Identification of host biomarkers from dried blood spots for monitoring treatment response in extrapulmonary tuberculosis

Abstract

Background

Extrapulmonary TB comprises of 16% of the 7.1 million incident cases of TB worldwide. The bacteriological confirmation of EPTB is challenging due to paucibacillary nature of the disease, and sampling difficulties. Therefore, treatment is often started empirically, especially in low resource settings. Hence, treatment monitoring is crucial for proper case management. Currently, there is a lack of objective tools for treatment monitoring in various forms of extrapulmonary TB. The aim of the study was to explore the utility of inflammatory biomarkers from the dry blood spots as a tool for treatment monitoring of EPTB.

Methods

A prospective cohort study was carried out at Gulab Devi Hospital, Lahore, Pakistan. A total of 105 presumptive extrapulmonary tuberculosis patients were recruited and samples were taken before, during (2 months) and after treatment (6 months). A total number of forty inflammatory biomarkers were investigated in dried blood spot samples using Biorad 40 plex Bio-Plex Pro^{TM} Human Chemokine Panel.

Results

A total of 14 inflammatory host biomarkers changed significantly with treatment. MIG, CCL23, IP10, CXCL1, and CXCL2 significantly declined in both, tuberculous pleuritis and tuberculous

lymphadenitis EPTB, at both time points. Levels of IP10, CCL23, CCL8, CCL3, and IL6 was 2 to 9 folds lower in the dried blood spots as compared to plasma, however the trend in the decline after treatment was the same. A biosignature consisting of MIG, CCL23, CXCL2, corresponded with the treatment response in 81% patients at the second month and 79% of patients at the end of treatment.

Conclusion

The change in the level of inflammatory biomarkers in the dried blood spots corresponds with the success of treatment and can be developed as a point of care test in low resource settings. A biosignature of MIG, CCL23, CXCL2 can be used to gauge treatment success in TB lymphadenitis and TB pleuritis patients.

Introduction

Tuberculosis is one of the top ten causes of death worldwide, caused by *Mycobacterium tuberculosis*, ranking above HIV and AIDS (1). WHO estimates shows that extrapulmonary TB comprises of 16% of the 7.1 million incident cases of TB worldwide (1). Extra pulmonary TB is a paucibacillary disease (2, 3), that involves anatomical sites that are often inaccessible for a laboratory confirmation to make a definitive diagnosis (4). Lymph nodes and pleura are two of the most common sites of extrapulmonary tuberculosis (5, 6), even though any organ can be infected (7). All methods for microbiological confirmation of extrapulmonary TB have their set of limitations (3), due to which the treatment of extrapulmonary TB is often started empirically (2). Empirical treatment is more common in resource constrained settings with high TB burden (8). Hence, treatment monitoring is crucial in identifying the EPTB patients at an earlier stage, for proper case management and avoid delay in case of alternative diagnosis. The current tools for

treatment monitoring have limited sensitivity and specificity (4). There is a lack of objective tools for treatment monitoring in various forms of EPTB. In the routine TB control programs, completion of treatment is used as a proxy for a successful treatment outcome. However, many cases despite taking full course of treatment do not respond in a satisfactory manner. This normally leads to unnecessary cycles of TB treatment, leading to delay in diagnosis and treatment of alternative diagnosis, increasing mortality and morbidity (9). An objective, robust, rapid and inexpensive method for treatment monitoring, needs to be employed that can help us gauge treatment response to the anti TB therapy, in a timely manner (10). Host biomarkers produced as a result of pathogen infection have shown to have potential to be used as a surrogate tool for monitoring therapy in EPTB (2, 11, 12). Using whole blood and plasma samples for treatment monitoring requires cold storage, expensive reagents and an uninterrupted supply of water and electricity that are absent in most of the high TB endemic settings (13). Using dried blood spots can resolve many problems as they offer a simple, robust, cost-effective method of sampling, involving simple storage and transportation methods (14, 15). This study is part of a larger project, in which the same forty inflammatory biomarkers were also investigated in unstimulated plasma, to monitor treatment response in EPTB (16). For this study, we intend to investigate the same set of biomarkers in dried blood spots as markers for treatment response in EPTB. We wanted to observe how many of these biomarkers change significantly with treatment and their potential to be developed as a point of care test.

Methodology

This study is a part of a larger project carried out at Gulab Devi Hospital, Lahore, Pakistan (16). Gulab Devi Hospital is a tertiary care hospital that specializes in TB care that also receives suspected TB patients from the surrounding suburban and rural areas. The recruitment for this

study was done during a period of one year, from April 2016 to August 2017. A total of 671 outpatients with presumptive extrapulmonary tuberculosis > 15 years of age were enrolled. All investigations were carried out at Gulab Devi hospital laboratory. The enrolled patients were followed up prospectively from the start to the end of the treatment. Blood samples of 5ml was collected from the patients at three different time points during treatment, that is, before the start of the treatment, and at the end of second and sixth months of treatment.

Laboratory Procedures

For patients with tuberculous lymphadenitis, samples were collected by excisional biopsy for performing histopathology, AFB smear, Xpert and mycobacterial culture. Whereas, for tuberculous pleuritis, aspirated fluid of the pleural effusion was sent for cytology and microbiological workup. The collected specimen was processed for smear examination, Xpert MTB/RIF assay (Xpert) and culture (17). The smear was visualized with both Ziehl Neilson stain and Auramine O-stained smears under Light emitting diode Fluorescence microscope. Xpert was performed according to the manufacturers protocol (18) . For culture, two slopes of Lowenstein Jensen culture and one mycobacterial growth indicator tube (MGIT^{TM} 960^{TM}; Becton Dickinson, Sparks, MD, USA) were inoculated (19) (5). The bacteriological confirmation was done either be culture or/and by Xpert MTB/RIF in conjunction with smear microscopy.

Preparation of Dried Blood Spots

A Whatman 903 paper was taken and labelled with the patients ID and date. The filter paper was placed on a flat surface to ensure drying and preventing the sample to be drawn below the surface. A 2 to 3 ml of blood sample was drawn into an EDTA tube. Only 25 microliter of blood was pipetted out from the EDTA tube, to make spots on the Whatman paper. Care was taken to make

an even distribution of blood in the spots. After that, each paper was placed in a drying rack and allowed for drying for 3-4 hours at room temperature. Ones dried, the filter papers were put in double sealed bags with a desiccant bag, as humidity significantly decreases stability. The double sealed bags were then stored at minus 20 degree Celsius until further use.

Elutes from the Dried Blood Spots

The dried blood spots were eluted before they were subjected to the multiplex assay. The blotting cards were taken out of the freezer and first kept at room temperature for thirty to forty minutes. Two punches of 6mm were made in dried blood spot (DBS). If more specimen was required, then a blank filter paper was punched twice or thrice between each punch of the specimen. 120µl of buffer (one tablet of protease in 10 ml of wash buffer) was added to the micro centrifuge tube and subsequently placed in the shaker for 15 minutes. After taking out of the shaker it was kept at room temperature for one hour. It was centrifuged at 2300 g for 10 minutes. The supernatant was eluted in the other micro centrifuge tube and then the elute was preserved as per assay specification.

Inflammatory biomarkers detection through Multiplex Microbead Immunoassay

Biorad 40 plex Bio-Plex Pro™ Human Chemokine Panel, was used on Luminex® xMAP™ to detect biomarkers from the elutes of dried blood spots (DBS). As seen in table 1, the biomarkers investigated included various pro and anti-inflammatory cytokines, chemokines and growth factors. 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 with the wash buffer. 50 ul of standards, controls, and samples were added in the respective wells. After one hour's incubation on shaker at room temperature, plates were washed three times and 25 ul of detection antibodies were added in each well. After an incubation of 30 minutes at

room temperature and washing thrice, 50 ul of streptavidin-E was added. Plates were incubated for another 10 minutes on the shaker at room temperature and after three washings, resuspended with 125 ul of assay buffer. Plates were read with a Luminex instrument (Luminex 200, Austin Luminex, USA) and data was analysed using MILLIPLEX Analyst 5.1 software (Merck Millipore Darmstadt, Germany), in accordance with the manufacturer's instructions.

Table1: List and classification of the biomarkers that were analysed using the Biorad 40 plex Bio-Plex Pro™ Human Chemokine Panel.

Pro-inflammatory cytokines	Interferon-gamma (IFN-γ), Tumor necrosis factor Alpha (TNF-α), IL-1β, IL-6 and IL-8, IL 16, MIF	
Anti-inflammatory cytokines	IL4, 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	

Response to treatment

A criterion was used as a tool to assess the treatment response of the EPTB patients. Response was considered good if two of the three criteria were fulfilled. Firstly, improvement of the presenting symptoms. Secondly, regression of the local signs of the disease such as, pleural effusion and enlarged lymph nodes, and lastly, weight gain.

Extrapulmonary Tuberculosis case confirmation

Participants were defined as confirmed or probable cases of EPTB by using a composite reference standard, including clinical, radiological and laboratory findings. Cases that were bacteriologically confirmed by culture and /or Xpert were defined as confirmed EPTB. Those not bacteriologically confirmed were defined as probable EPTB cases based on signs, symptoms and laboratory findings consistent with TB and a good response to anti TB treatment at 2 to 3 months or/and end of the treatment. The laboratory findings consistent with TB pleuritis include, lymphocytosis, fluid protein level > 3g/dl or plasma adenosine deaminase levels > 16 U/L or concomitant pulmonary TB suggested by positive acid-fast smear and/or chest radiograph. TB lymphadenitis was classified as probable if in addition to the typical clinical signs and symptoms, the histopathology findings, were also suggestive of TB lymphadenopathy.

Statistical Methods

The analysis was performed by using International Business Machine (IBM) – Statistical Package for Social Sciences (SPSS) Statistics version 26. Nonparametric statistical analytic methods were used to analyse the data. Wilcoxon signed rank sign test was performed for paired values. A p value < 0.05 was considered significant. Chi square test was used to compare groups on categorical data. Multiple biosignatures were extrapolated by selecting only those biomarkers that changed significantly with treatment at both time points. In order to formulate a biosignature, those patient samples were chosen that showed $\geq$ 20% change from the baseline levels. Python And Data Analysis was used to compute the various possible combinations. A combination consisting of least number of biomarkers and covering the maximum number of patients was selected.

Results

Patients Characteristics

As seen in figure 1, out of the total 364 EPTB patients registered, 105 patients were included in the study and were prospectively followed during their course of treatment. Rest of the patients could not be included due to noncompliance and unavailability for providing samples. Figure 1. shows the patients inclusion criteria and classification based on the type and bacteriological status of the cases. The cohort mainly comprises of two types of EPTB, 57 tuberculous lymphadenitis and 48 tuberculous pleuritis cases. A total of 61 patients were bacteriologically confirmed EPTB cases based on the positive culture results of their specimen. Rest of the 44 patients are clinically diagnosed based on the composite reference standard. All patients received the standard TB treatment (isoniazid, rifampicin, pyrazinamide, ethambutol) for at least 6 months. Blood samples

were collected before and during (2 and 6 months) of treatment. A total of 8 patients were loss to follow up after two months of treatment. At the end of the two months of receiving TB treatment, 45% cases of TB lymphadenitis and 77% of TB pleuritis responded to the treatment. However, the percentage of responders rose to 68% amongst the TB lymphadenitis and 89% amongst the TB pleuritis, at the end of 6 months of treatment. Almost half of the patients that responded partially to the treatment after the second month, ultimately responded to the treatment after 6 months of treatment completion. Treatment was extended for a total of 21 patients. All the cases showed clinical improvement at the end of their treatment duration.

As seen in table 2, our cohort had a higher percentage of females amongst the TB lymphadenitis patients while, a male predominance was seen amongst the tuberculous pleuritis cases with a median age of 20 and 25 years, respectively. Only one tuberculous pleuritis patient had a high glucose level above 200mg/dl, while the rest of the patients had no history of diabetes.

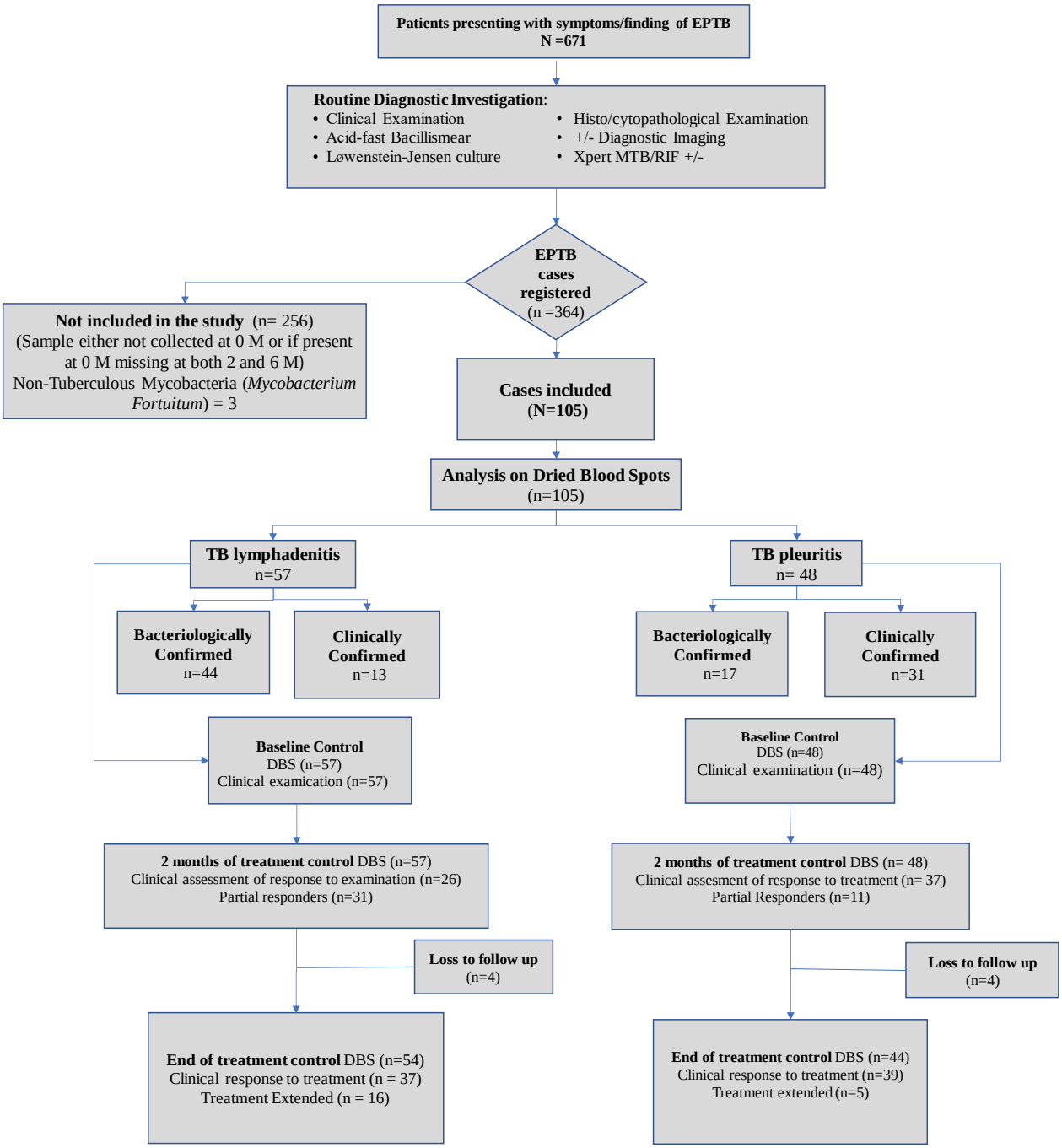


Figure 1. A flow chart showing the patients inclusion and the number of dried blood spots samples. A further classification of the DBS based on the type of EPTB and the bacteriological status.

Abbreviations, DBS: dried blood spot, DPS; dried plasma spot, TB; tuberculosis, EPTB: extrapulmonary tuberculosis, M: Month of the treatment, +ve; positive, -ve; negative

Table 2: Demographic and clinical characteristics of extrapulmonary tuberculosis patients:

Patient Characteristics		TB Lymphadenitis N=57	TB Pleuritis N=48	p value
Age in years, median (range)		*20 (11 – 72)*	*25 (15 – 70)*	*0.143*
Gender	Male	*16 (27.5)*	*35 (72)*	*0.00*
	Female	*42 (72.4)*	*13 (27.6)*	
HIV status n (%)	Positive	*0 (0)*	*0 (0)*	*insignificant*
	Negative	*57 (100)*	*48 (100)*	
History of Diabetes, n/N (%)	No	*53/57 (93)*	*45/48 (94)*	*0.407*
	Yes	*0/57 (0)*	*1/48 (2)*	
	N/A	*4/51 (7)*	*2/48 (4)*	
TB case Category, n/N (%)	Confirmed	*44/57 (77)*	*17/48 (13)*	*0.00*
	Probable	*13/57 (23)*	*31/47 (65)*	
Clinical response at 2nd month of treatment, n/N (%)	Responders	*26/57 (46)*	37/48 (77)	*0.011*
	Partial responder	*31/57 (54)*	11/48 (23)	
	N/A	0/57 (0)	0/48 (0)	
Clinical response at 6 month/end* of treatment, n/N (%)	Responders	*37/57 (73)*	38/48 *(81)*	*0.103*
	Partial responder	*16/52 (28)*	5/48 *(10)*	
	N/A	4/57 *(7)*	4/48 *(8)*	

N: Total number, n: number, %: percentage, NA: no information available or loss to follow up
TB: tuberculosis, M: month.

*Treatment was extended for 16 TB lymphadenitis and 5 TB pleuritis patients beyond 6 months.

Significantly changed inflammatory biomarkers with treatment in Tuberculous Pleuritis

All biomarkers were detected in the dried blood spots isolated from tuberculous pleuritis patients. Data is shown as the supplementary table 1. A total of 11 inflammatory biomarkers changed significantly in tuberculous pleuritis patients as shown in the box plots in figure 2. The biomarkers that declined significantly at both time points during treatment as compared to the

baseline before treatment were CXCL9/MIG (2M, p = 0.025, 6M, p = 0.04), CCL23 (2M, p = 0.023, 6M, p = 0.01), IP10 (2M, p=0.025, 6M, p= 0.021), CXCL1 (2M, p=0.04, 6M p= 0.015), CXCL2 (2M, p=0.00, 6M, p=0.003) and CCL2 (2M, p=0.08, p= 0.012). Only IL6 (p =0.01), CCL20 (p=0.026), CXC11 (0.011), CCL3 (p = 0.047), and CXCL13 (p=0.012) declined significantly at the end of the 6 months as compared to the baseline levels. The biomarkers that changed insignificantly with treatment in Tb pleuritis can be reviewed in supplementary figure 1.

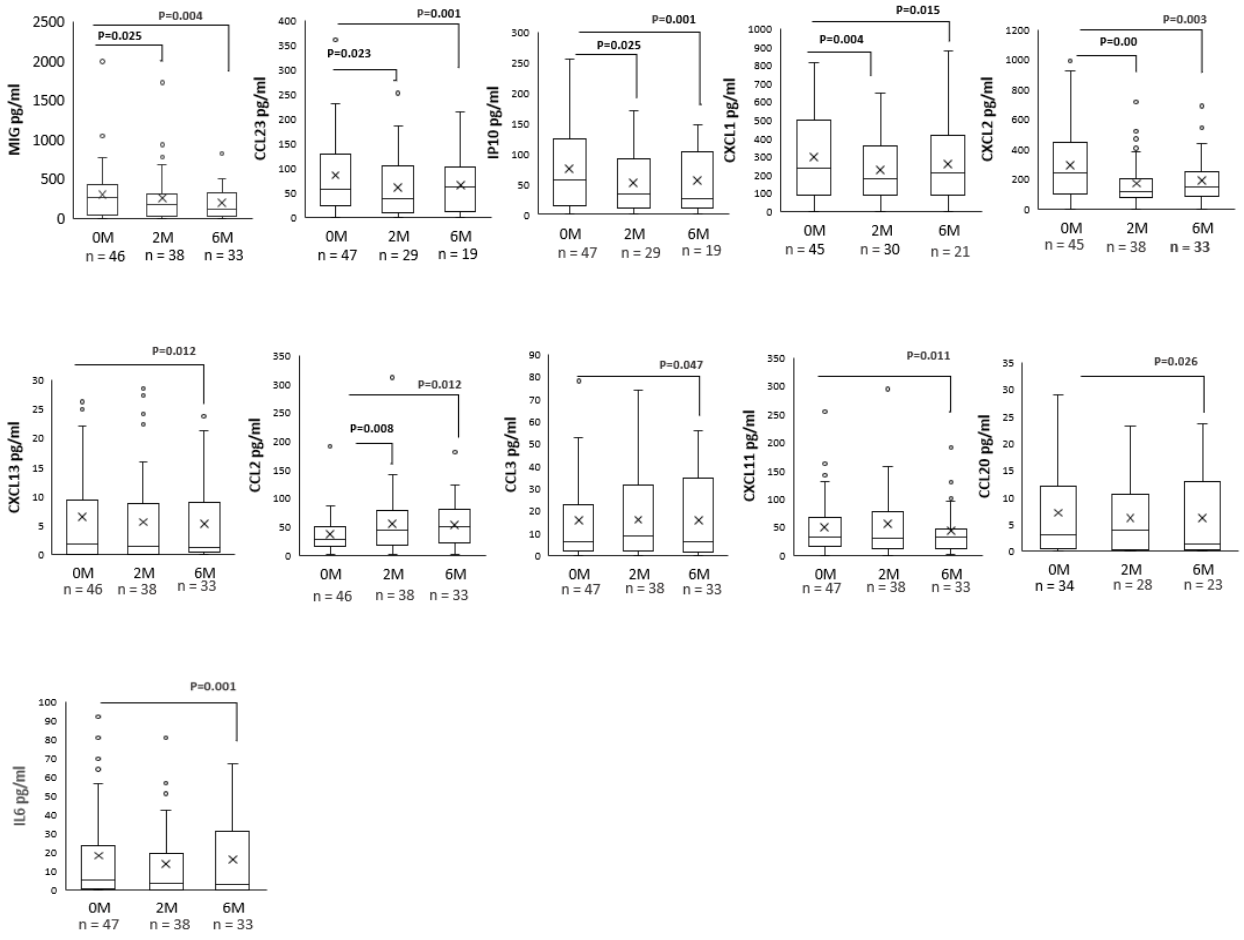


Figure 2. Box plots showing levels of inflammatory biomarkers in dried blood spots in TB pleuritis patients at baseline, 2nd and 6th months of treatment. Only significantly changing biomarkers are shown. A p-value < 0.05 was considered significant. The boxes represent the

median and interquartile range, while the whisker represents the minimum and maximum values. Different time points of treatment are shown on x axis.

0 M; the baseline levels before initiation of treatment, 2M; 2 months of treatment, 6 M; 6 months of treatment, n; number of patients

Significantly changed inflammatory biomarkers with treatment in Tuberculous lymphadenitis

Even though all forty biomarkers were detected in the dried blood spots (shown in supplementary figure 2) isolated from the tuberculous lymphadenitis patients, ten biomarkers were detected to change significantly amongst them as seen in figure 3. CXCL9/MIG (2M, p= 0.05 6M, p= 0.02), CXCL11 (2M, p= 0.03, 6M p=0.02), CCL23 (2M p=0.09 6M p=0.042), IP10 (2M p=0.04, 6M p= 0.04), CXCL2 (2M, p=0.00 6M, p=0.026), CXCL1 (2M, p=0.02, 6M, p=0.03), declined at the second and the sixth months after treatment as compared to the baseline after treatment. While CCL20 (p= 0.02), CCL15 (p=0.06), CCL8 (p= 0.01), and MIF (p= 0.009) significantly changed only at the second month of treatment.

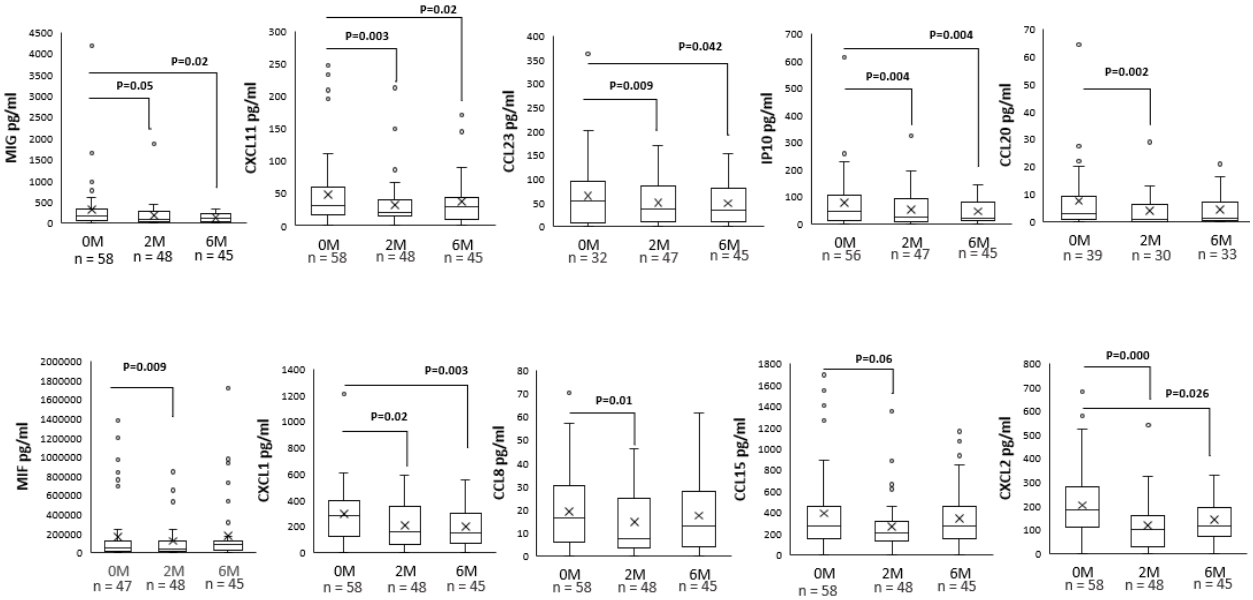


Figure 3. Box plots showing levels of inflammatory biomarkers in dried blood spots amongst the TB lymphadenitis patients at the baseline, 2^{nd} and 6^{th} months of treatment. Only significantly changing biomarkers are shown. A p-value < 0.05 was considered significant. Boxes represent the median and interquartile range, and the whisker represent the minimum and maximum values. Three different time points of treatment are shown on x axis.

0 M; baseline levels before initiation of treatment, 2M; 2 months of treatment, 6 M; 6 months of treatment, n; number of patients.

Common biomarkers in Tuberculous pleuritis and Tuberculous lymphadenitis

Overall biomarkers that changed significantly in both types of EPTB, after the second and sixth months of treatment were, MIG, CCL23, IP10, CXCL1 and CXCL2, as shown in figure 4. These inflammatory biomarkers decreased in levels at both time points during treatment, as compared to the baseline levels. In addition, MIF, CCL15 and CCL8 were observed to decline significantly after the end of two months of intensive phase of treatment, in tuberculous lymphadenitis group. While CCL3, CXCL13, IL6 changed significantly in tuberculous pleuritis patient at the end of the 6 months of treatment.

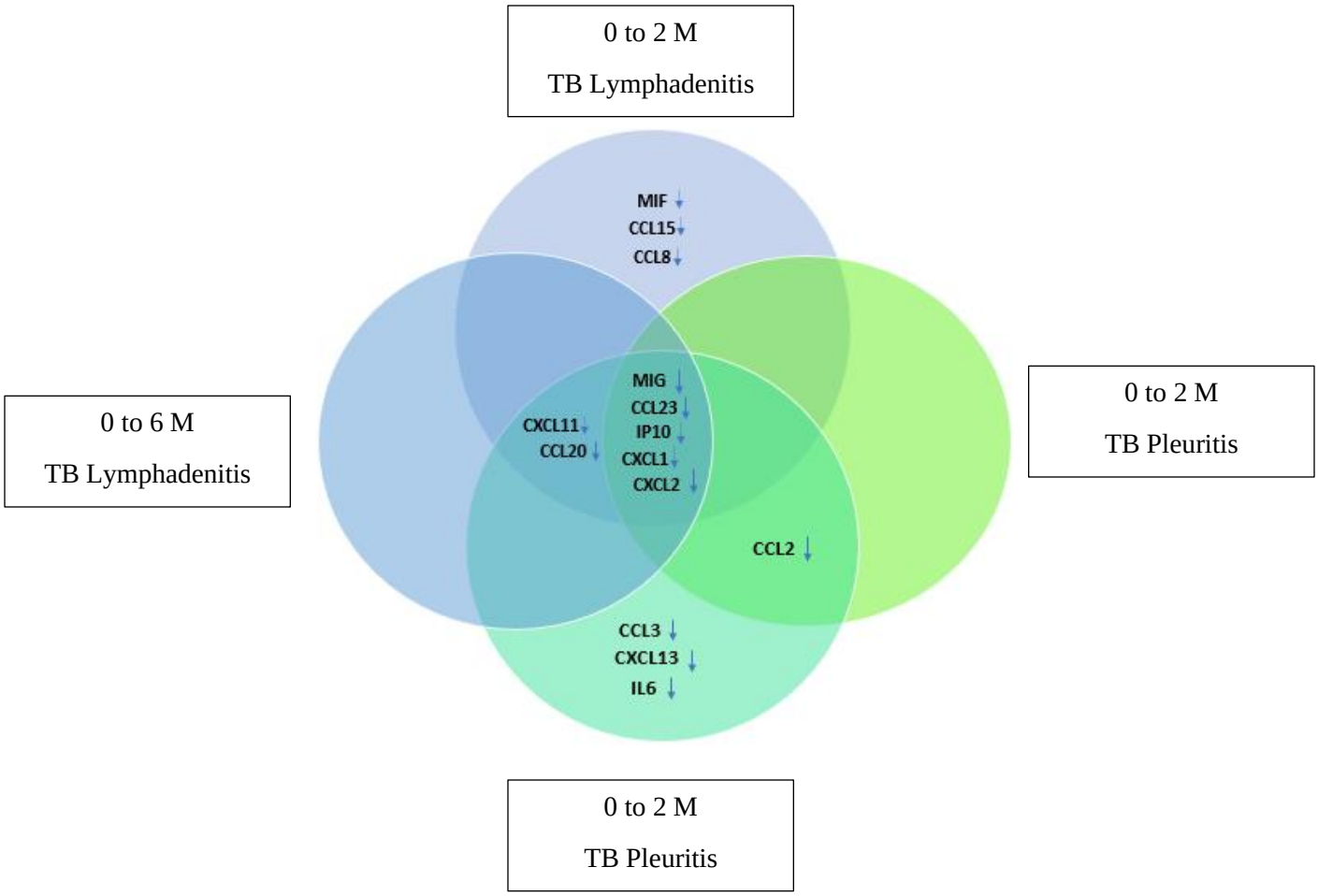


Figure 4. Common inflammatory biomarkers changing significantly with treatment in both types of TB pleuritis and TB lymphadenitis, at the second and the sixth month of treatment.

0 to 6 M: biomarkers changing significantly at 6 months as compared to the baseline, 0 to 2 M: biomarkers changing significantly at 2 months as compared to the baseline ↓: used to show a decline in the level of biomarker and ↑: used to show an increase in the level of biomarker.

Magnitude and proportion of biomarkers change with treatment

A total of 14 biomarkers changed significantly in both types of EPTB as shown in table 3. These biomarkers were not observed to change in all the patients. Table 3 shows the proportion of patients showing a $\geq$ 20% change in its levels of biomarkers at second and sixth months of treatment as compared to the baseline. The magnitude of change varied amongst the biomarkers. This was

depicted by the amount of fold change in the biomarkers when compared to the levels at the baseline. Overall, the fold change ranged from 2 to 13 folds. Amongst the tuberculous lymphadenitis patients, CCL20 and MIF underwent maximum fold change after the second month of treatment, 13-fold and 10 folds respectively. Followed by a 5-fold rise in the levels of MIF and decline in CCL20 at the end of the treatment. Amongst the tuberculous pleuritis patients, IL6 and CXCL13 were amongst the biomarkers with the highest fold change as compared to the baseline levels. IL 6 underwent a 5-fold and a 6-fold decline in its levels at the end of the 2^{nd} and 6^{th} months of treatment respectively, when compared to the baseline. CXCL13 showed an 8 folds decline in its level at the end of the treatment when compared to the baseline.

Table 3. Proportion of tuberculous pleuritis and tuberculous lymphadenitis showing a change of ≥20% in the levels of biomarkers as a response to treatment and the magnitude of fold change in the medians in the dried blood spots

Biomarkers	**TB lymphadenitis**				**TB pleuritis**			
	0-2 Months		**0-6 Months**		**0-2 Months**		**0-6 Months**	
	n/N (%)	**Median FC ↓/↑**	**n/N (%)**	**Median FC ↓/↑**	**n/N (%)**	**Median FC ↓/↑**	**n/N (%)**	**Median FC ↓/↑**
MIG	25/48 (52)	3↓	28/45 (62)	4↓	22/37 (59)	2↓	21/32 (65)	6↓
CCL23	42/57 (74)	3↓	36/57 (63)	3↓	18/38 (47)	3↓	23/33 (73)	3↓
IP-10	25/45 (56)	6↓	29/43 (67)	2↓	20/38 (53)	3↓	21/33 (64)	3↓
CXCL1	17/48 (35)	3↓	27/45 (60)	3↓	9/37 (24)	2↓	22/32 (69)	3↓
CCL20	18/29 (62)	13↓	18/35 (51)	5↓	16/28 (57)	3↓	15/23 (62)	5↓
CXCL2	33/48 (69)	5↓	27/45 (60)	2↓	27/37 (73)	2↓	22/33 (67)	2↓
MIF	14/46 (30)	10↓	12/45 (27)	5↑				
CXCL11	12/48 (25)	3↓	26/45 (58)	3↓	7/38 (18)	3↓	21/33 (64)	3↓
CCL8	27/48 (56)	3↓	23/45 (56)	6↓				
CCL15	25/48 (52)	2↓	22/45 (49)	2↓				
CCL2					22/37 (59)	2↑	21/32 (66)	2↑
IL-6					8/38 (21)	5↓	31/38 (81)	6↓

CCL3					12/38 (31)	2↑	16/33 (48)	2↓
CXCL13					11/37 (30)	4↓	22/32 (67)	8↓

N: total number of patients, n: number of patients showing significant change, FC: fold change

Comparison of biomarkers in Plasma from the DBS

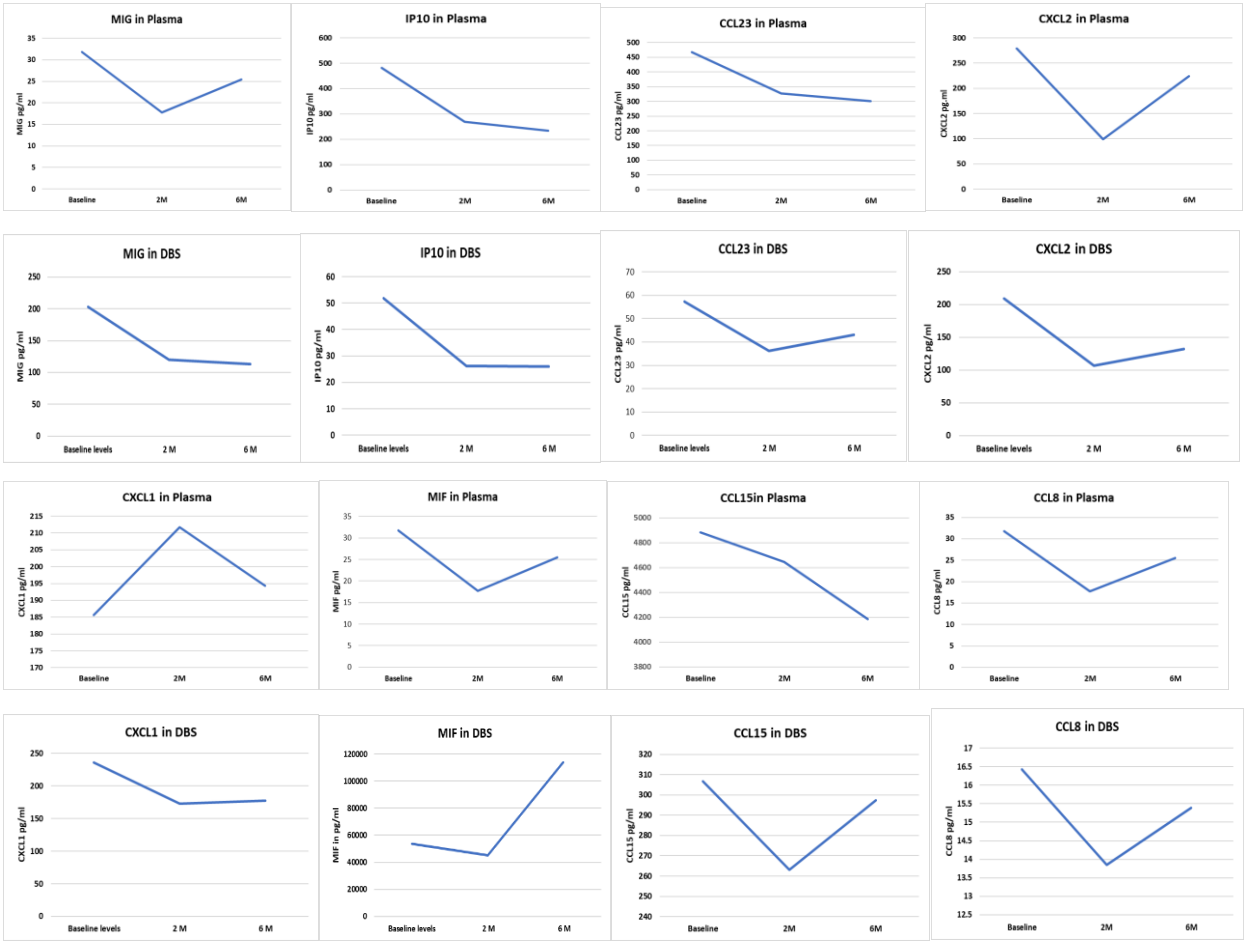
The levels of significantly changing biomarkers from the dried blood spots and the trend in their change during treatment was compared with plasma. As seen in table 4, the baseline levels of the biomarkers in plasma were far greater than in dried blood spots. Overall, the biomarkers underwent a change of 1 to 16 folds decline in DBS. CCL15 underwent a maximum decline of 16 folds in DBS. As shown by the line graphs in figure 5, irrespective of the difference in the levels between the two types of samples, the trend of change in the levels with treatment was quite similar for IP-10, CCL23, CCL8, CCL3 and IL6. IP10, CCL23, and IL6 underwent a decline of 9, 8, and 4 folds in dried blood spots, respectively. While CCL8, and CCL3 declined 2 folds in dried blood spots.

Table 4. Difference of the baseline levels of significantly changing biomarkers in plasma and DBS.

Biomarkers	***Baseline level in plasma (pg/ml)**	***Baseline level in DBS (pg/ml)**	**Difference in Levels**
MIG	2275	203	11 ↓
IP10	481	52	9 ↓
CCL23	468	57	8 ↓
CXCL2	279	209	1 ↓
CXCL1	186	236	1 ↑
MIF	13990	53548	4 ↑
CCL15	4882	307	16 ↓
CCL8	32	16	2 ↓

CCL2	30	31	1 ↑
CCL3	10	6	2 ↓
CXCL11	130	33	4 ↓
CCL20	12	3	4 ↓
CXCL13	17	1.4	12 ↓
IL6	14	4	4 ↓

*All values at the baseline are given as medians. ↓ indicates a decrease in the median levels of the biomarkers, ↑ indicates an increase in the median levels of the biomarkers



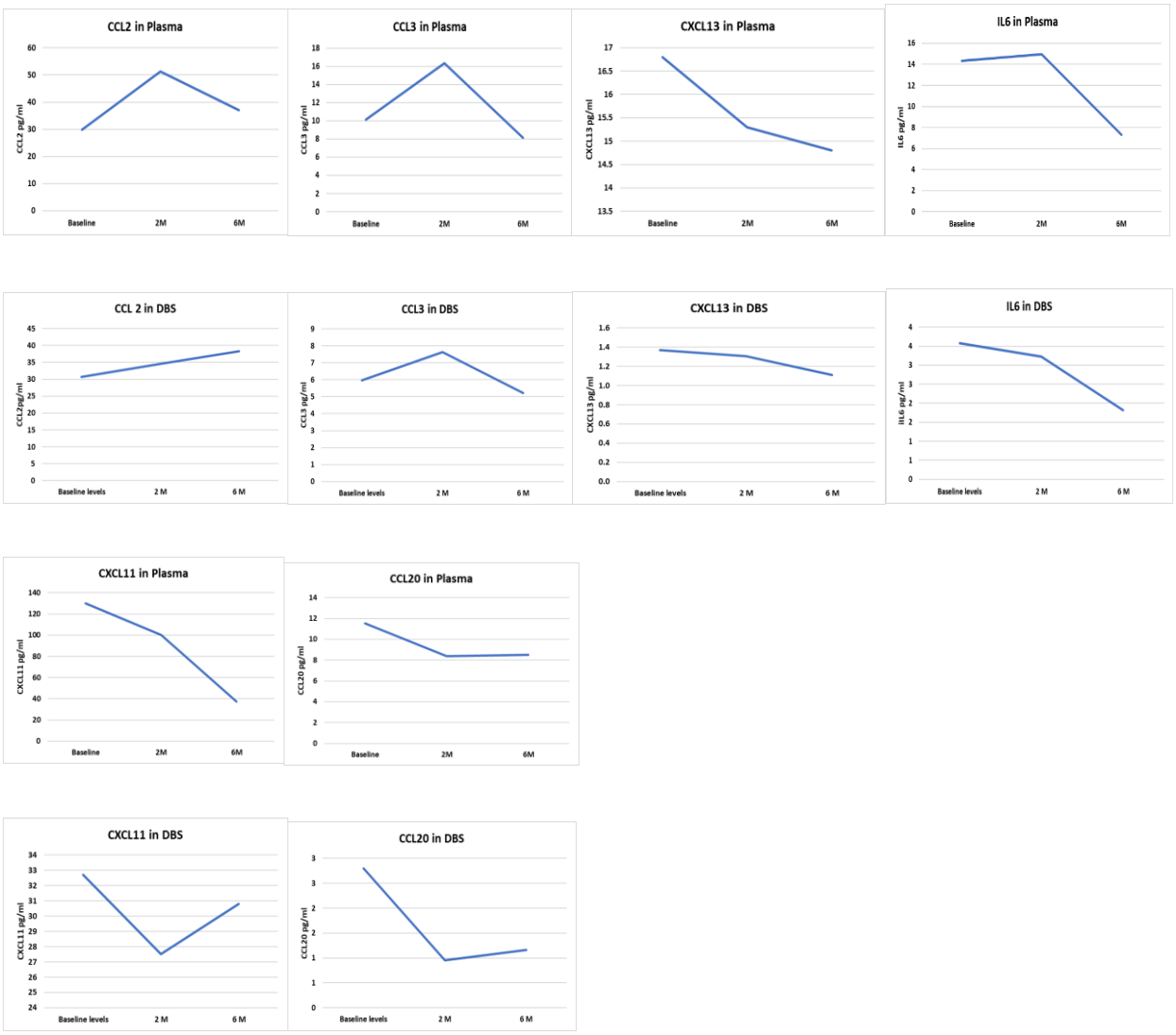


Figure 5: Line graphs showing trends of change in the biomarkers at the second and sixth month of treatment amongst plasma, and dried blood spots. The values shown are the medians of the biomarkers.

M; months, DBS; dried blood spots. The x axis represents the three timepoints, when the samples were taken.

<u>Comparison of change in biomarkers among Responders and partial responders at the end of second month of treatment</u>

Based on the response of the patients to the anti TB treatment, the patients were classified into responders and patrial responders. Figure 6 shows the comparison of biomarkers significantly changing in both the groups. Except for CCL20 that exclusively declined in partial responders, MIG, CCL23, IP10, CXCL2 and CXCL1 changed significantly with treatment in responders as well as partial responders.

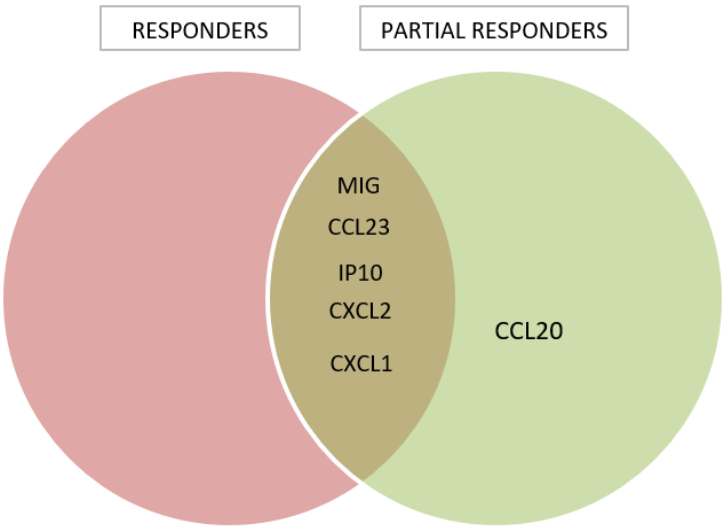


Figure 6. Common biomarkers changing significantly with treatment amongst the responders and partial responders. All biomarkers decreased in their levels.

Comparison of biomarkers based on the bacteriological status

The samples were grouped based on their bacterial load among both, tuberculous pleuritis and tuberculous lymphadenitis patients. At the baseline, there was no difference among the bacteriologically and clinically confirmed cases. As viewed in figure 7, CXCL1 and CXCL2 significantly changed in bacteriologically and clinically confirmed cases of both TB lymphadenitis and TB pleuritis. Whereas MIG and IP10 changed significantly amongst bacteriologically confirmed cases of both types of EPTB.

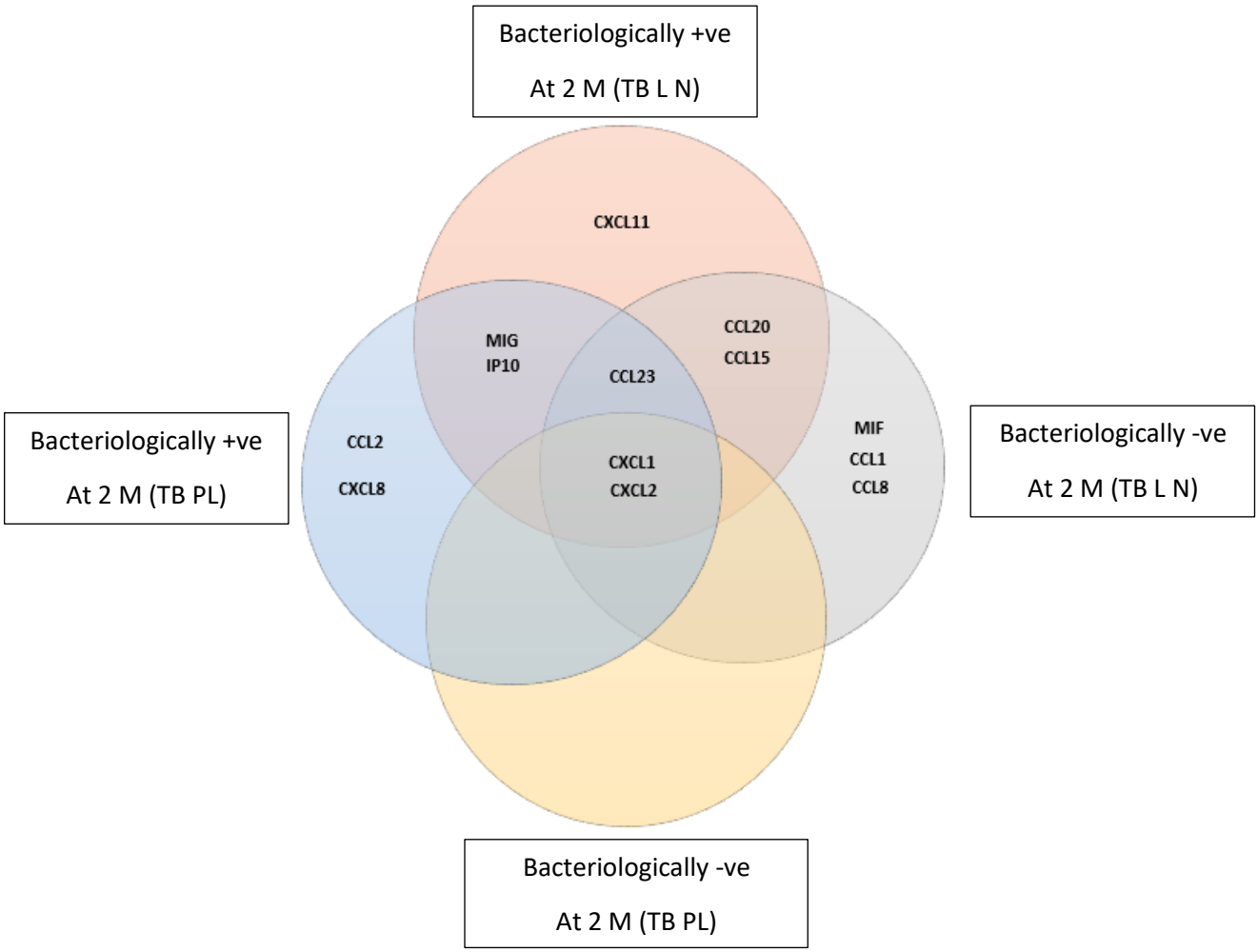


Figure 7. Common biomarkers changing significantly amongst the bacteriologically confirmed cases and non-bacteriologically confirmed cases. Bacteriologically +; bacteriologically confirmed, Bacteriologically -; clinically confirmed.

A Biosignature predicting response to treatment in majority of patients

Levels of inflammatory biomarkers, that declined consistently with treatment were selected to formulate a biosignature, that can be used for predicting treatment response in maximum number of patients by least possible number of biomarkers at both time points, and in both types of EPTB. As shown in table 5, we narrowed down our selection to CXCL2, MIG, IP10, CCL23 as these were in an easily detectable range based on their median levels in dried blood spots and showed

large differences amongst the low and high value change during treatment. As seen in table 3, the fold change of these biomarkers ranged from 2 to 6 folds.

Table 5. The number of patients showing a significant change in a combination of five biomarkers, constituting the biosignature, changing significantly with treatment at both time points, and the sensitivity of the biosignature.

Immune Biomarkers	**All samples**	**All samples**	**Responders**	**Partial responders**
(Median at 0–2-6 months pg/ml)	**(0 M–2 M)**	**(0 M−6 M)**	**(0 M−2 M)**	**(0 M−2 M)**
	N = 86	**N = 78**	**N = 52**	**N = 33**
	n (%)	**n (%)**	**n (%)**	**n (%)**
CXCL2 (209–107 –132)	60 (70)	49 (63)	37 (71)	22 (67)
CCL23 (57 – 36 – 43)	49 (60)	47 (60)	26 (50)	22 (67)
MIG (203–120 –113)	47 (55)	49 (63)	26 (50)	20 (61)
IP 10 (52–26 –26)	45 (52)	51 (65)	26 (50)	18 (54)
MIG + CXCL2	66 (77)	60 (78)	40 (77)	25 (76)
IP10 + CXCL2	66 (77)	61 (78)	40 (77)	25 (76)
CCL23 + CXCL2	69 (80)	58 (74)	41(79)	27 (81)
MIG + IP10 + CXCL2	69 (80)	62 (79)	41 (79)	26 (79)
MIG + CCL23 + CXCL2	70 (81)	62 (79)	41 (79)	27 (81)

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

As viewed in table 5, combination of biomarkers constituting, MIG, CCL23 and CXCL2 showed a significant decline in 81% patients at the second month of treatment and 79% of patients at the end of treatment.

Discussion

This prospective cohort study was designed to test the hypothesis whether it is possible to identify and harness novel host biomarkers from the dried blood spots for the purpose of monitoring treatment efficacy in extrapulmonary tuberculosis, and their potential to be used as a point of care test. All the candidate biomarkers have a well-established role in the pathogenesis of tuberculosis and were detected in the dried blood spots. Out of all the biomarkers that were detected, a biosignature of three biomarkers namely, MIG, CCL23 and CXCL2 was deduced to be a good candidate for developing a point of care test for therapy monitoring in EPTB. This biosignature, can predict response in two types of EPTB with the accuracy of 81% after 2 months of treatment and in 79% of patients, at the end of the treatment. Studies have shown that the use of a combination of biomarkers, for monitoring a disease process is more beneficial, than using a single biomarker (20) . Especially in a disease like EPTB whose pathogenesis involves interplay of so many pro and anti-inflammatory cytokines and chemokines (21, 22).

Role of inflammatory biomarkers as candidates for treatment monitoring has been explored in some previous studies. There is evidence that decline in plasma levels of MIG, IP10, G-CSF, IFN corresponds with successful TB treatment (23). The role of CFP-10, ESAT-6 and matrix metalloproteinases has also been investigated for the management of EPTB (24-26). So far, IP-10 is the only biomarker that has, demonstrated to be the most promising candidate for treatment monitoring of EPTB, in both plasma and in dried blood spots (2, 23, 27). Several studies are

supporting its role as a potential surrogate marker for management of EPTB, in both high and low endemic countries. In certain studies, an increase in the levels of MCP-1/CCL2 and eotaxin -1/CCL11 biomarkers with successful treatment has also been studied (23). However, very few studies have focused primarily on EPTB, majority of the work concentrating on pulmonary tuberculosis (23).

Even though all the biomarkers showed a change in their levels during treatment, not all changed significantly. A few prerequisites were taken under consideration while selecting a biosignature. Firstly, biomarkers that showed a persistent decline with treatment were selected. Secondly, they had high baseline levels and showed a high fold change with treatment in the maximum number of patients. A high baseline level ensures that biomarker of interest is easily detectable. These features could facilitate the future development of more robust ELISA test which is low cost and sustainable in routine clinical settings.

MIG, CCL23, IP10, CXCL2 and CXCL1 declined significantly with treatment amongst both, responders and partial responders. CCL20 declined significantly only amongst the partial responders. Observing the decline in the levels of host biomarkers after the second month of treatment was crucial. This is because the major bactericidal activity takes place during this intensive phase of treatment and the effect of treatment can be measured after the completion of two months of treatment (28). Hence, the cohort was classified into good responders and partial responders depending upon their response to treatment and partial responders to treatment were taken as proxy control groups.

We decided to study the host biomarkers in dried blood pots, as the use of plasma has an obvious set of limitations. Plasma sampling cannot be performed in low resource settings, in the absence

of made to purpose collection facilities, infrastructure and trained staff (29). The storage, handling and stability of biological proteins is quite crucial in plasma sampling. Certain biological proteins such as , CCL19, have shown degradation in there level especially during thawing processes, even if the cold storage is maintained (30) (31). Presence of anticoagulants, duration of storage, and dilutions make levels of biological proteins in the plasma samples vulnerable to change. We investigated the biomarkers in dried blood spots as their use can be revolutionary in the field of medical research, surveillance and delivery of health care, especially for the resource constraint areas of the world, that bear the maximum burden of communicable diseases (13, 32) (14). As seen in previous studies, they have been rendered quite accurate and reliable for the analysis and investigation purposes of biomarkers, with rapid results (13, 14). The proteins remain stable and can be transported to a centralized laboratory for further manipulation. Dried blood spots are a simple, robust, non-invasive, and an in expensive way of sample collection, that does not require a cold chain to be maintained for its storage or transportation (2, 14, 15, 33). A finger prick of blood spot on a filter paper is all the sample that is needed (15). In contrast to the venous blood sampling, where a trained technical staff and infrastructure is a prerequisite at the collection site, for its transport and further manipulation (13, 33) (14, 15).

When the results are compared with a sister study that analyzed the same forty inflammatory host biomarkers in plasma, the number and the concentration of biomarkers that changed significantly in plasma samples were greater than those in dried blood spots (16). Even though, the precision and reproducibility of the dried blood spots were comparable to the plasma sampling. Many reasons can attribute to this difference in the number of significantly changing biomarkers. Firstly, there is an obvious difference in the size of the sample. In contrast to plasma, a blood spot requires 50 micro liters of blood only. Secondly, lysis of certain cellular components occurs when blood

spots are applied to the filter paper (34). This biochemical change may alter the levels of biomarkers in the elutes prepared from the dried blood spots (35, 36). Analytes also behave differently in how they adhere and get off the filter paper (34). The form and size of the blood spots, matrix of the filter paper, the precision and protocols of the assay and internal standards are bound to have an effect on the number and concentration of analytes recovered from the dried blood spots (34). Greater sensitivity can be achieved by using a larger diameter of punch and multiple punches. However, there are no guidelines for cross validating the results obtained by using dried blood spots with plasma samples (34).

This study has some limitations. Firstly, there is no control group of the non-responders. During the development of study, it was assumed that some of the EPTB patients started empirically on treatment would not respond. However, all the patients responded to treatment in our cohort. This could be due to the Hawthorne effect, leading to change in the practices of empirical treatment in this cohort, and the clinicians seemed to have selected patients more cautiously.

Another limitation of this study is the use of venous blood for preparing the blood spots. Practically, in low resource settings, the capillary blood is used for making blood spots via a finger prick. However, we used venous blood to make the spots as this was a part of a larger study that involved investigating the levels of biomarkers in unstimulated plasma at first (16). There are numerous physiological differences between the capillary blood and venous blood samples. The concentration of hematocrit, hemoglobin, and cells like erythrocytes, thrombocytes are found to be higher in capillary blood. In addition, presence of certain viruses in the host may also affect the concentration of biomarkers based upon the sample type. Biomarkers present in lower concentration are more susceptible to these physiological differences (14). Hence, if the same study is performed by using capillary blood for making dried blood spots, the results may vary.

Conclusion

A biosignature of MIG, CCL23 and CXCL2, has a reliable predictive value in the treatment monitoring of the EPTB, at the second and sixth month of treatment, by using dry blood spots. The findings have the potential to be developed into a robust ELISA based test which can be used sustainably in low resource settings.

Funding

This work was partly supported by the Research Council of Norway through the Global Health and Vaccination Program (project number 234457). This project is part of the EDCTP2 program supported by the European Union.

Data availability

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

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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Supplementary Data

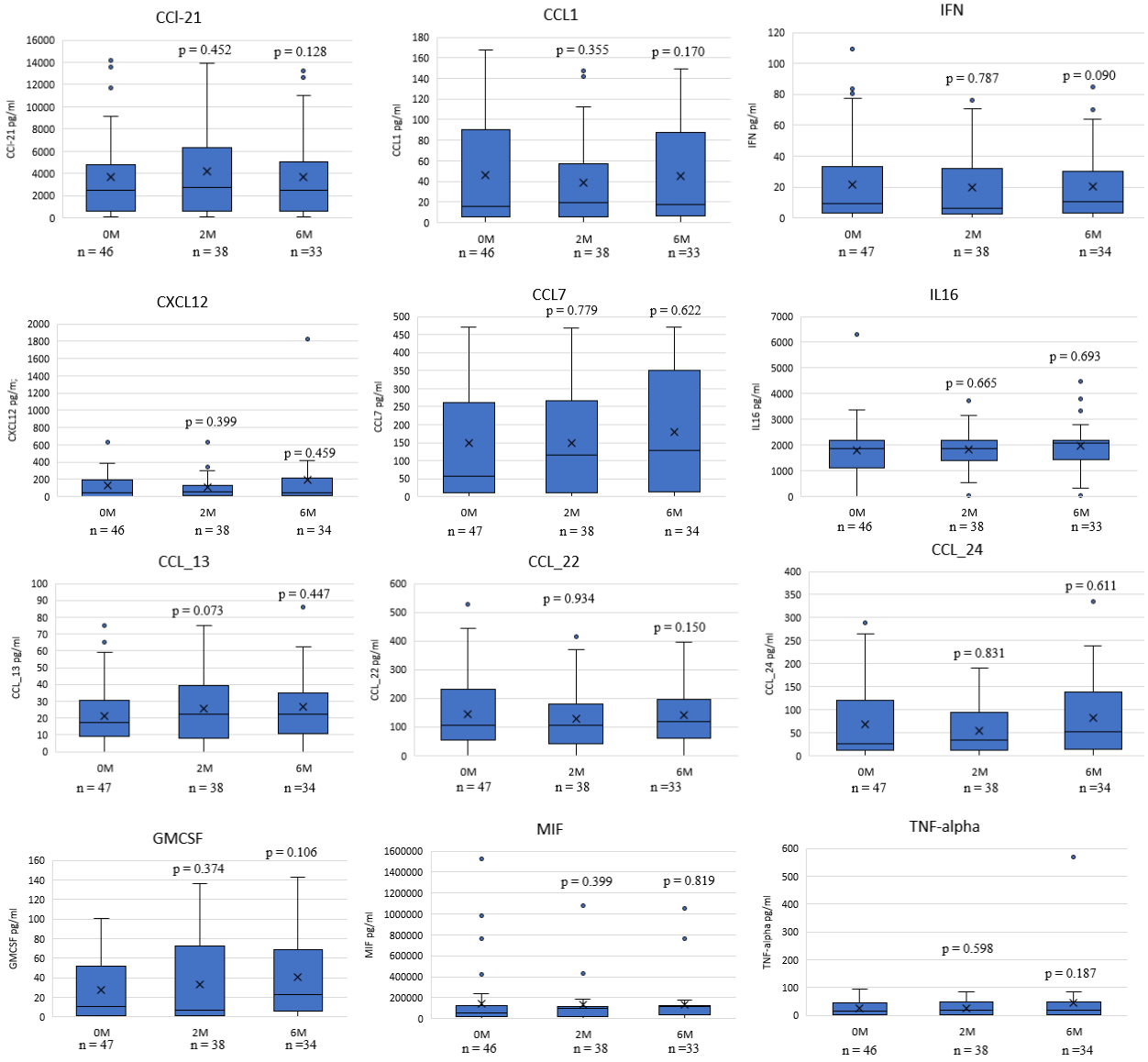
Supplemenatry Table 1: The significance level of the forty biomakers detected in dried blood spots. p value: significance < 0.05.

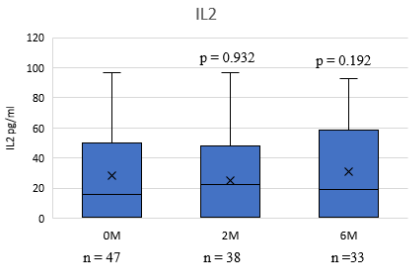
Biomarkers	Significance at 2 Months	Significance at 6 Months
CCl-21	0.452	0.128
CXCL9/MIG	0.025*	0.004*
CXCL6	0.688	0.204
IL6	0.078	0.001*
CCL1	0.355	0.170
IFN	0.787	0.090
CXCL12	0.399	0.459
CXCL11	0.983	0.011*
CCL7	0.779	0.622
IL16	0.665	0.693
CCL_13	0.073	0.447
CCL_22	0.934	0.150
CCL_24	0.831	0.611
GMCSF	0.374	0.106
MIF	0.399	0.819
TNF-alpha	0.598	0.187
CCL23	0.023*	0.001*
IL2	0.932	0.192
IL1B	0.778	0.781
CCL11	0.217	0.866
CCL25	0.330	0.501
IP10	0.025*	0.001*
IL4	0.881	0.218
CCL2	0.008*	0.012*
CXCL8	0.094	0.059
CCL3	0.851	0.047*
IL10	0.806	0.594
CCL8	0.870	0.428
CXCL1	0.004*	0.015*
CCL20	0.211	0.026*
CXCL16	0.167	0.313
CCL26	0.102	0.781
CCL15	0.777	0.376
CCL17	0.335	0.713
CCL27	0.159	0.454
CXCL5	0.970	0.382
CXCL13	0.829	0.012*

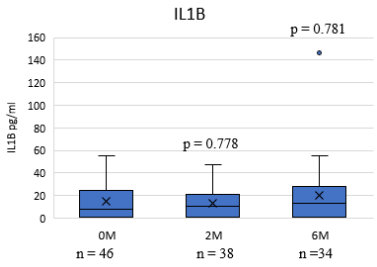
CCL19	0.688	0.433
CX3CL1	0.509	0.407
CXCL2	0.000*	0.003*

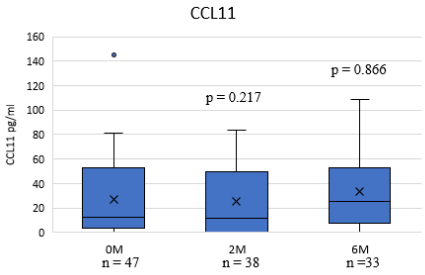
***significant values**

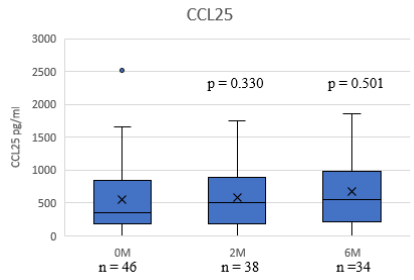
Supplementary Figure 1. Box plots of biomarkers changing insignificantly in Tb pleuritis.

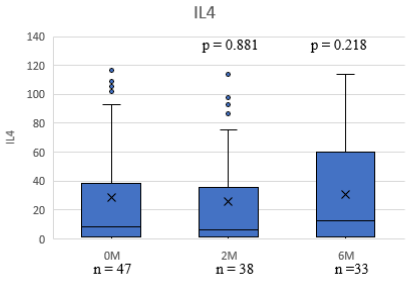


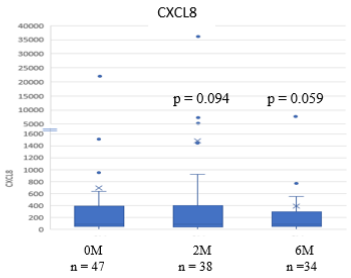

IL2
IL2 pg/ml
p = 0.932
p = 0.192
0M
2M
6M
n = 47
n = 38
n =33

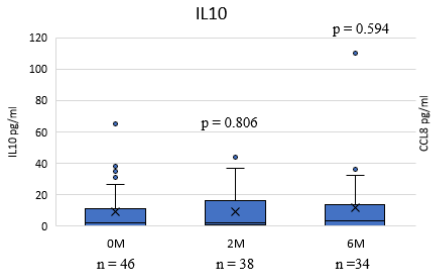

IL1B
IL1B pg/ml
p = 0.781
p = 0.778
0M
2M
6M
n = 46
n = 38
n =34

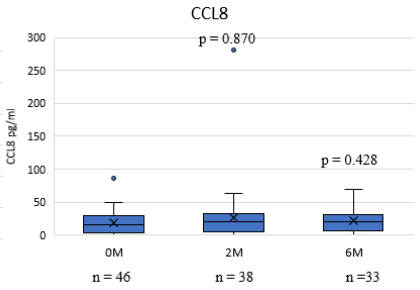

CCL11
CCL11 pg/ml
p = 0.866
p = 0.217
0M
2M
6M
n = 47
n = 38
n =33

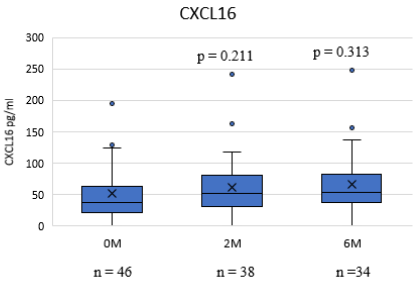

CCL25
CCL25 pg/ml
p = 0.330
p = 0.501
0M
2M
6M
n = 46
n = 38
n =34

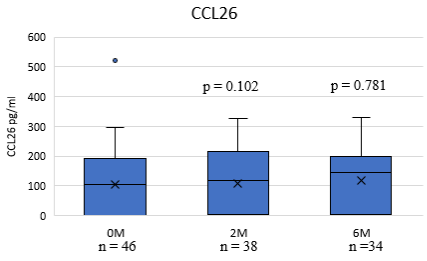

IL4
IL4
p = 0.881
p = 0.218
0M
2M
6M
n = 47
n = 38
n =33

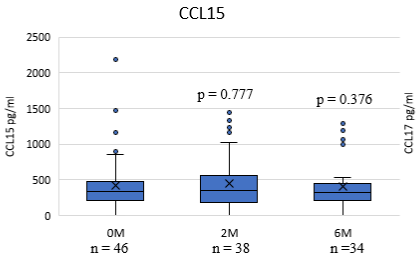

CXCL8
p = 0.094
p = 0.059
0M
2M
6M
n = 47
n = 38
n =34

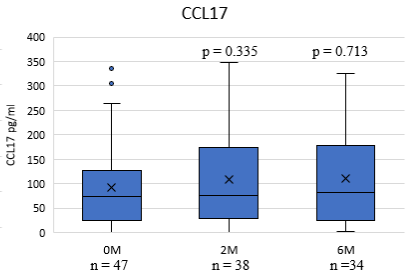

IL10
IL10 pg/ml
p = 0.594
p = 0.806
0M
2M
6M
n = 46
n = 38
n =34

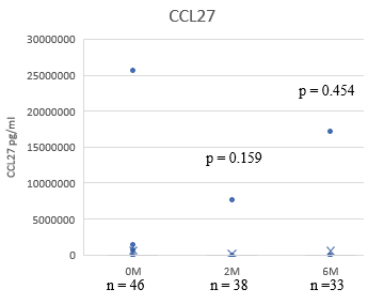

CCL8
CCL8 pg/ml
p = 0.870
p = 0.428
0M
2M
6M
n = 46
n = 38
n =33

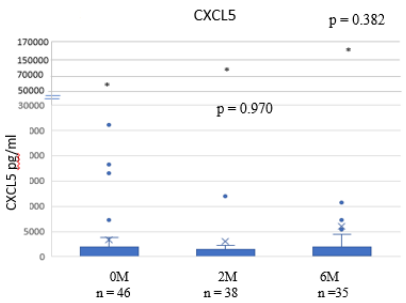

CXCL16
CXCL16 pg/ml
p = 0.211
p = 0.313
0M
2M
6M
n = 46
n = 38
n =34

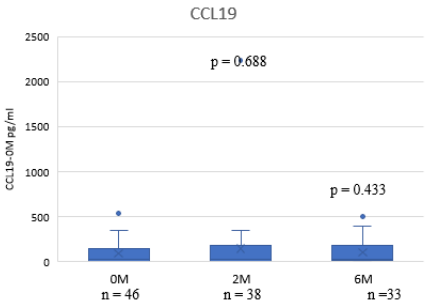

CCL26
CCL26 pg/ml
p = 0.102
p = 0.781
0M
2M
6M
n = 46
n = 38
n =34


CCL15
CCL15 pg/ml
p = 0.777
p = 0.376
0M
2M
6M
n = 46
n = 38
n =34


CCL17
CCL17 pg/ml
p = 0.335
p = 0.713
0M
2M
6M
n = 47
n = 38
n =34


CCL27
CCL27 pg/ml
p = 0.454
p = 0.159
0M
2M
6M
n = 46
n = 38
n =33

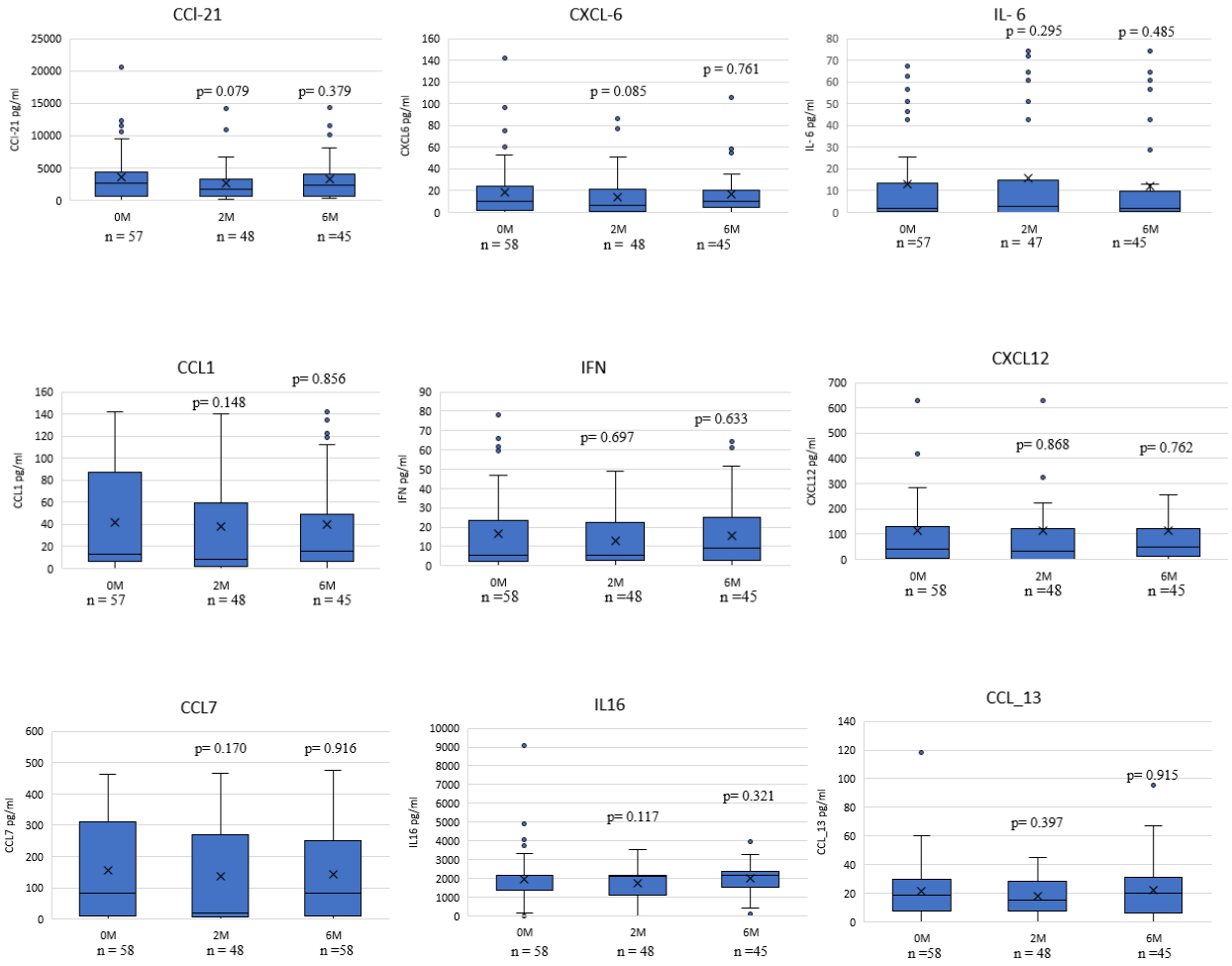

CXCL5
CXCL5 pg/ml
p = 0.382
p = 0.970
0M
2M
6M
n = 46
n = 38
n =35

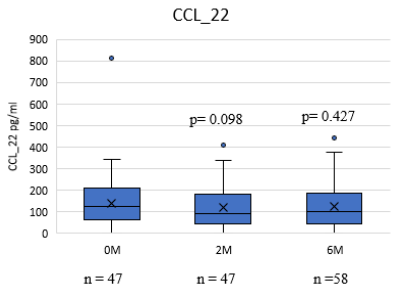

CCL19
CCL19-0M pg/ml
p = 0.688
p = 0.433
0M
2M
6M
n = 46
n = 38
n =33

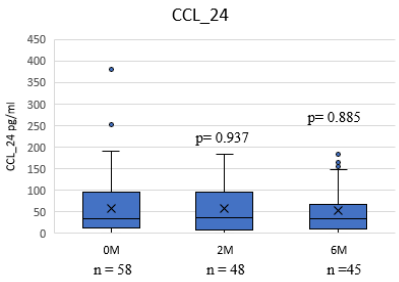
Box plots showing levels of inflammatory biomarkers in dried blood spots in TB pleuritis patients at baseline, 2nd and 6th months of treatment. A p-value < 0.05 was considered significant. The boxes represent the median and interquartile range, while the whisker represents the minimum and maximum values. Different time points of treatment are shown on x axis.

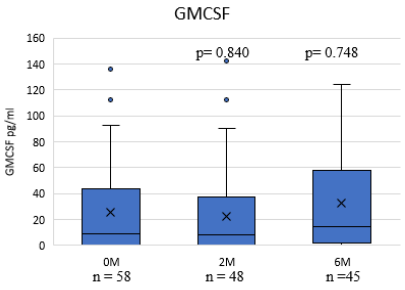
0 M; the baseline levels before initiation of treatment, 2M; 2 months of treatment, 6 M; 6 months of treatment, n; number of patients

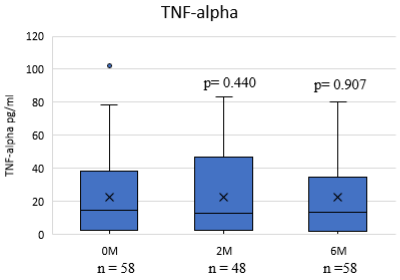
Supplementary figure 2. Box plots of biomarkers changing insignificantly in Tb Lymphadenitis

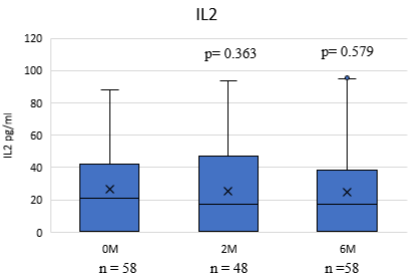


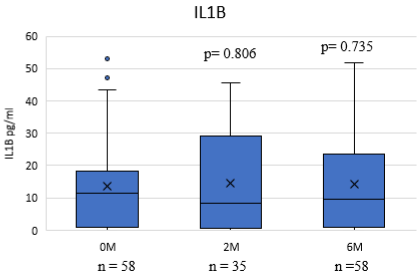

CCL_22
CCL_22 pg/ml
p= 0.098
p= 0.427
0M
2M
6M
n = 47
n = 47
n =58

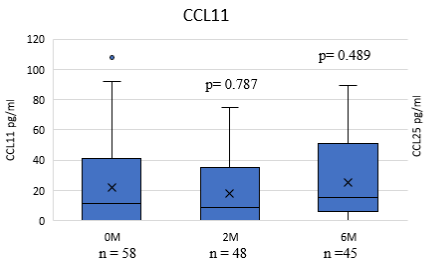

CCL_24
CCL_24 pg/ml
p= 0.937
p= 0.885
0M
2M
6M
n = 58
n = 48
n =45

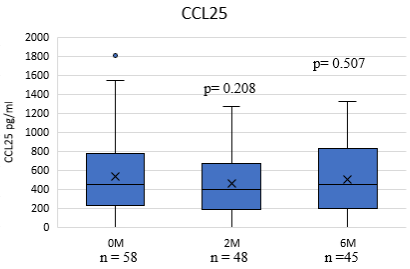

GMCSF
GMCSF pg/ml
p= 0.840
p= 0.748
0M
2M
6M
n = 58
n = 48
n =45

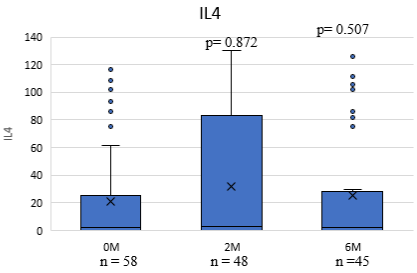

TNF-alpha
TNF-alpha pg/ml
p= 0.440
p= 0.907
0M
2M
6M
n = 58
n = 48
n =58

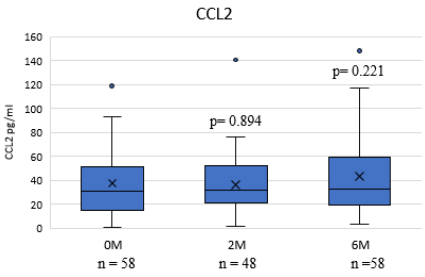

IL2
IL2 pg/ml
p= 0.363
p= 0.579
0M
2M
6M
n = 58
n = 48
n =58

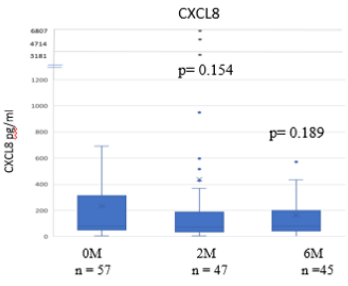

IL1B
IL1B pg/ml
p= 0.806
p= 0.735
0M
2M
6M
n = 58
n = 35
n =58

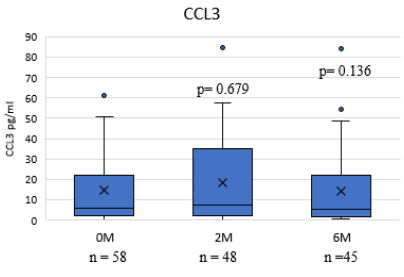

CCL11
CCL11 pg/ml
p= 0.787
p= 0.489
0M
2M
6M
n = 58
n = 48
n =45

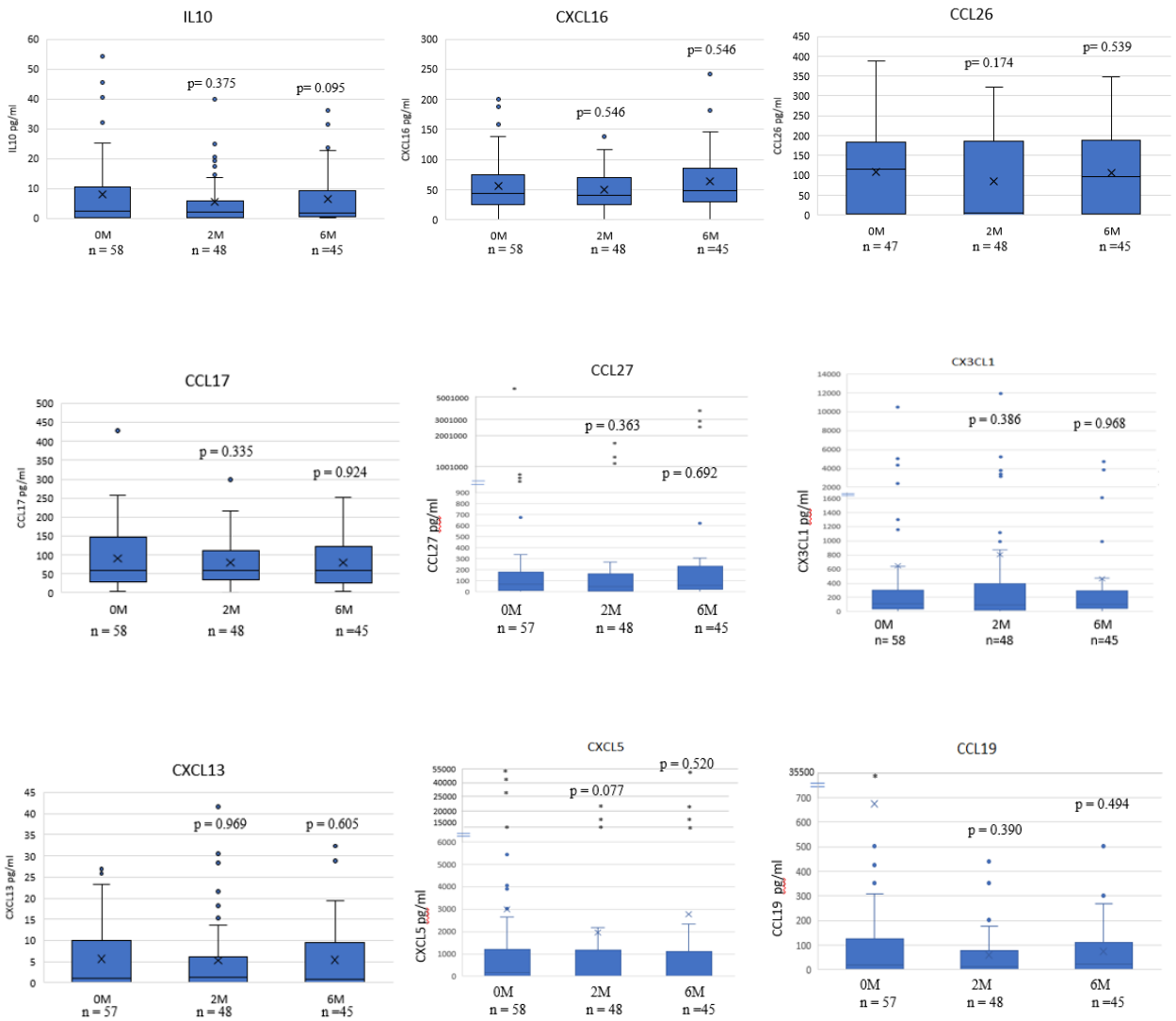

CCL25
CCL25 pg/ml
p= 0.208
p= 0.507
0M
2M
6M
n = 58
n = 48
n =45


IL4
IL4
p= 0.872
p= 0.507
0M
2M
6M
n = 58
n = 48
n =45


CCL2
CCL2 pg/ml
p= 0.894
p= 0.221
0M
2M
6M
n = 58
n = 48
n =58


CXCL8
CXCL8 pg/ml
p= 0.154
p= 0.189
0M
2M
6M
n = 57
n = 47
n =45


CCL3
CCL3 pg/ml
p= 0.679
p= 0.136
0M
2M
6M
n = 58
n = 48
n =45



Box plots showing levels of inflammatory biomarkers in dried blood spots in TB lymphadenitis patients at baseline, 2nd and 6th months of treatment. A p-value < 0.05 was considered significant. The boxes represent the median and interquartile range, while the whisker represents the minimum and maximum values. Different time points of treatment are shown on x axis.

0 M; the baseline levels before initiation of treatment, 2M; 2 months of treatment, 6 M; 6 months of treatment, n; number of patients

Supplementary Table 2. Inflammatory biomarkers changing significantly with treatment at 2nd and 6th months in both types of EPTB, in dried blood spots and unstimulated plasma.

Dried Blood Spots				Plasma Samples			
0 to 2M	**p value**	**0 to 6 M**	**p value**	**0 to 2M**	**p value**	**0 to 6 M**	**p value**
CXCL9/MIG	0.025	CXCL9/MIG	0.004	MIG	0.000	MIG	0.000
CCL 2	0.008	CCL 2	0.012	CCL2	0.001	CCL2	0.000
IP10	0.025	IP10	0.001	IP10	0.000	IP10	0.000
CXCL1	0.004	CXCL1	0.015	IFN γ	0.008	IFN γ	0.006
CXCL2	0.000	CXCL2	0.003	CXCL11	0.001	CXCL11	0.000
CCL23	0.023	CCL23	0.001	CCL13	0.003	CCL13	0.000
		IL6	0.001	IL4	0.012	IL4	0.007
		CXCL11	0.011	CCL1	0.002	CCL1	0.001
		CCL3	0.047	CCL20	0.010	CCL20	0.045
		CCL20	0.026	CCL26	0.000	CCL26	0.023
		CXCL13	0.012	CXCL2	0.000	CXCL1	0.034
				IL 8	0.005	CCL15	0.012
				CCL3	0.001	CCL17	0.044
				CCL8	0.000	CX3CL1	0.042
				CCL22	0.000	MIF	0.001
				CCL24	0.016	IL1	0.002
						IL6	0.000

M: months, p value: significance < 0.05

Supplementary Table 3. Comparison of the median levels of the biomarkers at the baseline between plasma and dried blood spots.

Biomarkers	Baseline level in Plasma (pg/ml)	Baseline levels in DBS (pg/ml)	Difference in Levels
CCL21	10043	2566	4 ↓
MIG/CXCL9	2276	203	11 ↓
CXCL6	28	9	3 ↓
IL6	14	4	4 ↓
CCL1	51	14	4 ↓
INFY	38	6	6 ↓
CXCL	508	41	12 ↓
CXCL	130	33	4 ↓
CCL	67	83	1 ↑
IL	624	2033	3 ↑
CCL	40	18	2 ↓
CCL	884	111	8 ↓
CCL	349	32	11 ↓
GMCSF	19	9	2 ↓
MIF	13,991	53,548	4 ↑
TNF- ALPHA	14	16	1 ↑
CCL23	468	57	8 ↓
IL2	3	18	6 ↑
IL1 - BETA	5	10	2 ↑
CCL	37	12	3 ↓
CCL	469	402	1 ↓
IP10	481	52	9 ↓
IL4	5	2	3 ↓
CCL2	30	31	1 ↑
IL8/CXCL8	18	89	5 ↑
CCL3	10	6	2 ↓
IL10	13	2	7 ↓
CCL	32	16	2 ↓
CXCL1	186	236	1 ↑
CCL20	12	3	4 ↓
CXCL16	497	41	12 ↓

CCL26	19	116	6 ↑
CCL15	4882	307	16 ↓
CCL17	133	60	2 ↓
CCL27	974	68	14 ↓
CXCL5	3	181	60 ↑
CXCL	17	1	17 ↓
CCL19	112	24	5 ↓
CX3CL1	331	111	3 ↓
CXCL	279	209	1 ↓

↓ indicates a decrease in the median levels of the biomarkers, ↑ indicates an increase in the median levels of the biomarkers