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

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Chapter

Quantitative lateral flow strip assays as user-friendly tools to detect biomarker profiles for leprosy

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Abstract

Leprosy is a debilitating, infectious disease caused by *Mycobacterium leprae*. Despite the availability of multidrug therapy, transmission is unremitting. Thus, early identification of *M. leprae* infection is essential to reduce transmission. The immune response to *M. leprae* is determined by host genetics, resulting in paucibacillary (PB) and multibacillary (MB) leprosy associated with dominant cellular or humoral immunity, respectively. This spectral pathology of leprosy compels detection of immunity to *M. leprae* to be based on multiple, diverse biomarkers.

In this study we have applied quantitative user friendly lateral flow assays (LFAs) for four immune markers (anti-PGL-I antibodies, IL-10, CCL4 and IP-10) for whole blood samples from a longitudinal BCG vaccination field-trial in Bangladesh.

Different biomarker profiles, in contrast to single markers, distinguished *M. leprae* infected from non-infected test groups, patients from household contacts (HHC) and endemic controls (EC), or MB from PB patients. The test protocol presented in this study merging detection of innate, adaptive cellular as well as humoral immunity, thus provides a convenient tool to measure specific biomarker profiles for *M. leprae* infection and leprosy utilizing a field-friendly technology.

Introduction

Leprosy, a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*) ranking second as the most pathogenic mycobacterial infectious disease after tuberculosis (TB), is still considered a major threat in developing countries (1). The condition is characterized by skin lesions and damage to peripheral nerves, the hallmark of leprosy pathology often resulting in severe, life-long disabilities and associated stigma (2,3). Despite the remarkable decrease in prevalence following introduction of multidrug therapy, it remains challenging to further reduce transmission as substantiated by the stable global annual incidence around 200,000 new cases for the past 10 years (4). This continued transmission is largely due to *M. leprae* infected individuals lacking clinical symptoms (5). In addition, identification of host-derived biomarkers for progression to disease is complicated by the low incidence and long incubation time requiring extensive, longitudinal studies. Furthermore, although molecular techniques to elicit strain differences within the leprosy bacillus are important diagnostic tools to enhance our understanding of the epidemiology of leprosy, differentiate between relapse and re-infection (6-10), these pathogen-derived profiles are not suitable to indicate development of leprosy in infected, asymptomatic individuals. These hurdles contributed to the current lack of tests for detection of asymptomatic *M. leprae* infection and diagnosis of early stage leprosy (11). As clinical resistance to commonly used antibiotics in leprosy treatment is increasingly occurring (12, 13), such tests should be highly specific to prevent redundant use of antibiotics.

Clinical manifestations closely parallel cellular immunity to *M. leprae* such that leprosy presents as a characteristic spectrum ranging from tuberculoid (TT) or paucibacillary (PB) leprosy to lepromatous (LL) or multibacillary (MB) leprosy (14). TT patients in general show strong T helper 1 cell (Th1) immunity with exacerbated levels of pro-inflammatory cytokines and develop localized granulomatous disease with bacilli scarcely detectable in their lesions. At the opposite pole of the spectrum are LL patients who predominantly generate Th2 and anti-inflammatory cytokines such as interleukin-10 (IL-10) resulting in disseminating, progressive infections (15). In between these two opposite poles of the leprosy spectrum, borderline states of leprosy [borderline tuberculoid (BT), borderline (BB) and borderline lepromatous (BL)] are positioned. Due to the diverse disease spectrum, detection of *M. leprae* infection in diagnostic tests requires multiple, diverse biomarkers specific for both cellular and humoral mediated immunity. In previous studies we have shown that IFN- γ -inducible protein 10 (IP-10) in response to a *M. leprae*-specific antigen (ML2478) correlates with *M. leprae* exposure and thereby the risk of infection and its subsequent transmission (16). Additionally, we demonstrated that chemokine (C-C motif) ligand 4 (CCL4) a component of the innate immunity, can be used to identify

pathogenic immunity against *M. leprae* since it was increased in patients, partly in household contacts but not in endemic controls (16). IL-10, on the other hand, is associated with suppression of Th1 cells in leprosy (17-19). Moreover, most lepromatous patients with high bacillary loads produce antibodies against the *M. leprae* specific phenolic glycolipid I (PGL-I) (20, 21), which are hardly detected in PB (22). Hence, sensitive tests that can simultaneously quantitate multiple analytes in one sample provide apt tools to characterize different clinical leprosy types. In particular, tests based on multicomponent host biomarker profiles that can identify *M. leprae* infected individuals (yet) without clinical symptoms of leprosy, will be useful for guidance of prophylactic treatment, thereby contributing to reduction of *M. leprae* transmission as well as prevention of disabilities.

Inherent to the situation in leprosy endemic areas is the absence of sophisticated laboratories. It is therefore imperative that new diagnostic tests are facilitated for application in the field. Up-converting phosphor lateral flow assays (UCP-LFAs) have previously shown to be robust, low-complexity assays, representing a field-friendly alternative for common laboratory-based ELISAs (23, 24), applicable for detection of multiple pathogens including food-borne pathogenic strains and potential biowarfare/bioterrorism agents (25-27). Field evaluation of UCP-LFAs for detection of IL-10, IP-10, CCL4 and anti-PGL-I IgM demonstrated high correlation with ELISAs using samples from cohorts of limited numbers of leprosy- or TB patients (28, 29).

In the current study UCP-LFAs were applied to a more extensive (five-fold) sample size compared to our previous studies, derived from a randomized BCG vaccination field trial in Bangladesh (30). Six test groups were included: MB patients, PB patients, healthy household contacts (HHC), HHC vaccinated with *Bacillus Calmette-Guérin* (BCG) (HHC&BCG), HHC who developed leprosy after BCG vaccination (new cases; NC) and endemic controls (EC) from the same area without known contact with leprosy patients. This extended cohort study allowed exploratory identification of biomarker profiles for *M. leprae* infection, leprosy disease per se, the type of leprosy and BCG vaccination as determined with UCP-LFAs for the above indicated targets.

Materials and methods

Study participants

Participants were recruited on a voluntary basis between January 2013 and December 2014 in leprosy endemic areas in Bangladesh as described previously (30). Leprosy was diagnosed based on clinical, bacteriological and histological observations and classified by skin smears according to Ridley and Jopling (14). Clinical and demographic data was

collected in a database. Participants were classified into six test groups; MB patients, PB patients, HHC, HHC&BCG, NC and EC. Control individuals from the same leprosy endemic area (EC) were examined for the absence of clinical signs and symptoms of leprosy and TB; staff of leprosy- or TB clinics were excluded.

Test group selection

A randomized sample selection was taken from 1110 participants (30). Individuals were randomly assigned for sample inclusion using the RAND formula (Excel 2010), aiming for a 50/50 male/female ratio and a 1:1:1 ratio of three age groups: 0-14, 15-29, and 30+ (Supplementary Table S1). In total 242 individuals were selected; MB patients (n=34), PB patients (n=45), HHC (n=54), HHC&BCG (n=50), EC (n=51) and NC (n=8; PB=7, MB=1). Patient characteristics are shown in Supplementary Table S2.

Leprosy prevalence

During this study the prevalence in the four districts (Nilphamari, Rongpur, Ponchagor and Thakurganch) was 0.82 per 10,000 with a new case detection rate of 0.98 per 10,000 (monthly report of Rural Health Program of 4 districts of Nilphamari, Bangladesh).

Whole blood assay (WBA)

Upon recruitment venous, heparinized blood (4 ml) was used directly in whole blood assays (WBA), using microtubes pre-coated with *M. leprae* whole cell sonicate (designated WCS), ML2478/ ML0840 recombinant proteins (designated Mlep) (16) or without antigen stimulus (designated Nil) (30). After 24 hour incubation at 37°C materials were frozen at -20°C, shipped on dry ice to the LUMC and stored at -80°C until analysis by ELISA or UCP-LFA (24).

PGL-I and *M. leprae* whole cell sonicate (WCS)

Synthesized disaccharide epitope (3,6-di-O-methyl-β-D-glucopyranosyl(1→4)2,3-di-O-methylrhamnopyranoside), similar to *M. leprae* specific PGL-I glycolipid, coupled to human serum albumin (synthetic PGL-I; designated ND-O-HSA) and *M. leprae* whole cell sonicate (WCS) generated with support from the NIH/NIAID Leprosy Contract N01-AI-25469 were obtained through the Biodefense and Emerging Infections Research Resources Repository (<http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx>)(47).

PGL-I ELISA

IgM antibodies against *M. leprae* PGL-I were detected as previously described (19).

Absorbance of horseradish peroxidase (HRP) was determined at a wavelength of 450 nm.

ELISA for IL-10, IP-10 and CCL4

IP-10 (851.870.015, Diaclone Research, Besancon, France), IL-10 (851.540.015, Diaclone Research, Besancon, France) and CCL4 (DY271-05, R&D systems, Minneapolis, USA) ELISA kits were used. ELISA testing was performed according to the manufacturer's protocol using coating antibody clones B-S10, B-C50 and #24006 and detection antibodies B-T10, BC-55 and BAF271 respectively for IL-10, IP-10 and CCL4. HRP absorbance was determined at wavelength of 450 nm.

UCP-LFA for IL-10, IP-10 and CCL4

UCP-LFAs for CCL4, IL-10 and IP-10 were prepared and performed as described previously (24, 28, 29). The same antibody pairs as used for ELISAs were applied, with the non-biotinylated variant of the detection antibodies (non-biotinylated CCL4: AF-271-NA). Briefly, mixtures of 100 ng cytokine-specific UCP reporter conjugate and diluted serum sample (1:4 for IL-10, 1:30 for IP-10 and 1:300 for CCL4) were incubated for 60 min on a thermoshaker at 37 °C and 900 rpm. The mixture was then applied to cytokine-specific LF strips (containing a Test line with an antibody complementary to the antibody on the UCP particles) and immunochromatography was allowed to continue until strips were dry. LF strips were scanned in a Packard FluoroCount microtiterplate reader adapted for measurement of the UCP label (980 nm IR excitation, 550 nm emission). Results are displayed as the ratio value between Test and Flow-Control signal based on relative fluorescence units (RFUs) measured at the respective lines (48). Ratio values were translated to concentration based on standard curves for each immunemarker. Lower limit of detection was 32 pg/ml for IL-10 and 316 pg/ml for IP-10 and CCL4.

To determine test positivity, similar wholeblood samples from a set of healthy, non-endemic control individuals (NEC) were analysed and UCP-LFA thresholds were calculated based on the average value of all NEC samples (Supplementary Table S3).

UCP-LFA for anti-PGL-I antibody

For detection of anti-PGL-I IgM antibodies, the same protocol as used for cytokine detection was applied utilizing 100-fold diluted serum and IgM-specific UCP conjugate (UCP^{algM}). Only unstimulated samples were analysed as the level of antibody levels does not change upon antigen stimulation. The threshold for positivity of 0.29 was determined by computing receiver operating characteristic (ROC) curves.

Ethics

This study was performed according to the Helsinki Declaration as described previously (30). The national Research Ethics Committee (Bangladesh Medical Research Council) has approved the study protocol (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.

ROC curves

Graphpad Prism version 6.02 for Windows (GraphPad Software, San Diego CA, USA www.graphpad.com) was used to plot ROC curves and calculate the area under curve (AUC); for IP-10, IL-10, and CCL4 the concentrations (pg/ml) were applied, whereas for anti-PGL-I IgM the OD₄₅₀ corrected for background (ELISA) and ratio value (UCP-LFAs) was used.

Statistical analysis

Differences in cytokine or antibody levels between test groups, as determined with UCP-LFA, were analysed with the One-way ANOVA for non-parametric distribution (Kruskall-Wallis) and Dunn's correction for multiple testing using GraphPad Prism. For IP-10, IL-10 and CCL4 the concentrations (pg/ml) and for anti-PGL-I IgM the UCP-LFA ratio values were utilized. The statistical significance level used was $p \leq 0.05$.

Results

Performance of the UCP-LFA versus ELISA

Whole blood samples (n=726) from all individuals were analysed using ELISA, as well as the field-friendly UCP-LFAs for IL-10, IP-10, CCL4 and anti-PGL-I antibodies. Comparison of UCP-LFA and ELISA results demonstrated significant correlation for all four biomarkers ($p < 0.0001$), confirming earlier observations (28, 29).

The diagnostic performance of the UCP-LFA in comparison to ELISA was further assessed through AUCs for the two most distinct phenotypes: MB patients (n=34) and EC (n=51). IL-10, IP-10 and CCL4 levels were determined in Nil, WCS and Mlep samples, as well as anti-PGL-I IgM levels (Figure 1). The IL-10 and IP-10 UCP-LFAs outperformed the corresponding ELISAs, whereas the CCL4 and anti-PGL-I IgM tests performed equally. For discrimination of MB patients from EC, the proposed diagnostic field-tool UCP-LFA provides an equally well or even better alternative for the conventional ELISAs.

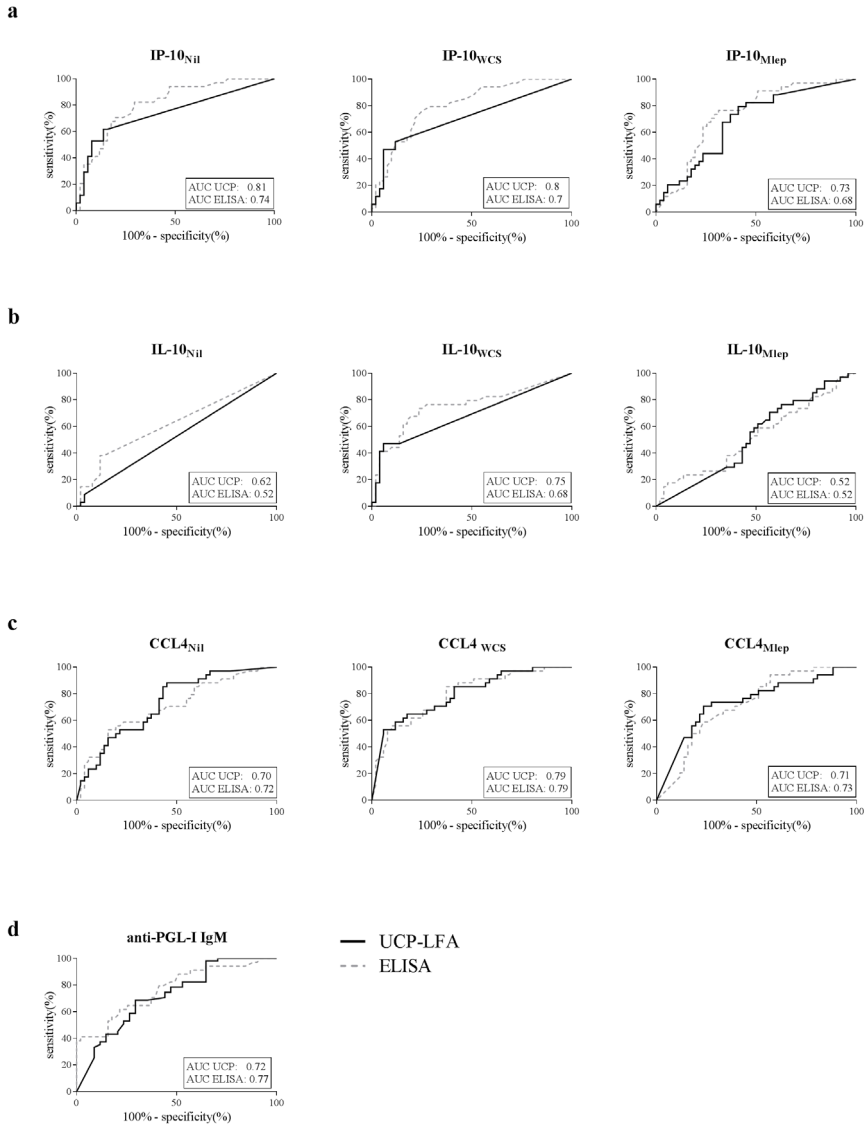


Figure 1: Discriminatory capacity of ELISA and UCP-LFA. To compare the ability of ELISA (dotted line) and UCP-LFA (solid line) to discriminate between individuals with or without disease ROC curves were computed using data of MB patients and EC. Areas under the curve (AUCs) were compared for all 10 conditions tested, shown in the lower right corner of each graph. (a) ROC curves for IP-10 stimulated and unstimulated samples based on concentration in pg/ml, showing an improved AUC for the UCP-LFA for IP-10_{Nil} and IP-10_{WCS}. (b) ROC curves for IL-10 stimulated and unstimulated samples based on concentration in pg/ml, showing an improved (IL-10_{Nil}) or equal AUC for UCP-LFA. (c) ROC curves for CCL4 stimulated and unstimulated samples based on concentration in pg/ml, showing comparable values for UCP-LFA and ELISA. (d) ROC curves for anti-PGL-1 IgM in unstimulated samples based on ratio, showing comparable AUCs for ELISA and UCP-LFA.

***M. leprae* specific responses based on single analyte UCP-LFA measurements**

In order to put the quantitative test results obtained with the four single UCP-LFAs in the context of their biomarker potential, we assessed each analyte/ stimulus combination by comparing median group levels. As indicated by the AUCs in Figure 1, MB patients can be distinguished from EC based on IP-10 and CCL4 (irrespective of stimulus), anti-PGL-I IgM and IL-10_{WCS}, therefore also showing significantly different median levels (Figure 2, Table 1). Moreover, median levels of anti-PGL-I IgM and IP-10_{Nil} differed between MB patients and (BCG-vaccinated) HHC, whereas median levels of IP-10_{WCS} and CCL4_{WCS} only distinguished the non-vaccinated HHC from MB patients.

BCG vaccination therefore affects the immune response in HHC, as reflected by the significant difference in IP-10_{WCS} levels between HHC and HHC&BCG ($p=0.018$) (Figure 2B). Furthermore, IL-10_{WCS} levels differed between HHC and EC, while median levels of IP-10_{WCS} and CCL4_{WCS} differed between HHC&BCG and EC as well. PB patients and EC showed significantly different median CCL4_{Nil} and CCL4_{WCS} levels (Figure 2C), as well as borderline significant different levels for IL-10_{WCS} ($p=0.07$) and IP-10_{WCS} ($p=0.06$).

The ability of each analyte/ stimulus combination to distinguish between two groups is summarized in Table 1, thereby reviewing the biomarker potential of the four individual host immune markers. Remarkably, the levels of IP-10 ($p<0.0001$), IL-10 ($p=0.003$) and CCL4 ($p<0.0001$) in WCS stimulated samples were more significantly different for MB and EC than anti-PGL-I IgM levels ($p=0.0042$). Moreover, anti-PGL-I IgM levels could not be used to discriminate PB patients or (BCG-vaccinated) HHC from EC, which clearly demonstrates the added value of IP-10, IL-10 and CCL4 in leprosy diagnostics.

Table 1: Discriminatory biomarkers with potential for leprosy diagnostics

	MB vs HHC	MB vs HHC&BCG	MB vs EC	PB vs EC	HHC vs EC	HHC&BCG vs EC	HHC&BCG vs HHC
anti-PGL-I IgM	++	++	++	-	-	-	-
IP-10	++	++	++	-	-	-	-
IP-10 WCS	++++	-	++++	-	-	++	+
IP-10 <i>M.leprae</i> proteins	-	-	+	-	-	-	-
IL-10	-	-	-	-	-	-	-
IL-10 WCS	-	-	+++	-	++	+	-
IL-10 <i>M.leprae</i> proteins	-	-	-	-	-	-	-
CCL4	-	-	+	+	-	-	-
CCL4 WCS	++	-	++++	+	-	++	-
CCL4 <i>M.leprae</i> proteins	-	-	+	-	-	-	-

Differences in IP-10, IL-10, CCL4 and anti-PGL-I IgM levels between various test groups detected by UCP-LFA are provided. Each row represents one of the 10 different analyte/ stimulus combinations measured. Each column shows the potential to distinguish the test groups indicated, only displaying the groups for which significant differences were observed: - $p \geq 0.05$ indicates inability to distinguish test groups, + : $p \leq 0.05$, ++ : $p \leq 0.01$, +++ : $p \leq 0.001$, ++++ : $p \leq 0.0001$ indicating increasing capacity to distinguish test groups. Using one or multiple analyte/ stimulus combination MB patients could be distinguished from (BCG-vaccinated) HHC and EC, whereas PB patients and BCG-vaccinated HHC could be distinguished from EC.

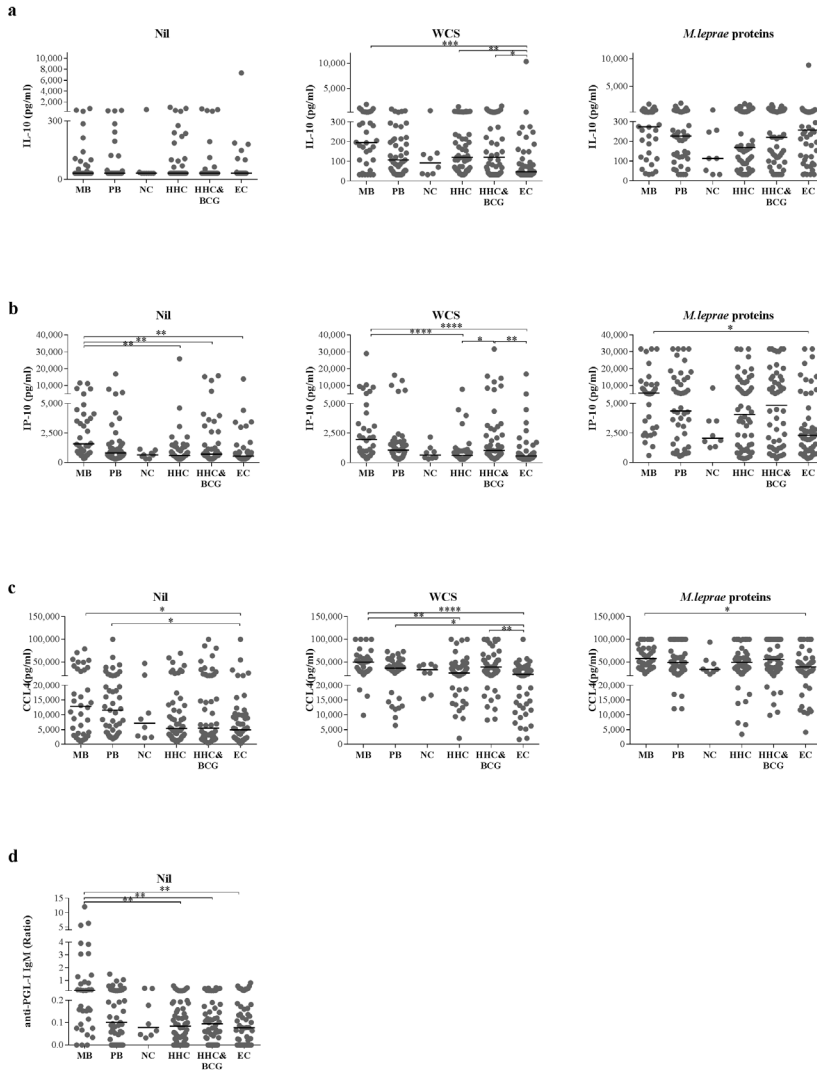


Figure 2: Identification of *M. leprae* specific IL-10, IP-10, CCL4 and anti-PGL-I IgM antibodies by UCP-LFA. (a) IL-10 concentrations (pg/ml) measured per group per stimulus show that MB patients, HHC and BCG-vaccinated HHC significantly differ from EC upon WCS stimulation. (b) IP-10 concentrations (pg/ml) measured per group per stimulus show that MB patients significantly differ from EC in both stimulated and unstimulated samples, from HHC in unstimulated and WCS stimulated samples and from BCG vaccinated HHC in unstimulated samples. BCG vaccinated HHC significantly differ from HHC and EC upon WCS stimulation. (c) CCL4 concentrations (pg/ml) measured per group, per stimulus show that MB patients significantly differ from EC in both stimulated and unstimulated samples and from HHC in WCS stimulated samples. PB patients significantly differ from EC in unstimulated and WCS stimulated samples and BCG vaccinated HHC significantly differ from EC in WCS stimulated samples. (d) anti-PGL-I IgM ratio measured per groups shows that MB patients have significantly higher levels of anti-PGL-I IgM compared to HHC, BCG vaccinated HHC and EC. P-values: *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

Biomarker signatures to specify *M. leprae* infection, leprosy or disease classification

Diagnostic tests that allow detection of *M. leprae* infection, leprosy per se and leprosy classification would be of great benefit to the general healthcare in leprosy endemic areas. The four host immune markers allowed distinction between two groups (Table 1). However, to distinguish *M. leprae* infected from non-infected individuals or patients from healthy contacts, we compared host immune markers for multiple groups (Figure 3).

First, in order to combine immune markers into multicomponent host biomarker profiles, positive UCP-LFA results for each analyte/ stimulus combination were collectively specified (Figure 3; Supplementary Table S3). Second, analyte/ stimulus combinations were selected such that they optimally distinguished individuals with a specified disease- or infection state (Figure 3), considering all HHC as *M. leprae* infected. This resulted in three specific profiles:

I. To indicate *M. leprae* infection we selected single test results obtained with $IP-10_{Mlep'}$, $CCL4_{WCS}$ and $IL-10_{WCS}$ UCP-LFAs as these analyte/ stimulus combinations individually showed the least positive test results for EC compared to the *M. leprae* infected test groups (MB, PB, HHC and HHC&BCG) (Figure 3). The combination of $IP-10_{Mlep'}$, $CCL4_{WCS}$ and $IL-10_{WCS}$ indeed was more frequently positive for MB/PB patients and (BCG-vaccinated) HHC than EC (Figure 4A). Moreover, AUCs confirmed discrimination between non-infected and *M. leprae* infected test groups based on this multicomponent host immune profile (AUCs: 0.84 (MB vs. EC), 0.75 (PB vs. EC), 0.7 (HHC vs. EC) and 0.71 (HHC&BCG vs. EC) (Supplementary table S4A).

II. To detect leprosy patients from healthy, though possibly *M. leprae* infected individuals, $CCL4_{WCS}$ and $IP-10_{WCS}$ were selected as immune markers since these single tests were more frequently positive in patients (MB and PB) compared to contacts (HCC and HCC&BCG) and are therefore associated with pathogenic immunity to *M. leprae*. The combination of $CCL4_{WCS}$ and $IP-10_{WCS}$ indeed demonstrated a positive test result more often in patients than in HHC or EC (Figure 4B), whereas the related AUCs were ≥ 0.66 thus confirming leprosy disease-specificity (Supplementary table S4B).

III. For classification of leprosy a signature consisting of anti-PGL-I IgM, $IL-10_{WCS}$ and $IP-10_{Nil}$ was applied, as each of these markers individually showed more positive test results in MB patients compared to PB patients (Figure 3). This profile proved to be specific for MB patients (Figure 4C) and thereby allowed the differentiation of MB and PB patients (AUC=0.73, Supplementary table S4C).

a

	Groups to differentiate	Potential Profile
Infection	MB, PB, HHC, HHC&BCG vs. EC	IP10 _{Mlep} , CCL4 _{WCS} , IL10 _{WCS}
Disease per se	MB, PB vs. HHC, HHC&BCG, EC	IP10 _{WCS} , CCL4 _{WCS}
Disease classification	MB vs. PB	anti-PGL-I IgM, IL10 _{WCS} , IP10 _{SI}

b

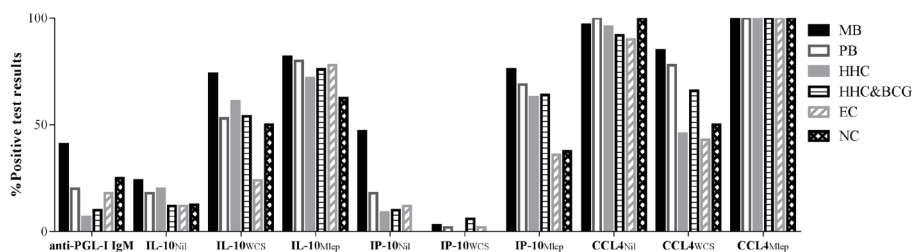


Figure 3: Positive test results per analyte/ stimulus combination used to construct potential biomarker profiles. The groups that should be differentiated to indicate *M. leprae* infection, disease per se and disease classification are shown. The potential profiles indicated are based on the percentage of positive individuals of these particular groups. The cut-off for positivity was based on values for NEC (Supplementary Table S3) per analyte/ stimulus combination the percentage of individuals with a positive test result per group is shown. Based on these data the optimal analyte/ stimulus combination to differentiate either infected from non-infected groups, patients and non-patients groups or MB and PB patients were selected to construct the potential profiles described.

Ideally only one multicomponent host biomarker profile for diagnosis of *M. leprae* infection, leprosy per se and leprosy classification would be more suitable for field-use. In this exploratory study, a 4 marker profile of IL-10_{WCS}, IP-10_{Mlep}, CCL4_{WCS} and anti-PGL-I IgM was selected for this purpose, enabling distinction of infected and non-infected individuals by IL-10_{WCS}, IP-10_{Mlep} and CCL4_{WCS}. MB and PB patients from HHC and EC by CCL4_{WCS} and MB from PB patients by anti-PGL-I IgM and IL-10_{WCS} (Figure 4D; Supplementary Table S4D). However, to distinguish MB from PB patients or PB patients from HHC profile III for leprosy classification showed a higher AUC compared to the 4 marker profile (0.73 vs. 0.65 and 0.66 vs. 0.62 respectively, Supplementary Table S4). These data indicate the importance of distinct phase-specific profiles, the application of which will depend on the nature of the diagnosis to be made.

Nonetheless, application of the 4 marker profile demonstrated the influence of multicomponent host biomarker profiles on test accuracy, showing increased AUCs compared to individual markers (Supplementary Figure S1). The added value of using various analytes indicates the potential of multicomponent host biomarker profiles for leprosy diagnostics to detect *M. leprae* infection, leprosy disease or disease classification.

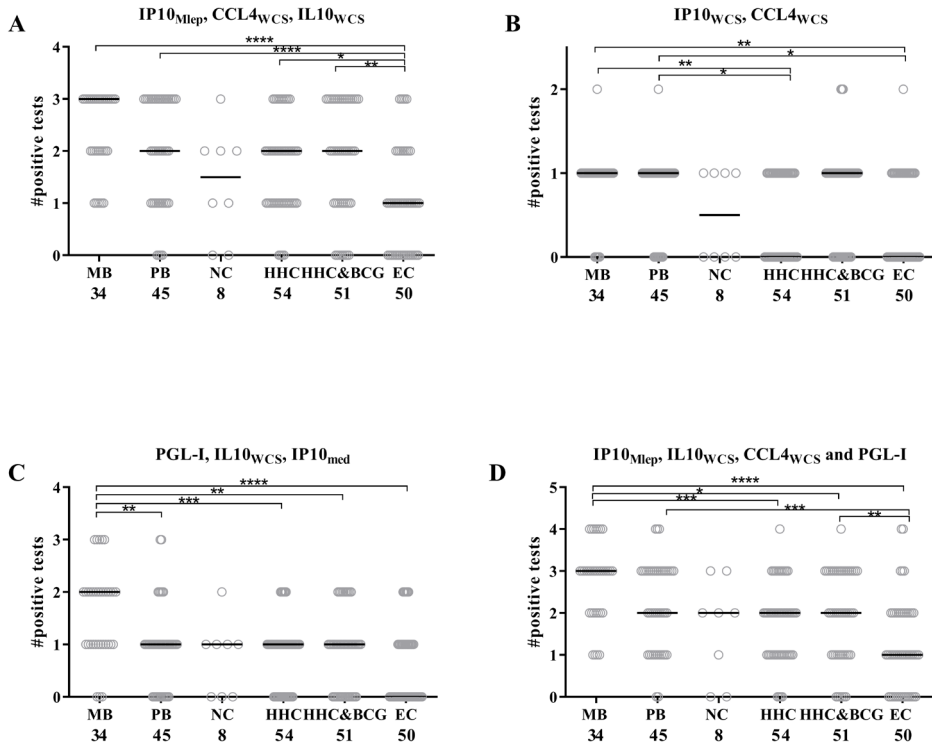


Figure 4: Potential of biomarker profiles to indicate *M. leprae* infection, disease per se and disease classification. The amount of positive test results per group is shown. (a) IP-10_{Mlep'}, CCL4_{WCS} and IL-10_{WCS} significantly differed in MB/PB patients and (BCG-vaccinated) HHC from EC, showing more positive test results in the groups that are exposed to *M. leprae* and thereby indicating *M. leprae* infection. (b) CCL4_{WCS} and IP-10_{WCS} enabled the distinction between patients and HHC, thereby indicating the pathogenic immune responses to *M. leprae* in patients. (c) Anti-PGL-I IgM, IL-10_{WCS} and IP-10_{Nil} showed more positive test results in MB patients thereby enabling the distinction between MB and PB patients. (d) A four marker profile of IL-10_{WCS}, IP-10_{Mlep'}, CCL4_{WCS} and anti-PGL-I IgM shows the majority of significant differences observed in A, B and C. P-values: *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

Discussion

The obvious incessant transmission of *M. leprae* has brought about increased focus in leprosy research on discovery of biomarkers to improve diagnosis. Nevertheless, thus far only few biomarkers for leprosy are recommended by expert panels (11). Consequently, there is a growing need for new and sensitive diagnostic tools based on specific biomarkers which should, ideally, allow straightforward translation into field-friendly tests.

In this exploratory study, we aimed to provide several multicomponent host immune-biomarker profiles which distinguish between distinct stages of *M. leprae* infection.

In this process we also emphasized the challenges that need to be tackled to allow application of these biomarkers in the field. As high-tech laboratories are often lacking in leprosy endemic areas, we examined the diagnostic potential of earlier developed field-friendly UCP-LFAs for detection of anti-PGL-I IgM antibodies and cyto/chemokines IP-10, IL-10 and CCL4 (24, 28, 31), in an extensive cohort in Bangladesh.

We demonstrated the biomarker potential of IP-10, IL-10, CCL4 and anti-PGL-I IgM measured by UCP-LFAs in whole blood, either in response to *M. leprae* specific stimuli or without stimulus. Moreover, multicomponent host biomarker profiles including selected analyte/ stimulus combinations could indicate *M. leprae* infection, leprosy per se or be used for classification of leprosy subtypes. A biomarker profile of IP-10_{Mlep'}, CCL4_{WCS} and IL-10_{WCS} was highly indicative of *M. leprae* infection, consistent with our previous finding that the IP-10 response to *M. leprae* specific proteins indicates exposure to *M. leprae* (16,32).

Leprosy per se, on the other hand, was indicated by CCL4_{WCS} and IP-10_{WCS'}, showing the potential to identify pathogenic immunity against *M. leprae* and confirming earlier observations on CCL4 (16). As current diagnostic assays for leprosy are antibody-based and only facilitate the diagnosis of MB cases (33-35), inclusion of the host immune markers CCL4 and IP-10 in the profile shows promise for diagnosis of PB patients and indicates the importance of measuring cellular markers simultaneously with humoral markers.

For leprosy classification, the combination of anti-PGL-I IgM, IL-10_{WCS} and IP-10_{Nil} was indicative for MB patients, enabling the distinction between MB and PB patients. Although IL-10 and particularly anti-PGL-I IgM have been identified as characteristic markers for MB leprosy (36, 37), we also identified IP-10 as a, seemingly counterintuitive, host immune marker for patients at this side of the spectrum who usually display decreased pro-inflammatory immunity. However, since T-cells are not the exclusive source of IP-10 (38), IP-10 may still be produced in MB patients by monocytes and neutrophils (39), as described for HIV-infected TB patients (40).

To detect *M. leprae* infection, leprosy per se, as well as leprosy classification simultaneously with only one biomarker profile, IL-10_{WCS'}, IP-10_{Mlep'}, CCL4_{WCS} and anti-PGL-I IgM demonstrated the most optimal 4 marker profile performance. However, it performed less optimal for the distinct stages of *M. leprae* infection than the phase-specific profiles. Other cyto-/ chemokines to identify pathogenic immunity to *M. leprae* (e.g. MCP-1 and IL-1 β (16)), leprosy classification (e.g. CCL17 and CCL18 (41)) or general mycobacterial infection (EN-RAGE (42, 43)) could therefore be included to achieve more optimal diagnostic accuracy (44) as distinct phase-specific profiles. In a multiplex UCP-LFA format multicomponent host immune biomarker profiles can be measured in one

single test. This format therefore provides a field-friendly diagnostic tool, facilitating the diagnosis of leprosy based on biomarker signatures.

Of note is the observation that CCL4 levels in response to *M. leprae* WCS were elevated for HHC who received BCG vaccination compared to those who did not. Thus, BCG vaccination may also cause increased pro-inflammatory immune responses which renders contacts more prone to development of over-reactive, pathogenic immunity to *M. leprae*. Indeed, in a recent vaccination study an unexpectedly high proportion of HHC presented with PB leprosy after BCG vaccination supporting this idea (45). In this respect, this vaccination study also shows the importance of immunomonitoring individuals at high risk to identify and treat patients at an early stage. In addition, since BCG vaccination or boost is a well-accepted prophylaxis against leprosy in contacts of newly diagnosed patients (46), it is relevant to distinguish BCG-induced immunity in healthy contacts from early stage leprosy in these individuals. To efficiently monitor contacts for this purpose, the different stages of infection and disease of leprosy should be covered in diagnostics tools. Through simultaneous measurement of all analytes of interest on a single lateral flow strip, this format allows assessment of multicomponent host biomarker profiles using a unique field-friendly technology (24, 29, 31). Thereby, the UCP-LFA format not only provides diagnostic tools for leprosy but similarly holds promise for TB diagnosis (28) and immunomonitoring of other chronic diseases (31).

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Competing Financial Interests

The authors declare that they have no conflict of interest.

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Supplementary material**Tables****Supplementary Table S1: Test group selection**

Test group	Gender	Age group		
		0-14	15-29	30+
EC	F	10	10	10
EC	M	10	10	10
HHC&BCG	F	10	10	10
HHC&BCG	M	10	10	10
HHC	F	10	10	10
HHC	M	10	10	10
MB	F	2	1	4
MB	M	0	4	20
PB	F	2	8	11
PB	M	0	15	15

Samples were randomly selected using a 50/50 ratio of males and females and a 1:1:1 ratio of three age groups (0-14 yrs; 15-29 yrs; 30 yrs or older) within each group. For the MB and PB patient groups samples could not be evenly distributed for age and gender, due to the limited number of samples present.

Supplementary Table S2: Patient characteristics according to Ridley-Jopling classification. The bacterial index (BI) is indicated for all MB patients.

	BI	Classification		Classification
MB1	5	BL	PB1	TT
MB2	5	LL	PB2	TT
MB3	4	BL	PB3	TT
MB4	4	BL	PB4	TT
MB5	4	LL	PB5	BT
MB6	4	LL	PB6	BT
MB7	3	BL	PB7	BT
MB8	3	BT	PB8	BT
MB9	1	BT	PB9	BT
MB10	0	BL	PB10	BT
MB11	0	BT	PB11	BT
MB12	0	BT	PB12	BT
MB13	0	BT	PB13	BT
MB14	0	BT	PB14	BT
MB15	0	BT	PB15	BT
MB16	0	BT	PB16	BT
MB17	0	BT	PB17	BT
MB18	0	BT	PB18	BT
MB19	0	BT	PB19	BT
MB20	0	BT	PB20	BT
MB21	0	BT	PB21	BT
MB22	0	BT	PB22	BT
MB23	0	BT	PB23	BT
MB24	0	BT	PB24	BT
MB25	0	BT	PB25	BT
MB26	0	BT	PB26	BT
MB27	0	BT	PB27	BT
MB28	0	BT	PB28	BT
MB29	0	BT	PB29	BT
MB30	0	BT	PB30	BT
MB31	0	BT	PB31	BT
MB32	0	BT	PB32	BT
MB33	0	BT	PB33	BT
MB34	0	BT	PB34	BT
			PB35	BT
			PB36	BT
			PB37	BT
			PB38	BT
			PB39	BT
			PB40	BT
			PB41	BT
			PB42	BT
			PB43	BT
			PB44	BT
			PB45	BT

Supplementary Table S3: Cut-off values for IL-10, IP-10 and CCL4

	IL-10 Nil	IL-10 WCS	IL-10 Mlep	IP-10 Nil	IP-10 WCS	IP-10 Mlep	CCL4 Nil	CCL4 WCS	CCL4 Mlep
NEC1	32	32	32	316	379	450	316	11621	1379
NEC2	32	32	32	316	1935	519	316	12732	566
NEC3	32	32	32	379	14771	790	316	8374	460
NEC4	32	32	32	1481	992	2015	316	2838	316
Threefold Average	96	96	96	1869	13557	2830	948	26673	680
Cut-off	100	100	100	1900	14000	2800	950	27000	700

IP-10, IL-10 and CCL4 concentrations (pg/ml) determined by UCP-LFA of 24h whole blood cultures of non-endemic healthy control individuals (NEC; n=4) without stimulus (Nil), in response to *M. leprae* whole cell sonicate (WCS) or *M. leprae* recombinant proteins [ML2478 and ML0840(Mlep)]. The cut-off for a positive test result was set as three times the average value of the four NEC in unstimulated samples, WCS and Mlep stimulated samples.

Supplementary Table S4: Areas under the curve for each biomarker profile

a

M. leprae Infection (IP10_{Mlep}, CCL4_{WCS}, IL10_{WCS})

AUC	MB	PB	NC	HHC	HHC&BCG	EC
MB		ns	0.76	0.7	0.63	0.84
PB	ns		ns	ns	ns	0.75
NC	0.76	ns		ns	ns	ns
HHC	0.7	ns	ns		ns	0.7
HHC&BCG	0.63	ns	ns	ns		0.71
EC	0.84	0.75	ns	0.7	0.71	

b

Leprosy (IP10_{WCS}, CCL4_{WCS})

AUC	MB	PB	NC	HHC	HHC&BCG	EC
MB		ns	ns	0.7	ns	0.71
PB	ns		ns	0.66	ns	0.67
NC	ns	ns		ns	ns	ns
HHC	0.7	0.66	ns		0.61	ns
HHC&BCG	ns	ns	ns	0.61		0.62
EC	0.71	0.67	ns	ns	0.62	

c

Leprosy Classification (PGL-I IL10_{WCS}, IP10_{med})

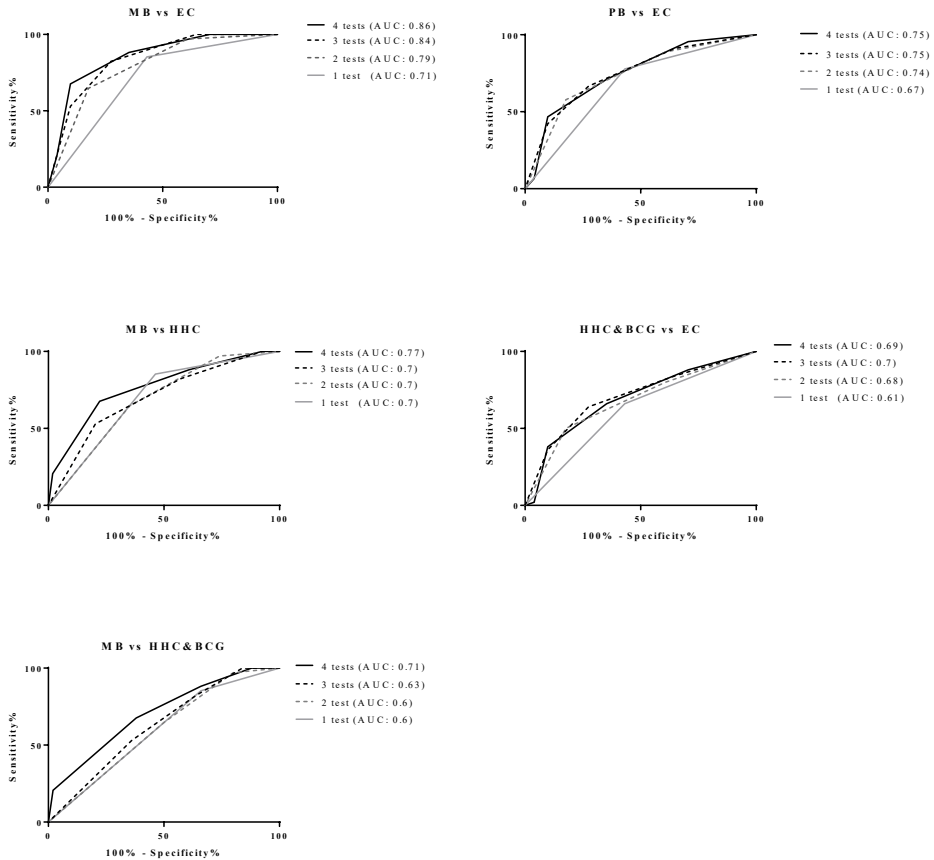
AUC	MB	PB	NC	HHC	HHC&BCG	EC
MB		0.73	0.77	0.77	0.73	0.82
PB	0.73		ns	ns	ns	0.64
NC	0.77	ns		ns	ns	ns
HHC	0.77	ns	ns		ns	0.61
HHC&BCG	0.73	ns	ns	ns		0.63
EC	0.82	0.64	ns	0.61	0.63	

d

AUC	Four marker profile (IP10 _{Mlep} , IL10 _{WCS} , CCL4 _{WCS} , PGL-I)					
	MB	PB	NC	HHC	HHC&BCG	EC
MB		0.65	0.77	0.77	0.7	0.86
PB	0.65		ns	0.62	ns	0.75
NC	0.77	ns		ns	ns	ns
HHC	0.77	0.62	ns		ns	0.67
HHC&BCG	0.7	ns	ns	ns		0.69
EC	0.86	0.75	ns	0.67	0.69	

Areas under the curve (AUC) were determined using Graphpad Prism version 6.02 for Windows (GraphPad Software, San Diego CA, USA www.graphpad.com) for M. leprae infection (a), leprosy (b), leprosy classification (c) and the four marker profile (d), displaying only AUCs that showed a significant p-value (ns=non-significant). AUCs were determined for each combination of groups. (MB: multibacillary patients; PB: paucibacillary patients; NC=new cases who developed leprosy after BCG vaccination; HHC: healthy household contacts; HHC&BCG: BCG-vaccinated HHC; EC: endemic controls). (a) Combined test results for IP-10_{Mlep}, CCL4_{WCS} and IL10_{WCS}; (b) Combined test results for IP-10_{WCS} and CCL4_{WCS}; (c) Combined test results for anti-PGL-I IgM IP-10_{Med} and IL10_{WCS}; (d) Four marker profile of IL-10_{WCS}, IP-10_{Mlep}, CCL4_{WCS} and anti-PGL-I IgM, showing the potential of this profile to indicate M. leprae infection, leprosy per se and leprosy classification.

Figures



Supplementary Figure S1: Influence of multicomponent host biomarker profiles on test accuracy. ROC curves and the corresponding areas under the curve (AUC) using UCP-LFAs for 1, 2, 3 or 4 marker profiles (CCL4_{WCS}, IP-10_{Mlep}, IL-10_{WCS} and anti-PGL-I IgM). Only the groups that showed significant differences in test results based on this 4 marker profile are shown. The AUC for the 4 marker profile test was increased compared to the AUC for 1 marker profile, showing improved diagnostic accuracy (44). 1 marker profile = CCL4_{WCS} (the most sensitive test condition for detecting patients); 2 marker profile = CCL4_{WCS} and IP-10_{Mlep}; 3 marker profile = CCL4_{WCS}, IP-10_{Mlep} and IL-10_{WCS}; 4 marker profile = CCL4_{WCS}, IP-10_{Mlep}, IL-10_{WCS} and anti-PGL-I IgM

