Short Report: Serum Levels of Neopterin during Antimicrobial Treatment for *Mycobacterium ulcerans* Infection

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Abstract. Neopterin is closely associated with activation of the cellular immune system. Neopterin levels differed between controls and patients with Buruli ulcer disease. No differences between patients with or without paradoxical responses were observed. Therefore, neopterin has no value in detecting paradoxical responses among patients with limited Buruli ulcer disease. Neopterin levels were lower in patients receiving clarithromycin. This finding might indicate a slower cellular immune recovery, with possible consequences in future therapy with clarithromycin.

Buruli ulcer (BU), which is caused by *Mycobacterium ulcerans*, is an emerging neglected Tropical Disease reported from more than 30 countries, mainly with tropical and subtropical climates. The region with the highest disease burden is western Africa. The treatment recommendation for BU issued by the World Health Organization is streptomycin in combination with rifampicin for eight weeks, with or without additional surgical debridement or skin grafting.^{1,2} In BU, paradoxical reactions are common³ and difficult to distinguish from treatment failure. Neopterin is a stable biomarker. Its production is closely associated with activation of the cellular immune system.⁴ Macrophages are stimulated to secrete neopterin by interferon- γ (INF- γ) derived from T lymphocytes. An improved cell-mediated immunity could be followed by a paradoxical response. The objective of this study was to evaluate the levels of neopterin in response to antimicrobial treatment and associated paradoxical responses during *M. ulcerans* infection.

Blood samples were collected and demographic and clinical information was obtained from a subset of patients participating in a randomized controlled trial to compare two antimicrobial regimens in two hospitals in Ghana (BURULICO).⁵ Participants had early, limited (cross-sectional lesion diameters ≤ 10 cm) disease and were followed-up for one year from start of treatment (ClinicalTrials.gov, identifier NCT00321178). Patients who showed treatment failure, received a skin graft, or were co-infected with human immunodeficiency virus were excluded from the study. In this way we were able to measure lesions over time after effective antimicrobial treatment, and any increase in lesion size would probably reflect a paradoxical response. For this study, a paradoxical response is defined to as a lesion that was larger at week 8 than at week 6 for which no intervention was required and healing occurred after one year.

Blood was obtained from every patient drawn at two time points: at the start of treatment (baseline T0), and after 8 weeks of antimicrobial therapy (T1). Blood was obtained from age- and sex-matched community controls. Serial acetate sheet tracings and digital images were recorded for every patient at T0 and thereafter once every 2 weeks until week 8. In instances

of more than one lesion, the largest lesion was measured for the study.³

Serum neopterin levels were measured by using an enzyme-linked competitive immunosorbent assay (Neopterin ELISA; Immuno Biological Laboratories, Hamburg, Germany). The assay was performed according to the manufacturer's instructions.

An independent *t*-test was performed to compare neopterin levels of patients (T0) and community controls by using SPSS version 18.0 software (SPSS, Chicago, IL). Only sets of patients and community controls with available serum samples were included. To compare neopterin levels obtained in patients before and after eight weeks of antimicrobial therapy, a paired *t*-test was used. To compare the trend between antibiotic regimens, an independent *t*-test was used. Patients were excluded from analysis if one of two values (T0 or T1) were missing. One-way analysis of variance was used to study paradoxical reactions and neopterin levels before and after antimicrobial therapy and the difference between concentrations at both time points. Patients with healed lesions before week 8 and patients with missing values were excluded.

In total 134 out of the 151 patients of the BURULICO trial were included in the analysis of neopterin and evolution of lesions during treatment. Ten patients showed treatment failure; three patients were co-infected with human immunodeficiency virus (one of these patients also showed treatment failure) and five patients received skin grafts. Of 134 clinically diagnosed patients, 130 were confirmed by either polymerase chain reaction, culture, histopathologic analysis, or microscopy. The basic characteristics of the patients have been reported by Nienhuis and others.³

Of 134 patients, 17 had missing neopterin values at T0 and 9 had missing neopterin values at T1. A total of 109 patients had samples available at the start of treatment and after 8 weeks. No differences were found between age of patients with and without neopterin measurements at T0 (median = 12, interquartile range [IQR] = 8–18 versus median = 13, IQR = 7–26.50) and at T1 (median = 12, IQR = 8–20 versus (median = 11, IQR = 9–16.50). No difference was found between sex of patients with (male 29.9%) and without (male 35.3%) neopterin values at T0 and with (male 30.4%) and without (male 33.3%) neopterin values at T1.

Of 117 BU patients, a community control was available for 100 patients to compare neopterin levels. For these 100 patients, neopterin levels were available at T0 and from a matched community control. Neopterin values at the start of treatment (mean $\pm$ SD = 17.0 $\pm$ 10.5 nmol/L, IQR = 10.42–19.60) were

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significantly higher ($P < 0.01$) than in community controls (mean $SD = 10.6 \pm 3.4$, IQR = 7.99–12.30). The neopterin levels of 109 patients after eight weeks of antibiotic treatment (mean $\pm SD = 18.0 \pm 9.9$, IQR = 11.80–22.25) were higher than levels measured at the start of treatment (mean $\pm SD = 16.5 \pm 9.2$, IQR = 10.58–19.53), but this difference did not reach statistical significance ($P = 0.06$). The neopterin values of patients receiving 8 week streptomycin/rifampicin (SR) (mean difference 3.6 [SD 8.0]) and patients receiving 4 weeks (SR) and 4 weeks clarithromycin/rifampicin (CR) (mean difference -1.0 [SD 8.2]) were different ($P = 0.03$); SR group (mean 17.3 [SD 9.5]) and SR/CR group (mean 15.5 [SD 8.9]) at start treatment and after 8 weeks of antimicrobial treatment SR group (mean 20.9 [SD 11.6]) and SR/CR group (mean 14.4 [SD 5.6]).

Individual neopterin levels before and after eight weeks of antimicrobial therapy with results of patients with or without paradoxical response are shown in Figure 1. Of 109 patients, 97 had information on paradoxical response, of which 14 were healed within 8 weeks, resulting in 83 eligible patients for this analysis. No significant differences were found in neopterin levels among patients with (mean $\pm SD = 14.7 \pm 8.9$) or without (mean $\pm SD = 17.0 \pm 10.2$), paradoxical response at the start of treatment and after eight weeks of therapy (with paradoxical response (mean $\pm SD = 17.5 \pm 8.2$) and without paradoxical response (mean $\pm SD = 18.9 \pm 12.2$). The use of other definitions for clinical definition of a paradoxical response³ led to similar outcomes. Neopterin levels were not influenced by age or sex.

Markers of a paradoxical response would be a useful asset to distinguish this response from treatment failure, with subsequent potentially erroneous decisions to operate or change antimicrobial treatment. We detected significantly increased neopterin levels in BU patients with early, limited disease compared with matched, healthy community controls. After

eight weeks of effective antimicrobial therapy, neopterin values increased further, although this increase was not statistically significantly ($P = 0.06$). The findings of generally increased neopterin levels after effective antimicrobial treatment are consistent with results of previous research showing recovery of a typical Th1-type anti-mycobacterial immune response, resulting in a lower bacterial load and a reduced concentration of the immune suppressing toxin produced by *M. ulcerans*, mycolactone.^{6,7} In patients who received antibiotic treatment including clarithromycin, neopterin did not increase as in the patients on streptomycin/rifampicin for eight weeks. This difference in Th1-type immunity response in the group containing clarithromycin treatment may be caused by immunomodulatory effects of clarithromycin.⁸ Although in the BURULICO trial no significant difference in treatment response was shown between the two treatment arms, with completely oral treatment with clarithromycin/rifampicin, this difference may become clinically relevant in the future. We found no significant differences in neopterin values among patients with or without paradoxical reactions. For leprosy, higher neopterin levels were found in patients with reversal reactions and in multi-bacillary leprosy compared with patients with paucibacillary leprosy.^{9,10}

A limitation of our study is that only patients with limited disease were included. For other infectious diseases, such as malaria¹¹ and pneumonia,¹² neopterin levels have been shown to depend on severity of disease. Among BU patients, INF- γ concentrations increase during treatment, and show a rapid increase in patients with large lesions and only a minor increase in patients with small lesions.^{6,13}

In conclusion, neopterin values appear to be of limited value in detecting paradoxical responses among patients with limited BU disease. Other ways to predict clinical response to antibiotic treatment and to distinguish between treatment failure and a paradoxical response are needed. The slower immune response among patients with antibiotic treatment containing clarithromycin may have clinical consequences when fully oral antibiotic treatment will be introduced.

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FIGURE 1. Neopterin levels in patients ($n = 83$) at different time points with ($n = 38$) or without ($n = 45$) paradoxical responses. No significant difference was found between differences at different time points of neopterin levels for the group with paradoxical responses (mean $\pm SD = 2.8 \pm 9.1$) and in patients without paradoxical responses (mean $\pm SD = 1.9 \pm 8.8$).

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