Serum enolase-2, high-sensitivity C-reactive protein, and serum cholesterol in smear-positive drug-naïve pulmonary tuberculosis

Suhail Ahmed Almani, Tariq Zaffar Shaikh, Haji Khan Khoharo[1], Ikramuddin Ujjan[2]

Department of Medicine, Liaquat University of Medical and Health Sciences, Jamshoro, Pakistan, [1]Faculty of Medicine and Allied Medical Sciences, Isra University, Hyderabad, Sindh, [2]Department of Pathology, Liaquat University of Medical and Health Sciences, Jamshoro, Pakistan

Background: Pulmonary tuberculosis (PTB) is a chronic granulomatous disease caused by *Mycobacterium tuberculosis*. The present study determined the serum human enolase-2 (ENO-2), high-sensitive C-reactive protein (hs-CRP), and serum cholesterol levels as biological marker of disease activity and treatment response in smear-positive drug-naïve PTB. **Materials and Methods:** This case–control study was done in the Department of Medicine, Liaquat University of Medical and Health Sciences (LUMHS), Jamshoro/Hyderabad, Sindh, from January 2015 to April 2016. Thirty-five sputum smear-positive drug-naïve PTB patients and thirty controls were studied. MTB culture and drug sensitivity were performed at the Diagnostic and Research Laboratory of LUMHS. Serum ENO-2, hs-CRP, and serum cholesterol were estimated at baseline, 3rd and 6th month of antituberculosis (TB) therapy. **Results:** Serum ENO-2 and hs-CRP were found raised in PTB compared to controls and showed decrease of 13% and 21.55%, 19.6% and 31.5% at 3rd and 6th month, respectively ($P = 0.0001$). Serum ENO-2 revealed positive correlation with hs-CRP ($r = 0.734$, $P = 0.0001$), and serum cholesterol revealed negative correlation with ENO-2 and hs-CRP ($r = -0.509$, $P = 0.0001$) and ($r = -0.566$, $P = 0.0001$), respectively. **Conclusion:** The present study reports the baseline ENO-2 and hs-CRP were raised, and serum cholesterol was low in smear-positive PTB patients and the ENO-2 and hs-CRP were reduced by anti-TB drug therapy.

Key words: Enolase-2, high-sensitive C-reactive protein, pulmonary tuberculosis, serum cholesterol

INTRODUCTION

Pulmonary tuberculosis (PTB) is a chronic granulomatous disease of bacterial origin. *Mycobacterium tuberculosis hominis* (MTB) is an acid-fast bacillus (AFB) implicated in the PTB.[1,2] In 2011, Pakistan ranked fifth among 22 countries in terms of absolute number of tuberculosis (TB) cases. Estimated incidence and prevalence rate were reported as 231/100,000 and 364/100,000 population, respectively.[3,4] Available drug therapy has problems in particular of multidrug resistance.[5,6] Sputum culture and sensitivity, Ziehl–Neelsen (ZN) staining, chest roentgenography, and MTB culture are common methods of diagnosis, but still many cases are not diagnosed by these methods.[7] Enolase-2 (ENO-2) is the phosphopyruvate hydratase enzyme of glycolysis of neuron origin; hence, it is also known as ENO-γ or the neuron-specific enolase (NSE).[8,9] ENO-2 is found in various tissues including the brain, macrophages, neuroendocrine cells, neuroendocrine cells tumors, such as the small cell lung cancer,[10,11] active PTB,[12] and poor neurological outcome in cardiac arrest patients.[13] The high-sensitivity C-reactive protein (hs-CRP) is an acute phase protein biomarker for inflammatory disease.[14] Diagnosis of PTB is still a diagnostic dilemma in developing countries, particularly the smear-negative cases. Therefore, alternative diagnostic methods should be searched for PTB. The present study postulated the ENO-2 of macrophage origin may prove a biological marker of PTB disease activity and treatment response because macrophage

Address for correspondence: Dr. Haji Khan Khoharo, Faculty of Medicine and Allied Medical Sciences, Isra University, Hyderabad, Sindh, Pakistan. E-mail: drhajikhan786@gmail.com

plays major role in granulomatous inflammation. The present study intended to measure the ENO-γ, hs-CRP, and serum cholesterol in smear-positive PTB as biological marker of disease activity and treatment response.

MATERIALS AND METHODS

The present case–control study was conducted at the Department of Medicine, Liaquat University of Medical and Health Sciences (LUMHS), Jamshoro/Hyderabad, Sindh, Pakistan. The study commenced from January 2015 to April 2016.

Inclusion criteria

Sputum smear-positive drug-naïve PTB patients, age 20–60 years, and either gender were included in this prospective study.

Exclusion criteria

Smear-negative PTB, extra PTB, chronic PTB cases, anti-TB drug defaulters, and multidrug resistance cases were excluded from the study. PTB with concomitant diabetes mellitus, chronic viral hepatitis, chronic kidney disease, renal failure, ischemic heart disease, and pregnancy were also excluded from the study.

Study groups

A sample of 35 smear-positive PTB and 35 age-matched controls was selected.
- Group A (*n* = 35). Smear-positive PTB patients
- Group B (*n* = 35). Normal healthy, age- and gender-matched participants taken as controls.

Mycobacterium tuberculosis culture and drug sensitivity

MTB culture and drug sensitivity were performed at the Diagnostic and Research Laboratory of LUMHS. Auramine–Rhodamine staining was employed for screening of smears by microscopy. A positive slide was confirmed by the ZN stain. Both liquid and solid media were used for the *Mycobacterium* cultures. The sediments were cultured on Lowenstein–Jensen (LJ) medium and MGIT (Becton Dickinson Diagnostic Instruments Systems) at 37°C temperature. 0.1 ml of concentrated specimen was inoculated on the LJ medium and incubated for 8 weeks.

0.5 ml of concentrated specimens was inoculated in the MGIT vials. Vials were supplemented with OADC and PANTA which contained the nalidixic acid, polymyxin B, amphotericin B, azlocillin, and trimethoprim. Vials were incubated at 37°C temperature. *Mycobacterium* growth from +ve LJ and MGIT vials were stained ZN stain. Identification of MTB was done by "BD BACTEC NAP TB" differentiation test (Becton Dickinson and Company, USA).

Drug susceptibility testing was performed by an agar proportion method on enriched Middlebrook 7H10 medium (BBL Microbiology Systems, Cockeysville, MD, USA) at the following concentrations: Rifampicin 1 μg/ml, isoniazid (INH) 0.2 μg/ml, ethambutol 5 μg/ml and streptomycin 2 μg/ml. Pyrazinamide (PZC) sensitivity was estimated on BACTEC 7H12 medium at 100 μg/ml (pH 6.0) (BACTEC PZA test medium, BD). Control reference strain of MTB H37Rv was used in drug susceptibility test batch.[15,16]

Anti-tuberculosis drug therapy

Standard anti-TB drug therapy was started. Initial intensive phase of 2 months of four-drug regimen, including INH, rifampin, ethambutol, and PZA followed by three-drug regimen (PZA discontinued) for another 4 months.

Blood sampling and biochemical testing

Blood samples were taken at baseline, 3rd and 6th month of anti-TB therapy. Ten-milliliter blood was taken by venepuncture into a disposable syringe (BD, USA) and processed properly. Blood centrifugation was performed at 4000 rpm for 15 min. Sera were stored at temperature of −20°C for the detection of serum ENO-2, serum hs-CRP, and serum cholesterol. ENO-2 was measured by "Quantikine Human ENO-2" solid phase ELISA assay. The quantitative hs-CRP was determined by Lumax CLIA Strip Reader (Model-4100) machine by the "chemiluminescence" immunoenzymometric assay. Roche cholesterol assay was carried out according to the NIH instructions 1992. The assay was carried out by standard method as per instructions using Roche Hitachi analyzer.

Ethical approval and consent form

Ethical approval was taken from the Advanced Review Board and Ethics Committee of LUMHS Jamshoro. The study was conducted according to the guideline of the "Declaration of Helsinki" for human studies. Consent form was mandatory for the participation of volunteer's participants.

Statistical analysis

Data were analyzed on Statistix 10.0 software (Tallahassee, FL 32317, USA). Paired sample *t*-test and Chi-square test were used for the analysis of continuous (ENO-2, serum cholesterol, and hs-CRP) and categorical (gender) variables, respectively, at 95% confidence interval ($P \leq 0.05$). Pearson's correlation was used for correlation of ENO-2, hs-CRP, and serum cholesterol.

RESULTS

Age, body weight, systemic blood pressure, random blood glucose, and erythrocyte sedimentation rate are shown in

Table 1. Gender distribution shows 27 (77.1%) males and 8 (22.8%) females, 26 (74.2%) males 7 (20%) females in control and PTB cases, respectively ($P = 0.92$).

Serum ENO-2, hs-CRP, and serum cholesterol showed statistically significant differences at 3rd and 6th month of anti-PTB therapy compared to baseline [Table 2]. The serum ENO-2 and hs-CRP showed decrease of 13% and 21.55%, 19.6% and 31.5% at 3rd and 6th month, respectively. Serum cholesterol showed a rise from baseline 105.3 ± 9.3 to 155.7 ± 15.5 mg/dl at 6th month (a 14.8% rise) ($P = 0.0001$).

Table 3 shows the correlation coefficient of ENO-2, hs-CRP, and serum cholesterol. Serum ENO-2 revealed positive correlation with hs-CRP ($r = 0.734$, $P = 0.0001$), while serum cholesterol revealed negative correlation with ENO-2 and

hs-CRP ($r = -0.509$, $P = 0.0001$) and ($r = -0.566$, $P = 0.0001$), respectively. Scatter plots 1 and 2 show the correlation distribution curve of ENO-2, hs-CRP and serum cholesterol.

DISCUSSION

The present study is the first research being reported from our tertiary care hospital, which caters thousands of patients a year. PTB is a major public health issue of Pakistan. A reliable biological marker of PTB and treatment response is major problem for clinicians in developing countries, which often result in treatment failures, drug resistance, and mortality. Chest roentgenographs, ZN staining, and cultures are available methods; however, in Pakistan, these facilities are not available except for the tertiary care hospitals. Mycobacteria culture takes more weeks to yield the results.[17] All these have limitations, for example, radiographical improvement of lung tissue does not accurately correlate with PTB disease activity. ZN staining has proved poor sensitivity in terms of sufficient number of bacteria be present in the specimen for positive acid-fast bacilli reaction.[18] MTB culture and sensitivity takes time of 8 weeks, and clinicians are waiting for several weeks, and patients also do not comply with this strategy.[19] This often leads to delayed treatment at the cost of loss of patient confidence because of symptoms aggravate and disease may become advanced. There is a practical gap for the clinicians what to do in such situations. This needs some novel biological disease marker for diagnosis to initiate the therapy at the earliest, progression or regression of disease activity, and treatment response.

Serum ENO-2 has been reported as a disease marker for small cell carcinoma lungs, neuroblastoma, retinoblastoma,[10,11] and meningitis.[20,21] At present, sufficient evidence is present to use ENO-2 as disease marker in malignant and nonmalignant disorders, such as PTB.[10,11] However, role ENO-2 in PTB diagnosis and treatment response needs

Table 1: Demographic characteristics of the study participants (*n*=70)

Study groups	Mean±SD	P
Age (years)		
Group A: Controls	51.9±4.8	0.50
Group B: PTB	52.8±6.0	
Body weight (kg)		
Group A: Controls	57.7±13.6	0.020
Group B: PTB	51.9±4.8	
Systolic BP (mmHg)		
Group A: Controls	129.4±10.4	0.0001
Group B: PTB	120.3±3.8	
Diastolic BP (mmHg)		
Group A: Controls	78.4±7.1	0.004
Group B: PTB	73.7±6.2	
RBG (mg/dl)		
Group A: Controls	136.9±11.2	0.0001
Group B: PTB	105.3±9.3	
ESR (mm)		
Group A: Controls	11.9±1.7	0.0001
Group B: PTB	54.0±27.0	

RBG = Random blood glucose; ESR = Erythrocyte sedimentation rate; PTB = Pulmonary tuberculosis; SD = Standard deviation; BP = Blood pressure

Table 2: Serum enolase-2, high-sensitive C-reactive protein, and serum cholesterol (*n*=70)

Variables	Study groups	Mean±SD	95% CI for mean		P
			Lower bound	Upper bound	
Enolase-2 (ng/dl)	Controls*	9.5±1.5	9.0	10.1	0.0001
	Baseline - PTB	22.0±3.1	21.0	23.1	
	3rd month - PTB	16.8±4.2	15.3	18.2	
	6th month - PTB*	10.2±1.8	9.6	10.8	
hsCRP (mg/L)	Controls*	1.8±0.9	1.5	2.1	0.0001
	Baseline - PTB	6.3±1.1	5.9	6.7	
	3rd month - PTB	3.2±1.2	2.8	3.6	
	6th month - PTB*	2.0±0.8	1.7	2.3	
Serum cholesterol (mg/dl)	Controls	136.9±11.2	133.1	140.8	0.001
	Baseline - PTB	105.3±9.3	102.1	108.5	
	3rd month - PTB	121.6±25.3	112.9	130.3	
	6th month - PTB	155.7±15.5	150.3	161.0	

*P-value nonsignificant. CI = Confidence interval; hsCRP = High-sensitivity C-reactive protein; PTB = Pulmonary tuberculosis; SD = Standard deviation

further exploration. The serum ENO-2 and hs-CRP showed decrease of 13% and 21.55%, 19.6% and 31.5% at 3rd and 6th month, respectively. Serum cholesterol showed a rise from baseline 105.3 ± 9.3 to 155.7 ± 15.5 mg/dl at 6th month (a 14.8% rise) [Table 2 and Graphs 1-3]. The findings of serum ENO-2 correlated with pulmonary disease activity and treatment response is in keeping with the previous study.[12]

Serum ENO-2 revealed positive correlation with hs-CRP (r = 0.734, P = 0.0001), while serum cholesterol revealed negative correlation with ENO-2 and hs-CRP (r = −0.509, P = 0.0001) and (r = −0.566, P = 0.0001), respectively. Nam *et al.*[12] also reported positive correlation of serum ENO-2 and hs-CRP; hence, findings are consistent.

Serum hs-CRP is a validated nonspecific marker of inflammation; hence, it was also measured. Serum hs-CRP is an acute phase protein produced by hepatocyte in response to inflammation.[22-24] Serum hs-CRP showed positive correlation with ENO-2 (r = 0.734, P = 0.0001) [Graph 1]. The findings are in keeping with a recently published study.[12] Serum ENO-2, hs-CRP, and serum

cholesterol showed statistically significant differences at 3rd and 6th month of anti-PTB therapy compared to baseline [Table 2 and Graphs 1-3]. Hypocholesterolemia is a risk factor for PTB, mortality in miliary TB, and poor therapeutic response.[25]

The evidence-based findings of the present study show the ENO-2 may be useful tool for PTB diagnosis, disease progression, and treatment response. Further research, is needed to set cutoff values of NSE and hs-CRP for PTB.

As the TB is rising in the developing countries like Pakistan, where latent and multidrug-resistant mycobacteria are prevailing and cultures and AFB staining often fail absolutely; hence, there is need for serological tests such as serum ENO-2 but it needs validation. Diagnosis and treatment of latent TB is an area of challenge for the treating physicians[26,27] if this problem is overcome by novel biological marker such as ENO-2 it will be a great success. The exact origins of ENO-2 are not certain in PTB; however, macrophages is supposed to secrete the ENO-2. The macrophage plays key role in granulomatous inflammation-like TB;[28] hence, it was hypothesized as an ultimate source of ENO-2.

The present study reports two important findings, first - the baseline ENO-2 and hs-CRP were raised, and serum cholesterol was low in smear-positive PTB patients compared to controls, second - a decrease in ENO-2 and hs-CRP with a concomitant increase in serum cholesterol was observed after anti-TB drug therapy. The findings point toward the fact that the ENO-2 and hs-CRP may be used as biological disease marker and treatment response for PTB. One of the main limitations of the present study is a small sample size; however, this study will prove a guide for future studies of ENO-2 in the diagnosis of smear-negative PTB.

Table 3: Pearson's correlation of enolase-2, high-sensitivity C-reactive protein and serum cholesterol (*n*=70)

|	Enolase	hs-CRP (mg/L)	SC (mg/dl)
Enolase-2 (ng/dl)			
$r^{††}$	-	0.734**	−0.713**
P	-	0.0001	0.0001
hsCRP (mg/L)			
$r^{††}$	0.734**	-	−0.566**
P	0.0001	-	0.0001
SC (mg/dl)			
$r^{††}$	−0.713**	−0.566**	-
P	0.0001	0.0001	-

**Correlation is significant at the 0.01 level (two-tailed), $^{††}r$ = Correlation co-efficient. SC = Serum cholesterol; hsCRP = High-sensitivity C reactive protein
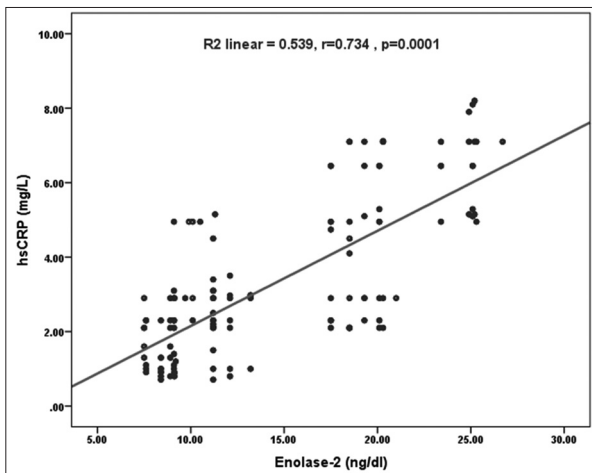
Graph 1: Scatter plot showing positive correlation of serum enolase-2 and high-sensitivity C-reactive protein
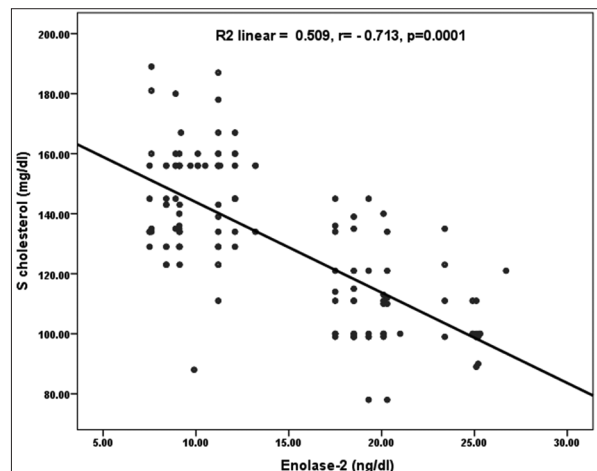
Graph 2: Scatter plot showing negative correlation of ENO-2 and S Cholesterol
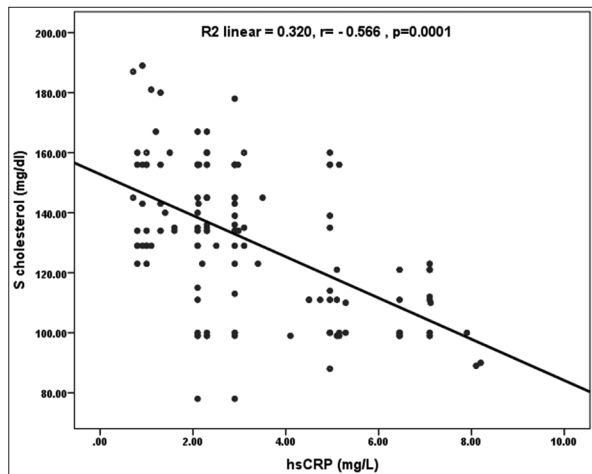
Graph 3: Scatter plot showing negative correlation of hs-CRP and S. cholesterol

CONCLUSION

The present study reports the baseline serum ENO-2 and hs-CRP were raised, and serum cholesterol was low in smear-positive PTB patients, and the serum ENO-2 and hs-CRP were decreased by anti-TB drug therapy. These both findings direct toward the fact that the serum ENO-2 and hs-CRP may be used as biological disease marker and treatment response for PTB.

Conflicts of interest
There are no conflicts of interest.

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