Development of immunodiagnostic tests for leprosy: from biomarker discovery to application in endemic areas

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Chapter

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

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Abstract

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 multi- and paucibacillary leprosy patients from control individuals in high- and low leprosy endemic areas. Pilot-testing of fingerstick blood showed similar MBT-performance in point-of-care 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 (1). 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 leprosy patients prevents irreversible nerve damage correlated with advanced stages of the disease (2). 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 WHO in 2000 that the global target of leprosy elimination had been reached (3), leprosy control activities received considerably less attention and leprosy care was integrated in general health care programs. This leads to diminished leprosy expertise amongst clinicians which currently results in frequent missed- or delayed diagnosis (4). Undiagnosed patients and *M. leprae* infected individuals (yet) without clinical symptoms are likely to contribute significantly to the ongoing transmission(5), 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 (6, 7). 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 (8, 9). Such diagnostic tests are not yet available (9, 10). 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 multibacillary (MB) patients (individuals with high bacillary loads) IgM antibody responses to phenolic glycolipid-I (PGL-I), a cell wall component of *M. leprae,* are frequently detected (11). In paucibacillary (PB) leprosy, this antibody response is generally absent, but instead biomarkers of (Th1-)cell-mediated immunity are observed (12, 13). 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 (14). Recently, we identified host biomarkers associated with leprosy in *M. leprae* antigen-stimulated whole blood assays (WBA) and plasma from a leprosy endemic population in Bangladesh. A host biomarker signature of αPGL-I IgM, IP-10, CRP, ApoA1 and S100A12 was identified, covering both the humoral- and cellular pole of the immunopathologic

leprosy spectrum (13, 15). High αPGL-I IgM, IP-10, CRP levels, relative to controls, were associated with MB leprosy, whereas ApoA1 and S100A12 levels were critical for identification of both patients groups. For PB patients, ApoA1 was identified as the most important biomarker (13). ApoA1 and S100A12 levels also differentiated highly-exposed contacts from endemic controls, identifying potentially *M. leprae*-infected individuals (16). In addition, CCL4 showed added diagnostic value in overnight stimulated WBA samples, particularly for PB leprosy patients (13) and was also associated with *M. leprae* infection among household contacts (16).

Utilizing the unique up-converting reporter particles (UCP), 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 (12, 13, 17). 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 (18-20). In contrast to most POC tests (21), 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 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 leprosy patients and *M. leprae* infected individuals, we analyzed banked plasma and serum samples of leprosy patients from a highly endemic area in Bangladesh 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 (22) but still reports new (import) leprosy cases annually (1). Finally, we pilot-tested the MBT in Bangladesh collecting and directly testing fingerstick blood (FSB) samples from leprosy patients and their contacts in the field to assess POC application of the MBT.

Materials and methods

Study participants

Leprosy was diagnosed based on clinical, histological and bacteriological observations and classified as MB or PB as described by the WHO (1) and the bacteriological index (BI) was determined (23). 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 (16, 23). 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 (1)).

Samples

Plasma and whole blood assay samples were collected in Bangladesh, shipped to the LUMC on dry ice and stored at -80 ^{0}C until further testing (24). 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 ^{0}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 (T_n) line comprised 300 ng capture molecules (Figure 1): T_1 = ND-O-HSA, obtained through the Biodefense and Emerging Infections Research Resources Repository (https://www.beiresources.org/) (25); 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_n) 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 (26) 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 T_n lines normalized to the respective FC_n 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 (27) 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: $R \geq 2x$ median of patient group, intermediate association: median of patient group $\leq R < 2x$ 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). 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 $p \leq 0.05$.

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 (αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 (13)) 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 (12, 13, 17) was included as the sixth biomarker. The read-out 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 MB and PB patients clearly showed the difference in peak height and concomitant R-values between the plasma samples of the MB patient and the EC, as well as the WBA sample of the PB patient (Figure 1A). Importantly, overnight stimulation with *M. leprae* antigens, as demonstrated for the PB patient, increased CCL4 levels which were undetectable in unstimulated samples/sera (13). Moreover, the dichotomy in αPGL-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 read-out, 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 cut-off R-value to discriminate leprosy patients from controls, which was done using the Youden's index (27) for each individual biomarker. The ALGO-score was based on an algorithm that tentatively indicates an association of the R-values with disease (Figure 1B). Using the biomarker median R-value of the patient group, individual R-values were classified as strongly, intermediately or not associated with disease. Fig. 1B illustrates both scoring methods, showing an MB and PB sample with higher NUM- and ALGO-scores as compared to the EC.

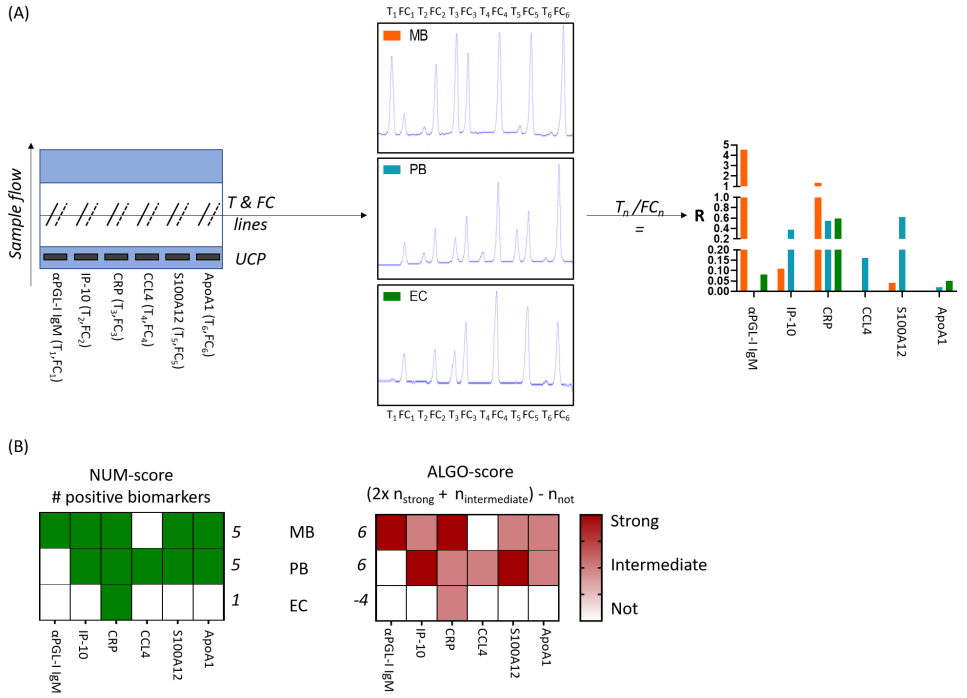

Figure 1: MBT schematic overview and scoring. (A) The MBT strip consists of 12 parallel lines, of which six are test lines (T_n) and six are flow controls (FC_n). Each T_n and FC_n pair measures a single biomarker: αPGL-I IgM, IP-10, CRP, CCL4, S100A12, ApoA1. The upconverting phosphor (UCP) particles 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 (T_n/FC_n). R-values for each biomarker are displayed for plasma samples of a multibacillary patient (MB; orange) and an endemic control (EC; green) and an *M. leprae* antigen-stimulated whole blood assay sample of a paucibacillary patient (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 cut-off). 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 leprosy patients.

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 (13), distinguished leprosy patients from controls effectively using 1000-fold dilutions, whereas for detection of IP-10 and S100A12, 10-fold

dilutions were required (Supplementary Figure S1). CCL4 could not be detected in unstimulated plasma samples, in line with what we have observed previously (13). For the other five individual biomarkers, AUCs observed in this cohort were comparable to results from an earlier study (13) using the same plasma samples but with multiple singleplex UCP-LF strips (Supplementary Figure S2).

The NUM-score as determined previously with singleplex UCP-LF strips accurately distinguished leprosy patients from EC (AUC:0.93) (13). 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 MBT as for singleplex strips, signifying the potential of this MBT read-out to identify leprosy patients in endemic areas.

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

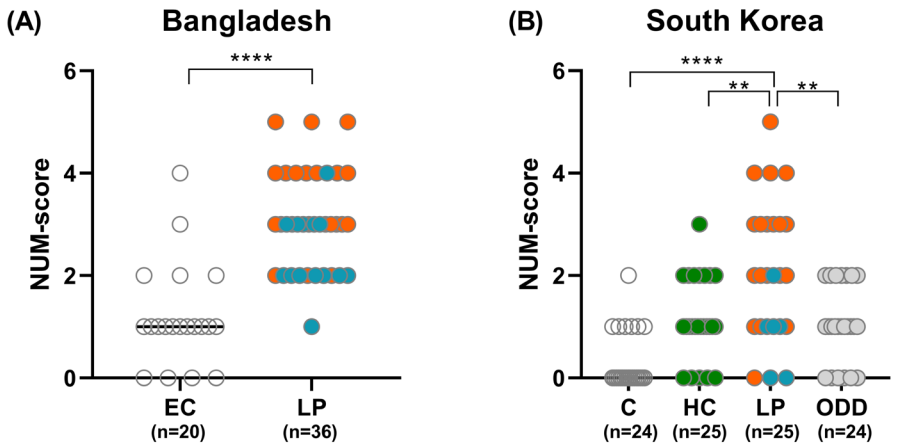

Figure 2: The MBT NUM-score identifies leprosy patients. The levels of αPGL-I IgM, IP-10, CCL4, CRP, S100A12 and ApoA1 were assessed by the MBT strip. The NUM-score, indicating the number of positive biomarkers based on the Ratio value was calculated per individual (*y*-axis). (A) NUM-scores observed in the cohort from Bangladesh (plasma) comparing leprosy patients (LP) to healthy endemic controls (EC). (B) NUM-scores in the South Korean cohort comparing LP to healthy controls (C), household contacts (HC) or patients with other dermatological diseases (ODD). Group differences were determined using Mann-Whitney U test, the statistical significance level used was $p \leq 0.05$. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Multibacillary leprosy patients are indicated with orange dots, paucibacillary patients with blue dots, HC with green dots, ODD patients with grey dots and healthy controls with white dots.

Although the NUM-score performed equally well in both cohorts to differentiate leprosy patients 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 leprosy patients group in each country (Supplementary Table S1). This showed that the earlier observed pattern for MB patients of high αPGL-I IgM, CRP and IP-10 R-values (13) was confirmed in both the Bangladeshi and South Korean cohort. Similarly, MBT data of both cohorts showed that the ApoA1 R-values in PB patients differed from those in healthy controls (Figure 3). Interestingly, contacts of leprosy patients in South Korea showed ApoA1 R-values similar to PB patients. This indicates not only the potential of this biomarker for discriminating PB from ODD, but also to detect *M. leprae* exposure/infection. An overview of the biomarkers differentiating controls from MB and PB patients, HC and ODD 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 MB to PB patients and healthy controls in Bangladesh (Figure 4). For the South Korean cohort, however, the ALGO-score of PB patients did not differ from the scores observed in controls and contacts. Interestingly, MB patients clearly showed the highest ALGO-scores, and scores ≥5 were uniquely observed in this patient group (Figure 4B). This observation implicates that the ALGO-score is associated with bacterial load in leprosy patients.

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, intermediately or not associated with disease. Application of these scoring methods facilitates interpretation of the quantitative MBT readout to identify leprosy patients and *M. 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

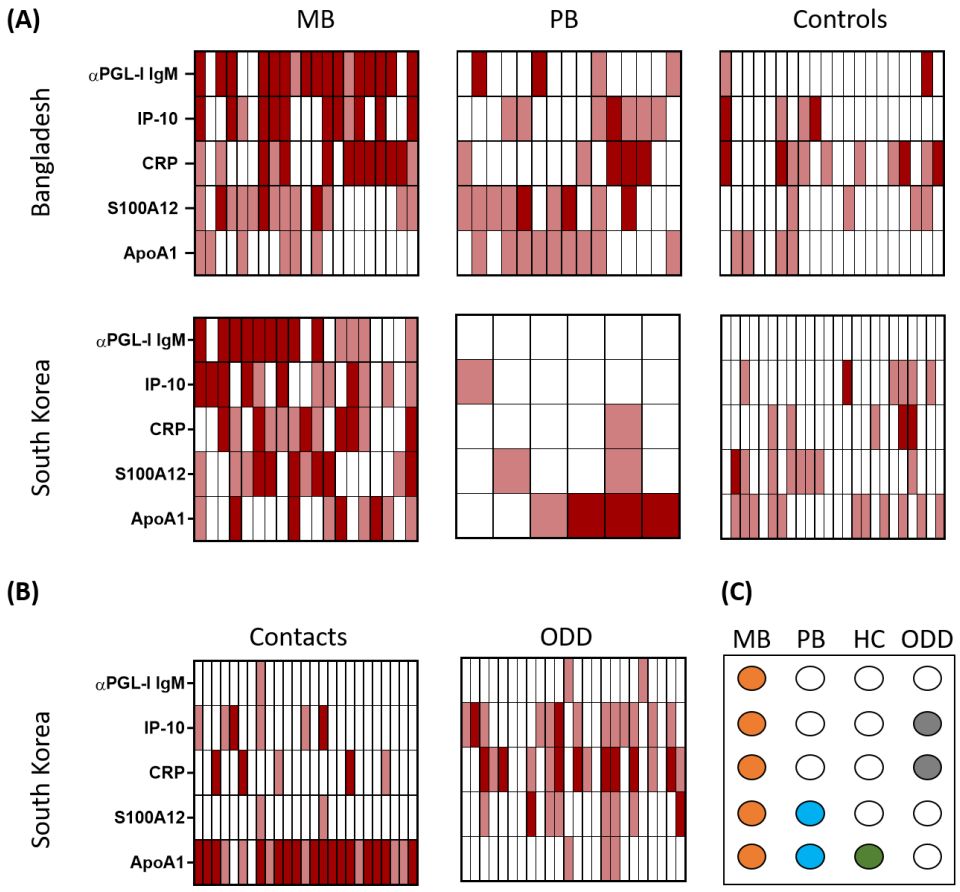

Figure 3: Stratification of biomarker levels using the MBT readout. Heatmap indicating per individual the αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 R-values classified in three groups based on the association with disease as strong (dark red), intermediate (pink) or not associated (white) in the Bangladeshi and South Korean cohort. 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 (Supplementary Table S1). (A) R-value classification of biomarkers in multibacillary (MB) patients (Bangladesh: 21; South Korea: 19), paucibacillary (PB) patients (Bangladesh: 15; South Korea: 6) and healthy controls (Bangladesh: 20; South Korea: 24). (B) R-value classification of biomarkers in household contacts (HC; n=25) and patients with other dermatological diseases (ODD; n=25) (South Korean cohort). (C) Dots indicating which biomarker showed a different pattern relative to controls in MB (orange) and PB (blue) patients, HC (green) and ODD (grey).

strips by 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 (Supplementary 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 leprosy patients compared

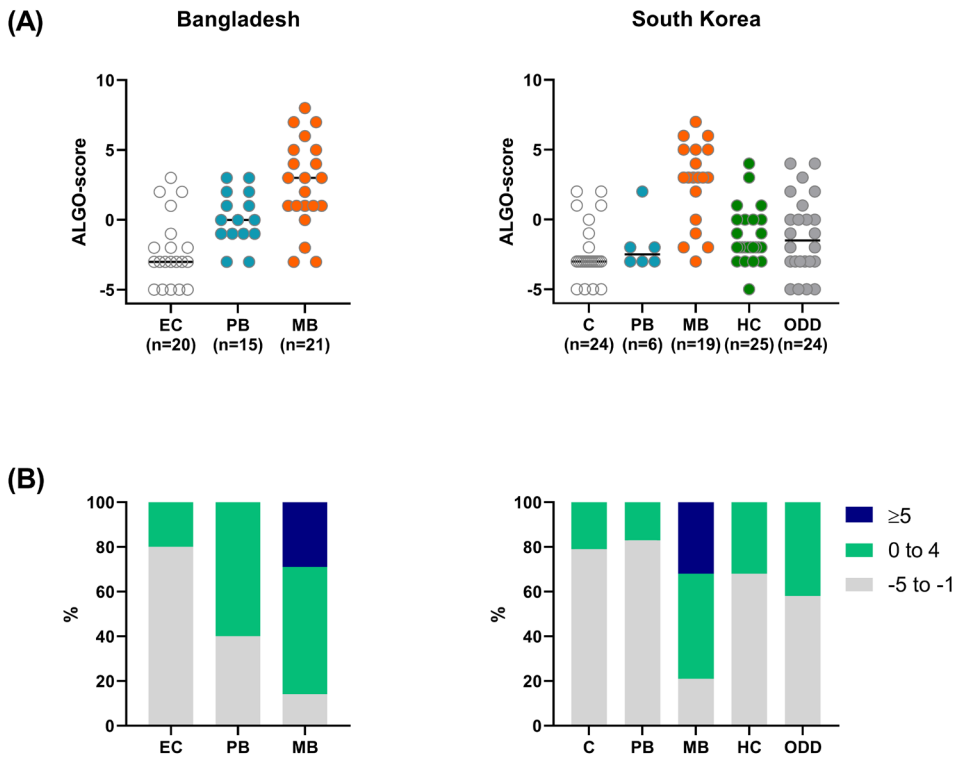

Figure 4: The MBT ALGO-score reflects disease severity and bacterial load. The ALGO-score is based on an algorithm that contributes higher weights to R-values associated with disease (Figure 1B). Based on the median R-value of the patient group, for each biomarker R-values were classified as strongly, intermediately or not associated with disease. 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}) ((2*nbiomarker^{strong}+nbiomarker^{intermediate})-nbiomarker^{not}). (A) ALGO-scores observed in the Bangladeshi and South Korean cohorts per test group. Untreated multibacillary (MB) leprosy patients (orange), untreated paucibacillary (PB) patients (blue), household contacts (HC;green), patients with other dermatological diseases (ODD; grey) and healthy controls ((E)C;white). (B) percentage (%) of individuals per test group with ALGO-scores ranging from -5 to -1 (grey), from 0 to 4 (green) or ALGO-scores ≥ 5 (blue) for the Bangladeshi plasma (left) and South Korean serum (right) cohort.

to HC (Supplementary 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 (14, 28, 29). This study showed POC testing of a biomarker signature covering humoral- and cellular immune responses against *M. leprae* (13). 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 towards 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 (13). 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 cut-off, allowed discrimination of leprosy patients 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, MB patients showed the highest ALGO-scores, confirming the association of these MBT-implemented biomarkers with disease severity and bacterial load (13). 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 (30, 31).

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. HC of MB patients are at the highest risk of acquiring *M. leprae* infection (32-34) and thus represent candidates for preventive drug administration in multiple studies (35-39) 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 (SDR) as PEP for leprosy prevention (40). 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 PB patients lacking anti-*M. leprae* antibodies is challenging using the currently available diagnostic methods leading to delayed or misdiagnosis. In Bangladesh the MBT result separated PB patients clearly from endemic controls, although in the low endemicity setting in South Korea the current biomarker signature could not distinguish the small-sized cohort of PB patients (n=6). Separate evaluation of biomarkers, however, indicated that ApoA1 differed significantly in these PB patients from controls, corroborating the potential of ApoA1 as a biomarker for PB leprosy (13). *M. leprae* exposed HC in South Korea showed a similar ApoA1 response as the PB patients, as observed previously in Bangladesh (16). In contrast to our findings in Bangladesh (16), R-values of S100A12 in contacts and PB patients 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.

We acknowledge several limitations to the findings in this study: the leprosy patients of which fingerstick blood was collected included patients treated with MDT, with varying treatment duration. As this study aimed at developing a test platform rather than evaluation of a biomarker signature relatively small sample sizes were tested. For the same reason, direct comparison of fingerstick blood and plasma/serum was not included, and intra-individual differences in biomarker R-values in these different samples could therefore not be determined. For global application including identification of PB patients, 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 (41-43). New techniques (44, 45) to identify disease markers in HC 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 (46, 47), rheumatoid arthritis (48) and inflammatory bowel

disease (49). More recently, it was also described for COVID-19 patients that cytokines play an important role in determining the outcome of infection besides SARS CoV-2-specific antibodies (50, 51).

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.

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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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Supplementary material

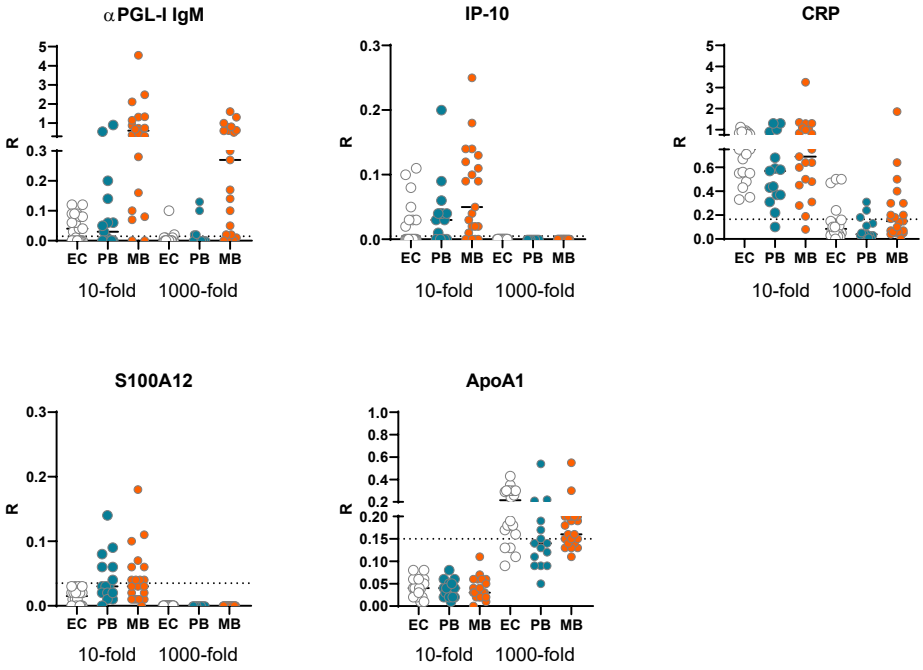
Tables

Supplementary Table S1: Study cohorts related to Figures 2 -4

	Group	N	BI (Mean)	Age range (years)	Male/Female (%)	MDT
Bangladesh (plasma)	*MB*	21	2.9+	18 to 60	91/9	0 (0%)
	PB	15	0	19 to 56	71/29	0 (0%)
	EC	20	NA	20 to 41	40/60	NA
	Biomarker characteristics	**αPGL-I IgM**	**IP-10**	**CRP**	**S100A12**	**ApoA1**
	Sample dilution	1000-fold	10-fold	1000-fold	10-fold	1000-fold
	AUC	0.75	0.7	0.52	0.78	0.69
	Cut-off	>0,015	>0,005	>0,165	>0,035	<0,23
	Sensitivity(%)	58	75	36	44	92
	Specificity(%)	90	60	80	100	50
	Median Ratio of patients	0.02	0.04	0.085	0.03	0.15
	Not	<0.02	<0.04	<0.085	<0.03	>0.15
	Intermediate	0.02-0.04	0.04-0.08	0.085-0.17	0.03-0.06	0.15-0.075
	Strong	>0.04	>0.08	>0.17	>0.06	<0.075
South-Korea (Serum)	**Group**	**N**	**BI (Mean)**	**Age range (years)**	**Male/Female (%)**	**MDT**
	MB	19	5.2+	21 to 82	63/37	0 (0%)
	PB	6	0	23 to 68	100/0	0 (0%)
	HC	25	NA	12 to 85	44/56	NA
	C	24	NA	24 to 95	71/29	NA
	ODD	24	NA	18 to 84	71/29	NA
	Biomarker characteristics	**αPGL-I IgM**	**IP-10**	**CRP**	**S100A12**	**ApoA1**
	Sample dilution	1000-fold	10-fold	1000-fold	10-fold	1000-fold
	AUC	0.77	0.6	0.67	0.6	0.54
	Cut-off	>0.03	>0.65	>0.205	>0.33	<0.105
	Sensitivity(%)	52	44	56	24	36
	Specificity(%)	100	92	79	96	100
	Median Ratio of patients	0.04	0.46	0.24	0.16	0.15
	Not	<0.04	<0.46	<0.24	<0.16	>0.15
	Intermediate	0.04-0.08	0.46-0.92	0.24-0.48	0.16-0.32	0.15-0.075
	Strong	>0.08	>0.92	>0.48	>0.32	<0.075
Bangladesh (FSB)	**Group**	**N**	**BI (Mean)**	**Age range (years)**	**Male/Female (%)**	**MDT**
	MB	11	1.4+	22 to 65	64/36	10 (91%)
	PB	16	0	17 to 70	38/62	2 (12,5%)
	HC	15	NA	17 to 60	53/47	NA
	Biomarker characteristics	**αPGL-I IgM**	**IP-10**	**CRP**	**S100A12**	**ApoA1**
	Sample dilution	1000-fold	10-fold	1000-fold	10-fold	1000-fold
	AUC	0.55	0.64	0.56	0.6	0.54
	Cut-off	>0.005	>0.175	> 0.375	> 1.06	< 0.225

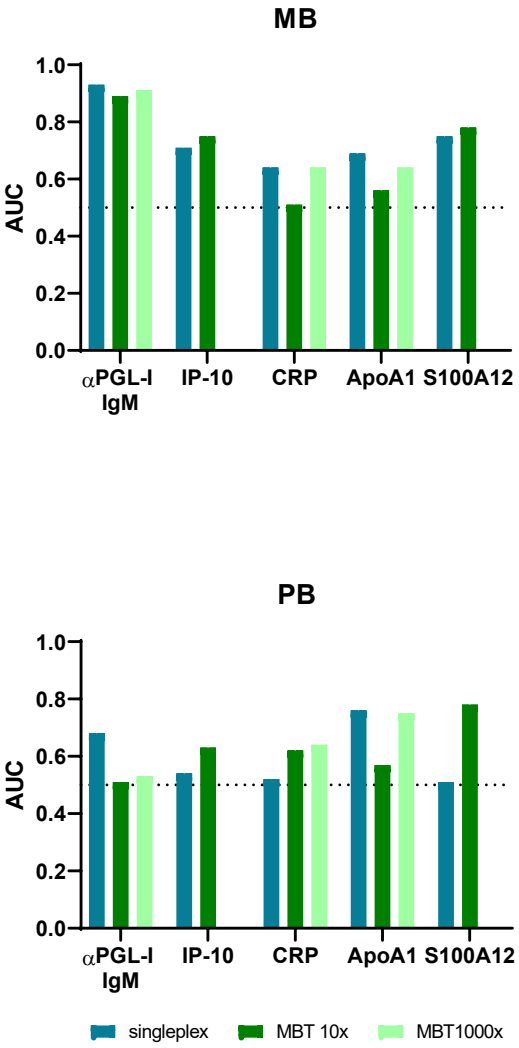
The percentages of males and females, individuals receiving multidrug therapy (MDT), the mean bacteriological index (BI) and the age range of the multibacillary (MB) and paucibacillary (PB) patients, household contacts (HC), healthy (endemic) controls ((E)C) and patients with other dermatological diseases (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 ≥ 2x median of patient group, intermediate association: median of patient group ≤ R < 2x median of patient group, no association: R < 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

Figures



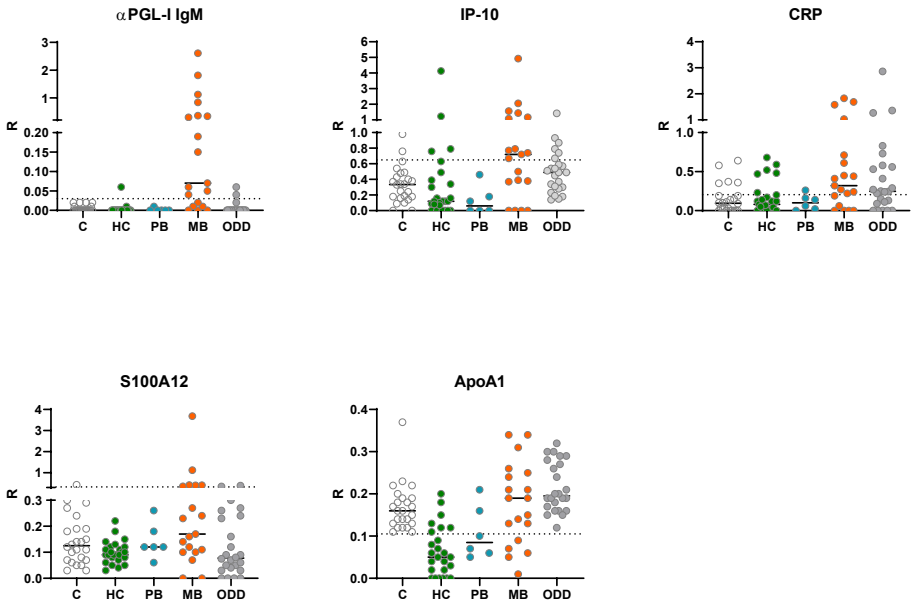
Supplementary Figure S1: Levels of αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 measured by multibiomarker test (MBT) strips (Bangladesh cohort) related to Figures 2 -4.

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; multibacillary (MB) = 21, paucibacillary (PB) =15) and endemic controls (EC; n=20) to MBT strips. CCL4 was not detected in these plasma samples, a graph was therefore not included. 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.



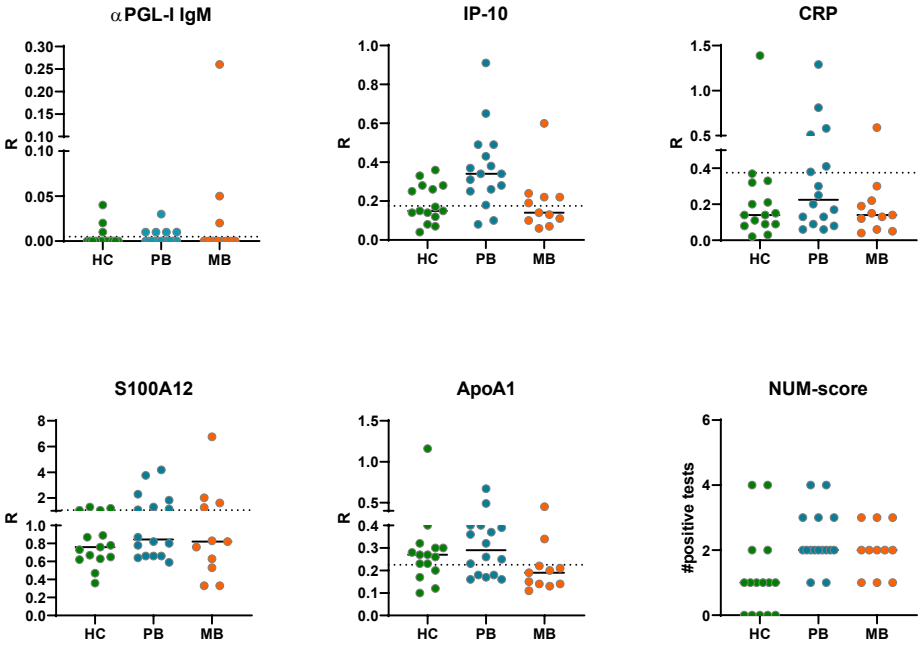
Supplementary Figure S2: Areas under the curve (AUCs) for αPGL-I IgM, IP-10, CRP, S100A12 and ApoA1 related to Figure 2.

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; multibacillary (MB) = 19; paucibacillary (PB) = 6), 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. 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.

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.