_5 = goat-anti-S100A12 pAb (AF1052; R&D systems); and T_6 = Goat-anti-ApoA1 pAb (AF3664; R&D systems). The respective Flow Control (FC) lines comprised 37.5 ng Goat-anti-Mouse (FC_2,3), 75 ng Goat-anti-Rabbit (FC_6) or Rabbit-anti-Goat (FC_1,4,5) antibody per 6 mm (Sigma-Aldrich, Saint-Louis, United States). Individual UCP conjugates (Corstjens et al., 2014) were prepared with mouse-anti-IP-10 (BC-50; Diaclone Research), mouse-anti-CRP (CRP135; Labned.com), goat-anti-CCL4 (AF-271-NA; R&D systems), goat-anti-human IgM (I0759; Sigma-aldrich), goat-anti-S100A12 pAb (AF1052; R&D systems, Minneapolis, USA) and Rabbit-anti-ApoA1 (Clone # 2083A; R&D systems) at a concentration of 50 μg antibody per mg UCP. Sodium yttrium fluoride upconverting nanomaterials (200 nm, NaYF_4:Yb^{3+},Er^{3+}) functionalized with polyacrylic acid were obtained from Intelligent Material Solutions Inc. UCP reporter conjugates were applied to the sample/conjugate pad at a density of 400 ng per 7 mm. After assembly a quality control was performed, testing the MBT strips with recombinant proteins and standardized sample.

**MBT assay:** Banked plasma and serum samples were thawed and 50 μl was mixed with 455 μl HSFS buffer to obtain a 10-fold dilution similar to the dilution of the FSB sample upon collection. A 1000-fold dilution was prepared by adding 5 μl of the 10-fold dilution with 495 μl HSFS buffer (1000-fold dilution). The diluted samples (500 μl) were added to a channel in a disposable tray, immunochromatography was initiated by placing the MBT strip into the channel and allowed to continue for at least 30 min. In South Korea this procedure was performed by local health care workers without prior training. Air dried MBT strips were scanned with a portable reader (ESEQuant LFR reader with 980 nm excitation and 550 nm
emission; QIAGEN Lake Constance GmbH, Stockach, Germany). The strip holder was in-house adapted to fit the MBT which requires scanning perpendicular to the sample flow. Test results are displayed as the Ratio (R) value of the signal (peak area) from individual Tn lines normalized to the respective FCn signal measured at the respective lines as determined by LF-Studio (ver. 3.3.8; QIAGEN Lake Constance GmbH) (Figure 1).

**Data analysis:** The R-value corresponds to the level of the biomarker present in the sample. Two different scores based on the MBT readout were evaluated.

i) **NUM-score:** Based on R-values, the optimal cut-offs to discriminate leprosy patients from their respective controls were determined using the Youden’s index (Fluss et al., 2005) per biomarker (Supplementary Table S1). Qualitative stratification of the biomarker result as positive or negative based on these cut-offs was used to calculate the number of positive biomarkers in the MBT per individual.

ii) **ALGO-score:** The ALGO-score was calculated using the median R-values of the individual biomarkers for the leprosy patients. First, scores were classified for each biomarker in three groups based on the association with disease as strong, intermediate or not associated (Supplementary Table S1). Strong association: R ≥ 2x median of patient group, intermediate association: median of patient group ≤ R < 2x median of patient group, no association: R < median of patient group. Second, the ALGO-score was set as 2x the number of strong biomarkers (2x n_{strong}), plus the number of intermediate biomarkers (n_{intermediate}), minus the number of biomarkers not associated with disease (n_{not}).

Note that for ApoA1, the 1000-fold dilution resulted in an inverse correlation of the Ratio values with the biomarker level a consequence of a distinct but reproducible high dose hook effect.

Statistical analysis was performed using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA; [http://www.graphpad.com](http://www.graphpad.com)). Receiver operating characteristic (ROC)
curves were computed in Graphpad Prism and the respective area under the curve (AUC) was calculated. Group differences were determined using Mann-Whitney U test. The statistical significance level used was $p \leq 0.05$. 

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