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Short communication

Interleukin-6 and -27 as potential novel biomarkers for human pleural tuberculosis regardless of the immunological status

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ABSTRACT

Tuberculosis (TB) is the leading cause of pleural exudative effusions. Inflammatory markers, such as IFNγ and ADA, have been used as proxies for its diagnosis. We evaluated *ex vivo* levels of several cytokines in 83 pleural effusion specimens from patients with TB (including 10 with HIV co-infection) and 26 patients with other pleuritis using multiplex and ELISA assays. IL-6 and IL-27 levels were higher ($p \leq 0.04$) in TB patients, regardless of the HIV status and the approach. IL-2, IL-4, IL-8, IFNγ, TNF and G-CSF showed variable results depending on the assay. This warranty these markers to be further validated.

Tuberculosis (TB) remains a serious global health problem and currently persists as an endemic scourge with high global prevalence and mortality [1]. Despite broad BCG vaccine campaigns in many settings and the use of effective antimicrobial treatment, TB continues to affect large populations, especially in low-income countries. Globally and according to the World Health Organization (WHO), in 2021, the disease affected 10.6 million people and 1.6 million died from it, including 187,000 with TB/human immunodeficiency virus (HIV) co-infection. Although the annual number of deaths from TB fell between 2005 and 2019, this trend was reversed in 2020 and 2021 [1].

Tuberculous pleuritis is a frequent clinical form of extrapulmonary TB. It is usually paucibacillary and often shows a spontaneous resolution. It is characterised by large effusions with predominance of lymphocytes, accompanied by a pulmonary infiltrate, and may result from primary TB or reactivation of infection (reviewed by Ref. [2]). During the inflammatory process, the bacilli reach the pleural space mostly by contiguity, and the interactions between *Mycobacterium tuberculosis* antigens and the previously sensitised host T lymphocytes result in a type IV delayed-type hypersensitivity reaction and accumulation of exudative fluid (reviewed by Ref. [2]).

The diagnosis of tuberculous pleuritis is challenging because *M. tuberculosis* is difficult to isolate in exudative fluids [3]. The diagnosis is usually established by indirect evidence of inflammation, such as the demonstration of granuloma in the pleural tissue, which requires an invasive procedure, or the demonstration of biomarkers of the immune response, which are non-specific [3]. Reliable, fast, low-cost, non-invasive, objective, highly sensitive and specific biomarkers that provide clinicians with a better overview for the diagnosis of tuberculous pleuritis are urgently needed. A better view of the host immune response at the cellular level in tuberculous pleuritis should improve our understanding of TB pathogenesis in humans.

Previous studies have shown that cytokines, such as interferon gamma (IFNγ), tumour necrosis factor (TNF), interleukin 6 (IL-6), and IL-12 (p40), among others, in pleural effusion are related to the disease [4]. On the other hand, one study has found high adenosine deaminase (ADA) and IL-27 levels in HIV-negative tuberculous pleurisy patients with improved specificity and diagnostic accuracy, but neither IL-6 nor IFNγ were included in that proposed biosignature [5]. Another additive value would be the inclusion of a comparative TB/HIV co-infection cohort.

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In this study, we evaluated the levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IL-23, IL-27, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), monocyte chemotactic protein 1 (MCP-1), macrophage-inducible protein 1β (MIP-1β), IFNγ, TNF, matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteinase 1 (TIMP1) in the pleural effusion of patients with tuberculous pleuritis, including patients with HIV co-infection, as well as in patients with pleural effusions due to other conditions.

1. Patients and methods

1.1. Study participants

Patients with clinically detected pleural effusion admitted for diagnostic purposes in an inpatient unit at a tertiary care general hospital were enrolled. This hospital is a referral centre for many primary care units in Rio de Janeiro. Most patients were from a 22-bed male internal medicine ward. Any patient with pleural effusion and clinical indication for thoracentesis was eligible for inclusion. Blood and pleural effusion were sampled by routine venesection and diagnostic pleural tap, respectively, after obtaining written informed consent from the participants. Patients without a final diagnosis were excluded. The study protocol was approved by the Santa Casa da Misericórdia do Rio de Janeiro hospital (protocol # 0002031200009) and FIOCRUZ (protocol # 36972314.6.0000.5248) ethics committees.

The diagnosis of tuberculous pleuritis (herein called case-patients) was defined as confirmed when at least one of the following criteria was met: (i) positive AFB staining of pleural effusion or sputum smears; (ii) a positive Lowenstein–Jensen (LJ) medium culture of sputum, pleural effusion or tissue followed by biochemical identification; or (iii) the finding of caseous granuloma in pleural tissue [3]. It should be noted that because the tuberculous pleurisy is paucibacillary by definition, microbiological methods, like AFB staining, lack sensitivity. Thus, the scarcity in bacillary load, the presence of amplification-inhibiting substances in the pleurisy, the amplified genomic sequence, and potential intracellular mycobacterial sequestration are all factors contributing to the low sensitivity [6]. Case-patients presenting with fever, night sweats for >3 weeks and a lymphocytic exudative pleural effusion, who had negative microbiologic and histopathologic examinations, but improved after two months of anti-TB treatment, were considered to have a clinical diagnosis of tuberculous pleuritis (reviewed by Ref. [2]). All patients were followed monthly after discharge.

All patients were submitted to tests for lactate dehydrogenase, glucose, cholesterol, amylase, protein and albumin concentrations, and they were tested for HIV infection by using three different methods. Pleural effusion was also assessed for total and differential cell count and submitted to Gram staining, AFB staining and LJ culture. The pleural effusion was considered exudative when the pleural effusion/serum protein ratio was >0.5 and lymphocytic when it contained >50 % lymphocytes (reviewed by Ref. [2]). Pleural effusion aliquots were collected with sterilised, single-use syringes and needles and were separated, numbered and frozen at −80 °C. Subsequently, four fragments of parietal pleura were obtained with a Cope needle: three for histopathologic examination and one for LJ culture.

1.2. Biomarker assays

A total of 21 cytokines and chemokines, including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IL-23 (specific for the p19/p40 heterodimers), IL-27 (specific for the EBI3/p28 heterodimers), GM-CSF, G-CSF, MCP-1, MIP-1β, IFN-γ and TNF, as well as MMP-9 and TIMP1 were measured in pleural effusions by using the Human Cytokine and Chemokine multiplex assay kit (Bio-Plex, Bio-Rad, USA) by the Luminex method, the Cytometric Bead Array kit (CBA, BD Biosciences, USA) and DuoSet enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA). All assays were performed according to the manufacturers' instructions, and for the BD CBA Human Th1/Th2 Cytokine Kit, the parameters were detected and analysed according to the core-laboratory facility standards, using the FCP array software (version 3.0.1). The three methods were used due to intrinsic limitation of each one; this approach provided robustness to the study. Samples were randomly selected for each method, and thus the sample size varied depending on the method. The purpose of employing the various methods was to compare them relative to the highest detection limit (i.e. ELISAs).

1.3. Statistical analysis

Statistical evaluations between different groups were done with Wilcoxon rank sum test using the SPSS version 16.0 software package (SPSS Lead Technologies Inc., USA). A value less than 0.05 was considered statistically significant. The diagnostic accuracy of those cytokines evaluated as potential biomarkers in the pleural effusion of case-patients *vs.* patients with other causes and etiologies of pleural effusion (herein called control-patients) was evaluated by Receiver Operating Characteristic (ROC) curve analyses by the GraphPad InStat version 5.0 software package (GraphPad Inc., USA).

1.4. Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

2. Results and discussion

During the study period, a total of 109 patients with a pleural effusion were admitted for diagnostic purposes. TB was diagnosed in 83 patients (76 %, case-patients), and among them 10 (12 %) were HIV positive. Tuberculous pleuritis was mostly confirmed by histopathological methods. The remaining 26 (24 %, control-patients) were diagnosed with a myriad of diseases other than TB, namely malignancies (n = 14), congestive heart failure (n = 3), cirrhosis (n = 1) and undefined (n = 8). The median follow-up time for all case-patients after completing anti-TB treatment was 13 months; all have remained well, free of pleural effusions. Other demographic details are shown in Table 1.

Seven of 21 cytokines were evaluated in both multiplex assays; among the 21 cytokines, IL-5, IL-17, IL-23, IL-27 and TIMP-1 were exclusively assessed by ELISA. Then, these cytokines were compared to the control-patients. The levels of IL-2, IL-4, IL-6, IL-8, IL-27, IFNγ, TNF and G-CSF were significantly higher ($p \leq 0.05$) in

Table 1
Characteristics of the participants in the study at baseline.

	Control	TB+/HIV-	TB+/HIV+
Gender M:F	19:7	59:24	10:0
Age (years)[a]	54.3 ± 9.5	42.1 ± 16.6	37.7 ± 6.3
Diagnosis (Pos:Neg)			
Histopathology	0:5	34:19	6:1
Culture - Biopsy	0:4	4:12	1:2
Culture - Pleural effusion	0:20	6:47	3:6
Culture - Smear	0:16	4:7	0:1
AFB - Pleural effusion	0:18	2:66	0:10
AFB - Smear	0:22	3:29	0:6

[a] Mean ± SEM.

both case-patient subgroups (with or without HIV co-infection, Table 2 and Fig. 1). Case-patients with TB/HIV co-infection had significantly higher levels ($p \leq 0.04$, Table 2) of IL-6 (from 4-fold by Luminex, to almost 42-fold by CBA), IFNγ (24-fold), G-CSF (23-fold) and TNF (almost 6-fold) compared with the control-patients; IL-27 was 2-fold higher ($p \leq 0.04$, Fig. 1). There were no significant differences between the groups for the levels of the other 13 cytokines.

Based on the receiver operating characteristic (ROC) curve analysis, the highest area under the curve (AUC) values were for IFNγ (TB without HIV subgroup = 0.89, $p < 0.001$; TB/HIV co-infection subgroup = 1.0, $p = 0.008$). The ROC curve for the TB/HIV co-infection subgroup presenting high IL-27 levels showed a very good fit: it had 100 % sensitivity, 80 % specificity and revealed that IL-27 if of great value to detect TB/HIV co-infection (TB without HIV subgroup = 0.77, $p = 0.001$; TB/HIV co-infection = 0.85, $p = 0.016$). Finally, there was lower specificity to detect TB without HIV cases for IL-6 (AUC = 0.91, $p = 0.013$). On the other hand, there was a different picture for TB/HIV co-infection: there was high sensitivity and specificity (AUC = 0.92, $p = 0.004$). A summary of these findings is shown in Fig. 2.

The gold standard for the diagnosis of tuberculous pleuritis remains the detection of *M. tuberculosis* by liquid culture and histopathological findings by means of pleural biopsy specimens. This diagnosis requires a quite invasive gesture [4]. However, new non-invasive approaches that might complement and improve current strategies for the early detection and clinical management of pleural TB are wanted, because in daily clinical practice, there are patients who refuse invasive examinations or are physically difficult to endure [7]. Therefore, effective detection of circulating biomarkers potentially can provide a solid basis for rapid diagnosis of pleural TB and accurate TB treatment. Particularly, the determination of biofluids, such as IFNγ and ADA, plus the concomitant use of nucleic acid amplification tests, such as the GeneXpert MTB/RIF system, should find their place in the diagnostic algorithm of TB pleurisy [8,9]. The challenge now is to determine which biofluids and detection approaches would be most suitable for application at different stages of daily clinical practice. Therefore, the potential investigation of biomarkers in exudates has the advantage of being simpler, faster and safer than histopathology of surgical biopsy fragments.

There are few reports regarding the IL-2, IL-6 and TNF levels in pleural effusions of HIV-positive patients with tuberculous pleuritis, but our study corroborates a previous one that reported higher levels of those three cytokines, but not IL-1β, in a cohort of HIV-negative patients [10]. In fact, similarly to our findings, that study also failed to find higher IL-1β levels in pleural effusions from

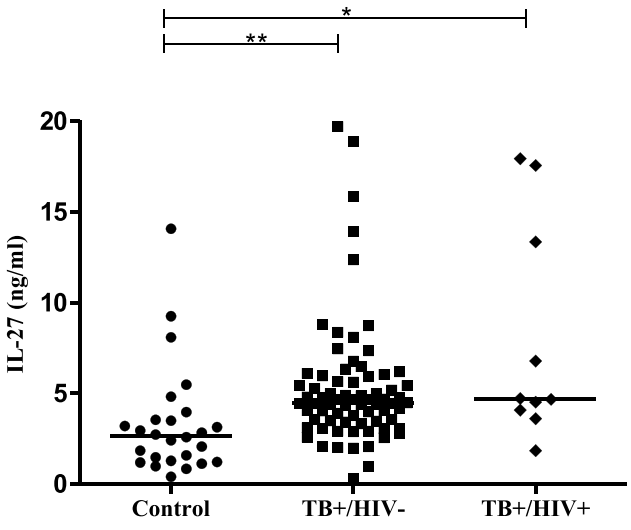

Fig. 1. The *ex vivo* human IL-27 levels (ng/ml) were determined in control-patients (n = 26), TB+/HIV- (n = 83) and TB+/HIV+ co-infected (n = 10) case-patients pleural effusion samples using a commercially available enzyme linked immunosorbent assay (ELISA) kit. The immunoassay was carried out according to the manufacturer' instructions, and horizontal bars represent median values. *$p \leq 0.04$ and **$p \leq 0.001$, based on statistical significance using the Mann-Whitney U test.

patients with TB/HIV co-infection compared with patients with cancer. Also consistent with our findings, prior studies have shown that the IL-2 levels in pleural effusions of patients with malignancies are lower than in patients with tuberculous pleuritis [10,11]. In addition, previous studies have reported the highest TNF levels in patients with tuberculous pleuritis compared with patients with malignancy [10,12]. In our study, the TNF levels in the pleural effusion of patients with tuberculous pleuritis were positively modulated in both case-patient subgroups, but mostly in the HIV-negative subgroup (8-fold higher than the TB/HIV co-infection group). Remarkably, the IL-6 levels were significantly higher in the tuberculous pleuritis group compared with the parapneumonic group (reviewed by Ref. [13]). In agreement with our study, Yang and colleagues [10] also found higher IL-6 levels in the pleural effusion of patients with tuberculous pleuritis, and those levels of pleural effusions in patients with TB/HIV co-infection were also higher than patients with other forms of pleuritis. Importantly, our results are rather reproducible regardless of the method employed.

Two recent reviews on the fascinating topic of *in situ* soluble factors as potential diagnostic biomarkers for to detect tuberculous

Table 2
Cytokine levels detected by two multiplex arrays as potential biomarkers in pleurisy of HIV- and HIV+ Case- and Control-patients.

	Luminex					CBA				
	Control (n = 17)	TB+/HIV- (n = 42)	p-value[a]	TB+/HIV+ (n = 5)	p-value[b]	Control (n = 9)	TB+/HIV- (n = 31)	p-value[a]	TB+/HIV+ (n = 5)	p-value[b]
IL-6	10.0 ± 0.3[c,d]	20.8 ± 0.7	**0.0056**	40.4 ± 0.2	**0.0022**	0.9 ± 0.5	33.8 ± 0.0	**0.0022**	37.4 ± 2.2	**0.0420**
IFNγ	50.3 ± 10.3	2068.1 ± 887.5	**<0.0001**	1214.4 ± 487.1	**<0.0001**	1.9 ± 0.0	298.3 ± 68.5	**0.0117**	38.8 ± 24.6	0.1055
TNF	7.8 ± 3.2	358.6 ± 262.2	**0.0006**	45.0 ± 4.6	**0.0003**	0.0 ± 0.0	0.2 ± 0.1	ND	0.8 ± 1.0	ND
IL-2	1.3 ± 0.6	34.6 ± 28.4	**0.0318**	17.4 ± 8.5	**0.0460**	0.0 ± 0.0	0.0 ± 0.0	ND	0.0 ± 0.0	ND
IL-4	0.3 ± 0.0	23.6 ± 22.2	**0.0074**	3.8 ± 2.8	**0.0229**	0.0 ± 0.0	0.0 ± 0.0	ND	0.0 ± 0.0	ND
IL-8	0.9 ± 0.4[c]	1.7 ± 0.3	**0.0266**	1.1 ± 0.4	**0.0543**	NA	NA	–	NA	–
G-CSF	14.0 ± 5.9	341.1 ± 158.5	**0.0009**	323.4 ± 162.1	**0.0022**	NA	NA	–	NA	–

Statistically significant values in bold.
[a] Control *vs.* TB+/HIV-.
[b] Control *vs.* TB+/HIV+.
[c] ng\mL, otherwise pg\mL.
[d] Mean ± SEM.

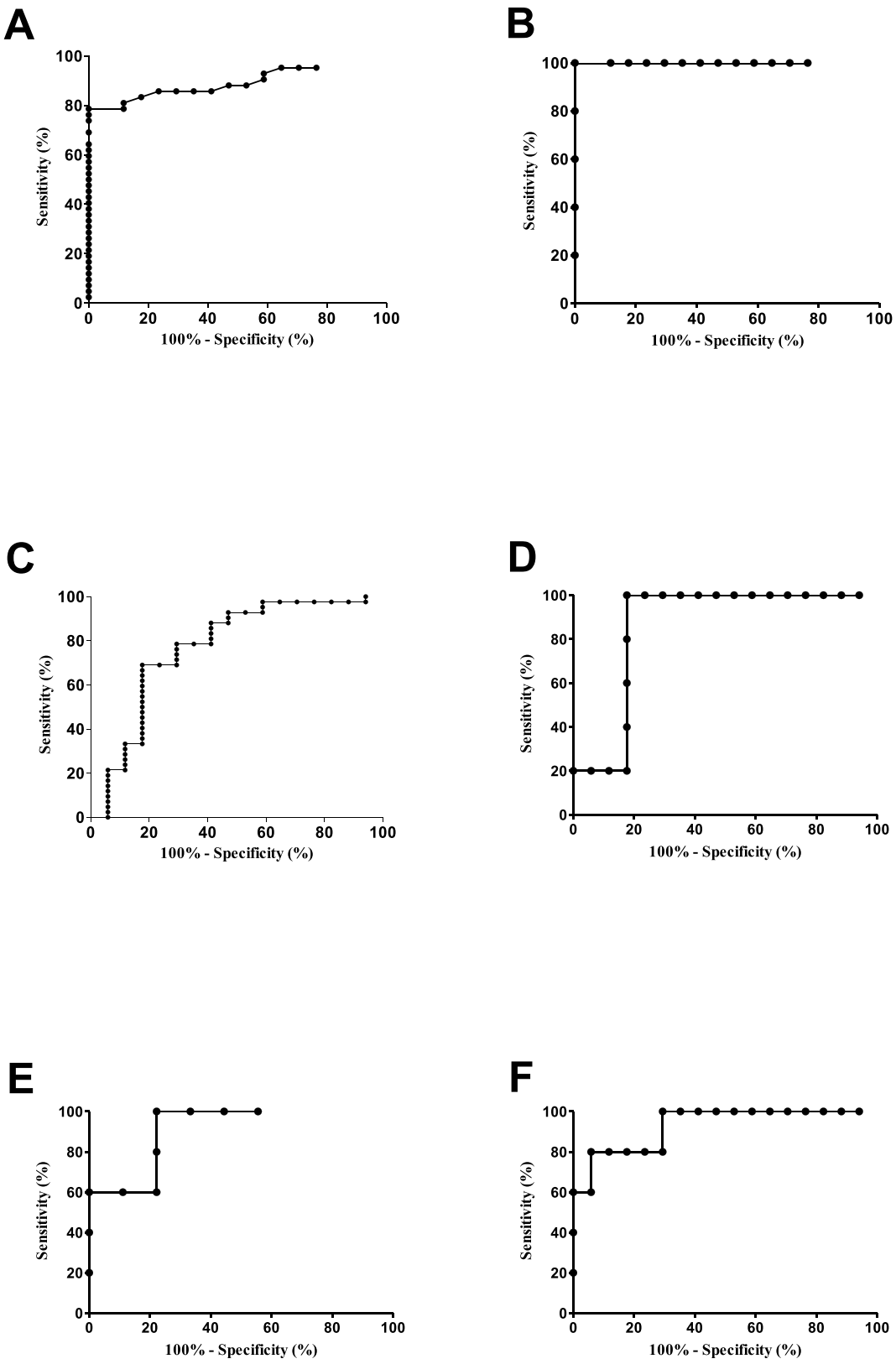

Fig. 2. The ROC curves by IFNγ (A and B), IL-27 (C and D) and IL-6 (E and F) associated with the TB+/HIV- (A, C and E) and TB+/HIV+ (B, D and F) case-patients when compared to the control-patients.

pleuritis have been published [14,15]. Until 2017, among the soluble biomarkers, IFNγ, total ADA and ADA2 seemed to the most relevant to detect tuberculous pleuritis, with a sensitivity of 89 %, 92 % and 97.2 %, respectively, and a specificity of 97 %, 90 % and 94.2 %, respectively [8]. Surprisingly, the IFNγ release assay (IGRA) seems to have moderate sensitivity (80 %) and specificity (72 %) to detect tuberculous pleuritis [8], and TNF shows poor diagnostic accuracy to detect this condition [16]. Other candidates, such as sFasL [17], angiotensin-converting enzyme (although cross-reactive in patients with rheumatoid arthritis), calpain-1, spectrin breakdown products, MMP-1 [18], sCD26 [19], sIL-2R [20], IL-32 [21], C1q [22], CXCL9/MIG, CXCL10/IP-10 and CXCL11/IP-9 [23,24] have proved to be rather specific and sensitive. Consistently, a multiprotein biosignature comprising CXCL1, CXCL2, CXCL8, CXCL9/MIG, CXCL10/IP-10, CCL20 and CCL23 has been shown to decline in patients with tuberculous pleuritis upon management [25]. More recently, the combination of CD46, CD55, CD59 and ADA have been reinforced as promising candidate biomarkers to provide a highly accurate tuberculous pleuritis diagnosis [26].

Two prospective biomarker studies and two meta-analyses have shown that higher IL-27 levels in pleural effusion seem to be sensitive and specific to differentiate tuberculous pleuritis from pleural effusions due to the other causes [27,28]. Compared with control individuals, IL-27, IFNγ and ADA all simultaneously increased in patients with tuberculous pleuritis but without HIV, suggesting that the applications of IL-27 detection, alone or with IFNγ and ADA, may contribute to more efficient diagnosis of tuberculous pleuritis [29]. However, IL-27 is less effective than ADA in the tuberculous pleuritis diagnosis [30]. In our study, the IL-27 levels in the pleural effusion of patients with TB/HIV co-infection were highly specific and sensitive. To the best of our knowledge, our study is the first to report this aspect.

We have shown that patients with tuberculous pleuritis, but not patients with other forms of pleuritis, have persistently higher levels of IL-6 and IL-27 in pleural effusions; these differences could be helpful to differentiate the diseases. The levels of those two cytokines were also higher in pleural effusions of patients with TB/HIV co-infection. By using the combinations of multiparametric approaches, we confirmed the significant association of IL-6 and IL-27 in patients with tuberculous pleuritis, regardless of the immunological status. In addition, IL-2, IL-4, IL-8, IFNγ, TNF and G-CSF were significantly higher in pleural effusion of patients with TB/HIV co-infection compared with controls in one detection approach, but there were discordant results with another approach.

The most obvious limitation of our study is the modest, limited sample size because we used the collected specimens for intensive immunological Multiplex testing as well as ELISAs. Nevertheless, we observed significant differences between the groups. Larger studies are needed to replicate previously identified biomarkers, as well as the biomarkers identified in this study. Another limitation is that the two Multiplex approaches yielded discordant results for six biomarkers, denoting an inherent constraint of the assays. In fact, for IL-6 levels there was an almost 10-fold difference between the methods. Although pro-inflammatory cytokines, like IL-6, are believed to be disease-specific [31], inflammatory biomarkers are generally thought of as non-specific. A panel of multiprotein biosignature, including non-specific inflammatory markers as well [32], would have higher accuracy for tuberculous pleuritis detection and for monitoring anti-TB treatment efficacy than a single biomarker. For instance, in *Mycobacterium leprae* infection, the diagnostic tests require multiple, diverse biomarkers, now specific for both humoral- and cell-mediated immune responses [33]. Like IL-6, Procalcitonin appears to be more specific for bacterial infection, being unsuitable as a general marker of inflammation [31]. Finally, we did not evaluate a group of patients with only HIV infection to check whether the virus *per se* modulates those biomarkers. At least for IL-6 and IL-27, it is worth further investigation [34]. Given the above, one should consider the results of this study to be hypothesis generating rather than hypothesis confirming.

In conclusion, we detected significantly associated biomarkers in patients with confirmed tuberculous pleuritis. We identified elevated IL-6 and IL-27 levels as promising novel candidate biomarkers to detect tuberculous pleuritis, regardless of the immunological status. These two biomarkers plus IFNγ may allow for a faster, more accurate diagnosis of extrapulmonary TB.

Declaration of competing interest

Authors have no competing interests.

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