Comparative Diagnostic Utility of Neopterin and IFN- γ /IL-2 in Extrapulmonary Tuberculosis

Nisha Goyal¹ · Bineeta Kashyap¹ · N. P. Singh¹ · Iqbal R. Kaur¹

Received: 19 August 2016 / Accepted: 9 November 2016 / Published online: 1 December 2016
© Association of Clinical Biochemists of India 2016

Abstract Resurgence of TB has emphasized the need for newer methods of diagnosis. Extrapulmonary tuberculosis (EPTB), being paucibacillary, is a diagnostic dilemma. The aim of the present study was to correlate IFN- γ /IL-2 with neopterin in diagnosis of EPTB. Extrapulmonary specimens from 69 clinically diagnosed cases were stained by Ziehl-Neelsen and cultured on Lowenstein–Jensen medium for *Mycobacterium tuberculosis*. ELISA was used to assess serum IFN- γ , IL-2 and neopterin levels. Median serum levels of IFN- γ /IL-2 and neopterin were 3.22 and 21.6 nmol/L in clinically diagnosed EPTB cases and 0.52 and 4.20 nmol/L in healthy controls respectively ($p < 0.001$). Both IFN- γ /IL-2 and neopterin were significantly higher in culture positive (14.64 and 49.8 nmol/L) than culture negative cases (3.01 and 17.5 nmol/L) respectively ($p < 0.05$). IFN- γ /IL-2 was significantly higher in AFB smear positive cases (8.63) than smear negative cases (3.04) ($p = 0.003$), whereas no significant difference in neopterin levels was seen ($p = 0.307$). A positive correlation between IFN- γ /IL-2 and neopterin was seen in EPTB cases (spearman's $\rho = 0.453$, $p < 0.001$), whereas in healthy controls no such correlation existed (spearman's $\rho = 0.018$, $p = 0.884$). An urgent need for research in the field of biomarkers exists to utilize them as point of care test in the diagnosis of EPTB.

Keywords Extrapulmonary tuberculosis · Neopterin · Interferon- γ · IL-2 · Biomarker · Cytokine

Introduction

The resurgence of tuberculosis (TB) in recent times has emphasized the immediate need for the newer methods for accurate and rapid diagnosis. Developing countries are still largely dependent on acid-fast staining or cultures of *Mycobacterium tuberculosis* for the diagnosis. These conventional methods of diagnosis often have low sensitivity, hence miss on a significant proportion of smear negative or extrapulmonary tuberculosis (EPTB) cases. EPTB due to its paucibacillary nature often presents with a diagnostic dilemma. Diagnosis of EPTB is made, in majority of cases, on the basis of clinical assessment and the response to treatment. The clinical markers generally used (i.e. extent of cavitation on chest radiography etc.) are unable to distinguish active from inactive TB and improvement on chest radiographs very often lags behind the overall response to treatment [1]. The recent association of EPTB with HIV has further outlined the need for accurate and timely diagnosis of EPTB.

Biomarkers involved in immuno-pathogenesis of TB can be helpful in monitoring the progression of disease activity, therapeutic outcome, reinfection or relapse. Host and pathogen biomarkers, detected in readily available body fluids, hold the potential to be useful as point of care diagnostic test in extrapulmonary tuberculosis. Interferon-gamma (IFN- γ) and Interleukin-2 (IL-2) are key pro-inflammatory cytokines involved in immuno-regulation of *M. tuberculosis* infection. These cytokines can be used as surrogate markers for T cell activation and thus can be utilized for the detection of disease activity. On stimulation

✉ Bineeta Kashyap
dr_bineetakashyap@yahoo.co.in

¹ Department of Microbiology, University College of Medical Sciences (UCMS), Guru Teg Bahadur (GTB) Hospital, Flat No. C-402, Vimal CGHS LTD., Plot-3, Sector-12, Dwarka, New Delhi, Delhi 100 078, India

with specific *M. tuberculosis* antigens, T cells release IFN- γ which is indicative of past or current TB infection. There is independent increase in the CD4 + IFN- γ effector T cells during active TB disease. This is supported by the recently published transcriptional signature for active TB, consisting mainly of an interferon gene profile [2]. IL-2 induces expansion in the pool of antigen specific lymphocytes and plays a pivotal role in generation of an immune response [3]. Thus the evaluation of IL-2 in subjects positive for IFN- γ may be helpful in discriminating active from latent TB [4]. The assessment of relative change in the serum levels of IFN- γ and IL-2 during the course of disease has been suggested to be more helpful tool for the discrimination of active tubercular disease [5, 6]. Active tuberculosis is characterized by increased rate of macrophage activation. Neopterin, being a macrophage activation marker, can prove to be a useful tool to monitor the disease activity. Macrophages secrete neopterin on activation through exposure to IFN- γ . Many studies have evaluated neopterin levels in various body fluids amongst TB cases as a marker to assess the extent, prognosis and response to anti-tuberculosis treatment [7, 8]. Moreover, the higher stability of neopterin in body fluids further substantiates its potential role in the diagnosis of EPTB [9].

We hypothesized that the ratio of serum levels of IFN- γ and IL-2 (IFN- γ /IL-2) correlated with neopterin levels in comparison to conventional diagnostic tests would be useful in the diagnosis of extrapulmonary tuberculosis. Thus, we correlated the IFN- γ /IL-2 with neopterin along with conventional diagnostic methods of AFB smear microscopy and mycobacterial culture in clinically diagnosed cases of extrapulmonary tuberculosis.

Materials and Methods

This study was carried out in a tertiary care hospital of east Delhi over duration of one year. Our study included 69 clinically diagnosed EPTB cases and an equal number of age and sex matched healthy subjects. This study was approved by the Institutional Ethical Committee and a written informed consent from each study subject prior to being included in the study was taken. A total of 207 specimens were collected including one serum specimen from all the 138 subjects enrolled in study and, one extrapulmonary specimen (depending on the site involved) from each of the 69 clinically diagnosed cases of EPTB. Aliquoted sera were stored at -80°C until further assessed for biomarkers under study. All sterile body fluid specimens were centrifuged and the sediments thus obtained were subjected to culture and ZN staining. FNAC specimens were minced and homogenised in sterile saline, then the sample was directly used for culture and ZN staining.

Urine specimens were decontaminated and centrifuged, before being subjected to conventional diagnostic procedures [10]. The extrapulmonary specimens obtained from cases were stained by Ziehl-Neelsen (ZN) technique for the detection of acid fast bacilli (AFB) and were simultaneously cultured on Lowenstein-Jensen (LJ) medium for the *M. tuberculosis* isolation.

Commercially available, Diaclone Human ELISA kit (France) was used for the detection of serum levels of IFN- γ and IL-2. The kits had the sensitivity of <5 pg/mL and 7 pg/mL respectively for individual markers. These assays utilized the principle of solid phase sandwich ELISA. IFN- γ /IL-2 was determined in all the clinically diagnosed EPTB cases and healthy controls by using the individual values of serum IFN- γ and IL-2 obtained by ELISA. Thereafter we analysed the ratio of serum levels of IFN- γ and IL-2 (IFN- γ /IL-2) and assessed it as a potential diagnostic circulating biomarker in our study. Serum neopterin levels were assessed with DRG, DRG Instruments GmbH, Human Neopterin ELISA kit (Germany) based on the principle of solid phase competitive ELISA. The interpretation of results in all the above assay was done by correlating the absorbance of reference controls and the samples.

Statistical Analysis

Analysis of differences in the distribution of biomarkers under study between the EPTB cases and healthy controls was performed by Mann-Whitney U test and p values were determined for analysing the statistical significance of the differences in biomarker distribution. Correlation between the concentrations of serum levels of biomarkers under study was evaluated by using the spearman's correlation coefficient (ρ).

Results

The study showed a slight female preponderance, with 36 (52.2%) of the 69 clinically diagnosed EPTB cases being females. The age range of EPTB cases extended from 18 years to 62 years with the largest proportion of 55.1% (38) being in age group 18–30 years followed by 27.5% (19) in 31–45 years age group, 14.5% (10) in 46–60 years age group and 2.9% (2) in 61–75 years age group. Maximum number of extrapulmonary specimens were of pleural fluid 26 (37.7%) and lymph nodes aspirate specimens 13 (18.8%), followed by CSF specimens 11 (15.9%), urine 9 (13%), ascitic fluid specimens 8 (11.6%) and pus specimens 2 (2.9%).

Of the 69 extrapulmonary specimens, AFB were demonstrated in 8 (11.6%) specimens by direct smear microscopy. Out of the total specimens positive by direct

demonstration of AFB (3 pleural fluid specimens, 3 urine specimens and 2 lymph node aspirate specimens), urine and pleural fluid specimens showed equal and highest positivity (37.5%). *M. tuberculosis* was isolated on culture in 9 out of the total 69 (13%) specimens from clinically diagnosed cases of extrapulmonary tuberculosis. 44.4% of the total isolates on culture were from pleural fluid specimens, followed by lymph node aspirates (33.3%) and pus (22.2%) specimens. Of the 8 specimens positive on direct demonstration of AFB by ZN technique, 3 (37.5%) were also positive by isolation of *M. tuberculosis* on culture. In the smear negative extrapulmonary specimens ($n = 61$), 6 specimens were positive for isolation of *M. tuberculosis* on culture. In 3 pleural fluid specimens that were positive for direct demonstration of AFB, only 1 could be isolated on culture. Among 4 pleural fluid specimens positive by isolation of *M. tuberculosis* on culture, 1 was also positive for direct demonstration of AFB. Out of the 3 lymph node aspirate specimens that were positive by isolation of *M. tuberculosis* on culture, 2 were also positive for direct demonstration of AFB. None of the urine samples were positive by isolation of *M. tuberculosis* on culture, though in 3 specimens AFB was demonstrated on direct demonstration by ZN technique. Similarly, the two pus samples positive by isolation on culture could not demonstrate AFB. None of the CSF or ascitic fluid specimens were positive either by culture or staining for AFB.

Comparison of Median serum Levels of IFN- γ /IL-2 and Neopterin Between the Cases and Healthy Controls

The median serum levels of IFN- γ /IL-2 in clinically diagnosed cases of EPTB ranged from 2.07 to 56.12 while in healthy controls it ranged from 0.05 to 1.54. Serum neopterin levels ranged from 4.2 nmol/L to >111 nmol/L in cases while 0.7–16.8 nmol/L amongst healthy controls. The median serum levels of IFN- γ /IL-2 and neopterin in clinically diagnosed EPTB cases and healthy controls were 3.22, 0.52, 21.6, 4.20 nmol/L respectively. The distribution

of median serum levels of IFN- γ /IL-2 and neopterin were found to significantly discriminate between the cases and the controls ($p < 0.001$).

Comparison of Median Serum Levels of IFN- γ /IL-2 and Neopterin with Conventional Diagnostic Methods

The median serum levels of IFN- γ /IL-2 was higher and significantly different in cases positive by direct demonstration of AFB by ZN staining or isolation of *M. tuberculosis* by culture than the cases that were negative by either of the conventional methods ($p < 0.05$).

Though distribution of median serum neopterin levels in the positive or negative cases by identification with direct demonstration of AFB was not significantly different ($p = 0.307$), levels were significantly different in the culture positive and negative cases ($p = 0.002$) (Table 1).

Correlation Between IFN- γ /IL-2 with Neopterin Among Cases and Controls

We have observed a positive correlation between the IFN- γ /IL-2 with serum levels of neopterin in the EPTB cases by spearman's correlation coefficient (spearman's $\rho = 0.453$, $p < 0.001$), whereas in healthy controls no such correlation existed (spearman's $\rho = 0.018$, $p = 0.884$) (Fig. 1).

Discussion

Tuberculosis remains one of the most important causes of death from an infectious disease. This disease is caused by the bacteria *Mycobacterium tuberculosis* and can involve almost every organ of the body. In our study direct demonstration of bacilli was done by acid-fast smear microscopy using ZN staining technique which is perhaps the easiest and most rapid procedure that can be performed in the laboratory. We documented AFB in 8 (11.6%) of 69

Table 1 Comparison of ratio of median serum levels of IFN- γ and IL-2 (IFN- γ /IL-2) and neopterin with various conventional diagnostic techniques ($n = 69$)

Conventional diagnostic technique	Ratio of median serum levels of IFN- γ and IL-2 (IFN- γ /IL-2)	p value	Median serum levels of neopterin (nmol/L)	p value
Direct demonstration of acid fast bacilli				
Positive group ($n = 8$)	8.63	0.003	30.4	0.307
Negative group ($n = 61$)	3.04		19	
Isolation of <i>M. tuberculosis</i>				
Positive group ($n = 9$)	14.64	<0.001	49.8	0.002
Negative group ($n = 60$)	3.01		17.5	

Fig. 1 Correlation between serum levels of IFN- γ /IL-2 with serum level of neopterin among the clinically diagnosed cases of extrapulmonary tuberculosis and healthy controls (n = 138)

extrapulmonary specimens and out of the total smear positive specimens, urine and pleural fluid showed the equal and highest positivity (37.5%). Makeshkumar et al. [11] in their study reported AFB smear microscopy positive in 10 out of 178 (5.6%) of extrapulmonary cases with highest positivity in lymph node aspirates followed by urine among all samples. However, another study observed 3 out of 141 (2.1%) of extrapulmonary samples to be positive by acid-fast smear microscopy with lymph node aspirate showing the highest positivity [12]. There must be at least 5000–10,000 bacilli/mL of specimen to allow detection of bacteria by AFB smear microscopy [13, 14]. The low positivity of smear microscopy could be due to the paucibacillary nature of extrapulmonary specimens and the irregular distribution of mycobacteria that have tendency to clump together in such samples [15].

We have demonstrated isolation of *M. tuberculosis* on culture in 9 of 69 (13%) extrapulmonary specimens with highest positivity in pleural fluid (44.4%), followed by lymph node aspirates (33.3%) and pus (22.2%) specimens. Rishi et al. [12] demonstrated isolation of *M. tuberculosis* in 4.96% of their EPTB samples with highest positivity in lymph node aspirates (50%) followed by pleural fluid (7.3%) and pus (5.9%) samples. However, Makeshkumar et al. [11] in their study could isolate *M. tuberculosis* in 6 out of 178 (3.4%) of samples with urine showing the highest positivity. Another study observed comparatively higher isolation rates of *M. tuberculosis* with 21.2% of specimens positive by isolation on culture, with pus samples showing highest isolation of *M. tuberculosis* in 12 out of 34 (35.3%) followed by pleural fluid samples (22.9%) [16]. Six samples in our study which were negative by ZN

staining, were positive for isolation of *M. tuberculosis* on culture which could be due to low mycobacterial load in these specimens as 10–100 bacilli/mL of the sample is sufficient for isolation on culture [15].

When the comparison between the two conventional diagnostic techniques of culture and acid fast staining was done in our study, isolation of *M. tuberculosis* on culture was found to be better (13%) than direct demonstration of AFB by ZN staining (11.6%). Similar finding of higher positivity by culture was reported by Iqbal et al. [16] in their study where 36 (21.2%) samples were positive by culture as compared to 12 (7.1%) samples that were positive by ZN staining out of 170 samples in EPTB. Another study however reported a very low positivity by both the conventional techniques (5% by culture as compared to AFB smear positivity of 2.1%) [12].

We have analysed the potential of IFN- γ /IL-2 and neopterin estimation in the diagnosis of EPTB. Our results revealed a higher median serum levels of IFN- γ /IL-2 in EPTB cases than the healthy controls. IFN- γ /IL-2 was significantly different between the two groups. In an extension to our present study, we have previously reported the diagnostic potential of IFN- γ and IFN- γ /IL-2 in EPTB, although IL-2 demonstrated a limited utility. The same study reported IFN- γ /IL-2 to be the best discriminatory biomarker with the highest area under the ROC curve rather than the individual IFN- γ or IL-2 serum levels [6]. Our findings are in agreement with the observations of Nemeth et al. [5] who have demonstrated a relative shift in T cell from the production of IL-2 towards IFN- γ in active tuberculosis. They further suggested that the estimation of IFN- γ /IL-2 may be a more useful tool than analysing any

of these single cytokine. Sargentini et al. [4] have also reported that there is an increase in number of IL-2 secreting central memory T cells or IL-2/IFN- γ and reduction in number of IFN- γ -secreting effector T cells in latent TB infection in comparison to active TB disease. Suter-Riniker et al. have suggested that the simultaneous assessment of IFN- γ and IL-2 in plasma supernatants can permit the detection of shifts in *M. tuberculosis*-specific T-cell signatures. They have further revealed that a significantly elevated IL-2/IFN- γ can be useful as a marker of elimination of *M. tuberculosis* infection following successful treatment [17].

Our analysis documented significantly higher levels of median serum levels of neopterin in the EPTB cases. Our results demonstrated a median serum neopterin level of 21.6 nmol/L in clinically diagnosed EPTB cases and 4.2 nmol/L in healthy controls. Our observations were in agreement with the findings of Cesur et al. describing the serum mean neopterin levels of 23.74 ± 21.8 nmol/L (median: 18.3) in newly diagnosed pulmonary tuberculosis patients and 4.03 ± 5.12 nmol/L (median: 5.1) in healthy subjects. The serum neopterin levels in this study were also significantly higher in patients with tuberculosis than the control group. They further demonstrated a statistically significant correlation between neopterin positivity and clinical symptoms of hemoptysis and weight loss [18]. Immanuel et al. [19] also reported a significantly higher serum neopterin levels in active tuberculosis than in healthy controls. Elevated neopterin levels in TB have been well reported in the literature and various studies have investigated neopterin levels in response to anti-tuberculosis treatment, its correlation with the disease activity or its comparison with healthy individuals or non-tuberculous diseases, such as pneumonia and malignancies [7, 8]. Significantly elevated levels of serum, pleural fluid and urinary neopterin have been documented in tubercular pleuritis patients as compared to patients with non-tuberculous pleural effusion [19].

Despite our exhaustive search for the previous research assessing the correlation of IFN- γ /IL-2 with neopterin in TB patients, we were unable to find any relevant data. Our study has revealed a positive correlation between the IFN- γ /IL-2 with neopterin in the EPTB case, whereas no such correlation was seen in healthy controls. Further research is needed to validate our findings.

Conclusions

This study has demonstrated a positive correlation between IFN- γ /IL-2 and neopterin in EPTB cases. We have observed a significant difference in the serum levels of biomarkers under study in culture positive and negative cases. IFN- γ /IL-2 was also significantly discriminatory in

AFB smear positive and negative cases, though same could not be demonstrated with neopterin. A significant discrimination between EPTB cases and healthy controls by the serum levels of these biomarkers suggest a potential for their use as point of care test in the diagnosis of EPTB as serum specimens can be collected much easily as compared to other extrapulmonary specimens. Our study has been done only in a single center with limited number of subjects. More exhaustive studies involving multiple centers and larger number of subjects are needed to substantiate our observations. There is urgent need for research in the field of biomarkers for the diagnosis of EPTB as they hold the potential of being used as point of care test.

Acknowledgements Dr. Rahul Sharma for statistical analysis and Dr. Rajat Jhamb for clinical specimens.

Compliance with Ethical Standards

Conflict of interest None.

References

- Perrin FM, Lipman MC, McHugh TD, Gillespie SH. Biomarkers of treatment response in clinical trials of novel antituberculosis agents. *Lancet Infect Dis*. 2007;7:481–90.
- Mori T, Sakatani M, Yamagishi F, Takashima T, Kawabe Y, Nagao K, et al. Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens. *Am J Respir Crit Care Med*. 2004;170:59–64.
- Blanchard DK, Michelini-Norris MB, Friedman H, Djeu JY. Lysis of mycobacteria-infected monocytes by IL-2 activated killer cells: role of LFA-1. *Cell Immunol*. 1989;119:402–11.
- Sargentini V, Mariotti S, Carrara S, Gagliardi MC, Teloni R, Goletti D, et al. Cytometric detection of antigen-specific IFN- γ /IL-2 secreting cells in the diagnosis of tuberculosis. *BMC Infect Dis*. 2009;9:99.
- Nemeth J, Winkler HM, Zwick RH, Müller C, Rumetshofer R, Boeck L, et al. Peripheral T cell cytokine responses for diagnosis of active tuberculosis. *PLoS ONE*. 2012;7:e35290.
- Goyal N, Kashyap B, Kaur IR. Significance of IFN- γ /IL-2 ratio as a circulating diagnostic biomarker in extrapulmonary tuberculosis. *Scand J Immunol*. 2016;83(5):338–44.
- McNerney R, Daley P. Towards a point-of-care test for active tuberculosis: obstacles and opportunities. *Nat Rev Microbiol*. 2011;9:204–13.
- Akiibinu MO, Ogunyemi EO, Shoyebo EO. Levels of oxidative metabolites, antioxidants and neopterin in Nigerian pulmonary tuberculosis patients. *Eur J Gen Med*. 2011;8:213–8.
- Tozkoparan E, Deniz O, Cakir E, Yaman H, Ciftci F, Gumus S, et al. The diagnostic values of serum, pleural fluid and urine neopterin measurements in tuberculous pleurisy. *Int J Tuberc Lung Dis*. 2005;9:1040–5.
- Tille PM. Mycobacteria and other bacteria with unusual growth requirements. In: Bailey & Scott's diagnostic microbiology. 13th ed. Missouri: Mosby Elsevier Inc; 2014. p. 487–512.
- Makeshkumar V, Madhavan R, Narayanan S. Polymerase chain reaction targeting insertion sequence for the diagnosis of extrapulmonary tuberculosis. *Indian J Med Res*. 2014;139:161–6.

12. Rishi S, Sinha P, Malhotra B, Pal N. A comparative study for the detection of mycobacteria by BACTEC MGIT 960, Lowenstein Jensen media and direct AFB smear examination. *Indian J Med Microbiol.* 2007;25(4):383–6.
13. Watt B, Rayner A, Harris G. *Mycobacterium*. In: Collee JG, Marmion BP, Fraser AG, Simmons A, editors. *Mackie and McCartney practical medical microbiology*. 14th ed. New Delhi: Elsevier, Reed Elsevier India Private Limited; 2012. p. 329–42.
14. Allen S, Janda W, Koneman E, Procop G, Schreckenberger RC. Introduction to microbiology: part II. In: Winn W Jr, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, et al., editors. *Koneman's color atlas and textbook of diagnostic microbiology*. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2006. p. 67–110.
15. Chawla K, Gupta S, Mukhopadhyay C, Rao PS, Bhat SS. PCR for *M. tuberculosis* in tissue samples. *J Infect Dev Ctries.* 2009;3:83–7.
16. Iqbal S, Ahmed R, Adhami SZ, Mumtaz A. Importance of polymerase chain reaction in diagnosis of pulmonary and extra-pulmonary tuberculosis. *J Ayub Med Coll Abbottabad.* 2011;23(1):73–6.
17. Suter-Riniker F, Berger A, Mayor D, Bittel P, Iseli P, Bodmer T. Clinical significance of interleukin2/gamma interferon ratios in *Mycobacterium tuberculosis*-specific T-cellsignatures. *Clin Vaccine Immunol.* 2011;18(8):1395–6.
18. Cesur S, Aslan T, Hoca NT, Çimen F, Tarhan G, Çifçi A, et al. Clinical importance of serum neopterin level in patients with pulmonary tuberculosis. *Int J Mycobacteriol.* 2014;3(1):5–8.
19. Immanuel C, Rajeswari R, Rahman F, Kumaran PP, Chandrasekaran V, Swamy R. Serial evaluation of serum neopterin in HIV seronegative patients treated for tuberculosis. *Int J Tuberc Lung Dis.* 2001;5(2):185–90.