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## Serum neopterin levels in HIV infected patients with & without tuberculosis

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**Background & objective :** Three categories of prognostic markers are best documented as having significance in relation to prognosis of HIV infection. These include HIV viral load, CD4 T-cell levels and plasma levels of soluble markers of immune activation. The plasma activation markers, like neopterin, tumor necrosis factor alpha (TNF- $\alpha$ ), interleukins *etc.*, are products of cytokine activity and represent immunologic changes throughout the body. There is not much information available on serum neopterin estimation in patients infected with both HIV and tuberculosis (TB), though neopterin levels are known to be elevated in pulmonary TB patients. In this study we attempted to correlate neopterin levels with the presence of tuberculosis in HIV infected and uninfected individuals and studied the changes after anti-tuberculosis treatment.

**Methods :** Serum neopterin concentrations were measured by high performance liquid chromatography (HPLC) in 25 HIV-seropositive (HIV-TB) and 10-seronegative (TB) patients with tuberculosis before, during and at the end of antituberculosis therapy (ATT). S-neo was also measured in 10 HIV-seropositive asymptomatic individuals and 10 healthy controls. The results were correlated with clinical, bacteriological and immunological status.

**Results :** All TB patients regardless of HIV status had elevated s-neo concentrations at diagnosis, which declined gradually during treatment. Patients with HIV/TB with CD4 counts  $< 200/\text{mm}^3$  had the highest levels at baseline with a steep fall during treatment. The median level at the end of treatment was significantly higher in HIV/TB than in TB patients, despite clinical improvement and bacteriological clearance of *Mycobacterium tuberculosis*. HIV infected asymptomatic individuals had neopterin levels that were higher than healthy controls but lower than HIV-TB patients.

**Interpretation & conclusion :** Serum neopterin levels are elevated in HIV-positive patients, with the highest levels in those with tuberculosis and CD4 counts  $< 200/\text{mm}^3$ . Though the levels decrease with anti tuberculosis therapy, persistently elevated levels indicate progressive HIV disease and a poor prognosis.

**Key words :** HIV - neopterin - opportunistic infections - tuberculosis

The use of surrogate end points has been more intensely examined in HIV/AIDS than in any other disease. Many studies have shown the value of prognostic markers such as CD4+ T-lymphocyte

count, the viral load and the soluble immune activation markers in predicting the course of the illness<sup>1-5</sup>. The CD4+ T-lymphocyte count has been the principal biological marker utilized for clinical evaluation of

disease. However, measuring CD4 counts and viral load requires expensive equipments, separate space, trained personnel, *etc.* Hence there is a need to study other markers for use in monitoring HIV disease progression and response to treatment.

Activation of all components of the immune system is a major feature of HIV infection and one manifestation of immune activation is increased cytokine production. However, limitation in the ability to quantitate circulating cytokines has led to the assessment of downstream products that reflect cytokine activity in lymphoid tissues<sup>2</sup>. Such assessment includes level of soluble markers and one such marker is neopterin. Neopterin is a pteridine derivative of guanosine triphosphate released into circulation from activated macrophages. Serum neopterin (s-neo) level is an indicator of both macrophage activation and interferon gamma (IFN- $\gamma$ ) activity, a major macrophage activating factor<sup>1,3,4</sup>. Neopterin can be measured more easily and accurately than IFN- $\gamma$  levels in serum.

Tuberculosis is one of the commonest opportunistic infections in HIV infected patients, in India. Previous studies have shown that s-neo levels are elevated in patients with pulmonary tuberculosis<sup>5,7</sup>. Serial evaluation of s-neo in patients on antituberculosis treatment (ATT) is useful in assessing the response to therapy<sup>8,9</sup>. As HIV and TB both stimulate the cellular arm of immune system, effective treatment of tuberculosis would reduce some of the excessive immune activation. However, there are not much data on serial estimation of neopterin in patients infected with both HIV and tuberculosis. In the present study, we have tried to correlate changes in s-neo levels with clinical and bacteriological status of patients infected with HIV and TB, and compared these results with HIV-negative TB patients. The changes in s-neo levels after antituberculosis treatment were also studied in all these groups of patients.

### Material & Methods

*Study subjects* : Patients admitted to various controlled clinical trials at Tuberculosis Research Centre (TRC) clinics and subcentre at Chennai and

Madurai, between January and June 2001, were randomly selected for serum neopterin assays. A thorough history and clinical examination, chest X-ray and three sputum smear examinations for acid-fast bacilli (AFB) and culture for *Mycobacterium tuberculosis* were done. After pretest counselling and obtaining informed consent from the patients, HIV status was determined by using two different rapid tests (Comb Aids-RS, Span diagnostics, India and TRI-DOT, J. Mitra & Co, India) followed by an ELISA (Labsystems, UK).

Twenty five HIV-positive tuberculosis patients aged between 22-50 yr (mean  $\pm$  SD 31.0  $\pm$  4.6 yr) and 10 newly diagnosed HIV-negative smear positive pulmonary tuberculosis patients aged between 20 to 50 yr (mean  $\pm$  SD 26.8  $\pm$  9.8 yr) were included in the study. All the HIV seronegative patients had culture confirmed pulmonary TB.

HIV-TB group comprised of patients with newly diagnosed pulmonary and extra pulmonary forms of tuberculosis. Of the 25 patients, 19 had pulmonary tuberculosis while 6 had extrapulmonary tuberculosis, including 2 with TB lymphadenitis (proven by histopathology) and 4 with pleural effusion; 11 had sputum smears positive for AFB while 17 had sputum culture positive for *M. tuberculosis*. Seven patients had associated opportunistic infections like diarrhoea, oral candidiasis, scabies, *etc.* during pre-treatment period. The 25 HIV-TB patients were categorized on the basis of CD4 T cell counts at presentation as less than or greater than 200 cells/mm<sup>3</sup>. 15 patients had CD4 T cells <200/mm<sup>3</sup> ranging from 22-195 cells/mm<sup>3</sup> (mean  $\pm$  SD: 92.8  $\pm$  58.8) and 10 had >200 cells/mm<sup>3</sup> ranging between 204 to 513 cells/mm<sup>3</sup> (mean  $\pm$  SD 334.6  $\pm$  127.5).

Tuberculosis patients were started on a 6-month short-course intermittent chemotherapy regimen containing rifampicin (450 mg), isoniazid (600 mg), pyrazinamide (1500 mg) and ethambutol (1200 mg) thrice weekly for two months followed by rifampicin (450 mg) and isoniazid (600 mg) thrice weekly for 4 months. In addition, all HIV-positive patients with CD4 cell count < 200 cells/mm<sup>3</sup> were given corimoxazole double strength one tablet daily. Ten asymptomatic HIV-seropositive individuals aged between 20 to 50 yr (mean  $\pm$  SD 30.3  $\pm$  8.0 yr) and

10 healthy seronegative individuals aged 20-40 yr (mean  $\pm$  SD 28.8  $\pm$ 3.2 yr), mainly TRC laboratory staffs, were also included in the study. All the patients with TB became smear and culture negative by the end of treatment and showed clinical and radiological improvement.

The study proposal was approved by the Institutional Ethics committee.

**Study design :** Blood samples (10ml) were collected before, during and at the end of treatment for HIV-TB and TB patients and at one time point for HIV seropositive asymptomatic individuals and healthy controls. Serum was separated and stored frozen at  $-70^{\circ}\text{C}$  until use.

**Neopterin assay :** Neopterin in serum was measured by high performance liquid chromatography (HPLC) as described elsewhere<sup>10</sup>. The HPLC system used was Shimadzu vp series (Shimadzu corporation, Japan) equipped with two pumps (LC-10ATvp), spectrofluorimetric detector (RF-10AXL) and system controller (SCL-10Avp). Ultra filtrate of serum samples were prepared using the ultrafree-microcentrifuge filters (Sigma Chemical Co., USA). The mobile phase was a 15mM potassium phosphate buffer (pH 6.5) with a flow rate of 1ml/min. Standards ranging from 0 to 160nmol/l and the protein filtrate of samples were analysed by injecting 20 $\mu$ l directly to a reverse phase C<sub>8</sub> column (LiChroCART 5 $\mu$ m 250x4.0mm, Merck KgaA, Germany); neopterin was measured by its native fluorescence (350nm excitation, 440nm emission).

**Measurement of CD4+ T-lymphocyte count :** CD4 lymphocyte enumeration in whole blood samples from HIV-positive patients was done by flow cytometry

(FACSsort, Becton-Dickinson, USA) before and at the end of treatment.

**Statistical analysis:** The SPSS (version 8.0) was used for statistical analysis. Using the non-parametric Mann-Whitney U test and Wilcoxon rank test, comparison between the groups was performed;  $P < 0.05$  were considered significant. The correlation between s-neo and CD4+ T-lymphocyte count was assessed with use of Spearman's rank correlation coefficient.

## Results & Discussion

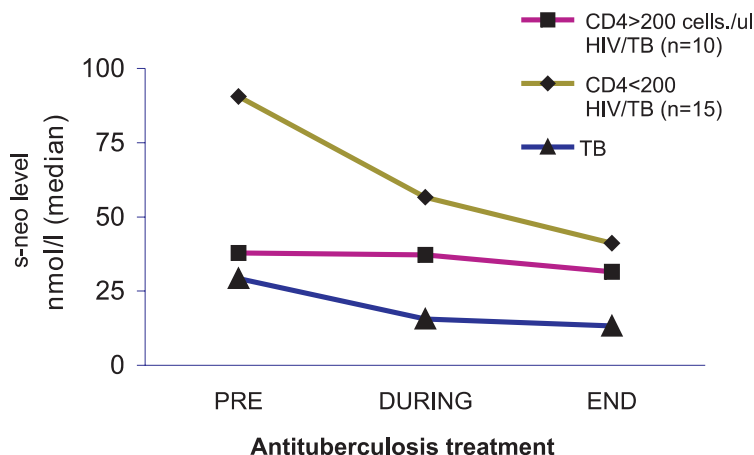
The median s-neo level was significantly elevated above the normal level in patients with active tuberculosis regardless of their HIV status. The HIV-positive asymptomatic group had significantly lower level (median: 13.5 nmol/l,  $P < 0.001$ ) than that of HIV-TB patients (median: 37.8 nmol,  $P < 0.001$ ), but higher than healthy controls ( $P < 0.001$ ). Among patients with tuberculosis, the median levels of s-neopterin in HIV-TB group with CD4 count of  $>200/\text{mm}^3$ , was similar to that of HIV uninfected TB patients. Patients with CD4 cell count of  $<200/\text{mm}^3$ , had the highest median level and the range of 25<sup>th</sup> and 75<sup>th</sup> percentile did not overlap with the other two groups (Table).

There was a gradual decrease in s-neo level in all the three groups (HIV/TB with CD4  $< 200$  cells/ $\mu$ l, HIV/TB with CD4  $>$  no cells/ $\mu$ l, and TB patients) in response to chemotherapy. However, the median level (25<sup>th</sup>-75<sup>th</sup> percentile) at the end of treatment was significantly higher in both the HIV-TB groups than in TB patients [41.1 (33.2-72.2), 31.5 (19.4-41.8) vs 13.2 (10.7-22.1) nmol/l] with a  $P < 0.001$ ,  $P < 0.01$ , respectively (Fig.). The median values for both HIV

**Table.** Baseline serum neopterin levels in different study groups

Serum Neopterin (nmol/l)	HIV-TB (CD4 $<200/\text{mm}^3$ ) (n=15)	HIV-TB (CD4 $>200/\text{mm}^3$ ) (n=10)	Pulmonary TB (n=10)	Asymptomatic HIV positive (n=10)	Healthy control (n=10)
Median	90.7	37.8	29.2	13.5	7.4
25 <sup>th</sup> -75 <sup>th</sup> percentile	54.7-124.8	25.0-48.5	18.9-42.0	11.8-14.5	6.0-8.1

All groups significantly ( $P < 0.001$ ) different from each other except pulmonary TB and HIV-TB with CD4 T cell count  $>200$  cells/ $\text{mm}^3$



**Fig.** Median s-neo levels in HIV/TB (n=10) and 10 TB patients before, during and end of treatment.

groups were higher than the 75<sup>th</sup> percentile for TB patients at the end of treatment. Four patients, three with CD4+ T-lymphocyte counts < 200 and one with  $\geq 200/\text{mm}^3$  had increased neopterin level at the end of treatment after a decline during treatment.

In HIV-TB patients with CD4+ T-lymphocyte counts < 200/mm<sup>3</sup>, the median decrease in neopterin was 34 and 45 per cent during and at the end of treatment respectively; the corresponding decrease in patients with CD4+ T-lymphocyte counts > 200/mm<sup>3</sup> was 6 and 19 per cent; and in TB patients it was 45 and 55 per cent. The decrease was statistically significant only in HIV-TB patients with CD4+ T cell counts < 200/mm<sup>3</sup> ( $P < 0.01$ ) and in TB patients ( $P < 0.05$ ).

Neopterin concentrations were inversely related to CD4+ T-cell counts, both initially and at the end of treatment, ( $r = -0.484$  and  $-0.449$  respectively,  $P < 0.05$ ) in patients with HIV-TB.

Immune activation is an important feature in HIV infection because the extent of immune activation is correlated to the clinical outcome of the disease<sup>11</sup>. In HIV associated tuberculosis, cellular activation is prominent, despite the deleterious effect of HIV on immune response<sup>12</sup>. This activation can be quantified by measurement of plasma levels of soluble markers. It would be useful to have a marker that could predict prognosis when HIV and TB occur together. In the present study, an attempt was made to examine whether neopterin, a surrogate marker for interferon gamma (IFN- $\gamma$ ) would correlate with clinical and

bacteriological response during treatment for tuberculosis in HIV-seropositive patients. Other indices such as CD4 T-lymphocyte counts were correlated to s-neo concentrations both at presentation and at the end of ATT.

S-neo level was significantly increased initially in all the patients with tuberculosis irrespective of HIV status. These findings confirm our previous report<sup>7</sup> and of others that TB patients have elevated neopterin levels<sup>6-8</sup>. The median neopterin level was markedly elevated in HIV-TB patients and this was higher than that reported in Zambian patients<sup>8</sup>; this could be because 60 per cent of our patients had CD4+ counts < 200/mm<sup>3</sup>. However, our results for the median neopterin level in patients with CD4 count  $\geq 200/\text{mm}^3$  was comparable to that of Zambian patients. The median s-neo level of HIV-TB patients with < 200 CD4 T-lymphocyte counts  $\mu\text{l}$  was the highest of all the groups, indicating that immune activation was maximum in patients with severe immunosuppression.

*In vitro* experiments to investigate the possibility that HIV-1 infection of mononuclear phagocytes directly induces enhanced neopterin production, showed that the observed 6 to 12 fold increase in neopterin was not caused by HIV infection but might be a result of the normal response by mononuclear phagocytes to increased level of IFN- $\gamma$ <sup>13</sup>. Further, acute primary HIV-1 infection was characterized by CD4 lymphocytopenia with elevated serum level of IFN- $\gamma$  and neopterin<sup>14</sup>. Aziz *et al*<sup>15</sup> had reported progressive increase in the levels of IFN- $\gamma$  and

neopterin when HIV-seropositive individuals were stratified on the basis of CD4 cell counts, and the highest levels were seen in CD4 category of  $<200/\text{mm}^3$ . Similarly, circulating plasma level of IFN- $\gamma$  is shown to be increased in newly diagnosed tuberculosis patients<sup>16</sup>. In HIV-TB patients, plasma IFN- $\gamma$  level is maximum<sup>17</sup> in patients with CD4 T cell counts of less than  $200/\text{mm}^3$ . These findings clearly indicate that s-neo level is an indicator of IFN- $\gamma$  activity and thus is a good surrogate marker for this cytokine.

In this study, persons with asymptomatic HIV infection had s-neo level higher than healthy controls but much lower than patients with tuberculosis. These results are in agreement with the findings of Fahey *et al*<sup>18</sup>, that HIV infection produces immune activation even in early stages of the disease. During antituberculosis treatment, s-neo concentrations started to decline but this decline was statistically significant in HIV-TB patients with CD4 + T-lymphocyte counts of  $<200/\text{mm}^3$  and in TB patients. It is interesting to note that three patients in HIV-TB group had rising s-neo levels after the decline during treatment with a corresponding decrease in CD4+ counts. Two of these patients who had s-neo levels  $>100\text{nmol/l}$  at the end of treatment died within nine months after the completion of ATT due to causes other than TB (one patient died due to AIDS wasting and the other developed atypical mycobacterial infection) and one patient relapsed after four months. Similarly, one patient with CD4 counts of  $>200/\text{mm}^3$  at presentation, had an increased serum neopterin level at the end of treatment ( $120\text{nmol/l}$ ) with a corresponding decrease in CD4 cell counts to  $<200/\text{mm}^3$  and this was associated with the development of multiple opportunistic infections, over the period of her treatment. She developed recurrent episodes of diarrhoea, oral thrush and skin infestations. Although we observed a declining trend in other patients, the level of s-neo at the end of treatment did not reach the normal range and it was also higher than that found in HIV-negative TB patients despite good clinical and bacteriological response to treatment. This shows that there is persistent activation of cell-mediated immunity and the higher level at the end of treatment in HIV-TB patients indicates either co-existing opportunistic infections or progressive HIV disease.

The advantage of combining CD4+ T-lymphocyte counts with neopterin levels as a prognostic marker has been documented previously<sup>12</sup>. Our data and earlier reports show that s-neo level does not correlate very closely with CD4+ T-lymphocyte counts ( $r=0.45$  vs  $-0.39, -0.43, 0.41$ )<sup>11,19</sup>. The increase in the s-neo level has been shown to be largely independent of changes in CD4 T-lymphocyte counts in HIV infection. The CD4 levels showed an inverse relationship to the s-neopterin levels in most patients, both at baseline and at end of treatment. The number of patients in this study was not adequate to test the sensitivity of s-neopterin levels for predicting the decline in CD4 T cell count. Though s-neo estimation is easier to perform and less expensive as compared to CD4 count measurement, it does not replace CD4 count estimation. Combination of a single plasma activation marker measurement like neopterin with CD4 T-cell levels has been shown to improve the prognostic capability<sup>17</sup>. However, these markers, including neopterin are non specific and could be elevated due to a variety of causes in individuals with HIV infection. The limitations of our study are small sample size and a relatively short period of follow up. More studies are required to evaluate s-neopterin and other serum immune markers in patients with HIV and TB and to correlate the changes with CD4 T cell counts.

In conclusion, though neopterin is a non specific marker of cellular immune activation, we have shown that s-neo levels are elevated in HIV-seropositive compared to seronegative patients with tuberculosis. Further, the levels decline with effective anti-TB treatment, but remain well above the normal range in patients with HIV co-infection. The decrease in s-neopterin is most marked in patients with severe immunosuppression with CD4 T cell counts  $< 200$  cells/ $\text{mm}^3$ , indicating the benefits of early diagnosis and treatment of tuberculosis in this group. Further, rising levels of neopterin in serum are indicative of presence of another opportunistic infection or disease progression itself. Neopterin is stable in stored serum and is technically easy and cheaper to measure compared to viral load. More studies are required to assess its role in the monitoring of patients with HIV in developing countries, including those on anti-retroviral therapy.

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