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# Study of Inflammatory Markers in Sputum Positive Patients of Pulmonary Tuberculosis and its Response to Anti-tubercular Treatment

Authors
**Mohd Yousoof Dar*[1], B K Menon[2], Sarfaraz Jamal[3]**
[1]Senior Resident (MD, DM)*, [2]Professor (MD), [3]Resident (MBBS)
Department of Pulmonary Medicine, VPC (Vallabhai Patel Chest Institute), Delhi University 10009
*Corresponding Author
**Mohd Yousoof Dar**
Permanent Address: 1/3/SRS/Lane 16, House Number -044, Syed Rehman Sahabbrein, Srinagar Kashmir Pin Code - 191123 INDIA
Email: *yousufhumi@gmail.com*

**Abstract**
*Studies have shown that active tuberculosis is associated with elevated systemic inflammatory markers like ESR, CRP, IL-6, SAA, TNF-a, IFN-γ and many others. We aim to evaluate the serum levels of serum CRP and IFN-γ in active pulmonary tuberculosis (APTB) and the possible effects of ATT treatment on these cytokine levels. A total of 54 patients with the diagnosis of APTB and 20 healthy volunteers as controls were taken for study. The study showed that patients of APTB have significantly elevated baseline levels of serum CRP levels (mean+SD) of 21.71± 6.37mg/dl and serum IFN-γ (mean+SD) of 41.47±43.25 pg/ml compared to controls with serum CRP levels of ≤ 5mg/dl and serum IFN-γ of 1.81±1.6pg/ml pg/ml respectively. Both serum CRP and serum IFN-γ levels decreased significantly with duration of treatment with CRP levels of 7.7±4.23 mg/dl at 2 months vs 2.41±1.5mg/dl at end 6 months and serum IFN-γ of 12.53±8.33 pg/ml at 2 months and 2.47±1.55 pg/ml at end of 6 months of ATT respectively with a statistically significant of p value < 0.00. Sputum IFN-γ levels were also elevated in cases with baseline values of 24.92±16.53 pg/ml which decreased to 7.68±5.17 pg/ml after 2 months and further to 3.86±3.12 pg/ml at end of 6 months of ATT respectively with a statistically significant of p value < 0.00.*
*In conclusion, our observations reveal that patients with APTB have significantly elevated serum CRP and IFN-γ levels compared to normal healthy individuals and these levels decrease significantly with duration of treatment. Therefore serum CRP and IFN-γ levels may be useful adjuvant markers in cases of APTB to monitoring response to treatment besides clinical and radiological feature who cannot produce adequate sputum in follow up.*
**Keywords**: *APTB: active pulmonary tuberculosis, ATT: anti-tubercular treatment, CRP: C-reactive protein, IFN-γ: Interferon gamma.*

## Introduction

Tuberculosis (TB) remains one of the world's deadliest communicable diseases caused by various strains of mycobacteria, usually *Mycobacterium tuberculosis*.[1] One third of the

world's population is estimated to be infected with *M. tuberculosis*,[2] with new infections occurring in about 1% of the population each year.[3] India is the highest TB burden country in the world with estimated incidence figure of 2.2 million cases of TB out of a global incidence of 8.7 million cases.[4]

The interaction of T cells with infected macrophages is central to protective immunity against M tuberculosis and depends on the interplay of cytokines produced by each cell.[5] TH^1 cells and natural killer (NK) cells secrete IFN-γ, which activates alveolar macrophages to produce a variety of substances (e.g; TNF-α) involved in growth inhibition and killing of mycobacteria. At physiological levels, TNF-α has anti-microbial activities through the apoptotic effect. Excessive production of TNF-α have been implicated in immunopathogenesis of tuberculosis. Macrophages also secrete IL-12, which drives antigen-naive TH^ cells towards development into TH^1 cells thus amplifying this pathway in a positive feedback loop.[6,7]

**Material and Methods**

This study was conducted at the Clinical Research Centre, Vallabhbhai Patel Chest Institute (VPCI), University of Delhi over a period of one year after ethical clearance from research committee. A total of 54 patients with the diagnosis of sputum positive pulmonary tuberculosis were recruited after taking a written consent from all patients. Six patients were excluded form study as they withdrew the consent. Also 20 healthy volunteers were taken as normal control for measurement of Serum CRP levels and serum IFN-γ.

The diagnosis of all cases was considered on clinical history and examination consistent with disease. Diagnosis was confirmed by sputum microscopy for AFB and culture sensitivity. Also all patients underwent CBNAAT (cartridge based nucleic acid amplification test), called Gene expert- MTB. Patients who turned out to be rifampicin resistant were excluded from the study. In order to prevent effect of other confounding factors, patients with following characterised were excluded from study: patients with any systemic disease such as hypertension or patient on antihy-pertensive medications, cardiovascular disease, diabetes mellitus/ acute hyper-glycemia, hepatic, renal diseases or HIV positive patients, pregnant and lactating female, past history of tuberculosis or ATT intake or extra-pulmonary disease.

Serum high sensitive C-reactive protein (hs CRP - mg/l) as well as sputum and serumInterferon-gamma (IFN γ - pg/ml) were measured by ELISA method in all the cases at baseline, at 2 months (completion of intensive phase) and at completion of treatment (6 months).

All the subjects received standard Anti-tubercular therapy as per RNTCP (cat-1) guidelines on daily basis. All subjects were followed up and parameters assessed were clinical, radiological and bacteriological response at completion of intensive phase (2 month) and again at completion of treatment. Majority of patients developed mild gastrointestinal problems in the form of nausea, aversion to food and indigestion which resolved in majority within 1-2 weeks with symptomatic treatment. Two patients developed symptomatic liver dysfunction at start of treatment which resolved within 10-14 days after withholding treatment. Both patients received again same first line treatment without any modification.

**Methods**

Sputum samples were collected and processed with some modifications of Bhownick's method.[8] Sputum samples were examined for adequacy as soon as possible, within two hours. Sputum around 2ml was taken and then mucus was digested with Petroff's method.[9] This sputum was then mixed with equal amounts of balanced salt solution (BSS). This was vortexed for 15 seconds and then centrifuged for 15 minutes and stored at -80 degree. Later supernatant was used (1ml) was used to measure Interferon- γ levels.

For measurement of serum CRP and serum IFN-γ levels, 10 ml of blood was withdrawn under all aseptic precautions and levels were measured on

TECAN automated ELISA reader using respective ELISA kits based on Quantitative sandwich immunoassay as per the manufacturer's instructions.

**Statistical Analysis**

The data analysis was performed using SPSS statistical package version 15.0 windows (SPSS, Chicago IL, USA). The data was examined for distribution and homogeneity of variance was checked before applying parameters tests, ANOVA, unpaired t –test, Mann –Whitney, chi-square test and Fischer exact test were used for data analysis. Level of significance was set at ≤0.05.

**Results**

Mean age of cases (33.02±16.9 years) and controls (30.30±11.86 years) was comparable with p value of 0.308. There was almost equal number of males (48 %) and females (52%) in the study group. Almost 2/3rd of cases were having leucocyte counts within normal limits and around 1/3rdwere having leukocytosis (>11,000/ul) with only 4.17% were having leukocyte count above 15,000/microliter. Leukocytosis was correlating with disease severity, those presenting with bilateral disease and cavitatory disease on chest x-ray and significant constitutional symptoms were having leukocytosis compared to other cases with less disease burden. X-ray was correlated with clinical status of the patient. Overall consolidation was most common presentation followed by cavitation which was seen in 16 % of cases.

Normal chest x- ray was seen in 2 cases despite being sputum positive for MTB.(figure 1)

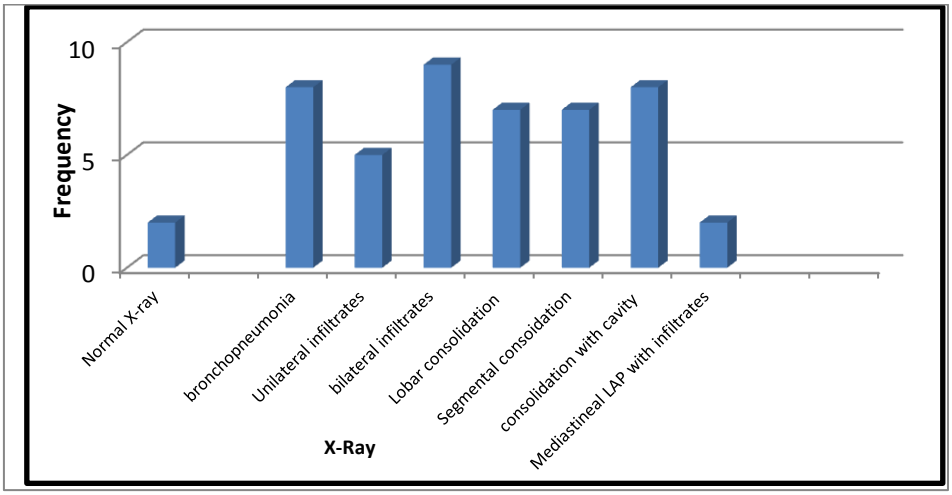


**Figure 1** X-ray features in the study population at base line

Serum CRP levels (Table-1 below) were done in controls and cases at baseline and were repeated in cases at 2 months and 6 months after ATT. About 75% of cases were having CRP levels above 25mg /dl and there is statistically significant difference between cases and controls (p value < 0.001), as 98 % ( 47/48) of cases are having serum CRP levels > 5mg/dl while 100% (20/20) of controls are having serum levels ≤ 5mg/dl(figure 2).

**Table 1** Serum CRP levels in controls and cases at baseline

| CRP mg/dl (O month) at baseline | Cases | | Controls | | p-value |
|---|---|---|---|---|---|
| | N | % | N | % | |
| ≤ 5 | 1 | 2.08% | 20 | 100.00% | <0.001 |
| 5 – 15 | 8 | 16.67% | 0 | 0.00% | 0.082 |
| 16 – 25 | 3 | 6.25% | 0 | 0.00% | 0.208 |
| > 25 | 36 | 75.00% | 0 | 0.00% | <0.001 |
| **TOTAL** | **48** | **100%** | **20** | **100%** | |

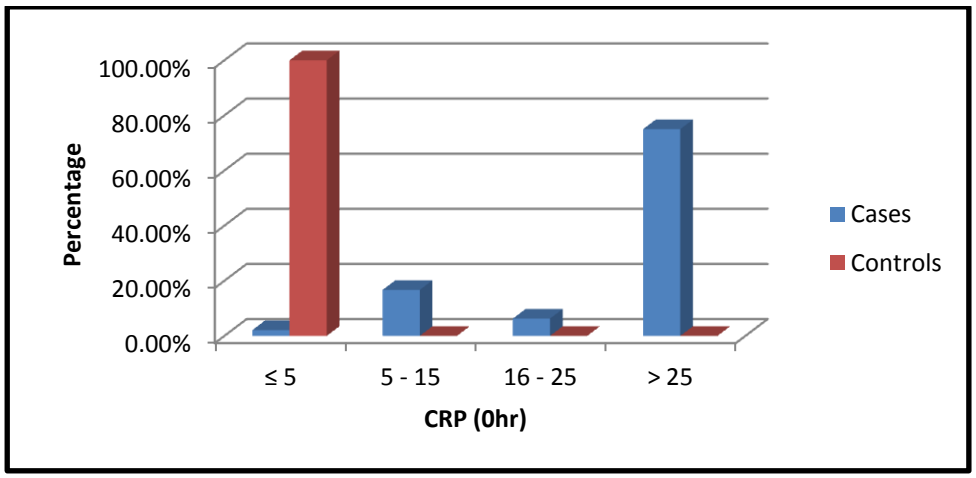


**Figure 2** Distribution of Serum CRP levels in controls and cases at base-line

Serum CRP was repeated at 2 months and 6 months after ATT in cases to look for impact on serum CRP levels with treatment (Table 2). Serum CRP levels after 2 months (7.70±4.29 mg/dl) and at end of 6 months (2.41±1.50 mg /dl) of treatment where significantly lower than at baseline (21.71±6.37 mg/dl) with a statistically significant of p value <0.001. (Figure 3)

**Table 2** Serum CRP levels in cases and correlation with treatment duration.

| **CRP (mg/dl)** | | **0 mths** | **2 mths** | **6 mths** |
|---|---|---|---|---|
| **Cases** | Mean<br>±sd | 21.71<br>6.37 | 7.70<br>4.29 | 2.41<br>1.50 |
| | p-value (vs. 0hr) | - | <0.001 | <0.001 |
| **Controls** | Mean<br>±sd | 2.15<br>1.30 | 2.15<br>1.30 | 2.15<br>1.30 |
| **p-value (Cases Vs. Controls)** | | <0.001 | <0.001 | 0.307 |

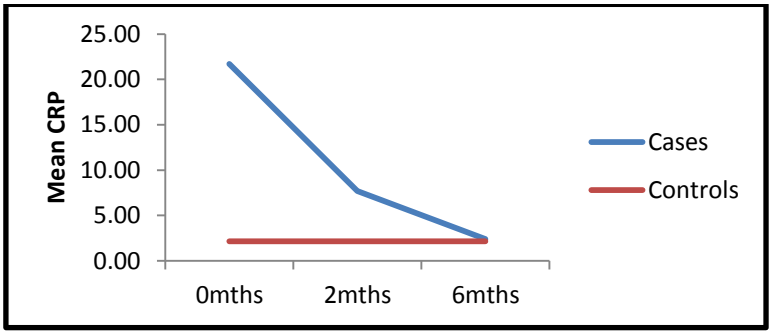


**Figure 3** Distribution of Serum CRP levels in controls and in cases at base-line and response with duration of treatment.

Sputum IFN-γ was measured in cases at baseline and were repeated at 2 months and 6 months after ATT. (Table 3) Sputum IFN-γ levels at baseline were (24.92±16.53 pg/ml) and decreased significantly after 2 months (7.68±5.17 pg/ml) and decreased further at end of 6 months (3.86±3.12 pg/ml) of treatment with a statistically significant of p value < 0.001 both at 2 months and at end of 6 months when compared to baseline.[Fig.4]

**Table 3** Sputum IFN-γ levels in cases at base-line and response with duration of treatment

| Sputum IFN -γ(pg/ml) | | 0mths | 2mths | 6mths |
|---|---|---|---|---|
| Cases | Mean | 24.92 | 7.68 | 3.86 |
| | ±sd | 16.53 | 5.17 | 3.12 |
| | p-value (vs. Baseline | - | <0.001 | <0.001 |

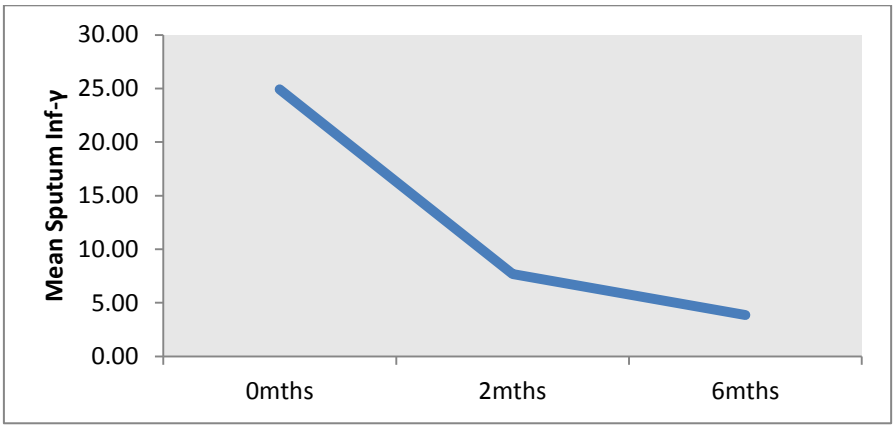


**Figure 4** Sputum IFN-γ of cases at baseline and declining levels with duration of treatment

Serum IFN-γ was measured in controls and cases at baseline and was repeated in cases at 2 months and 6 months after ATT and was compared to controls. Serum IFN-γ levels in cases at baseline (41.47±43.25 pg/ml) were significantly elevated compared to controls (1.81±1.6pg/ml) with a p value of < 0.003.[fig. 5]Further-more serum IFN-γ levels decreased significantly after 2 months (12.53±8.33 pg/ml) and decreased further at end of 6 months (2.47±1.55 pg/ml) of ATT with a statistically significant of p value < 0.001 both at 2 months and at end of 6 months when compared to baseline.

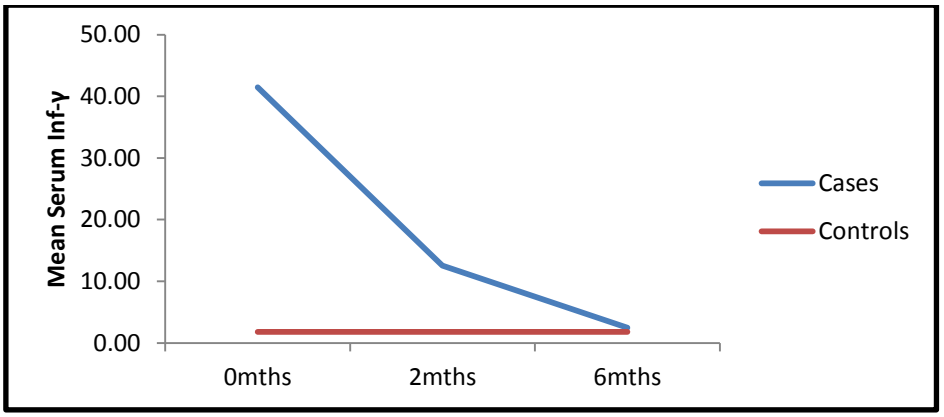


**Figure 5** Serum IFN-γ levels in controls and cases at base-line and response with duration of treatment

It was observed that mean serum IFN-γ in cases was very high at base-line compared to controls and decreased substantially with treatment and there is a negative correlation with mean serum IFN-γ levels and duration of treatment. Also it is clear that serum IFN-γ levels are approaching to baseline comparable to controls, possibly explaining cure in cases with 6 months of treatment.

**Discussion**

In this study we observed increased levels of circulating IFN-γ and CRP levels in patients with APTB and levels were higher in those with extensive radiographic infiltrates, consistent with prior studies. Therapy caused statistically significant decreases in the concentrations of these cytokines levels and declining levels of these

cytokines correlated with disease cure. Although interferon gamma and IL-18 are believed to contribute to effective immunity to tuberculosis, Yamada and coworkers found highest serum levels of these cytokines in patients with severe disease, high fever, and extensive radiographic infiltrates. These clinical findings show that type 1 cytokines, while necessary for protective immunity, can also mediate immune-pathology and levels correlate with disease burden. [10]

The present study showed overall high levels of CRP in patients of APTB at baseline. Majority of patients (75%) showed level of CRP above 25 mg/ml. Patients with high disease burden as seen clinically and by area of involvement on X-ray, were having higher levels of CRP. Study by Khalid et al [11]in pulmonary TB patients showed a similar pattern with raised overall mean CRP in patients with a mean of 9.87±4.83 in cases and serum CRP was 2.76±1.34 in controls (p 0.01). Shameem et al [12] in their study from north India found results comparable to our study. They found serum-CRP levels to be significantly higher at baseline (43.65 ± 23.68), as compared with the follow-up patients (9.88 ± 5.23).This study also studied changes in serum CRP concentration during treatment with regimen of anti-tubercular therapy. It was observed that there was a marked decrease in the CRP level with treatment. Mean serum CRP levels at baseline (0 month) was 21.71± 6.37mg/dl, at 2 months of ATT was 7.7±4.23 mg/dl and at completion of treatment (6 months) was 2.41±1.5mg/dl with a significant p value of <0.001 between baseline and 2 months and between 2 and 6 months of ATT. Khalid et al [11] also showed similar trend in CRP levels with ATT with mean serum CRP at baseline was 45.86± 7.52mg/dl and at the end of treatment was 9.86±2.21mg/dl respectively. Kaminskaia et al [13] found similar results of declining levels of CRP with treatment as in our study.

One of the prime aims of our study was to study of kinetics of IFN-γ with ATT. This study showed that serum IFN-γ levels were significantly higher in patients of APTB with mean + SD of 41.47±43.23 pg/ml compared to healthy controls with mean +SD of 1.81±1.6 pg/ml with p value of < 0.001.Hussainet al [14] also noticed raised levels of results IFN-γin their study of APTB patients, with mean + SD of IFN-γ of 48.69 + 28.78 pg/ml in cases while it was 12.99 + 5.70 pg/ml in the control group (p <0.001). Study by Verbon et al [15] showed similar results.

Present study also showed treatment effect on levels of serum IFN-γ with results showing mean + SD of IFN-γ of 41.47±43.23 pg/ml at baseline , 12.53±8.3pg/ml after 2 months of ATT and 2.47±1.55pg/ml at completion of 6 months of ATT while it was 1.81±1.6 pg/ml in the control group (p <0.001). Study by Ryu et al [16] found similar trend in IFN-γ levels in patients with APTB when put on anti-tubercular therapy. They observed a mean IFN-γ levels 41± 52.8 pg/ml at baseline which decreased to 22+/-23.9 pg/ml (p<0.05), respectively, after completing 2 month of ATT treatment. Won lee et al [17] also showed similar decreasing levels of serum IFN-γin patients of active pulmonary TB on ATT with baseline values of 5.31 ± 5.34 pg/ml, and the levels at 1, 3, and 6 months were 3.95 ± 4.30, 1.82 ± 2.14, and 1.50 ± 2.12 IU/ml, respectively.

Our study also evaluated changes in sputum IFN-γ during treatment with anti-tubercular therapy. We observed increased levels of IFN-γ at baseline of 24.92±16.52 pg/ml compared 7.68±5.17 pg/ml after 2 months and 3.86±3.12 pg/ml after 6 months of treatment with significant p value of < 0.001. This is probably one of few studies to measure sputum IFN-γ in patients of pulmonary TB and its kinetics with ATT treatment.

Rodrigo Ribeiro-Rodrigues et al [18] in study of sputum cytokine kinetics in patients with pulmonary tuberculosis found IFN-γ levels of (9 - 481)pg/ml in spontaneous sputum and (7 - 413)pg/ml in induced sputum. Levels in both spontaneous sputum and induced sputum were highest at the time of diagnosis and before initiating treatment.

They also noticed that IFN-γ was detectable only at low levels in induced sputum from control subjects (12 ± 2 pg/ml [mean ± standard error of the mean {SEM}]; range, 0 to 27 pg/ml) and was undetectable in sputum specimens from all six pneumonia controls.

There was some variation in sputum IFN-γ levels between our study and study by Rodrigo Ribeiro-Rodrigues et al.[21] This could be possibly due variation due to ethnicity/genetic response to tubercule bacilli or due to variation in detection range of ELISA kit used to measure sputum IFN-γ.

Drawback of study: All cases could not produce adequate produce sputum in follow up. At 2 months of ATT 40/ 48 (aprox. 83%) and at 6 months of ATT only 17/48(35 %) of cases could produce adequate sputum respectively.

## Conclusion

In conclusion, our observations reveal that patients with APTB have significantly elevated serum CRP and IFN-γ levels compared to normal healthy individuals and these levels decrease significantly with duration of treatment. Therefore serum CRP and IFN-γ levels may be useful adjuvant markers in cases of APTB to monitoring response to treatment besides clinical and radiological feature who cannot produce adequate sputum in follow up.

We also suggest that for individuals with sputum smear negative pulmonary tuberculosis, CRP and IFN-γ may serve as potential adjuvant markers of pulmonary TB besides clinical and radiological features. However there is need to evaluate IFN-γ levels in other conditions mimicking pulmonary TB, so that it could be used as a diagnostic as well as monitoring marker in cases of sputum negative pulmonary TB put on ATT.

**Conflict of interest:** none

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