Leptin response in patients with tuberculous pleuritis

C. Prabha, P. Supriya, Sulochana D. Das, B. Sukumar* & S. Balaji**

*Departments of Immunology & *Statistics, Tuberculosis Research Centre (ICMR) & **Institute of Thoracic Medicine, Chennai, India*

Background & objectives: Tuberculous pleuritis is used as a model to understand the protective immune response in tuberculosis. It is predominated by Th1 response at the site of infection, where a possible role for the leptin, a known enhancer of Th1 response, could be speculated. Hence, we investigated leptin levels in pleural effusions in patients with both tuberculous (TP) and non-tuberculous (NTP) pleural effusion.

Methods: Leptin and cytokine levels were assessed in serum and pleural fluid of TP and NTP patients (N = 20 each) by ELISA. Multivariate regression analysis were performed to find the possible determinants of leptin taking leptin as the dependent and body mass index (BMI), gender, source of leptin [*i.e.*, serum or pleural fluid (PF)], age and disease status as independent variables.

Results: PF leptin levels were significantly higher than serum leptin levels in both the groups however the PF leptin levels were significantly lower in TP subjects compared to NTP. The results showed that the leptin was found to be dependent on BMI but not on the other parameters. However, regression analysis based on the source of leptin showed males to be a better predictor of leptin. No correlation was observed between leptin and measured immune parameters.

Interpretation & conclusions: Our findings demonstrated that the decreased leptin levels were associated with reduction in BMI but not with the disease status in tuberculous pleuritis.

Key words BMI - leptin - tuberculosis - tuberculosis pleuritis

Leptin has been shown to regulate food intake, promote basal metabolism and the β-oxidation of fatty acids. Its structural similarity permits it to function like cytokines[1]. Leptin biases the T cell responses towards a pro-inflammtory phenotype and its levels increase rapidly during acute infection and sepsis and decrease during chronic inflammation[2,3]. Leptin levels are also reduced during starvation and malnutrition which are associated with impaired immune response and thymic atrophy and are shown to reverse the starvation-induced immuno-suppression. Because of the dual role in energy metabolism and immune response, leptin mediates immune-endocrine crosstalk and is hypothesized to act as a key link between nutritional status and an optimal immune response[4,5].

Starvation is known to be associated with a high frequency of infectious diseases. Tuberculosis (TB) is a classical example of such infectious disease associated with wasting and weight loss. Pro-inflammatory immune response predominates in TB and brings down the metabolic changes that result in TB-associated wasting[6,7]. In search of the missing link between the

pro-inflammatory immune response and wasting in TB, leptin emerged as a possible candidate. However, little is known about the determinants of leptin concentration such as pro-inflammatory cytokines and nutritional status in TB. Our previous study on patients with TB with HIV infection showed that the leptin levels were strongly associated with the gender and body mass index (BMI) but not with the disease status or the circulating cytokine levels[8]. Our observations together with the other reports[8-11] suggest that the local immune response differ from the systemic immune response during tuberculosis. Hence, as an extension of these studies, we conducted the present study of assessing leptin levels at the site of infection (ie pleural fluid) using tuberculous pleuritis as a model.

Tuberculous pleuritis (TP), an extrapulmonary form of TB (EPTB) is a condition predominated by Th1 type of immune response, characterized by the presence of interferon gamma positive (IFN-γ^+) CD4$^+$T cells[12-14]. The observation that Th1 response is compartmentalized in TP[12,13], suggests a possible role of leptin, the known enhancer of Th1 response in this disease. Hence, the present study was undertaken to measure the leptin levels in TP patients. Both systemic and local levels (*i.e.*, serum and pleural fluid, PF) of leptin were measured in TP patients. Also, the possible determinants of leptin concentration were studied taking BMI, sex, age and the disease status as independent variables.

Material & Methods

Study subjects: The subjects with pleural effusion were recruited from Institute of Thoracic Medicine (ITM), Chennai, from January-June 2007. The study was approved by the Institutional Ethical Committee. A written and informed consent was obtained from all the study subjects. The patients with pleuritis were grouped into tuberculous (TP) and non-tuberculous (NTP). These patients were negative for HIV and did not have diabetes or hormonal disorders. The blood and the pleural fluid samples were collected before the start of the treatment. Of the total 42 subjects enrolled, two were excluded from the study as they had empyema.

Tuberculous pleuritis (TP) group: Patients who were diagnosed to have tuberculous pleural effusion based on the smear, culture and the clinical picture of the chest X-ray were included for the study (N=20). Of the 20 subjects, two were positive for smear, 6 by culture and the remaining 12 were diagnosed based on the clinical symptoms and the picture of the chest

X-ray suggestive of TB pleuritis. The diagnosis was retrospectively proven as these patients responded to the anti-tuberculosis treatment. The mean age of the study subjects was 36 yr (range 19-60 yr).

Non-tuberculous pleuritis (NTP) group: This group consisted of 20 subjects who had effusion due to causes other than tuberculosis, such as malignancy, para-pneumonia, congestive cardiac failure and an exudative effusion due to secondary infection in systemic lupus erythematosis (SLE). The diagnosis for malignancy was based on the histology and cytology. The secondary infection in SLE and para-pneumonic effusions were diagnosed based on compatible clinical, bacteriological and radiological findings. The diagnosis was retrospectively proven as the patients responded to the appropriate therapy.

Sample preparation: The serum (BL) and PF samples were collected before the start of the treatment and were centrifuged at 1100 *g* for 10 min to get cell free supernatants which were stored at -70°C till assayed for various cytokines and leptin. For the enumeration of CD4$^+$T cells, peripheral and pleural fluid mononuclear cells were separated as described elsewhere[9].

Leptin measurement: Leptin levels were determined by ELISA, according to the manufacturer's instructions (R&D Systems, USA). The serum samples were brought to the room temperature and diluted in the ratio of 1:3 with dilution buffer containing 1xPBS and 0.1 per cent bovine serum albumin (BSA).

CD4 count and cytokine ELISA: The percentage of CD4$^+$T lymphocytes in the patients with pleuritis was analyzed by flow cytometry (FACS calibur, Becton Dickinson, USA) using FITC-conjugated anti-CD3 and PE conjugated anti-CD4 monoclonal antibodies (BD Biosciences, USA). The cytokines, IFN-γ, tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4) and IL-12 were also measured by ELISA as per the R&D systems protocol.

Statistical analyses: The data were analyzed using SPSS software, version 11.0 (SPSS Inc, USA). ANOVA with post HOC test (LSD) was carried out to estimate the significance of the observed differences. The relation between BL leptin concentration with BMI and CD4 counts were analyzed by univariate regression analysis. Multivariate regression analysis was performed by taking leptin concentration as dependent variable and BMI, sex, and disease group as independent variables. $P<0.05$ was considered to be significant.

Results

The study subjects in the two groups were not age-matched as the NTP patients were older than TP patients. The BMI of the TP patients was significantly lower than that of NTP patients (*P*<0.01) and the difference was primarily contributed by the males. The CD4⁺T cells were significantly higher in PF of TP subjects when compared to the NTP subjects (Table I).

The mean leptin levels of PF were significantly (*P*<0.01) higher than the BL in both TP (1.43 ± 0.18 *vs*. 0.97 ± 0.2 ng/ml) and NTP groups (2.59 ± 0.3 *vs*. 1.49 ± 0.2 ng/ml). However, the mean leptin levels in TP PF were significantly (*P*<0.01) lower when compared to the NTP PF. The female patients of TP group had significantly higher (*P*<0.01) leptin levels than males in both BL (1.98±1.0 *vs*. 0.6 ± 0.06) and PF (2.47 ± 0.8 *vs*. 1.05±0.7). The difference (female *vs*. male) was not significant in NTP subjects (1.52 ± 1.0 *vs*. 1.4 ± 0.9 in BL and 2.75 ± 1.4 *vs*. 2.41 ± 1.0 in PF).

Since the sample size of the study was small, we performed ad-hoc power analyses using G*Power version 3[15]. The effect size was calculated from the mean and standard deviation values. Power was calculated using studied sample size, effect size and the alpha level of 0.05. For all the comparisons the power ranged between 82-95 per cent except the comparison of BL leptin levels between the groups, which gave a
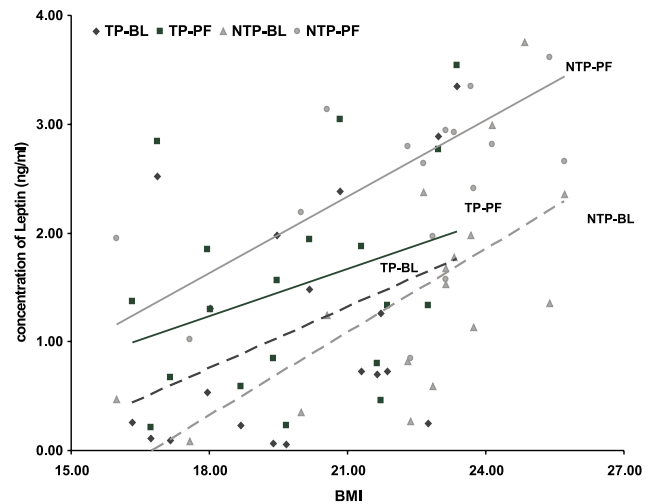

Fig. Correlation between serum and pleural fluid leptin concentration and body mass index.
Scatter plots of serum (BL) and pleural fluid (PF) leptin concentration against BMI in the two study groups (TP and NTP, N=20 in each group) are compared in the graph. The lines represent linear regression analysis. The correlation coefficients were : r² = 0.1626 (*P*=0.04), r²= 0.116 (*P*=0.08) for BL and PF of TP subjects respectively and r²=0.426 (*P*=0.001), r²=0.187 (*P*=0.04) for BL and PF of NTP subjects respectively.

power of 42 per cent. The analysis suggested that the selected sample size was adequate for this study to compare the systemic and local levels of leptin and to determine its predictors using multivariate regression analysis.

There was a positive correlation between BMI and leptin and the correlation was significant for the BL samples of both the groups and PF of NTP subjects. Although the PF of TP subjects showed a positive correlation, it was not significant (*P*=0.08) (Fig.). When multivariate regression analysis was performed taking leptin as dependent and BMI as independent variable, only the BL leptin levels of NTP subjects showed a significant relation (Table II). The gender was shown to have a significant effect on both BL and PF levels of leptin in TP subjects. For every unit increase in leptin levels in males, the leptin levels in females increased ~1.2 times in both BL and PF of TP subjects. In step-wise regression analysis, BMI was found to be significantly associated with the BL but not with PF leptin levels, despite of the disease status (Table III).

Correlation analysis of leptin and age showed a significant correlation in PF of TP group (r= 0.4, *P*= 0.05) and in BL (r=0.523, *P*=0.026) and PF (r= 0.536, *P*= 0.022) of NTP group. The BL leptin levels in TP subjects did not show significant correlation (r=0.262,

Table I. Demographic characteristic and leptin levels of pleuritis subjects

	Non-TB pleuritis (NTP) (N=20)	TB pleuritis (TP) (N=20)
Age (yr)	53±12 (25-75)	36±13* (19-60)
Sex (Female/Male)	15/5	12/8
BMI (kg/m²)	22.6±3.7 (16.6-28.2)	19.8±2.3* (16.3-23.3)
BMI-male (n=15) (kg/m²)	22.6±2.4 (16-25.7)	19.2±2.0* (16.3-22.8)
BMI- female (n=5) (kg/m²)	21.9±3.96 (17.6-25.4)	20.7±2.5 (16.9-23.3)
CD4 cells in blood (%)	34.5±14.8 (24-67)	28.8±9.28 (12-39)
CD4 cells in pleural fluid (%)	30±17.5 (14-65)	54±16.5#* (21-71)

Values are expressed as mean ± SD. The figures within the brackets represent the range. *P*<0.01 compared with NTP group and # *P*<0.01 compared with blood.

Table II. Independent associations of various variables with serum and pleural fluid leptin concentration

		Non-tuberculous pleuritis (N=20)		Tuberculous pleuritis (N=20)	
		Serum	Pleural fluid	Serum	Pleural fluid
BMI	β	0.198	0.141	0.127	0.049
	P value	0.034*	0.305	0.222	0.588
Age	β	0.028	0.039	-0.014	0.008
	P value	0.131	0.176	0.394	0.952
Sex	β	0.049	-0.081	-1.188	-1.23
	P value	0.922	1.000	0.017*	0.007*

Multivariate regression analysis was performed taking leptin concentration as dependent variable and BMI, age and sex as independent variables. Effect estimates are represented as regression coefficient β with its corresponding P value

P=0.187). However, multivariate regression analysis of independent association of age with leptin did not show any significant association (Table II).

A multivariate regression model was constructed separately for males and females (taken together from both the disease groups) to find out whether the leptin levels were dependent on its source (BL or PF). Leptin was dependent on BMI in both the genders, whereas it was significantly dependent on age and the source only in males (Table IV). In males, PF had better association with leptin levels than BL, showing that the PF is a better predictor of leptin in this model.

In the second model (Table V), which was constructed to find out whether the leptin levels were associated with the disease status (TP or NTP), leptin was strongly associated with the BMI of both males and females and not with the disease status. Inclusion of disease status weakened the association, indicating that the leptin levels are independent of disease status in both sexes.

Table III. Independent contributors to the explained variation in leptin concentration based on step-wise regression model

Contributors	Serum			Pleural fluid		
	Cumulative R^2	β	P value for the Coefficient β	Cumulative R^2	β	P value for the Coefficient β
BMI	0.288 [a]	0.197	0.006*	0.263 [a]	0.130	0.113
Sex	0.358 [b]	-0.551	0.112	0.292 [b]	-0.595	0.147
Age	0.362 [c]	0.006	0.622	0.366 [c]	0.020	0.188
Disease group	0.364 [d]	-0.06	0.753	0.396 [d]	0.276	0.228

Multivariate regression analysis was performed by taking leptin as the dependent and BMI, sex, age, and disease group (TP and NTP) as the independent variables. In multivariate regression analysis sex was coded as 1 for female and 2 for male and disease status was coded 1 for TP and 2 for NTP. R^2 is the coefficient of determination and β is the regression coefficient.
[a] predictor: BMI, [b] predictor: BMI, Sex, [c] predictor: BMI, Sex, Age, [d] predictor: BMI, Sex, Age, Disease group

Table IV Independent contributions of BMI, age and leptin source [serum and pleural fluid (SP)] to the explained variation in leptin concentration based on step-wise regression model

Contributors	Females			Males		
	Cumulative R^2	β	P value for the Coefficient β	Cumulative R^2	β	P value for the Coefficient β
BMI	0.145 [a]	0.245	0.030*	0.304 [a]	0.174	0.003*
Age	0.215 [b]	-0.028	0.194	0.387 [b]	0.025	0.009*
SP	0.310 [c]	-0.639	0.133	0.486 [c]	-0.775	0.005*

A multivariate regression analysis was performed by taking leptin as the dependent and BMI, age and sources of leptin [serum and pleural fluid (SP)] as the independent variables. In multivariate regression analysis, source of leptin was coded 1 for serum and 2 for pleural fluid. R^2 is the coefficient of determination and β is the regression coefficient.
[a] predictor: BMI, [b] predictor: BMI, Sex, [c] predictor: BMI, Sex, source of leptin [serum and pleural fluid (SP)].

Table V. Independent contributions of BMI, age and disease status (TP and NTP (TN)) to the explained variation in leptin concentration based on step-wise regression model

Contributors	Females			Males		
	Cumulative R^2	β	*P* value for the Coefficient β	Cumulative R^2	β	*P* value for the Coefficient β
BMI	0.145 [a]	0.245	0.039*	0.304 [a]	0.146	0.031*
Age	0.215 [b]	-0.025	0.279	0.387 [b]	0.020	0.078
TN	0.226 [c]	0.258	0.617	0.399 [c]	-0.384	0.340

A multivariate regression analysis was performed by taking leptin as the dependent and BMI, age and disease status [TP and NTP (TN)] as the independent variables. In multivariate regression analysis, disease status was coded as 1 for TP and 2 for NTP. R^2 is the coefficient of determination and β is the regression coefficient.
[a] predictor: BMI, [b] predictor: BMI, Sex, [c] predictor: BMI, Sex, and disease status [TP and NTP (TN)]

Table VI. Cytokine levels of patients with tuberculous and non-tuberculous pleuritis

	Non-tuberculous pleuritis		Tuberculous pleuritis	
	Serum	Pleural fluid	Serum	Pleural fluid
IFN-γ (pg/ml)	19±7	24±11	70±41	290±65*#
TNF-α (pg/ml)	4±2	42±16*	12±5	54±31*
IL-12 (pg/ml)	28±14	26±8	49±23	340±90*#
IL-4 (pg/ml)	2±0.4	6±3	3±0	13±4

Values are expressed as mean ± SEM (n=20)
*$P<0.05$ compared to serum level; #$P<0.05$ compared to NTP
IFN-γ, interferon- γ; TNF-α, tumor necrosis factor-α; IL, interleukin

The CD4^{+}T cells were higher in PF than BL of the TP patients indicating the compartmentalization of these cells as observed and reported previously[12]. This compartmentalization was unique to TP group and was not observed for NTP group (Table I). A partial correlation between leptin and CD4 percentage was performed after controlling for BMI and there was no significant correlation indicating that the leptin levels do not correlate with the CD4-T cell response (data not shown).

The levels of IFN-γ, TNF-α and IL-12 were significantly higher in PF than BL in TP subjects (Table VI). To find the relation of leptin levels with the cytokine response, a linear regression analysis was performed between these parameters. There was no significant association of leptin levels with the cytokines in BL and PF of both the groups, except a significant negative correlation of leptin with IL-4 in PF of NTP subjects (r =-0.484, *P* =0.04).

Discussion

The two important outcomes of the study were (*i*) the leptin levels in PF of TP patients were lesser than NTP patients despite the higher pro-inflammatory response observed in TP, and (*ii*) leptin levels correlated well with BMI but not with the studied immune parameters.

Recently, Celik *et al*[16] have studied the diagnostic value of leptin in tuberculous pleural effusion, and reported that the PF leptin levels were lower in tuberculous pleural effusion than other exudates. The finding of the present study is in accordance with this report as despite higher Th1 cytokine levels, the PF leptin levels were significantly lower in TP subjects. This reduced level of leptin in TP might be due to the prolonged inflammatory response that downregulates or exhausts leptin production, especially at the site of infection. Moreover, the leptin levels in TP subjects were lower than the previously observed leptin levels in healthy control subjects[8]. Such an observation has already been reported by other studies conducted in patients with pulmonary TB[6,17,18]. Interestingly, the PF leptin levels were higher than serum and this increase was not specific for TB. Although the cause is not known, we speculate that the local production of leptin by the fat tissues surrounding the pleura might be a cause of the increased PF leptin levels. Buyukoglan *et al*[19] have demonstrated that leptin levels are suppressed in TB patients and low leptin levels may contribute to increased susceptibility to infection and recovery with sequela lesions. van Lettow *et al*[20] have also shown that leptin concentrations do not predict wasting in human TB. Leptin-deficient mice have been shown to be more susceptible to *Mycobacterium tuberculosis* than wild-type mice, characterized by reduction in IFN-γ secreting

T cells in lungs[21], indicating that leptin contributes to protection against TB. However, in this study, although IFN-γ, TNF-α and IL-12 were increased in PF of TP subjects, no correlation was found with the increased leptin levels indicating no association between these parameters and leptin. While Cakir *et al*[18] have shown a positive correlation between TNF α and, Buyukoglan *et al*[19] did not find any significant association between these two parameters, and our results concords with the latter. The negative correlation of leptin with IL-4 in NTP subjects although had shown statistical significance, it is difficult to give explanation for this observation as the aetiologies for the pleural effusion varies in the NTP subjects. However, it can be speculated that low IL-4 levels might be due to the fact that leptin decreases IL-4 production by peripheral blood cells[22,23].

The influence of age on the immune status and the BMI is wellknown. In this study, NTP group differed significantly in age from the TP group which implies that decreased BMI in TP group cannot be fully explained by the presence of TB but may also be contributed by the difference in age between the groups. However, this was ruled out by performing multivariate regression analysis where age did not show any association with the leptin levels despite the fact that serum and PF leptin levels showed a positive correlation with age.

To ascertain the determinants of leptin levels, three different step-wise regression models were constructed. In model 1, BMI was shown to strongly influence the serum leptin levels and the leptin levels in both serum and PF were found to be independent of the disease status. To find whether gender plays a role in determining the leptin levels, the second model was constructed. In both the sexes, BMI was found to be a strong determinant of leptin. In males, age and the source of leptin were also significantly associated with the leptin levels. Pleural fluid in males was shown to be a better determinant of leptin levels regardless of the disease status. Since model 2 revealed that the determinants of leptin levels were gender-biased, model 3 was constructed based on model 1 to address the question whether the disease status (TP or NTP) might be a determinant of leptin in any of the genders. Again in this model, BMI but not the disease status showed a significant association with the leptin levels in both the sexes. Males showed increased predisposition to change in leptin levels with respect to variables like BMI, age and the source of the sample. However, such a study need to be done with a larger

sample to understand the association of leptin with age and gender and the influence of this association in TB.

In conclusion, the present study showed that the leptin levels were markedly decreased in TP group than NTP. The leptin levels in patients with TP were strongly influenced by BMI and not by the disease status. No correlation of leptin with the pro-inflammatory cytokine response and the CD4+T cell response of pleural fluid was seen indicating that leptin might not have any role in immunity against TB, particularly at the site of infection.

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Reprint requests: Dr Sulochana D. Das, Assistant Director, Department of Immunology, Tuberculosis Research Centre (ICMR)
Mayor V.R. Ramanathan Road, Chetput, Chennai 600 031, India
e-mail: sulochanadas@gmail.com, dsulochana@trcchennai.in