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Host biomarkers for monitoring therapeutic response in extrapulmonary tuberculosis

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ABSTRACT

Purpose: The aim of this study was to explore the utility of inflammatory biomarkers in the peripheral blood to predict response to treatment in extrapulmonary tuberculosis (EPTB).
Methods: A Luminex xMAP-based multiplex immunoassay was used to measure 40 inflammatory biomarkers in un-stimulated plasma of 91 EPTB patients (48 lymphadenitis, and 43 pleuritis) before and at 2 and 6 months of treatment.
Results: Overall a significant change was observed in 28 inflammatory biomarkers with treatment in EPTB patients. However, MIG/CXCL9, IP-10/CXCL10, and CCL23 decreased in all patients' groups with successful treatment at both time points. At 2 months, 29/64 (45%) patients responded partially while 35/64 (55%) showed complete regress. Among good responders, a higher number of biomarkers (16/40) reduced significantly as compared to partial responders (1/40). Almost half (14/29) of partial responders required longer treatment than 6 months to achieve satisfactory response. The levels of MIG, IP-10, MIF, CCL22 and CCL23 reduced significantly among 80, 74, 60, 71, 51% good responders, as compared to 52, 52, 52, 59, 52% partial responders, respectively. A biosignature, defined by a significant decrease in any one of these five biomarkers, corresponded with satisfactory response to treatment in 97% patients at 2 month and 99% patients at 6 months of treatment.
Conclusion: Change in inflammatory biomarkers correlates with treatment success. A five biomarker biosignature (MIG, IP-10, MIF, CCL22 and CCL23) could be used as an indicator of treatment success.

1. Introduction

Tuberculosis (TB) continues to be a major cause of morbidity and mortality in low and middle-income countries [1]. The global burden of the disease is estimated to be around 10 million as per World Health Organization and the prevalence of extrapulmonary TB (EPTB) is up to 24% of all notified TB cases [2]. The diagnosis of EPTB is challenging due to high variability in clinical presentation and difficulty in obtaining a representative sample from the disease site for microbiological confirmation. Moreover, due to the paucibacillary nature of the disease, sensitivity of the routine microbiological tests is low [3,4]. This often leads physicians to diagnose EPTB on clinical basis followed by administration of anti-TB treatment without bacteriological confirmation [5]. Monitoring response early during treatment is, therefore, critical to reduce overtreatment, development of drug resistance, morbidity, and mortality. For patients that have bacteriologically

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Table 1
Cytokine and chemokine panel used on plasma samples of the TB pleuritis and lymphadenitis patients.

Pro-inflammatory cytokines	Interferon-gamma (IFN-γ), Tumor necrosis factor Alpha (TNF-α), IL-1β, IL-6, IL-8, IL-16, MIF	
Anti-inflammatory cytokines	IL-4, IL-10	
Chemokines	CCL	6Ckine/CCL21, CTACK/CCL27, Eotaxin/CCL11, Eotaxin-2/CCL24, Eotaxin-3/CCL26, 309/CCL1, MCP-1/CCL2, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13, MDC/CCL22, TECK/CCL25, TARC/CCL1, MIP-3β/CCL19, MIP-3α /CCL20, MPIF-1/CCL23, MIP-1δ/CCL15
	CXCL	BCA-1/CXCL13, ENA-78/CXCL5, GCP-2/CXCL6, Gro-α/CXCL1, Gro-β/CXCL2, IL-8/CXCL8, IP-10/CXCL10, I-TAC/CXCL11, MIG/CXCL9, SDF-1α+β/CXCL12, SCYB16/CXCL16
	CX3CL	Fractalkine/CX3CL1
Growth factors	Granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2	

confirmed EPTB, monitoring response during treatment is equally important as the treatment involves a prolonged administration of multiple antimicrobials and the decision on duration of treatment often depends on the patients' response to prevent relapse. In case of smear-positive pulmonary TB, smear conversion is an important criterion for assessing response to treatment [6]. However, in case of EPTB, it is difficult to obtain repeated samples from the disease site during treatment, and response to treatment often relies on clinical criteria [7]. Currently, there is a lack of reliable objective criteria that can be used in the routine clinical practice for monitoring response to treatment in EPTB [7,8]. Some studies have explored the change in the levels of different immune biomarkers for this purpose [9,10]. However, most of the studies have small sample sizes [11,12], or use stimulation of immune cells [13–15]. Measurement of biomarkers in un-stimulated patient's plasma provides a direct means to observe the changes in biomarkers in response to treatment [16]. The aim of this study was to investigate change in the levels of inflammatory biomarkers in the unstimulated plasma of EPTB patients during the treatment and their utility to accurately predict the response to treatment.

2. Material and methods

The study was conducted at Gulab Devi Hospital, a private not-for-profit tertiary care hospital located in Lahore, Pakistan, and provides specialized TB care. Presumptive and diagnosed TB patients are referred from various districts for consultation and/or treatment at Gulab Devi Hospital Lahore. Patients of all ages with presumptive EPTB attending outpatient clinics were enrolled from April 2016 to August 2017. All patients received standard anti-TB treatment and followed up till the satisfactory response to treatment. Blood samples (5 ml) were collected at before initiation of anti-TB treatment (baseline) and at 2 and 6 months after treatment, centrifuged for 10 min at 1000*g*, plasma collected and frozen at −20 °C for a few months and then shifted to −80 °C until use.

2.1. Laboratory methods

For patients with enlarged lymph nodes, an excision biopsy was performed, and the sample was sent for histopathology and microbiological examination. For patients with pleural effusions, aspirated fluid was sent for cytology and microbiological workup. The specimens were processed for smear examination, Xpert MTB/RIF assay (Xpert), and culture [17]. Auramine O-stained smears were examined using a light-emitting diode fluorescence microscope [18]. Xpert was performed according to manufacturer's protocols [19]. Two slopes of Lowenstein-Jensen medium and one Mycobacteria Growth Indicator Tube (MGIT™ 960™; Becton Dickinson, Sparks, MD, USA) were inoculated for culture [17].

2.2. Inflammatory biomarkers detection through multiplex microbead immunoassay

Biorad 40 plex Bio-PlexPro™Human Chemokine Panel (Table 1), was used on Luminex® xMAP™ to detect cytokines/chemokines from the plasma (Referred to as inflammatory biomarkers in the text). Frozen plasma samples were thawed, mixed by vortexing, and centrifuged for 10 min at 10,000*g* to remove particulates before the assays were performed. Plasma samples were analyzed in duplicates in the first experiment and singlets in the succeeding experiments as inter-assay variability was in the acceptable range. Blanks and standards were run in duplicates in all experiments. Assays were performed as per manufacturer's instructions (BioRad, Hercules, CA). Briefly, after pre-wetting the plates, 50 µl of 1x beads were added to wells, plates were washed twice and 50 ul of standards, controls, and samples were added to the respective wells. After one hour's incubation on a shaker at room temperature, plates were washed 3 times and 25 ul of detection antibodies were added to each well. After an incubation of 30 min at room temperature and washing thrice, 50 ul of streptavidin-E was added to each well. Plates were incubated for another 10 min on the shaker at room temperature and after three steps of washings, re-suspended with 125 ul of assay buffer. Plates were read with a Luminex instrument (Luminex 200, Austin Luminex, USA). Data was analyzed using MILLIPLEX Analyst 5.1 software (Merck Millipore Darmstadt, Germany), as per the manufacturer's instructions.

2.3. Case definition

Using a combination of clinical, radiological, and laboratory findings, cases were defined as confirmed and probable EPTB cases. A confirmed case was defined based on the bacteriological confirmation either on culture or Xpert. A probable TB pleuritis case was defined if the symptoms and findings were consistent with TB pleuritis (lymphocytosis and fluid protein level more than 3 g/dl or plasma adenosine deaminase levels more than 16 U/L or concomitant pulmonary TB suggested by positive acid-fast bacilli smear and/or chest radiograph) and with good response to anti-TB treatment at 2–3 months and/or end of treatment. A probable TB lymphadenitis case was defined if the symptoms, clinical findings, and histopathology were consistent with TB lymphadenitis and with good response to anti-TB treatment at 2–3 months and /or end of the treatment.

2.4. Response to treatment

The response to treatment was considered as good if two of these three criteria were fulfilled, 1) regression of symptoms, 2) regression of local signs of disease; regression of lymph nodes among lymphadenitis cases and regression of pleural effusion assessed by ultrasound among the pleuritis cases, 3) weight gain.

2.5. Statistical analysis

International Business Machine (IBM) – Statistical Package for Social Sciences (SPSS) version 23 and R studio were used for data analysis. Data were evaluated for normality using Shapiro-Wilk and Kolmogorov-Smirnov tests. For normally distributed data, paired *t*-test was used, otherwise non-parametric Wilcoxon signed rank test was employed for analysis of data. Chi-square test was done for categorical data. A p-value < 0.05 was considered statistically significant. Furthermore, Linear Discriminant Analysis was used to classify cytokines at baseline and at two points after treatment. Ratio (or pirate) plots with 95% highest density interval were used to visualize the ratio of change in the biomarker levels for individual patients.

Different biosignatures were synthesized by making a combination of the inflammatory biomarkers which showed statistically significant change in the median levels at two timepoints during treatment as

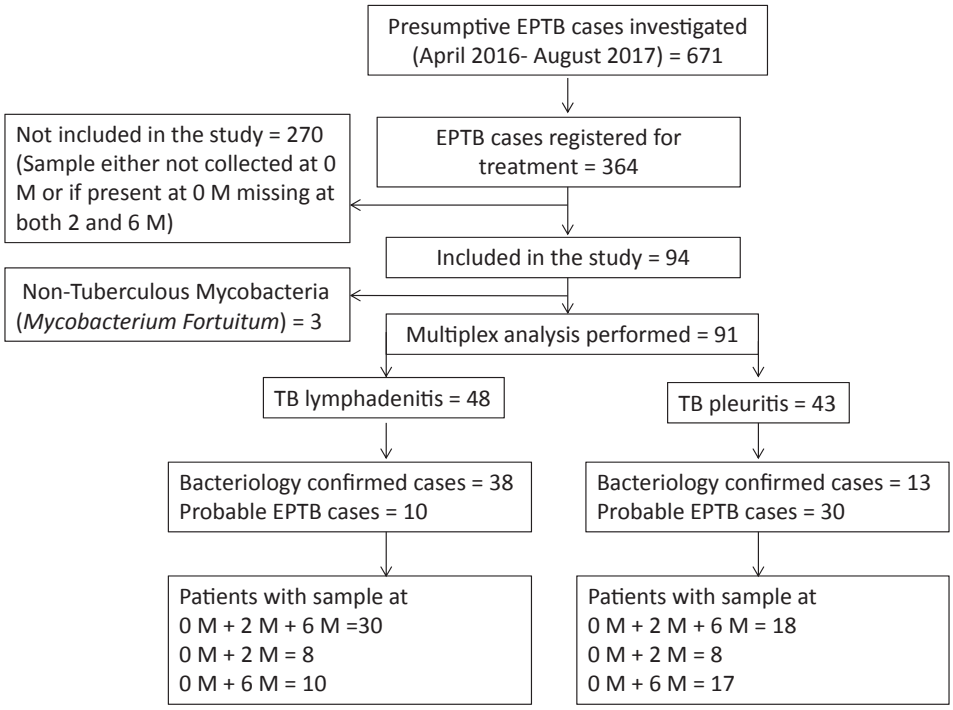


Fig. 1. Flow chart showing patients included in the study and the number of plasma samples obtained at different time points during treatment. Abbreviations, TB: tuberculosis, EPTB: extrapulmonary tuberculosis, M: Month of the treatment.

Table 2
Demographic and clinical characteristics of extrapulmonary tuberculosis patients.

Patient Characteristics	TB lymphadenitis N = 48	TB pleuritis N = 43
Age in years, median, (range)	20 (11–72)	25 (15–70)
Sex, n (%)		
Male	12 (25)	30 (70)
Female	36 (75)	13 (30)
HIV status, n (%)		
Positive	0 (0)	0 (0)
Negative	48 (100)	43 (100)
History of Diabetes, n/N (%)		
Yes	1/45 (2)	3/37 (8)
No	44/45 (98)	34/37 (92)
NA	3/48 (6)	6/43 (14)
Patient Categorization, n/N (%)		
Confirmed TB	38/48 (79)	13/43 (30)
Culture positive	11/38 (29)	10/13 (77)
Xpert Positive	3/38 (8)	1/13 (8)
Both positive	24/38 (63)	2/13 (15)
Probable TB	10/48 (21)	30/43 (70)
Clinical response at 2 M of treatment, n/N (%)		
Responders	20/48 (42)	32/43 (74)
Partial responders	28/48 (58)	11/43 (26)
Clinical response at 6 M/end* of treatment, n/N (%)		
Responders	44/44 (100)	41/41 (100)

N: Total number, n: number, %: percentage, NA : no information available TB: tuberculosis, M: month.
* Treatment was extended for 15 lymphadenitis and 5 pleuritis patients beyond 6 months.

compared to the baseline, and each biomarker showed ≥20% change from the baseline in an individual patient. A software library, Python And Data Analysis (Pandas), was used for basic data manipulation and all the possible combinations of members of the given biomarkers set were computed. The biomarker combinations covering the greatest number of patients and the least number of biomarkers were selected.

3. Results

3.1. Patients characteristics

Fig. 1 shows the patients included in the study. A total of 671 presumptive EPTB cases were investigated during the study period. Among them, 364 were registered at Gulab Devi Hospital. A total of 91 patients were included in the study. The demographic and clinical characteristics of study participants are shown in Table 2. There were 48 TB lymphadenitis and 43 TB pleuritis cases. Using a composite reference standard, 51 patients were classified as confirmed, and 40 as probable EPTB cases. The median age was 20 years for lymphadenitis and 25 years for pleuritis patients. There was a preponderance of females (75%) among lymphadenitis patients while the majority (70%) of patients presenting with pleuritis were males. None of these patients was positive for human immunodeficiency virus. One lymphadenitis and 3 pleuritis patients had a history of having diabetes. All other patients had random blood sugar levels less than 200 mg/dl. At the second month of treatment 20/48 (42%) lymphadenitis and 32/43 (74%) pleuritis patients showed satisfactory response to treatment with improvement of clinical and subjective criteria. Rest of the patients showed clinical improvement but not complete resolution of signs and symptoms. At 6 months of treatment, 44/48 lymphadenitis and 41/43 pleuritis patients turned up for follow-up. Treatment was extended for 20 patients (15 lymphadenitis and 5 pleuritis), 16 of these 20 patients showed partial response at 2 months of treatment. Clinical improvement was recorded in all patients at the end of the treatment.

3.2. Inflammatory biomarker profile change with treatment in TB lymphadenitis

Fig. 2 shows the 16 biomarkers that changed significantly with treatment as compared to the baseline. After 2 months of treatment, there was a significant decrease in the plasma levels of MIG ($p = .007$), IP-10 ($p = .025$), CXCL2 ($p = .042$), CCL8 ($p = .048$), CCL22 ($p = .003$), and CCL23 ($p = .009$), and an increase in the plasma levels of TNF-α ($p =$

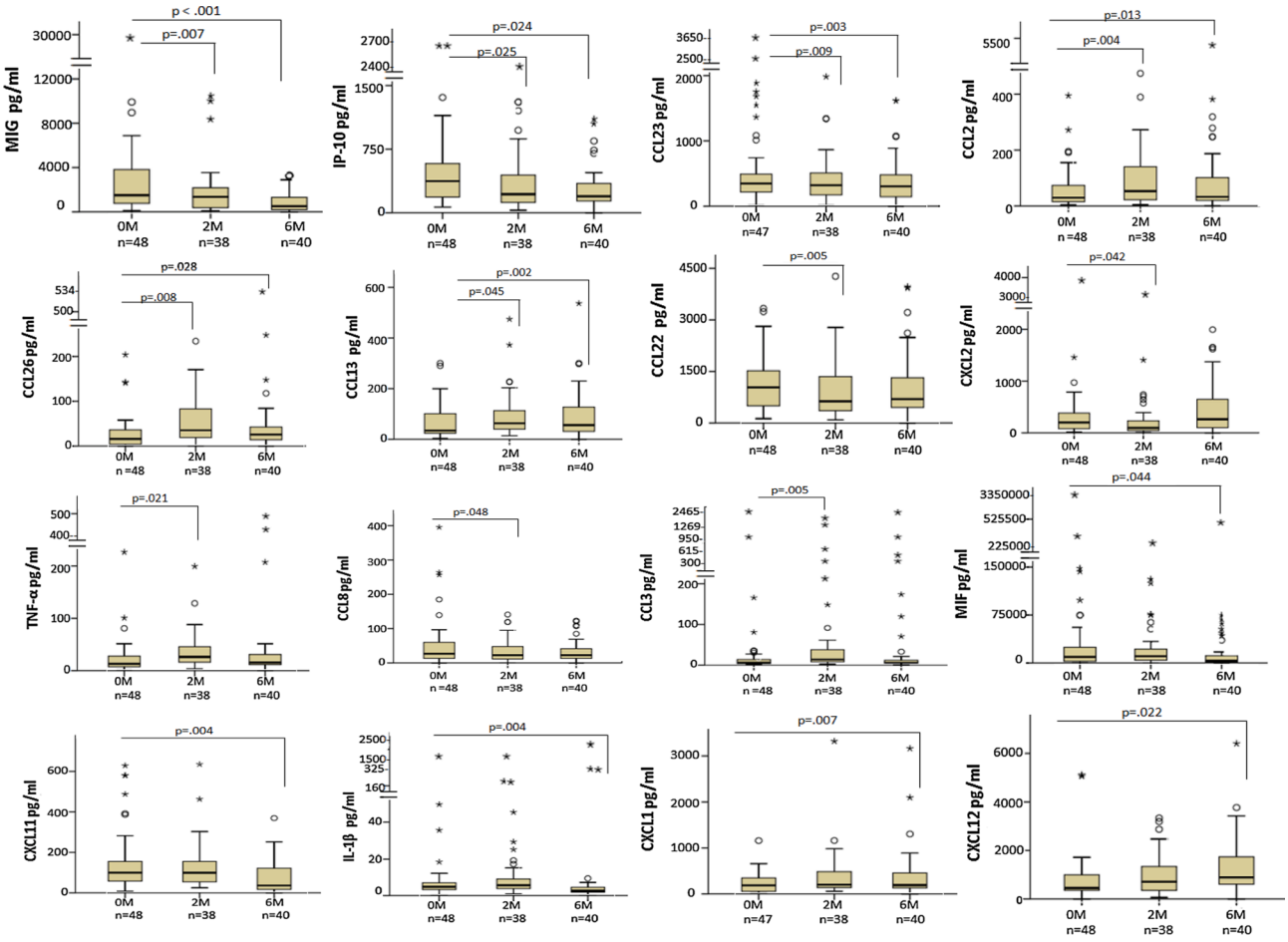
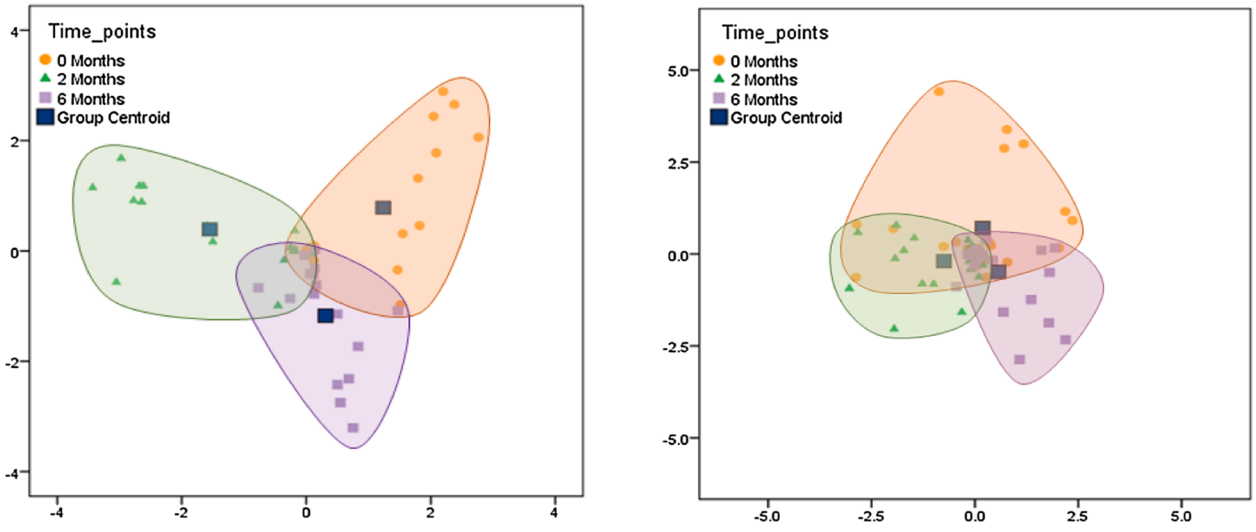


Fig. 2. Box plots showing plasma levels of inflammatory biomarkers in lymphadenitis patients at baseline, 2, and 6 months of treatment. Biomarkers that changed significantly with treatment are shown. The Wilcoxon signed rank test was used to compare biomarkers expression at different time points. A p-value < 0.05 was considered significant. Boxes represent the median and interquartile range, and the whisker represents minimum/maximum values. Outliers are shown by a broken axis. n = number of patients.



a: Tuberculous lymphadenitis patients

b: Tuberculous pleuritis patients

Fig. 3. Linear discriminant analysis (LDA) at baseline and at 2 & 6 months of treatment. a: LDA for tuberculous lymphadenitis patients (n = 30). LDA gave significant classification (Wilk Lambda = 0.016) with a membership of 100% at 6 months. b: LDA for tuberculous pleuritis patients (n = 18). Although statistical significance was not obtained (Wilk Lambda = 0.119, with a membership of 62.5% at 6 months) due to small sample size, there is a visible trend of separate clustering at 0, 2, and 6 months.

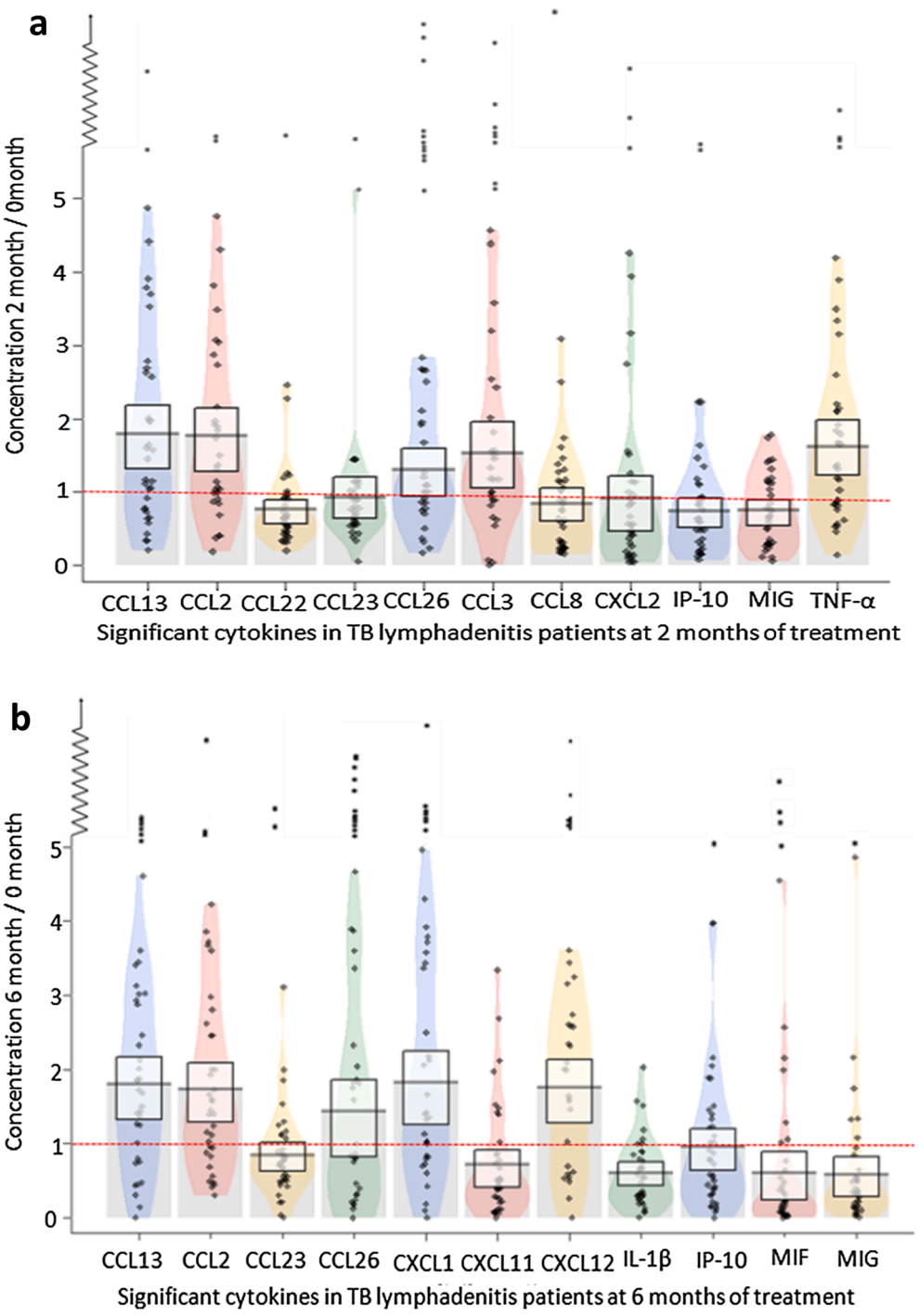


Fig. 4. Ratio/pirate plots to visualize the decrease or increase in plasma levels of inflammatory biomarkers in the individual tuberculous (TB) lymphadenitis patients. Only those inflammatory biomarkers that changed significantly with treatment are shown. a: Ratio of plasma level of inflammatory biomarkers at 2 month of treatment to their levels at baseline (n = 38) *Outliers: CCL13: 7 & 159, CCL2: 6; 14; 16 & 27, CCL22: 20, CCL23: 14, CCL3: 6; 8; 14; 22; 22; 26; 27; 76 & 209, CCL8: 1300, CXCL2: 8; 10 & 139, CCL26: 7; 8; 8; 10; 12; 22; 25; 164; 241 & 351, IP10: 6 & 8, TNF-α: 6; 9; 9 & 40. b: Ratio of plasma levels of inflammatory biomarkers at 6 months of treatment to their levels at the start of treatment (n = 40) *Outliers: CCL13: 6; 7; 7; 9 & 9, CCL2: 8 & 166, CCL23: 7 &15, CCL26: 6, 8, 9, 9, 10, 14, 42, 76, 104, 164 & 176, CXCL1: 6, 6, 8, 9, 12, 15, 17 & 551, CXCL12: 6, 6, 7, 8, 8, 26 & 251, IP10: 6, MIF: 10, 18, 31, 80 & 116.

.021), CCL2 ($p = .004$), CCL3 ($p = .005$), CCL13 ($p = .045$) and CCL26 ($p = .008$). After 6 months of treatment, levels of MIG ($p < .001$), IP-10 ($p = .024$), MIF ($p = .044$), IL-1β ($p = .004$), CXCL11 ($p = .004$), and CCL23 ($p = .003$) decreased significantly, while levels of CCL2 ($p = .013$), CCL13 ($p = .002$), CCL26 ($p = .028$), CXCL1 ($p = .007$), and CXCL12 ($p = .022$) increased significantly as compared to the baseline.

Linear discriminant analysis (Fig. 3a) gave a significant classification of patients with respect to their above-mentioned inflammatory biomarkers' levels at baseline, 2, and 6 months, further depicting significant change in the levels of inflammatory biomarkers after treatment at both time points, with a membership of 100% at 6 months.

Fig. 4 shows the ratio of change in the levels of above-mentioned biomarkers in individual patients at both time points showing wide variation between individual patients. All the above-mentioned biomarkers did not change in all patients. Table 3 shows the proportions of patients in which the individual biomarkers showed ≥20% change from the baseline in an individual patient. The magnitude of change (fold change) also varied between biomarkers ranging from 2- to12-fold change (Table 3).

3.3. *Inflammatory biomarker profile change with treatment in TB pleuritis*

Fig. 5 shows the 24 inflammatory biomarkers that changed significantly with treatment as compared to the baseline. After two months of treatment, a significant decrease in plasma levels of MIG ($p = .002$), IP-10 ($p < .001$), IFN-γ ($p = .004$), IL-4 ($p = .022$), CCL1 ($p < .001$), CCL8 (p

Table 3
Proportion of tuberculous lymphadenitis and pleuritis patients showing ≥20% change in the levels of biomarkers in response to treatment and the magnitude of change (fold change) in median plasma levels.

Biomarkers	TB lymphadenitis				TB pleuritis			
	0-2[ϕ]		0-6[¥]		0–2 [ϕ]		0-6[¥]	
	n/N (%)	FC ↓/↑	n/N (%)	FC ↓/↑	n/N (%)	FC ↓/↑	n/N (%)	FC ↓/↑
MIG	22/38 (58)	3↓	31/40 (76)	6↓	21/26 (81)	2↓	29/35 (83)	7↓
IP-10	21/38 (55)	3↓	22/40 (55)	3↓	20/26 (77)	3↓	27/35 (77)	3↓
CCL23	19/38 (50)	2↓	21/39[*] (54)	3↓	14/26 (54)	3↓	26/35 (74)	2↓
CXCL11			28/40 (70)	4↓	19/26 (73)	2↓	29/35 (83)	6↓
CCL22	24/38 (63)	2↓			18/26 (69)	2↓		
CCL2	23/38 (61)	3↑	25/40 (63)	3↑			19/35 (54)	2↑
CCL26	22/38 (58)	5↑	24/40 (60)	5↑	18/26 (69)	4↑		
CCL13	21/38 (55)	3↑	30/40 (75)	2↑	13/26 (50)	3↑		
CXCL2	20/38 (53)	5↓			22/26 (85)	7↓	21/35 (60)	5↓
CCL8	20/38 (53)	3↓			22/26 (85)	3↓	21/35 (60)	2↓
CCL3	23/38 (61)	7↑					23/35 (66)	2↓
TNF-α	24/38 (63)	3↑						
IFN-γ					19/26 (73)	2↓	19/35 (54)	3↓
CCL1					21/26 (81)	2↓	23/35 (66)	2↓
IL-4					17/24[*] (71)	3↓	19/32[*] (54)	8↓
CXCL12			26/40 (65)	3↑				
CXCL1			25/39[*] (64)	4↑				
MIF			27/40 (68)	12↓			25/35 (71)	5↓
IL-1β			27/40 (68)	3↓				
CCL20					18/26 (69)	3↓	24/35 (69)	4↓
CX3CL1					18/26 (69)	3↓	19/35 (54)	2↓
CCL24					19/26 (73)	12↓		
IL-8						12↑		

Table 3 (*continued*)

Biomarkers	TB lymphadenitis				TB pleuritis			
	0-2[ϕ]		0-6[¥]		0–2 [ϕ]		0-6[¥]	
	n/N (%)	FC ↓/↑	n/N (%)	FC ↓/↑	n/N (%)	FC ↓/↑	n/N (%)	FC ↓/↑
					16/26 (62)			
CXCL6							19/35 (54)	5↓
CCL15							15/35 (43)	2↓
CCL17							25/35 (71)	3↓
CCL19							21/35 (60)	3↓
IL-6							29/35 (83)	3↓

FC: fold change, n: number of patients showing significant change, N: total number of patients, ↓: decrease, ↑: increase.
[ϕ] From baseline to 2 months after treatment.
[¥] From baseline to 6 months after treatment.
[*] Valid available values.

< .001), CCL20 ($p = .005$), CCL22 ($p = .002$), CCL23 ($p = .001$), CCL24 ($p = .012$), CXCL2 ($p < .001$), CXCL11 ($p = .001$), and CX3CL1 ($p = .016$), and an increase in levels of IL-8 ($p = .012$), CCL26 ($p = .028$), and CCL13 ($p = .034$) was observed. After 6 months of treatment, a significant decrease was seen in the levels of 18 inflammatory biomarkers as compared to the baseline. These included MIG ($p < .001$), IP-10 ($p < .001$), IFN-γ ($p = .002$), MIF ($p = .013$), IL-4 ($p = .035$), IL-6 ($p < .001$), CCL1 ($p < .001$), CCL3 ($p = .038$), CCL8 ($p = .003$), CCL15 ($p = .041$), CCL17 ($p = .013$), CCL19 ($p = .036$), CCL20 ($p = .011$), CCL23 ($p < .001$), CXCL2 ($p = .027$), CXCL6 ($p = .021$), CXCL11 ($p < .001$), and CX3CL1 ($p = .023$). However, a significant increase was seen for only CCL2 ($p = .012$).

Linear discriminant analysis as shown in Fig. 3b gave a significant classification of patients with respect to their above-mentioned inflammatory biomarkers' levels at 2 months but not at 6 months. However, at 6 months, there was a visible trend depicted by clustering when compared with the baseline.

Fig. 6 shows the ratio of change in the levels of above-mentioned biomarkers in individual patients at both time points. As in lymphadenitis, a wide variation was seen between individual patients. All the above-mentioned biomarkers did not change in all patients. Table 3 shows the proportions of patients in which the individual biomarkers showed ≥20% change from the baseline in an individual patient. The magnitude of change (fold change) also varied between biomarkers ranging from 2- to12-fold change (Table 3). A biomarker with higher fold change implies its robustness as an indicator of treatment response.

3.4. Common inflammatory biomarkers changing in both TB pleuritis and lymphadenitis

Fig. 7 shows the common biomarkers that changed significantly in both patient groups at both time points. After two months of treatment, MIG, IP-10, CXCL2, CCL8, CCL22, and CCL23 decreased, while CCL13, and CCL26 levels increased in both groups. At 6 months, levels of MIG, MIF, IP-10, CCL23, and CXCL11 decreased and the level of one biomarker (CCL2) increased in both groups of patients.

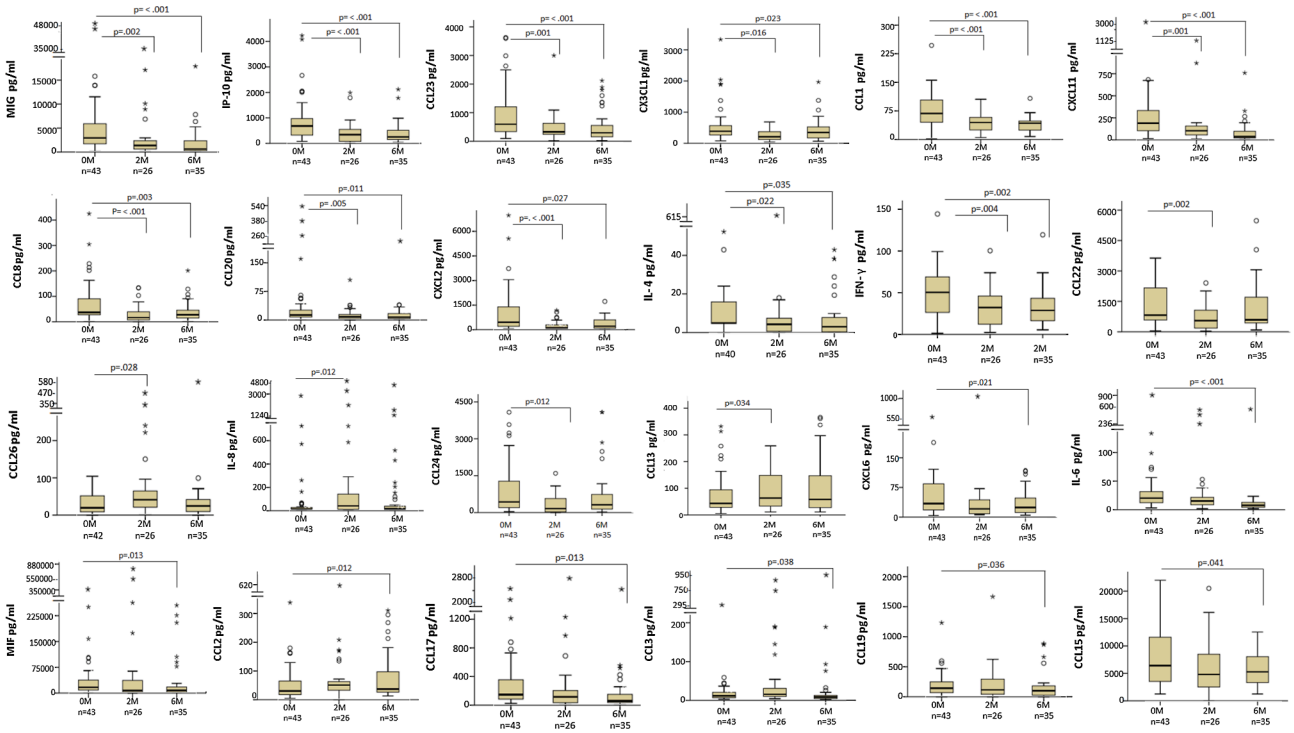


Fig. 5. Box plots showing changes in plasma levels of inflammatory biomarkers in tuberculous pleuritis patients at baseline and 2, and 6 months of treatment. Biomarkers that changed significantly with treatment are shown. The Wilcoxon signed rank test was used to compare biomarkers expression at different time points. A p-value < 0.05 was considered significant. Boxes represent the median and interquartile range, and the whisker shows minimum/maximum values. Outliers are shown by a broken axis. n = number of patients.

3.5. *Biosignature predicting response to treatment in all patients*

Biosignatures were synthesized by different possible combinations of biomarkers on the condition that change in any one of the biomarkers in the biosignature would predict response to treatment. The goal was to have a biosignature with minimum number of biomarkers changing in maximum number of patients and in both forms of TB. When all significantly changed biomarkers were used, the number of possible combinations became too high. We, therefore, selected five biomarkers, MIG, IP-10, MIF, CCL22, CCL23, based on their high baseline levels, and their change in higher proportion of patients. All possible combinations with these five biomarkers are shown in (Online Resource 1) Supplementary Tables 1, 2, & 3. Table 4 shows the selected biosignatures and their sensitivity to predict response to treatment at 2 and 6 months of the treatment. A biosignature (MIG + IP-10 + MIF + CCL22 + CCL23), could predict response to treatment in 97% patients at 2 months and 99% patients at 6 months of treatment.

3.6. *Change in plasma inflammatory biomarkers correlates with early clinical response during treatment*

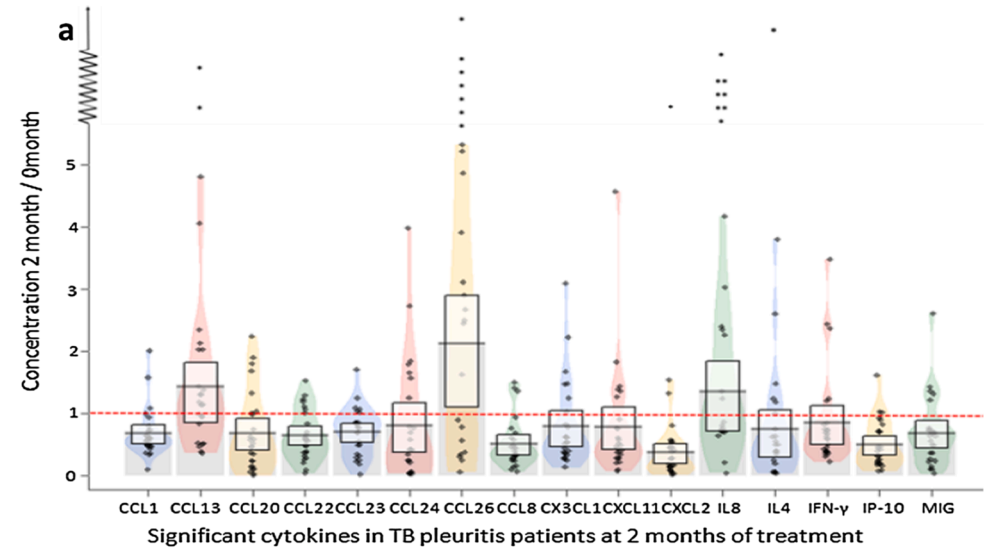
After 2 months of treatment, 35/64 (55%) patients (16 lymphadenitis and 19 pleuritis) showed good clinical response (responders), while 29/64 (45%) patients (22 lymphadenitis and 7 pleuritis) improved clinically but clinical signs did not settle completely (partial responders). Fig. 8 shows the levels of biomarkers among responders and partial responders. A total of 16 inflammatory biomarkers decreased significantly in responders while only one decreased in partial responders, indicating that a decrease in plasma levels of these biomarkers at 2 months correlates with good clinical response. Furthermore, responders showed significant decline in four of the biomarkers (MIG, IP-10, CCL22, CCL23) included in the biosignature as compared to the partial responders who showed significant change in only one (CCL23). Extension of treatment was required for 20 patients and 16/20 were partial responders. Plasma samples were available for 14/16 patients at 2 months. This implies that these biomarkers can also be used to predict which patients would require prolonged treatment beyond 6 months. Although decrease in MIF levels did not reach statistical significance at 2 months, $\geq$ 20% decrease was seen in 60% responders and 52% partial responders (Fig. 8).

4. Discussion

In this prospective cohort study, we have shown that plasma levels of several inflammatory biomarkers change with treatment. The patients' response to infection and treatment would depend on a variety of host and bacteriological factors and is expected to vary among individuals and between different disease sites. A single inflammatory biomarker is, therefore, not expected to give a satisfactory response in all patient categories, while a combination of biomarkers can predict response to treatment with reasonable certainty in many patients as shown in our study. Individually, the levels of IP-10, MIG, and CCL23 changed significantly in majority of TB lymphadenitis and TB pleuritis patients at both 2 and 6 months after treatment. A combination of five inflammatory biomarkers (MIG, IP-10, MIF, CCL22 and CCL23) could predict response to treatment in 97% of our study patients at 2 and 99% at 6 months after treatment.

Several studies have shown that MIG, MIF, IP-10 and CCL22 plasma levels increase in active TB and decline with successful treatment and have been proposed as surrogate markers for the evaluation of treatment response in pulmonary and EPTB. However, the main focus of these studies has been pulmonary TB with relatively few EPTB cases [20–24]. Few other studies have shown various combinations of biomarkers in plasma [9] or saliva [25] to assess response to treatment in pulmonary TB. To our knowledge this is the first study to evaluate the role of 40 inflammatory biomarkers in >90 EPTB patients at two time points after treatment.

Although several biomarkers changed with treatment, and many



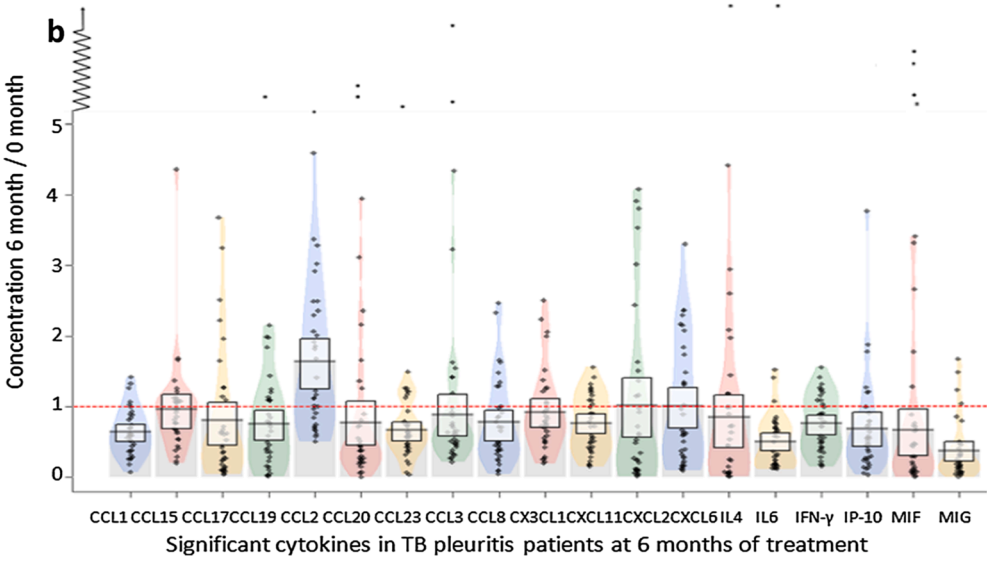


Fig. 6. Ratio/pirate plots to visualize the decrease or increase in plasma levels of inflammatory biomarkers in the individual tuberculous pleuritis patients. Only those inflammatory biomarkers that changed significantly with treatment are shown. a: Ratio of plasma level of inflammatory biomarkers at 2 months of treatment to their levels at the start of treatment (n = 26). *Outliers: CCL13: 11 & 13, CCL26: 6; 9; 9; 16; 21; 28; 29& 37, CXCL2: 11, IL8: 7; 8; 11; 12; 25; 30; 43; 44 & 77. IL4: 130. b: Ratio of plasma levels of inflammatory biomarkers at 6 months of treatment to their levels at the start of treatment (n = 35). *Outliers: CCL19: 14, CCL2: 6, CCL20: 12 & 19, CCL23: 7, CCL3: 10 & 36, IL4: 36, IL6: 47, MIF: 15; 16; 19 & 21.

combinations gave good sensitivity in assessing response to treatment, we selected a biosignature comprising of the biomarkers with, i) a persistent trend (downward or upward) throughout the treatment, ii) high plasma levels, iii) high fold change and iv) giving coverage to maximum number of patients. The relatively high plasma levels make them potential candidates for detection by less sensitive techniques than multiplex assay, and development into tests for routine clinical use. Furthermore, their higher levels in plasma imply that these biomarkers would be detectable in the dried blood spots as shown by previous studies on IP-10 on dry blood spots [26,27]. This opens the possibility of developing a point-of-care test based on these biomarkers. Dried blood spots are easy to make and can be transported at ambient temperature to a reference laboratory.

After 2 months of treatment, patients that responded well to treatment showed marked change in biomarkers levels as compared to the partial responders, indicating that significant change in plasma levels of inflammatory biomarkers correlates with good clinical response and can be used to predict response to treatment during initial months of the therapy. Since majority of the partial responders at 2 months required prolongation of treatment, these biomarkers can also be used to predict the need for prolonged duration of treatment to achieve satisfactory response. Previous studies have shown that inflammatory markers can be used for the evaluation of the early treatment response [20,28].

All patients in our cohort showed good response to treatment at the end of treatment. This is not in agreement with our clinical experience and experience from other cohorts [29]. Usually, some of the patients started on anti-TB treatment on clinical suspicion do not show satisfactory response to treatment. This could be due to the bias introduced by the study design, as the attending physician knew about the ongoing study and was extra careful in the selection of patients before the start of anti-TB treatment. Whereas in routine practice more liberal prescription of anti-TB treatment is done in presumptive EPTB cases due to lack of a reliable diagnostic test, leading to overdiagnosis and overtreatment.

Diabetes mellitus has been documented as a risk factor for TB [30,31], and the prevalence of diabetes in Pakistan is reported to be around 26% [32]. However, only 4 patients (5%) in our cohort were diabetics. This is in agreement with our previous studies on EPTB where the prevalence of diabetes has been shown to be low among EPTB patients in Zanzibar (2%) [33], and India (2%) [34]. We also reported low prevalence of diabetes among pulmonary TB patients from Pakistan (5%) [35]. In agreement, another study from India has also reported low prevalence (5.4%) of diabetes among 37 EPTB patients [36], implying that diabetes might not be a risk factor for EPTB. However, this study was not designed to study the correlation between diabetes and EPTB,

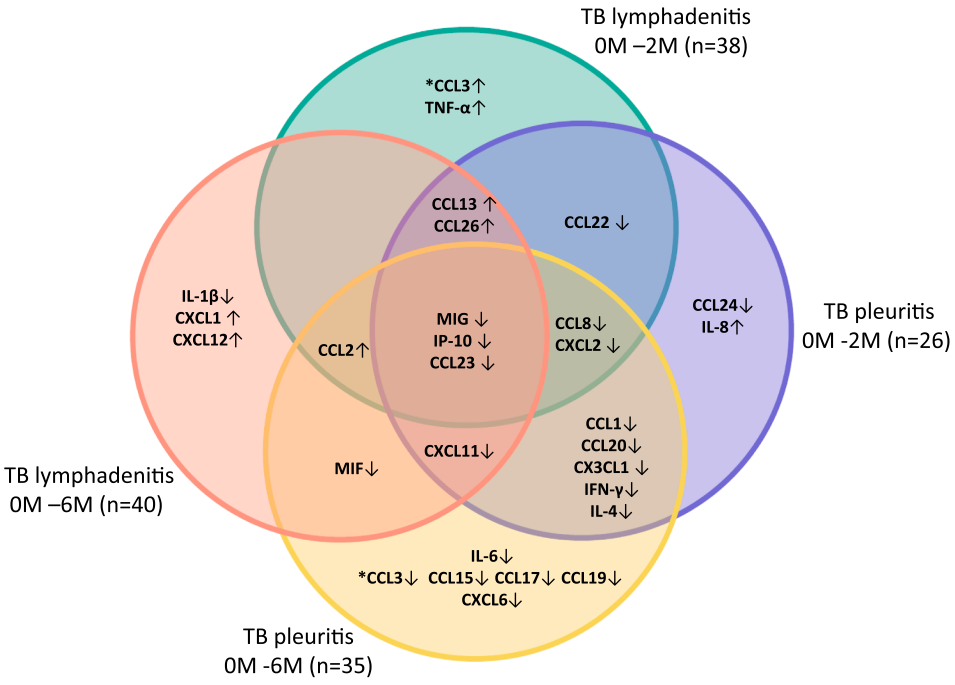


Fig. 7. Venn diagram showing common inflammatory biomarkers that changed significantly at different time points in tuberculous (TB) lymphadenitis and pleuritis patients, n: number of patients, 0 M–2 M: Significant change at 2 months of the treatment as compared to baseline, 0 M–6 M: Significant change at 6 months of the treatment as compared to baseline, ↑: significant increase in plasma levels with treatment, ↓: significant decrease in plasma levels with treatment, *CCL3 levels increased significantly at 2 M in lymphadenitis patients, whereas it decreased significantly in pleuritis patients.

and further studies are needed to address this.

Our study has few weaknesses, i) all patients included in the study did not have bacteriologically confirmed TB. However, this reflects the situation in routine clinical practice due to the paucibacillary nature of the EPTB, making our results more generalized for a heterogeneous group of patients. ii) Relapse rate was not studied as patients were not followed-up after completion of treatment. iii) Our sample size was not as large as we had anticipated, as many EPTB patients registered for anti-TB treatment refused to give blood samples for research purposes or were lost to follow-up. The results from this study need to be validated in larger patient populations as well as in different epidemiological settings.

Table 4
The proportion of EPTB patients showing significant change in the levels of five biomarkers constituting the biosignatures at 2 and 6 months of treatment, and the sensitivity of the biosignatures to predict response to treatment.

Immune Biomarkers (Median at 0–2-6 months pg/ml)	All samples (0 M–2 M) N = 64 n (%)	All samples (0 M–6 M) N = 75 n (%)	Responders (0 M–2 M) N = 35 n (%)	Partial responders (0 M–2 M) N = 29 n (%)	P-value**
MIG (2276 – 1418 – 568)	43 (67)	60 (80)	28(80)	15 (52)	.016
IP-10 (481 – 269 – 233)	41 (64)	49 (65)	26 (74)	15 (52)	.061
MIF (13990 – 9407 – 5773)	36 (56)	52 (69)	21 (60)	15 (52)	.506
CCL22 (884 – 626 – 670)	42 (66)	32 (43)	25 (71)	17 (59)	.283
CCL23 (468 – 327 – 301)	33 (52)	47/74* (64)	18 (51)	15 (52)	.910
MIG + IP-10	51 (80)	66 (88)	32 (91)	19 (66)	.010
MIG + CCL23	51 (80)	70 (93)	31 (89)	20 (69)	.052
MIG + MIF	54 (84)	68 (91)	31 (89)	23 (79)	.310
MIG + CCL22	54 (84)	64 (85)	33 (94)	21 (72)	.016
MIG + IP-10 + CCL22	57 (89)	67 (89)	34 (97)	23 (79)	.023
MIG + MIF + CCL23	59 (92)	74 (99)	33 (94)	26 (90)	.492
MIG + MIF + IP-10	58 (91)	72 (96)	34 (97)	24 (83)	.049
MIG + IP-10 + CCL23	54 (84)	70 (93)	32 (91)	22 (76)	.088
IP-10 + MIF + CCL23	59 (92)	70 (93)	33 (94)	26 (90)	.492
MIG + IP-10 + MIF + CCL22	61 (95)	72 (96)	35 (100)	26 (90)	.051
MIG + MIF + IP-10 + CCL23	60 (94)	74 (99)	34 (97)	26 (90)	.218
MIG + MIF + IP-10 + CCL22 + CCL23	62 (97)	74 (99)	35 (100)	27 (93)	.114

n: number of patients showing significant change, N: total number of patients, M: month of treatment.

* Valid available values.

** Chi-square test was done to see difference of biomarkers coverage among responders and partial responders at 2 months of treatment. A p-value < 0.05 was considered statistically significant

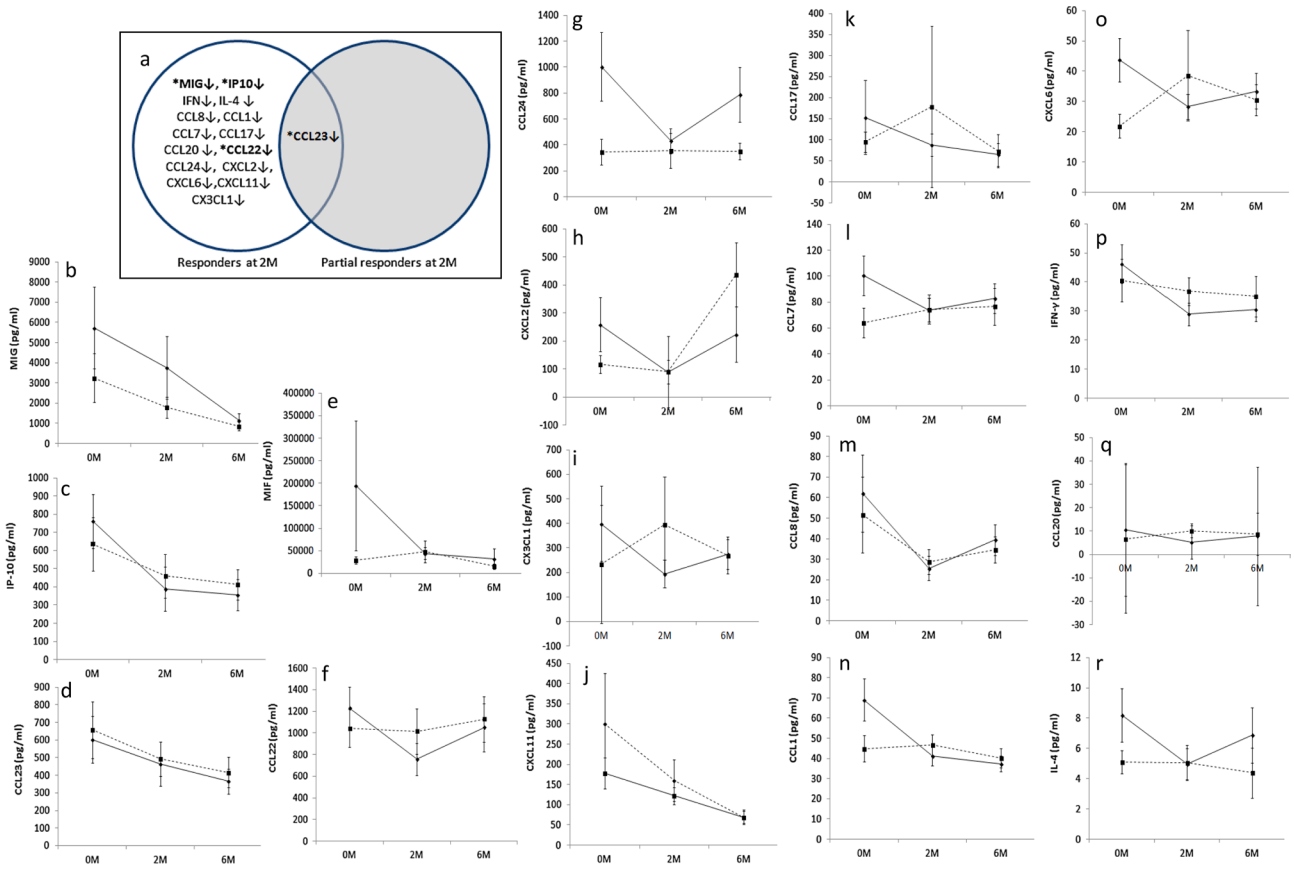


Fig. 8. a: Venn diagram showing inflammatory biomarkers that changed significantly in lymphadenitis and pleuritis patients showing good clinical response (responders), and partial clinical response (partial responders). A paired *t*-test ($p < 0.05$) revealed that a total of 16 inflammatory biomarkers decreased significantly in responders while only one decreased in partial responders. *Inflammatory biomarkers included in the biosignature ↑: significant increase, ↓: significant decrease, M: month of treatment. **b-r:** Mean concentrations of inflammatory biomarkers that changed significantly with treatment in responders n = 23 (continuous line) and partial responders n = 25 (dotted line). The vertical bars show standard error of the mean.

5. Conclusion

A biosignature including MIG, IP-10, MIF, CCL22, and CCL23 was reliable in predicting response to treatment in EPTB at 2 and 6 months after standard anti-TB treatment in our study cohort. Relatively high plasma levels of biomarkers included in the biosignature imply the possibility of developing it further into a test for routine use by using less sensitive ELISA method and using less invasive sampling as dry blood spots.

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7. Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

8. Author's contributions

Concept and study design: TM, AA. Acquisition of funds for the study: TM. Performing the experiments: AA, AK, SM. Analysis and interpretation of data: TM, AA, AT, SUC, SM, MM. Drafting and revising the manuscript: AA, TM, SM, SUC, AK, AT, MM, SZHN. All authors read and approved the final manuscript.

Ethics declarations

The study was approved by the Institutional Review Board, Al. Aleem Medical College & Gulab Devi Educational Complex Lahore (GDEC/18-322) and Regional Committee for Medical and Health Research Ethics, Western-Norway (2018/2392/REK vest). All study participants provided informed consent.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2021.155499.

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