Complement in neuroinflammation
Bahia El Idrissi, N.

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Maria Rita (70, hier met kleinzoon) is genezen van lepra. Door de ziekte heeft ze geen gevoel meer in haar handen, maar wat lepra precies is, vindt ze moeilijk te omschrijven. Haar vrienden heeft ze niets verteld.
Complement Activation In Leprosy: A Retrospective Study
Shows Elevated Circulating Terminal Complement Complex In
Reactional Leprosy

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Summary

*Mycobacterium leprae* (*M. leprae*) infection gives rise to the immunologically and histopathologically classified spectrum of leprosy. At present several tools for the stratification of patients are based on acquired immunity markers. However, the role of innate immunity, particularly the complement system, is largely unexplored. The present retrospective study was undertaken to explore whether the systemic levels of complement activation components and regulators can stratify leprosy patients, particularly in reference to the reactional state of the disease.

Serum samples from two cohorts were analyzed. The cohort from Bangladesh included multibacillary (MB) patients with (*n*=12) or without (*n*=46) reaction (R) at intake and endemic controls (*n*=20). The cohort from Ethiopia included paucibacillary (PB; *n*= 7) and MB (*n*= 23) patients without reaction and MB (*n*=15) patients with reaction.

The results showed that the activation products terminal complement complex (TCC) (*p* ≤0.01), C4d (*p* ≤0.05) and iC3b (*p* ≤0.05) were specifically elevated in Bangladeshi patients with reaction at intake compared to endemic controls. In addition, levels of the regulator Clusterin (*p* ≤0.001 without R; *p*<0.05 with R) were also elevated in MB patients irrespective of a reaction. Similar analysis of the Ethiopian cohort confirmed that irrespective of a reaction, serum TCC levels were significantly increased in patients with reactions compared to patients without reactions (*p* ≤0.05).

Our findings suggests that serum TCC levels may prove to be a valuable tool in diagnosing patients at risk of developing reactions.

**Keywords.** Complement, Leprosy, Reactions
Complement in serum of leprosy patients

Introduction

Leprosy is a chronic debilitating disease caused by *Mycobacterium leprae* (*M. lepra*e), an obligate intracellular parasite with tropism for macrophages and Schwann cells. Effective treatment with multi drug therapy (MDT) has reduced the prevalence around the world, although new case detection has remained stable at around 200,000 per annum. Nevertheless, the disease is still endemic in several parts of the world, including parts of Bangladesh, Ethiopia, Brazil and Nepal (http://www.who.int/mediacentre/factsheets/).

Although the infection is asymptomatic for a prolonged period, the disease eventually presents with nerve damage, which is the major cause of patients’ disability and deformities. On the basis of clinical, histopathological and immunological criteria leprosy is recognized as a spectral disease [1]. The inter-individual variability of acquired immune response to *M. lepra*e and its antigens dictates the clinical, histopathological and immunological spectrum of leprosy [2]. As a result, it is now well established that the leprosy spectrum fluctuates between two poles: tuberculoid leprosy (TT) with a strong *M. lepra*e specific T-helper 1 (Th1) cell-mediated immunity associated with negligible bacillary load, and lepromatous leprosy (LL) with a strong antibody response to *M. lepra*e, phenolic glycolipid-1 (PGL-I) accompanied by complete absence of *M. lepra*e specific Th1 response. The polar LL patients show high bacillary load in relation to T-cell anergy to *M. lepra*e not only in the lesions, but also in other tissues in a disseminated manner. Between the two polar forms, the majority of patients belong to immunologically unstable borderline categories that are classified as borderline tuberculoid (BT), mid-borderline (BB) and borderline lepromatous (BL) with variable degree of bacillary load with an increasing trend from
BT towards BL/LL. On the basis of bacillary indices of the lesions, LL together with BB and BL are collectively grouped as multibacillary (MB), whereas the BT and TT forms are grouped as paucibacillary (PB) [1].

MDT is effective in curing leprosy to a large extent as, in the majority of MB patients, the dead bacilli are cleared steadily. However, a considerable number of patients show a changing clinical and immunohistopathological status in the course of the disease as well as during and post-treatment either as a result of treatment or as a natural evolution of the disease. Such episodic disease status is widely recognized as reactional state, resulting in clinical and pathological alterations accompanied by exacerbation of tissue (particularly) nerve damage [3,4].

The change in immunological response results in one of two types of reactions: i) reversal reaction (RR, also called type 1 reaction) primarily encountered with patients with BT and BL category or ii) erythema nodosum leprosum (ENL, also called type 2 reaction), especially in the borderline and lepromatous region of the spectrum. Both these episodic reactions appear to be due to the persistence of antigens like lipoarabinomannan (LAM) or PGL-I [5]. Interestingly, the localization of persisting \( M. \text{leprae} \) antigens in leprosy patients with nerve damage was also demonstrated by Shetty VP et al [6].

In general, RR or type 1 reactions are due to the polarization of \( M. \text{leprae} \) specific T-cell activity with the cytokine characteristic of Th1 profile [7,8], and usually occur early in the course of treatment and result in an increased cellular immune response to mycobacterial antigens.
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On the other hand, ENL or type 2 reactions are due to the increased T-cell dependent antibody production (specifically to *M. leprae* antigens or to treatment drugs) resulting in immune complex formation and complement activation [9-13].

Accordingly, efforts to establish sets of biomarkers for laboratory diagnosis and prognosis of leprosy spectrum and leprosy reactions has concentrated on acquired immunity-based cytokine and antibody profiling of the patients [14-17]. In contrast, biomarkers of innate immunity in leprosy pathomechanism have received little attention. Indeed, studies linking biomarkers of innate immunity in regards to the role of complement in leprosy disease state, particularly to the reactional state, are rare in literature.

The complement system is an integral part of innate immunity, comprising more than 30 serum and cell-associated proteins and plays an important role in host immunity and inflammation[18]. Its activation and regulation occurs via multiple pathways. Complement activation can be triggered by antigen-antibody complexes (classical pathway), foreign surfaces (alternative pathway) or bacterial sugars (lectin pathway). Regardless of the trigger, activation results in the cleavage of C3, generating the anaphylatoxin C3a and the opsonin C3b, the latter of which binds pathogens thereby mediating clearance by phagocytes. C3b is also required for the formation of the C5 convertase, to cleave C5 into C5a and C5b. C5b initiates activation of the terminal pathway, which results in the formation of the membrane attack complex (MAC) comprising a heteropolymer of C5b, C6, C7, C8 and multiple C9 molecules that forms transmembrane channels in the target cell, resulting in lysis.

Deposits of MAC or the soluble terminal complement complex (TCC) were demonstrated in association with damaged nerve in leprosy patients[19]. In this
context, we recently showed the association between persistence of the *M. leprae* antigen LAM and complement activation in the damaged nerve. This finding strongly suggests that complement activation plays a causal role in nerve damage in leprosy. Since nerve damage is prominent in reactional episodes it is rational to speculate that complement activation and formation of TCC or MAC can be valuable in diagnosing leprosy patients without reaction from those with reactions. There are only a few studies on serology of complement activation in leprosy reported in the literature [20-23]. Previous serological studies showed 1) reduced complement hemolytic activity and reduced levels of C4 in LL patients suggesting consumption of complement in the circulation via activation of the classical or lectin pathway [23,24]; 2) an increased C1q binding activity (the initiator of classical pathway activation) in LL patients with ENL reactions suggesting involvement of the classical pathway in these patients, and increased C3d levels in 70% of patients with ENL and 18% of patients with uncomplicated LL. Such findings suggested that C3d could be a marker of complement activation which may be of some practical interest in the early diagnosis of reactional state [12]. Thus, taking the literature findings with our own, we decided to reinvestigate the use of serological markers of complement activation in leprosy reaction, which is the major cause of leprosy-associated nerve damage.

In this retrospective study we used a state-of-the-art multiplex assay set (Meso Scale) for the quantification of complement activation products and regulators in serum of patients and controls to test whether the quantification of complement products might be valuable in diagnosing leprosy patients without reaction from those with reactions.
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Materials and methods

Serum samples

Serum samples were obtained from stored serum bank collected for a prospective cohort study in two leprosy endemic regions, Bangladesh and Ethiopia [25]. Ethical approval of the study-protocol was obtained through appropriate ethics committees and written informed consent was obtained from the patients before the samples were collected.

These samples were transported to the LUMC, Leiden, Netherlands for storage. It should be stated with clarity that the serum samples were collected and stored under conditions found suitable for another study [25]; complement assay require special addition of EDTA which was not included in the collection of these sera. We have refrained from dealing with the types of reactions, as the patients’ samples are obtained from archive of stored specimens and not with the aim of longitudinal study. Serum samples were stored at -20°C or below and shipped frozen on dry ice.

Briefly, in this study we used biobanked serum samples of untreated leprosy patients without clinical reactions (and before MDT) and newly diagnosed patients who visited clinics with reactions and sera were collected in a similar fashion and before starting treatment. In addition, we had the opportunity to follow up four of these patients with reaction and also collected samples after the completion of treatment. Serum samples from the Bangladesh cohort consisted of MB patients with (n=12) or without (n=46) reactions at intake and endemic controls lacking clinical signs and symptoms of leprosy or TB (n=20); a replication cohort comprised serum samples of PB (TT and BT) (n=7), multibacillary (BL and LL) without (n=23) and with reaction
(n=15) from Ethiopia (Table 1). No endemic control samples were tested for the Ethiopian cohort.

Table 1. Demographic and clinical data of cases and controls for serological studies

<table>
<thead>
<tr>
<th>Number of cases</th>
<th>Female/male ratio</th>
<th>Age</th>
<th>Ethnicity</th>
<th>Leprosy Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>7:13</td>
<td>22-45</td>
<td>Bangladesh</td>
<td>Endemic controls</td>
</tr>
<tr>
<td>46</td>
<td>12:22</td>
<td>20-41</td>
<td>Bangladesh</td>
<td>MB, No reaction</td>
</tr>
<tr>
<td>47</td>
<td>5:14</td>
<td>18-44</td>
<td>Bangladesh</td>
<td>MB, Reaction</td>
</tr>
<tr>
<td>7</td>
<td>3:4</td>
<td>24-46</td>
<td>Ethiopia</td>
<td>PB (TT/BT), No reaction</td>
</tr>
<tr>
<td>23</td>
<td>10:13</td>
<td>16-41</td>
<td>Ethiopia</td>
<td>MB (BL/LL), No reaction</td>
</tr>
<tr>
<td>15</td>
<td>3:13</td>
<td>18-44</td>
<td>Ethiopia</td>
<td>MB, Reaction</td>
</tr>
</tbody>
</table>
Complement in serum of leprosy patients

Meso Scale Discovery (MSD) platform

All the complement assays were performed on a novel multiplex developed using the MesoScale Discovery Platform (MSD; Gaithersburg, MD; www.mesoscale.com). The multiplex set comprised C1s, the activation markers C4d, Bb, iC3b and TCC, and the regulators FH (using FH Y402, FH H402 monoclonal antibodies which quantifies the concentration of total factor H as described previously [26]) and clusterin (Table 2).

All the in-house antibodies were initially established as ELISA assays for evaluating the level of complement activation products on the MSD platform. The assays were extensively validated, and the results were described in details previously [26,27].

Antibody-coated plates were blocked with BSA/EDTA/PBS, serum samples and standards diluted in BSA/EDTA/PBS added to wells and incubated for 1 hour at RT on a shaker. Plates were washed, the detecting antibody cocktail added to the plate (Table 2) and incubated for 1 hour at RT on a shaker. After washing, the reading buffer (R92TC-2; MesoScale Diagnostics) was added and plates were read on a MSD Sector Imager 6000 instrument. The data were analyzed using SoftMax Pro 4.6 Enterprise Edition (Molecular Devices LLC, Sunnyvale, CA, USA).
Table 2. Antibodies for the MSD assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Coating Antibody (source)</th>
<th>Detection Antibody (source)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1s</td>
<td>M81 (Hycult)</td>
<td>F33 (in-house, BPM)</td>
</tr>
<tr>
<td>C4d</td>
<td>Neo C4d (A251, Quidel)</td>
<td>C4d (A213, Quidel)</td>
</tr>
<tr>
<td>Bb</td>
<td>Neo Bb (A252, Quidel)</td>
<td>JC1 (in-house, BPM)</td>
</tr>
<tr>
<td>iC3b</td>
<td>Neo iC3b (Hycult)</td>
<td>C3-30 (in-house, BPM)</td>
</tr>
<tr>
<td>TCC</td>
<td>aE11 (Hycult)</td>
<td>E2 (in-house, BPM)</td>
</tr>
<tr>
<td>FH Y402</td>
<td>MBI-6 (in-house, BPM)</td>
<td>Ox-24</td>
</tr>
<tr>
<td>FH H402</td>
<td>MBI-7 (in-house, BPM)</td>
<td>Ox-24</td>
</tr>
<tr>
<td>Clusterin</td>
<td>Polyclonal-Anti-Apolipoprotein J (AB825, Milipore)</td>
<td>MBI-40 (in-house, BPM)</td>
</tr>
</tbody>
</table>

Statistical analysis

Data analysis was performed using GraphPad Prism version 5.0 (GraphPad Software Inc, San Diego, CA, USA) statistical package. Student’s t test was performed for statistical analyses comparing two groups. For comparison of more than two groups, one-way ANOVA with Bonferroni multiple comparison post-hoc test was used when the data was normally distributed. For non-normally distributed data the Kruskal-Wallis test was used. Differences were considered statistically significant when $p \leq 0.05$. The data is presented and expressed as standard error (SE) of the mean.
Results

Complement activation and regulation in multibacillary leprosy patients with or without reaction from Bangladesh

Selected complement components, activation products and regulators were measured in serum samples of leprosy patients with or without a reaction from Bangladesh and endemic controls collected under similar conditions and all samples were collected before start of MDT. Analytes were selected in part to interrogate different parts of the complement activation cascade in order to assess the contributions of different activation pathways. Complement components, regulators and activation products were measured on a novel multiplex built on the MSD platform. The Bangladesh leprosy population showed no significant difference in serum C1s values compared to controls (p= 0.442) (data is shown in table 3). Levels of the classical/lectin pathway activation fragment C4d (p <0.001 without R; p <0.001 R) and the alternative pathway fragment Bb (p <0.01 without R; p <0.001 R) were significantly increased in MB leprosy patients with or without reaction compared to endemic controls (Figure 1A and B). C4d levels were also significantly increased in patients with reaction compared to patients without a reaction (p <0.05) (Figure 1A). The increased serum levels of C4d in the leprosy reaction patients suggest the involvement of the classical and/or lectin pathway in the reaction process.

The levels of the activation pathway fragment iC3b and the terminal pathway activation marker TCC were significantly raised in leprosy patients with a reaction compared to endemic controls (p <0.05 and p <0.01, respectively), showing further evidence for increased complement activation in reactions (Figure 1C and D). Four patients from Bangladesh that were followed up after treatment showed that the TCC
levels stay high even after treatment (supplement figure 1). This suggests that treatment with either multidrug therapy or steroids does not lower complement serum levels in reaction patients.

Serum levels of Factor H (FH), the principle plasma regulator of the alternative pathway, and clusterin, a plasma regulator of the TCC, were also analyzed in serum samples of MB patients with or without reaction from Bangladesh. Total FH levels did not show a significant difference between leprosy patients with or without a reaction compared to controls (p=0.149) (data is shown in table 3).

Clusterin levels in leprosy patients with or without reaction were significantly higher than endemic controls (p <0.05 without R; p <0.001 with R) (Figure 1E), but not different between the patient groups.
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Levels stay high even after treatment (supplement figure 1). This suggests that treatment with either multidrug therapy or steroids does not lower complement serum levels in reaction patients.

Serum levels of Factor H (FH), the principle plasma regulator of the alternative pathway, and clusterin, a plasma regulator of the TCC, were also analyzed in serum samples of MB patients with or without reaction from Bangladesh. Total FH levels did not show a significant difference between leprosy patients with or without a reaction compared to controls ($p=0.149$) (data is shown in table 3).

Clusterin levels in leprosy patients with or without reaction were significantly higher than endemic controls ($p <0.05$ without R; $p <0.001$ with R) (Figure 1E), but not different between the patient groups.

![Fig. 1. Complement activation and regulation in multibacillary leprosy patients with and without reactions from Bangladesh. MSD platform for measuring complement activation products C4d (A), Bb (B), iC3b (C), TCC (D) and clusterin (E) in serum from endemic controls ($n=20$) and multibacillary leprosy patients with ($n=12$) or without ($n=46$) reaction at intake, showing a significant increase in multibacillary leprosy patients with and without reaction compared to controls for all measured components ($p=<0.05$). C4d levels are specifically and significantly increased in patients with reaction compared to those without reaction (B) [mean C4d no reaction 8.83 mg/l (SE 0.77) versus mean C4d...](image)
Reaction 13.41 mg/l (SE 1.60); \( P < 0.05 \). In addition, Clusterin levels were significantly increased in serum of multibacillary leprosy patients compared to endemic controls, but no difference between patients with or without reactions (E) [mean clusterin no reaction 398.0 mg/l (SE 10.15); \( P = <0.001 \) versus mean clusterin Reaction 378.9 mg/l (SE 15.87); \( P =<0.05 \)]. The error bars represent the standard error of the mean.

### Table 3. Complement levels in the Bangladeshi cohort

<table>
<thead>
<tr>
<th></th>
<th>Endemic controls</th>
<th>MB patients without reaction</th>
<th>MB patients with reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bagladeshi cohort</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C1s</strong></td>
<td>Mean (mg/l)</td>
<td>SE (mg/l)</td>
<td>Mean (mg/l)</td>
</tr>
<tr>
<td></td>
<td>94.69</td>
<td>2.77</td>
<td>99.41</td>
</tr>
<tr>
<td><strong>C4d</strong></td>
<td>3.46</td>
<td>0.39</td>
<td>8.83</td>
</tr>
<tr>
<td><strong>Bb</strong></td>
<td>16.53</td>
<td>1.92</td>
<td>24.26</td>
</tr>
<tr>
<td><strong>iC3b</strong></td>
<td>5.42</td>
<td>0.63</td>
<td>8.63</td>
</tr>
<tr>
<td><strong>TCC</strong></td>
<td>1.87</td>
<td>0.15</td>
<td>2.27</td>
</tr>
<tr>
<td><strong>Factor H</strong></td>
<td>253.7</td>
<td>15.82</td>
<td>277.3</td>
</tr>
<tr>
<td><strong>Clusterin</strong></td>
<td>297.3</td>
<td>19.74</td>
<td>398.0</td>
</tr>
</tbody>
</table>
TCC levels are increased in serum of multibacillary leprosy patients with reaction compared to patients without reaction from Ethiopia

In the preceding section results using the leprosy serum samples from Bangladesh data indicate that levels of complement activation products were higher in patients with reaction compared to those without – significantly so for C4d and trending for iC3b and TCC. We therefore surmised that these markers could be used as an indicator for identifying patients with reactions. In order to substantiate this finding we measured the levels of these complement activation products in serum samples of an Ethiopian leprosy cohort. These samples were also collected before start of MDT and classified as PB (TT and BT) or MB (BL and LL) leprosy patients without reaction and MB patients with reaction. In these samples, like those of Bangladesh, no significant difference was found in the level of C1s in serum of MB compared to PB patients without reaction (p= 0.384) (data is shown in table 4). Serum levels of the complement activation products in MB patients compared to PB patients without a reaction were significantly increased for C4d (p= 0.04), Bb (p= 0.03) iC3b (p= 0.02 ) and TCC (p= 0.003) (Supplement figure 2). No significant difference was found in the levels of C4d and Bb in the MB leprosy patients without reaction compared to the patients with a reaction (p= 0.392 and p=0.143 respectively) (data is shown in table 4). Levels of the common complement pathway marker iC3b were not significantly different between MB leprosy patients without reaction compared to those with a reaction (Figure 2A). However, levels of TCC in Ethiopian cohort MB patients with a reaction were significantly raised in comparison to PB (p=<0.01) patients and to MB patients without reaction (p=<0.05) (Figure 2B). This latter result independently confirms that raised plasma levels of TCC represent a potential tool for identifying leprosy patients with reactions.
Fig. 2. TCC levels are increased in serum of multibacillary leprosy patients with reaction compared to patients without reaction from Ethiopia. MSD assay for measuring the common complement pathway activation fragment iC3b (A) and the terminal pathway activation marker TCC (B), in serum samples of paucibacillary (n=7) and multibacillary patients without a reaction (n=23) or with a reaction (n=15). Although no significant difference was found in the levels of iC3b in serum of paucibacillary and multibacillary patients without and with a reaction, TCC levels were significantly increased in multibacillary leprosy patients with a reaction compared to paucibacillary and multibacillary patients without a reaction [mean TCC no reaction 4.03 mg/l (SE 0.28) versus mean TCC Reaction 7.49 mg/l (SE 1.48); P =<0.01 and P =<0.05]. The error bars represent the standard error of the mean.
Fig. 2. TCC levels are increased in serum of multibacillary leprosy patients with reaction compared to patients without reaction from Ethiopia. MSD assay for measuring the common complement pathway activation fragment iC3b (A) and the terminal pathway activation marker TCC (B), in serum samples of paucibacillary (n=7) and multibacillary patients without a reaction (n=23) or with a reaction (n=15). Although no significant difference was found in the levels of iC3b in serum of paucibacillary and multibacillary patients without and with a reaction, TCC levels were significantly increased in multibacillary leprosy patients with a reaction compared to paucibacillary and multibacillary patients without a reaction [mean TCC no reaction 4.03 mg/l (SE 0.28) versus mean TCC Reaction 7.49 mg/l (SE 1.48); P =< 0.01 and P =< 0.05]. The error bars represent the standard error of the mean.

Table 4. Complement levels in the Ethiopian cohort

<table>
<thead>
<tr>
<th></th>
<th>PB patients without reaction</th>
<th>MB patients without reaction</th>
<th>MB patients with reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ethiopian cohort</strong></td>
<td><strong>Mean (mg/l)</strong></td>
<td><strong>SE (mg/l)</strong></td>
<td><strong>Mean (mg/l)</strong></td>
</tr>
<tr>
<td>C1s</td>
<td>112.4</td>
<td>9.71</td>
<td>128.2</td>
</tr>
<tr>
<td>C4d</td>
<td>17.99</td>
<td>1.99</td>
<td>27.15</td>
</tr>
<tr>
<td>Bb</td>
<td>24.91</td>
<td>2.39</td>
<td>28.62</td>
</tr>
<tr>
<td>iC3b</td>
<td>13.15</td>
<td>1.94</td>
<td>22.39</td>
</tr>
<tr>
<td>TCC</td>
<td>2.02</td>
<td>0.23</td>
<td>4.03</td>
</tr>
<tr>
<td>Factor H</td>
<td>385.0</td>
<td>26.85</td>
<td>301.9</td>
</tr>
<tr>
<td>Clusterin</td>
<td>457.9</td>
<td>34.08</td>
<td>493.8</td>
</tr>
</tbody>
</table>
Discussion

Nerve damage leading to deformity is a major problem in the course of leprosy and progression of the disease among susceptible hosts. In the absence of the peripheral neuropathy, leprosy would be an innocuous inflammatory skin disease rather than one that, even today in the 21st century, is one of the most stigmatized diseases often associated with severe social repercussions for the patient [28,29]. The etiology of leprosy reactions and nerve damage has largely been attributed to the consequence of granulomatous reactions due to the host immune response to *M. leprae*. Variability in the hosts’ immune response has a causal relationship to the spectral pathology of the disease. Timely diagnosis followed by optimum and evidence based treatment would reduce risks of permanent tissue damage and assist regeneration of the damaged tissue [30]. Despite continued efforts in the refinement of diagnostic methods, reactions are often misdiagnosed even by experienced health workers and clinicians [31]. Unquestionably, a reliable test or combination of tests for the prompt diagnosis of reactions, particularly RR or type 1, would contribute positively to the clinical outcome of the patients. Biomarkers for identification of patients developing reactions have focused on circulating parameters of adaptive immunity like antibodies, cytokines and chemokines as well as gene expression profiles [32]. However, a recent report described the importance of innate immunity in orchestrating leprosy immunopathology and in the initiating phase of host defense against the pathogen [33]. Innate immune effectors include cellular systems that are now being studied in relation to mycobacterial pathology, and also humoral effectors, including the complement system, critical in initiating and orchestrating the innate immune response to infection [34,35]. These findings imply that the analysis of
the complement system might be of value for the diagnosis and prognosis of leprosy disease status.

It is now well appreciated that complement activation can be induced by pathogen-associated molecular patterns (PAMPs) [18,34-36]. Particularly in the context of leprosy, we recently showed that the membrane attack complex (MAC) of complement is generated upon cognate interaction of the axon of the peripheral nerve with the \textit{M. leprae} specific PAMP lipoarabinomannan (LAM) [19]. In addition, we showed that MAC formation results in nerve damage and its inhibition is neuroprotective in a mouse model for \textit{M. leprae}-induced nerve damage. We also demonstrated that MAC is deposited in nerve lesions of leprosy patients, paralleling the observed nerve degeneration. Our findings indicate an important role for complement in the disease, and therefore we examined whether increased systemic levels of complement activation could be detected in leprosy patients and whether complement products and regulators might be useful markers of leprosy disease state.

The present study reports for the first time the quantification of a panel of complement activation products and regulators in serum of leprosy patients in multiplexed assays on the Meso Scale Discovery (MSD) platform. The study was performed retrospectively using serum samples from PB and MB patients with or without reaction collected in Ethiopia and Bangladesh for a previous study [25].

Here we show significantly increased levels of complement activation products C4d, Bb, in serum of Bangladeshi leprosy patients compared to endemic controls. The increased levels of C4d in the patients supports activation of the lectin and/or classical pathway of complement, while elevated Bb supports a significant
involvement of the alternative pathway in leprosy patients. Earlier studies had already suggested a role for the classical pathway of the complement system in leprosy across the spectrum by measuring the ability of circulating immune complexes isolated from sera of leprosy patients to activate complement, but none of these studies measured pathway-specific activation products [23, 24, 37].

Bacterial antigens either in the free form or by slow multiplication of bacilli in the reaction patients could initiate complement activation via the lectin pathway, initiated by the binding of the pattern recognition molecule mannose-binding lectin (MBL) to bacterial surfaces [38]. In support of the involvement of the lectin pathway in MB leprosy patients, one study found that MBL serum levels were significantly increased in LL form of leprosy compared to other leprosy types [23]. However, it should be noted that the association of MBL with leprosy pathophysiology is controversial, because some investigators found increased levels of MBL in the patients undergoing reaction. For this reason, no confirmatory conclusion can be drawn from our present limited study on the role of MBL pathway in contributing higher levels of complement activation products in leprosy reactions.

iC3b levels in the Bangladeshi population were significantly increased in leprosy patients with reaction compared to controls. The levels were also higher compared to patients without a reaction although not significant.

The present study is limited in identifying the major contribution of either the classical or lectin pathway for complement activation, TCC levels in serum of leprosy patients from Bangladesh were significantly higher in patients with reaction compared to endemic controls. No significant difference was found between leprosy patients without reactions and endemic controls for TCC. This reflects that complement TCC
level may be considered as a promising marker for patients developing a reaction. It also suggests that treatment with either MDT or steroids does not lower complement activation in reaction patients, reinforcing the possibility that complement contributes to the nerve damage in these patients.

Similar to the findings encountered with Bangladesh cohort, TCC levels in serum samples of leprosy patients from Ethiopia also showed a significant increase in reactions compared to patients without reactions. Since complement activation is under control of several regulatory molecules, we also measured the levels of factor H, regulator of the alternative pathway of complement, in leprosy patients with or without reactions; previous studies showed that factor H is associated with a variety of diseases, including neurodegenerative disease [39-42]. Changes in factor H levels, might suggest altered regulation of activation of the alternative pathway of complement during disease. We did not detect any significant change in the total level of Factor H in serum of leprosy patients with and without a reaction. Clusterin, a fluid-phase regulator of the MAC, is increased in tissue and plasma in different diseases including neurodegenerative conditions [43-48]. Hence, we determined the circulatory levels of Clusterin in leprosy patients in association with disease activities. Clusterin levels were higher in leprosy patients compared to controls. No difference was found in Clusterin levels in patients with or without reaction, suggesting no increase in regulatory status of the terminal pathway of complement by Clusterin in reaction patients.

Unfortunately, due to the nature of the study, 1) being carried out with the samples collected in the field situations and 2) the lack of EDTA in the serum samples, any elaborate interpretation regarding the mechanism of high level of TCC in leprosy patients with reaction should be avoided at this stage of the study. The lack of
especially EDTA might result in in vitro generation of TCC which continues in serum. This limitation is considered equally applicable to all the samples studied; therefore, any comparative values relating the disease state is considered as true reflection.

Despite such limitations, the present datasets have demonstrated convincingly, that systemic activation of complement occurs in leprosy and likely involves multiple pathways. The increased serum levels of TCC and other activation markers in leprosy patients with reaction warrants a prospective and sequential study on complement activation in leprosy, to confirm conclusively whether circulating complement activation products such as TCC can be applied as biomarkers for diagnosis of patients at a risk of developing a reaction.

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**Author’s contribution.** PKD, VR and FB formulated the project; AG provided access to the stored serum samples collected for a different prospective study; BPM provided access to the in-house complement assays and advised on the project; SH performed assays; NBEI analyzed the data and wrote the manuscript.

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**Competing financial interests.** FB and VR are inventors on a patent that describes the use of inhibitors of the terminal complement pathway for therapeutic purposes. FB and VR are shareholders of Regenesance BV. The remaining authors declare no competing financial interests.
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Chapter 3


Sup. Fig. 1. TCC serum levels of leprosy patients followed at reaction and after treatment. TCC serum levels of leprosy patients at reaction do not change after treatment, indicating that treatment does not affect complement activity in leprosy.
Sup. Fig. 2. Complement activation in paucibacillary and multibacillary leprosy patients from Ethiopia. MSD platform for measuring complement activation products C4d (A), Bb (B), iC3b (C) and TCC (D) in serum from paucibacillary (n=7) and multibacillary (n=23) leprosy patients, showing a significant increase in multibacillary compared to paucibacillary leprosy patients for C4d (mean PB 17.99 mg/l SE 1.99 versus mean MB 27.15 mg/l SE 4.04; p=0.04), Bb (mean PB 24.91 mg/l SE 2.39 versus mean MB 28.62 mg/l SE 1.99; p=0.03), iC3b (mean PB 13.15 mg/l SE 1.94 versus mean MB 22.39 mg/l SE 1.92; p=0.02) and TCC (mean PB 2.02 mg/l SE 0.23 versus MB 4.03 mg/l SE 0.28; p=0.003). The error bars represent the standard error of the mean.