



23 **ABSTRACT**

24  
25 Pleural tuberculosis (PITB), a form of extrapulmonary TB, remains as a challenge in the  
26 diagnosis among many causes of pleural effusion. We recently reported that the combinatorial  
27 analysis of interferon-gamma (IFN- $\gamma$ ), IFN- $\gamma$ -inducible protein 10 (IP-10), and adenosine  
28 deaminase (ADA) from the pleural microenvironment was useful to distinguish pleural effusion  
29 caused by TB (microbiologically or not confirmed cases) among other etiologies. In this  
30 prospective cohort study, a set of inflammatory mediators was quantified in blood and pleural  
31 fluid (PF) from exudative pleural effusion cases, including PITB (n = 22) and non-PITB (NTB; n  
32 = 17) patients. The levels of IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- $\gamma$ , TNF, IP-10, TGF- $\beta$ 1, and  
33 ADA were measured and a principal component analysis was applied in order to identify the  
34 mediators who contributed most for the variance in data. IFN- $\gamma$ , IP-10, TNF, TGF- $\beta$ , and ADA  
35 quantified in PF showed significantly higher concentrations in PITB patients when compared to  
36 NTB ones. When blood and PF were compared, we have identified significantly higher  
37 concentrations of IL-6 and IL-10 in PF, in both groups. TGF- $\beta$ , solely, showed significantly  
38 increased levels in PF and blood from PITB when both clinical specimens were compared to  
39 NTB patients. Principal components analysis from PF revealed that the ADA, IP-10, TGF- $\beta$ , and  
40 IFN- $\gamma$  contributed most for the discriminatory capacity between TPIB and NTB. Our findings  
41 showed that important inflammatory mediators in PF may discriminate TB cases from other  
42 causes of exudative effusion, the main diseases considered in the differential diagnosis of PITB.

43  
44 **KEYWORDS:** pleural tuberculosis, pleural effusion, adenosine deaminase, cytokines in pleural  
45 effusion

46

## 47 INTRODUCTION

48  
49 Tuberculosis, caused by *Mycobacterium tuberculosis* (Mtb), is currently endemic in the  
50 world and represents an important public health problem every year. Globally, in 2017, more than  
51 10 million new cases of TB were reported with an estimated 1.3 million deaths. Among  
52 infectious diseases, TB is the leading cause of death from a single agent, surpassing the human  
53 immunodeficiency virus (HIV) infection (1). Although TB affects mainly the lungs,  
54 extrapulmonary forms can appear as an initial manifestation in approximately 25% of adults with  
55 TB, of which the pleural space is the second site of involvement followed only by the lymph  
56 nodes (2). In Brazil, a high burden TB country, PITB is responsible for more than 40% of cases  
57 among many clinical sites of extrapulmonary TB (3) and still imposes a challenging diagnosis  
58 due to, mainly, its paucibacillary nature and the need of invasive procedures (4).

59 Cellular immune response (Th1 immunity) involving CD4<sup>+</sup> T-lymphocytes, classically  
60 studied and associated with the containment of Mtb in pulmonary parenchymal TB, is also  
61 predominant in TB pleuritis, which is confirmed by the higher levels of interferon-gamma (IFN-  
62  $\gamma$ ) and other inflammatory cytokines (e.g., IL-12) in pleural fluid in comparison to peripheral  
63 blood (2, 5–7). IFN- $\gamma$  promotes cell differentiation, stimulates an increased phagocytic activity  
64 and intermediate nitrogen and oxygen species production, which are bactericidal and participate  
65 in resistance to Mtb infection (8, 9). In addition, other T-cell effector patterns are involved in  
66 Mtb control in the pleural microenvironment, such as Th17, which express the retinoic acid-  
67 related orphan receptor gamma t (ROR $\gamma$ t), and are characterized by secretion of large quantities  
68 of IL-17 (also known as IL-17A), IL-21, and IL-22 (9, 10). Th17 cells induce the expression of  
69 many pro-inflammatory factors, chemokines, ultimately involved in granulopoiesis and  
70 recruitment of innate cells, mainly neutrophils, especially in the early stages of infection (11, 12).

71 It is well described that patients at early stages of the PITB (less than 2 weeks duration) or those  
72 who present pleural effusion with high complexity (e.g., loculated pleural effusion, TB  
73 empyema) are more likely to have a neutrophilic exudate (reviewed by 13), which may contribute  
74 to injuries and decreases pleuro-pulmonary functions.

75 Since that the gold standard for the diagnosis of PITB which is the detection of Mtb in the  
76 sputum, pleural fluid or pleural biopsy has a discrete and variable yield, the histological  
77 demonstration of caseating granuloma even in the absence of acid-fast bacilli can be sufficient for  
78 anti-TB treatment (14, 15). Additionally, values  $> 40$  IU/L of adenosine deaminase (ADA) in  
79 pleural effusion, a purine-degrading enzyme, associated with a predominantly lymphocytic  
80 exudate, and clinical suspicious of TB, altogether, indicates that the most likely diagnosis is  
81 tuberculosis (16, 17). However, high pleural fluid ADA values can also be found in certain  
82 conditions, such as adenocarcinoma, lymphoma, rheumatoid arthritis, and pleural empyema of  
83 bacterial etiology, making the differential diagnosis very hard (18, 19).

84 Considering the difficulty in the differential diagnosis already mentioned and the current  
85 knowledge about the products of the immune response against Mtb in pleural space, the present  
86 study aimed to identify biomarkers among Th1, Th2, and Th17 T-cells subsets and other  
87 inflammatory mediators in peripheral blood and pleural fluid which could present high potential  
88 of utility for the PITB diagnosis among exudative pleural effusion from other etiologies. Based  
89 on a principal component analysis (PCA), we could demonstrate a pattern of mediators which  
90 was able to discriminate TB from non-TB pleural effusion.

91

92

93 **MATERIAL AND METHODS**

94

95 **Study population and settings.** Patients aged  $\geq 18$  years with pleural effusion under  
96 investigation with thoracentesis indication were recruited in this cross-sectional prospective study  
97 which was conducted at the Pulmonology and Tisiology Service, Pedro Ernesto University  
98 Hospital/Rio de Janeiro State University (HUPE/UERJ), a tertiary care center at Rio de Janeiro,  
99 RJ, Brazil. Patients who were under 18 years of age, pregnant, or refused consent were not  
100 recruited. Of 49 recruited patients, 10 were excluded: 8 patients had transudative pleural effusion  
101 (cardiac or renal failure), and 2 patients were HIV-seropositive. Thus, 39 patients with exudative  
102 pleural effusion were enrolled in the study: 22 PITB and 17 non-TB (NTB) patients. *PITB cases*  
103 were defined by the patient history reviewed, followed by a detailed physical examination, and at  
104 least one diagnostic criteria: i) positive results in the microbiological and/or histopathological  
105 tests (acid-fast bacilli smear microscopy, mycobacterial culture, or Xpert MTB/RIF<sup>®</sup>) on pleural  
106 fluid or pleural tissue; ii) presence of granuloma with or without caseous necrosis; iii) clinical  
107 manifestations suggesting TB (fever, pain, dyspnea, cough, night sweats, hyporexia, and/or  
108 weight loss) in combination with a lymphocytic pleural effusion, followed by a full recovery after  
109 at least six months of anti-TB treatment. *Non-TB cases* consisted of patients with pleural or  
110 pleuro-pulmonary diseases, excluding active TB based on clinical, laboratory, radiological,  
111 microbiological and/or pathological features. Malignant pleural effusions were diagnosed by a  
112 positive pleural fluid cytology result or malignant cells identified in the pleural fragment. Even  
113 when both of these tests results were negative, malignant effusion was diagnosed when a primary  
114 cancer was known to have disseminated and no other cause of pleural effusion was identified.  
115 Patients who did not fit the criteria used for PITB diagnosis as above and with unknown cause of  
116 pleural effusion were classified as “undefined” pleural effusion and considered as non-PITB.

117 Medical information, peripheral blood, and pleural fluid sample collection were obtained from all  
118 study subjects after signing a written consent. The study protocol was approved by the respective  
119 institutional ethics committee (HUPE/UERJ; number 1.100.772).

120  
121 **Sample collection.** Ultrasound-guided thoracentesis was performed by a trained pulmonologist  
122 who collected pleural fluid which was directly drawn into collection tubes for routine diagnostic  
123 tests, including chemistry panel, total, and differential cell count, ADA measurement by Hermes  
124 Pardini laboratory according Giusti's method (20), cytopathology, microbiological analysis  
125 (bacteria, fungi and mycobacteria), and inflammatory biomarkers for the purpose of the present  
126 study. During collection, whole blood and pleural fluid were sampled in appropriated collection  
127 tubes without anticoagulant additive. After collection, whole blood and pleural fluid tubes were  
128 centrifuged at 1000 x g for 10 min and 25 °C or 4 °C, respectively. Then, serum and pleural fluid  
129 (without cells) samples were aliquoted and stored frozen at -20 °C until cytokines quantification.

130  
131 **Cytokines assays.** Cytokine levels in clinical samples were assessed using the following  
132 commercially available kits: i) human Th1/Th2/Th17 Cytokine Kit (BD Bioscience, San Jose,  
133 CA, USA) based on the principle of cytometric bead array (CBA) technology for simultaneous  
134 detection of seven cytokines (IL-2, IL-4, IL-6, IL-10, TNF, IFN- $\gamma$ , and IL-17A). Briefly, capture  
135 beads labeled with distinct fluorescence intensity (allophycocyanin; APC) conjugated to specific  
136 antibodies for cytokines were incubated around 3 hours in the dark at room temperature with the  
137 undiluted samples, and fluorescent detection antibody (phycoerythrin; PE). All unbound  
138 antibodies were washed and samples acquired on a BD fluorescence-activated cell sorting  
139 (FACS) analyzer FACSCanto II. Cytokine standard curves ranged 0-5,000 pg/mL. ii) IP-10 and  
140 TGF- $\beta$  levels were measured by enzyme-linked immunosorbent assay (ELISA) sandwich using

141 human CXCL-10/IP-10 DuoSet ELISA (R&D Systems Inc, MN, USA) and human/mouse TGF  
142 beta 1 ELISA Ready-SET-Go! Kit (2<sup>nd</sup> Generation; Affymetrix, eBioscience), respectively,  
143 following the manufacturer's instruction. The range of these assays was 31.3-20,000 pg/mL for  
144 IP-10 and 15.6-1,000 pg/mL for TGF- $\beta$ . Readings greater than the upper limit were set at 20,000  
145 (IP-10) or 1,000 (TGF- $\beta$ ) pg/mL for the purpose of analysis.

146

147 **Statistical analysis.** For the description of the population included in the study, according to  
148 their sociodemographic and clinical characteristics among the individuals with exudative pleural  
149 effusion due to PITB or other causes (non-TB), non-parametric Mann-Whitney test were used for  
150 continuous variables or Fisher's exact tests for comparison of the relative frequencies of the  
151 different levels of nominal/categorical variables. In the comparison between the levels of log-  
152 transformed expression (bases 10) of proteins in peripheral blood/serum and pleural fluid (tissue  
153 effect) between individuals with or without TBPI (TB effect), the expected mean marginal values  
154 obtained from multiple linear regression (log-linear) models of fixed effects were used with the  
155 inclusion of first-order interactions between the main tissue and TB effects. For the adjusted  
156 models, graphical analysis of residuals was performed to confirm their randomness. In the  
157 comparisons between expected mean marginal values obtained from linear regression models,  
158 adjustments of the confidence level were made by Sidak's method, and p-value adjustments by  
159 multiple comparisons by Tukey's method. Finally, for log<sub>10</sub>-transformed protein and ADA  
160 expression data, a multivariate principal component analysis (PCA) was performed to visualize  
161 the distribution of sample individuals in 2D dimensional spaces. Ellipses of the quantiles 68% of  
162 the normal distribution adjusted to the individuals of the different interest groups in these new  
163 dimensional spaces are presented. The level of significance,  $P \leq 0.05$ , was used in the analysis,  
164 and all analyses were performed in R software version 3.5.2.

165

166 **RESULTS**

167

168 **Patients and characteristics.** Study population was composed by 39 individuals who were  
169 diagnosed as PITB (n = 22) or non-TB (n = 17) according previously described. Their  
170 sociodemographic and clinical data are shown in Table 1. We observed a significant difference  
171 between the age distributions between the groups, which presented medians corresponding to 65  
172 years (IQR: 20) in the non-TB group, and 41 years (IQR: 14) in the PITB group ( $p < 0.0001$ ).  
173 Smoking and alcoholic habits among participants did not show statistical differences. Similarly,  
174 symptoms presentation was not dissimilar among groups. Fourteen (82%) patients in the non-TB  
175 group had one or more comorbidities, showing that this group had a significantly higher number  
176 of patients with comorbidities than observed in the PITB group, which had 4 individuals (18%)  
177 with others diseases ( $p = 0.0217$ ). The most prevalent comorbidity was hypertension, which was  
178 reported in 6 (35%) non-TB patients and 2 (9%) PITB patients. Among non-TB patients, 13 were  
179 malignancies, 1 autoimmune disease (systemic lupus erythematosus), and 3 undefined pleural  
180 effusion.

181



182 **Table 1. Sociodemographic and clinical characteristics of the study population.**

<b>Characteristics /Group</b>	<b>Non-TB (n=17)</b>	<b>PITB (n=22)</b>	<b>p-value</b>
<b>Age, years median (IQR)</b>	65 (14)	41 (20)	0.0001
<b>Gender (%)</b>			
Female	7 (17.9)	8 (20.5)	1
Male	10 (25.6)	14 (35.9)	
<b>Current smoker (%)</b>	2 (5.1)	3 (7.7)	0.2836
<b>Alcohol use (%)</b>	2 (5.1)	9 (23.1)	0.1032
<b>Comorbidities (%)</b>	10 (25.6)	4 (10.3)	0.0172
Hypertension	6 (15.4)	2 (5.1)	0.059
Diabetes	3 (7.7)	0 (0)	0.0744
Cardiac Insufficiency	3 (7.7)	0 (0)	0.0744
Hepatitis	2 (5.1)	1 (2.6)	0.5703
<b>Symptoms (%)</b>			
Fever	3 (7.7)	9 (23.1)	0.1612
Cough	13 (33.3)	9 (23.1)	0.0516
Chest Pain	5 (12.8)	10 (25.6)	0.5
Dyspnea	13 (33.3)	12 (30.8)	0.3068
Night Sweats	3 (7.7)	4 (10.3)	1
<b>Definitive cause of PE (%)</b>			
Tuberculosis		22 (56.4)	
Malignancy	13 (33)		
Autoimmune disease	1 (2.5)		
Undefined	3 (7)		

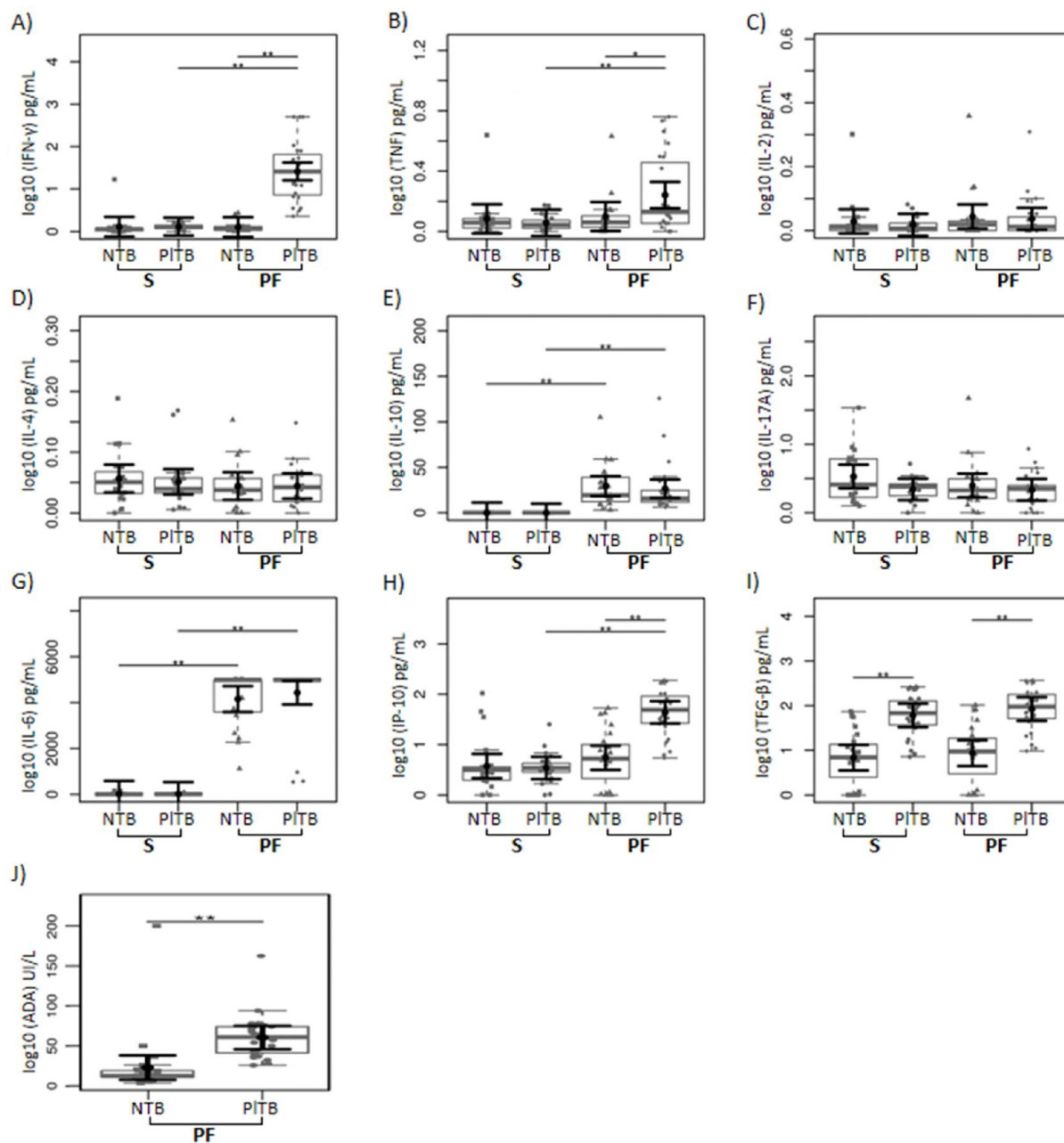
183 PITB, Pleural tuberculosis; PE, pleural effusion; IQR, Interquartile range. Values expressed as  
 184 n (%; from the total population) unless otherwise stated.

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 186  
 187 **Cytokines measurement in blood and pleural fluid from PITB and non-TB patients.** In order  
 188 to evaluate the potential diagnosis of cytokines Th1/Th2/Th17, IP-10 chemokine, and ADA in  
 189 exudative cases of pleural effusion, serum and pleural fluid samples from PITB and non-TB  
 190 patients were analyzed. As recently reported by our group (7) and others (21–23), IFN- $\gamma$  and IP-  
 191 10 levels were significantly increased ( $p < 0.0001$  in both) in pleural fluid comparison to serum

192 in PITB group (Figure 1A and H). As shown in Figure 1B, TNF concentration also showed a  
193 significant increase in the pleural fluid when compared to serum in PITB patient ( $p = 0.0016$ ).

194 When these cytokines were compared with discriminatory objectives between PITB and  
195 non-TB patients, we predominantly observed significant differences in pleural fluid. IL-6 and IL-  
196 10 levels presented the same behavior when serum and pleural fluid were compared in PITB or  
197 non-TB groups (Figure 1G and E, respectively). Both IL-10 and IL-6 concentrations show that  
198 patients in both PITB ( $p < 0.0001$  in both) and NTB ( $p < 0.0001$  in both) groups show increased  
199 concentrations of this cytokine in pleural fluid when compared to serum in their respective  
200 groups. As expected, ADA levels were significantly higher in pleural fluid of PITB patients  
201 compared to non-TB ( $p < 0.0001$ ). Interestingly, TGF- $\beta$  concentrations were significantly higher  
202 in the serum ( $p < 0.0001$ ) and pleural fluid of PITB patients, compared to concentrations found  
203 in non-TB patient samples ( $p < 0.0001$ ). Concentrations of this growth factor showed no  
204 significant serum and pleural fluid difference when compared in the same group (Figure 1I).

205 Finally, IFN- $\gamma$ , TNF, IP-10, TGF- $\beta$  and ADA concentrations in the pleural fluid presented  
206 a differentiated profile between PITB and non-TB patients. Cytokines IL-17A, IL-4, and IL-2 did  
207 not show significant differences in their concentrations.



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211 **Figure 1. Cytokines and ADA levels in serum and pleural fluid from PITB and NTB**  
212 **patients.** Cytokines were dosed by CBA (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A). The  
213 levels obtained from each cytokine were analyzed on a logarithmic (base = 10) scale and  
214 illustrated using boxplots to compare serum (S) and pleural fluid (LP) data between the non-TB  
215 (NTB) and TBPI groups. The small grey dots represent individual cases and the boxplots  
216 represent the interquartile range and the median of the sample (solid grey central line). Larger

217 black dots and vertical bars represent expected mean marginal values estimated by the linear  
218 model and its 95% confidence intervals (95% CI). Comparisons of means between groups were  
219 performed by contrasts/differences obtained after linear bi and multivariate models, adjusted by  
220 regressions by ordinary least squares. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

221  
222  
223 **Principal component analysis of pleural fluid cytokines.** Finally, it was observed whether the  
224 overall cytokines profile was able to discriminate PITB and non-TB cases. In the principal  
225 components analysis (PCA), 57.21% of the total variance in response to 9 cytokines and  
226 biomarkers was expressed by 2 principal components (Table 2; Figure 2). The first component  
227 accounted for a total of 40.58%, while the second accounted for 16,63% of the total variance  
228 (Figure 2). Altogether, these 9 cytokines were able to discriminate between PITB and non-TB.  
229 The most determinant variables of each of these two principal components were respectively  
230 ADA, IP-10, TGF- $\beta$ , IFN- $\gamma$ , and TNF, for the first principal component (PC1), and IL17A, IL-4,  
231 and IL-2 for the second principal component (PC2).

232

233 **Table 2. Principal components analysis.**

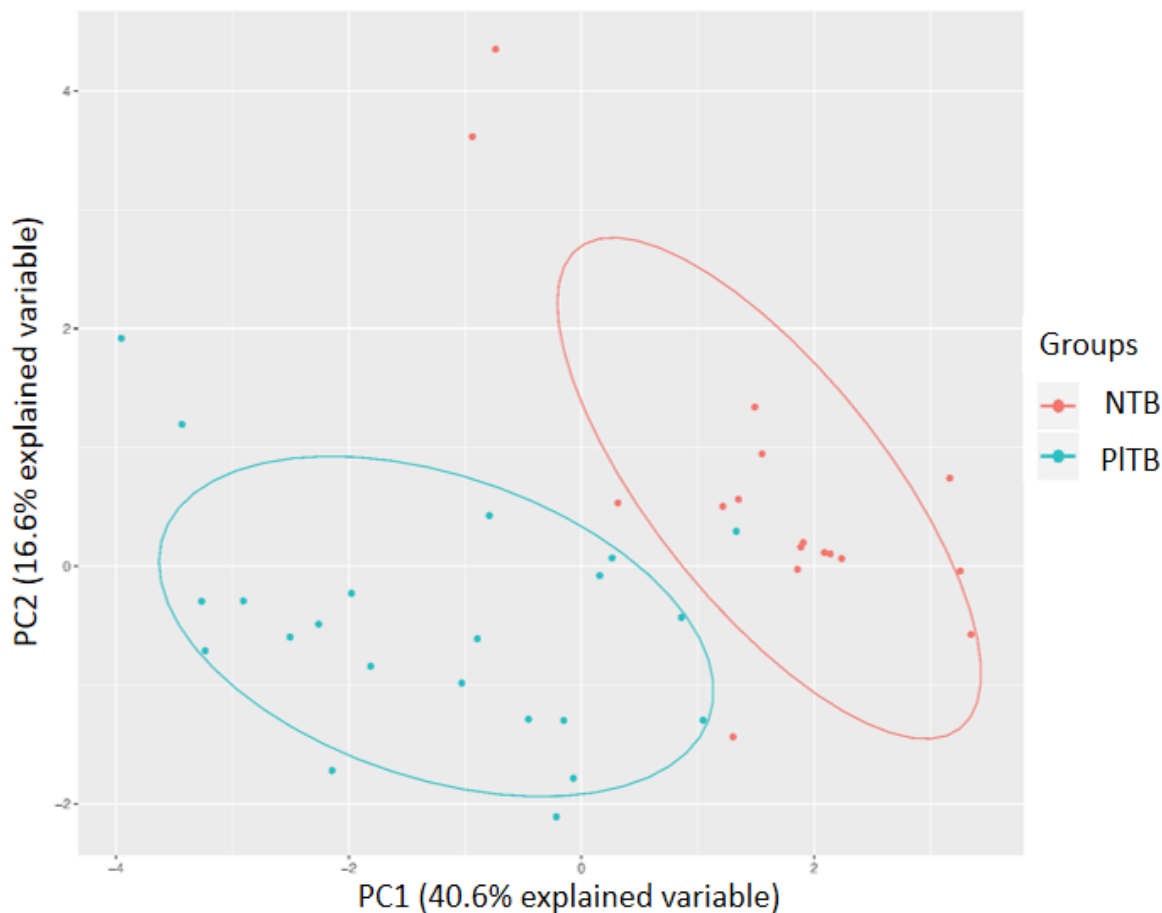
<b>Component</b>	<b>Eigenvalue</b>	<b>Difference</b>	<b>Proportion</b>	<b>Cumulative</b>
Component 1	3.95	2.33	40.58	40.58
Component 2	1.62	0.31	16.63	57.21
Component 3	1.32	0.42	13.50	70.70
Component 4	0.90	0.10	9.22	79.92
Component 5	0.79	0.30	8.16	88.08
Component 6	0.50	0.15	5.08	93.16
Component 7	0.35	0.10	3.55	96.71
Component 8	0.24	0.16	2.48	99.19
Component 9	0.08	0.07	0.79	99.98
Component 10	0.00	NA	0.02	100.00

<b>Variable</b>	<b>Component 1</b>	<b>Component 2</b>
ADA	-0.41429	-0.21968
IP-10	-0.44817	-0.19145
TGF- $\beta$	-0.4445	-0.18435
IL17A	-0.0793	0.394041
IFN- $\gamma$	-0.44469	-0.18198
TNF	-0.38718	0.253531
IL-10	-0.1654	0.330127
IL-6	-0.03609	-0.02504
IL-4	-0.16608	0.56734
IL-2	-0.1405	0.443796

234 Principal component analysis of inflammatory biomarkers in pleural fluid from patients with  
 235 pleural effusion by pleural tuberculosis and other diagnoses. Shaded values represent the most  
 236 important biomarkers in the component definition. NA: not applicable.  
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241 **Figure 2. A pattern of inflammatory biomarkers in pleural fluid discriminates PITB from**  
242 **NTB patients.** The analysis of variance of cytokine concentrations by CBA (IL-2, IL-4, IL-6, IL-  
243 10, TNF, IFN- $\gamma$ , and IL-17A) and ELISA (TGF- $\beta$  and IP-10) ADA were evaluated by the PCA  
244 method, with the objective to finding heterogeneity between the cytokine profile of patients with  
245 pleural tuberculosis (PITB) and other diagnoses non-TB (NTB). Two components were used to  
246 explain most of the total variation of these data.

247

248

249 **DISCUSSION**

250

251 Among many known causes of pleural effusion, heart failure, malignant conditions,  
252 pneumonia, and PITB are responsible for three-quarters of all cases (24). Currently, there is  
253 scarce literature comparing PITB with other causes of exudative pleural effusions, which  
254 contributes to the difficulty of establishing criteria for the differential diagnosis of PITB. The  
255 present study sought to find elements that are capable of differentiating the tuberculous effusion  
256 from other agents causing pleural effusion. Analyzing the pleural microenvironment, the  
257 quantification of cytokines showed higher concentrations of IFN- $\gamma$ , TNF, TGF- $\beta$ , IP-10 and ADA  
258 in LP of PITB patients, when compared to LP of non-TB patients. Analysis of principal  
259 components revealed that these cytokines and inflammatory mediators showed the largest  
260 variations associated with a partial distinction between PITB and non-TB patients.

261 As mentioned, ADA dosage is routinely used as a marker of PITB, although it does not  
262 define the differential diagnosis (17, 18). As for the general characteristics of pleural effusions, as  
263 expected, the median value corresponding to ADA concentrations was significantly higher in the  
264 PITB group, compared to the non-TB group. Although very useful in the differentiation of  
265 tuberculous effusion, several authors diverge about the true diagnostic value of the ADA, often  
266 setting other cutoff values. However, although they are found in greater amounts in the pleural  
267 fluid, these mediator have not been used, alone, as differential markers in exudative pleural  
268 effusions. In a recent work published by our group (7), we proposed a model where the values of  
269 IP-10, IFN- $\gamma$ , and ADA in a revised cutoff value, analyzed together, can be used in the  
270 differential diagnosis of PITB with high performance in microbiologically unconfirmed cases of  
271 PITB.

272           The immune system plays a pivotal role in the evolution of Mtb infection. To contain the  
273 infection, the defense cells together with inflammatory mediators generated at the site of the  
274 infection produce a potent and aggressive response, which can generate important tissue lesions  
275 (25). In addition, the direct action on the mesothelial cells and vascular endothelium, present  
276 great participation of the tissue repair and fibrosis processes, causing functional impairment of  
277 the pleural and lungs (26, 27). At the same time, an insufficient immune response may allow the  
278 multiplication and dissemination of the bacillus (25). Otherwise, the immune system products can  
279 be used to identify pleural effusions caused by TB.

280           Classically, the Th1 response is the most studied in TB, being responsible both for the  
281 containment of Mtb and for the tissue injury caused by the excessive response to the bacillus  
282 (reviewed by 28). As expected, Th1-related cytokines IFN- $\gamma$  and TNF, as well as the biomarker  
283 IP-10, are increased in the pleural fluid of PITB patients compared to blood (Figure 1). Recently,  
284 the dosage of IFN- $\gamma$  in pleural effusion raised importance as an auxiliary method for the diagnosis  
285 of PITB, becoming an example of a test used for this purpose, since this cytokine is at high levels  
286 during the active phase of the disease (23, 28). The IFN- $\gamma$ -release assay (IGRA) has also been  
287 highlighted in this context. The test evaluates the activity of T lymphocytes under stimulation of  
288 Mtb ESAT-6 and CFP-10 antigens. However, as reviewed by Aggarwal and collaborators (2015)  
289 there are many conflicting results regarding this diagnostic method of active tuberculosis, both in  
290 pulmonary and pleural forms (29). Moreover, as recently delineated by our group, IGRA has a  
291 poor meaning in PITB (7), perhaps due to their paucibacillary nature or due to the enrichment of  
292 inflammatory mediators in pleural space, without needing of an additional antigen-stimuli. TNF  
293 is another important mediator in the response against Mtb and it is directly related to the  
294 maintenance of the granuloma structure, maintaining the colonization of the bacillus and necrosis  
295 area in a restricted manner (30). Other evidence shows that patients treated with an anti-TNF



296 antibody developed active tuberculosis after reactivation of latent infection (31). In addition, TNF  
297 is important in the intracellular control of Mtb (Review by 25). Li et al. (2014) found a higher  
298 diagnostic value in TNF measurements than that found in ADA values (22). IP-10 is well studied  
299 as a possible biomarker in TB and is directly associated with INF- $\gamma$  since its production is mainly  
300 induced by this cytokine. As revised by Porcel (32), IP-10 is not an essential biomarker for the  
301 PITB diagnosis but has been the subject of several studies in this context, based on its  
302 participation in the immunopathogenesis of the disease and their correlation with IFN- $\gamma$  (7, 21).

303         Recently, the Th17 response also gained prominence in the immunopathology of  
304 tuberculosis, especially in the early stages of infection (9, 10). Particularly in PITB, Ye et al.  
305 (2011) showed an increase of lymphocytes with Th17 profile in pleural fluid compared to blood.  
306 Our results, presented here, show high concentrations of IL-6 and TGF- $\beta$  in pleural fluid of PITB  
307 patients compared to serum (34). These two biomarkers are critical in the differentiation of Th17  
308 cells (35). Therefore, although our study did not focus on the characterization of Th17 cells, it is  
309 quite probable that the microenvironment, through the high concentration of IL-6, TGF- $\beta$ , and the  
310 low concentrations of IL-2 is favoring the differentiation of this T-lymphocytes effector  
311 phenotype in the PLTB group.

312         IL-10 is a cytokine involved in the suppression of the immune response (36). In the case  
313 of TB, it has been associated with suppression of dendritic cell activity, the formation of foamy  
314 macrophages and defective formation of granuloma (37–39). The production of IL-10 is one of  
315 the more classic mechanisms of suppression by T regulatory cells, however, this cytokine can be  
316 produced by many other cells of the immune system, such as macrophages, B lymphocytes and  
317 Th2 lymphocytes (40). Our study has shown higher concentrations of IL-10 in the pleural fluid of  
318 patients with PITB compared to serum, and in the same way in the NTB group. However, the  
319 methodology used in this study was not able to identify which cells present in the pleural fluid

320 were responsible for the increase of IL-10 concentrations, as well as the other cytokines. Geffner  
321 et al. (2013) showed an increased IL-10 production after stimulation of mononuclear cells in  
322 pleural fluid and peripheral blood with Mtb antigens, and decrease of this cytokine after removal  
323 of culture Treg cells provides evidence that Treg is also responsible for the production of IL-10  
324 from the pleural cavity (41).

325 The cytokine pattern related to the Th2 effector phenotype was also evaluated. In the  
326 methodology employed, but did not detect significant levels of IL-4. This finding confirms the  
327 literature data that show little influence of this effector phenotype in cases of tuberculosis (2, 33),  
328 although IL-4 concentrations in miliary TB have already been reported (5).

329 Another important finding in our study was the quantification of TGF- $\beta$  in serum and  
330 pleural fluid. This growth factor, secreted by monocytes, is a chemotactic agent for fibroblasts,  
331 plays an important role in extracellular matrix remodeling (42). One of the possible contributions  
332 of TGF- $\beta$  to the pathophysiology of PITB is its ability to induce fibrosis, as shown in the study by  
333 Sasse et al (2003), where animals infected with Mtb showed increased pleural thickening in  
334 proportion to the increase in TGF-  $\beta$  (43). Seiscento et al (2007) also found elevated TGF- $\beta$   
335 levels in serum and pleural fluid of PITB patients, associating with the degree of pleural  
336 thickening in these patients (44). Our findings, together with the evidence found in the literature,  
337 reinforce the hypothesis that this mediator may be related to the development of pleural effusions  
338 in TBP1 patients since TGF- $\beta$  levels were found to be significantly higher in the pleural fluid of  
339 these patients, compared to the results found in non-TB patients. Although the cited studies found  
340 a significant increase of TGF- $\beta$  in pleural fluid and serum, the comparison group in the  
341 experimental model of these studies was composed of patients with transudative pleural effusion.  
342 Our work was able to detect the increase of TGF- $\beta$  in the serum and pleural fluid of PITB  
343 patients, compared to blood and pleural fluid in patients with other causes of exudative effusion.

344 This finding may contribute to future investigations, associating TGF- $\beta$  as a possible biomarker  
345 to aid in the differential diagnosis of PITB.

346 In fact, the cytokines analyzed alone are not able to provide data of high specificity and  
347 sensitivity, especially in comparison to exudative effusions. However, when analyzed together,  
348 they can provide high diagnostic value (7, 22). Therefore, in the present study, PCA was  
349 performed in an attempt to establish a pattern of cytokines and mediators in blood and pleural  
350 fluid capable of discriminating the PITB and non-TB cases. Our results show that 9 cytokines and  
351 biomarkers, measured in blood and pleural fluid, were reduced to two principal components  
352 (Table 2). Together, they were able to discriminate the PITB and non-TB cases and explained  
353 57% of the variation. The cytokines with determinant values were ADA, TGF- $\beta$ , IP-10, IL-17A,  
354 IFN- $\gamma$ , TNF, IL-4, and IL-2. These results provide new data in the search for new markers  
355 capable of differentiating the causes of exudative pleural effusion.

356 Some limitations should be considered in our study. First, it was conducted in a single-  
357 center, imposing a validation in other reference centers and in different populations. Another  
358 consideration is regarding the relatively low number of patients included per group. However,  
359 patients were included prospectively, in a real routine of clinical practice in a tertiary reference  
360 center which reflected in variable clinical characteristics inherent of each group of study, as can  
361 be observed in Table 1. Moreover, we have analyzed only exudative cases of pleural effusion, the  
362 main confounders in the differential diagnosis of TB. Also, we have excluded transudative cases  
363 which could add some bias in our analysis.

364 In summary, the analysis of a panel of inflammatory mediators previously highlighted in  
365 the TB literature was useful to provide new hypotheses and better comprehension about  
366 microenvironment of the pleural cavity during the immunopathology of Mtb infection. In  
367 addition, the screening in pleural fluid identified biomarkers with high potential for use alone or

368 in combination, which is able to increase the sensitivity of diagnosis and prompt the TB  
369 treatment, especially in cases of hard identification and distinction by conventional diagnostic  
370 methods.

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