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Plasma levels of C-reactive protein, matrix metalloproteinase-7 and lipopolysaccharide-binding protein distinguish active pulmonary or extrapulmonary tuberculosis from uninfected controls in children

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ABSTRACT

The immune profile associated with distinct clinical forms of tuberculosis (TB) has been extensively described for adult populations. Nevertheless, studies describing immune determinants of pulmonary or extrapulmonary TB (PTB or EPTB, respectively) in children are scarce. Here, we retrospectively assessed plasma levels of several mediators of inflammation in age and sex-matched children from South India presenting with PTB (n = 14) or EPTB (n = 22) as well as uninfected healthy controls (n = 19) to identify biomarkers that could accurately distinguish different TB clinical forms. Furthermore, we performed exploratory analyses testing the influence of sex on the systemic inflammatory profile. The analyses identified a biosignature of 10 biomarkers capable of distinguishing the three clinical groups simultaneously. Machine-learning decision trees indicated that C-reactive protein (CRP), matrix metalloproteinase (MMP)-7 and lipopolysaccharide-binding protein (LBP) were the markers that, when combined, displayed the highest accuracy in identifying the clinical groups. Additional exploratory analyses suggested that the disease signatures were highly influenced by sex. Therefore, sex differentially impacted status of systemic inflammation, immune activation and tissue remodeling in children with distinct clinical forms of TB. Regardless of such nuances related to biological sex, MMP-7, CRP and LBP were strong discriminators of active TB and thus could be considered as biomarkers useful in discrimination different TB clinical forms. These observations have implications on our understanding of the immunopathology of both clinical forms of TB in pediatric patients. If validated by other studies in the future, the combination of identified biomarkers may help development of point-of-care diagnostic or prognostic tools.

1. Introduction

Tuberculosis (TB) is a major cause of death worldwide [1,2]. Most studies on TB diagnosis are performed primarily in adult populations and then applied to children. Since both groups are described to present distinct immune and inflammatory profiles [3], the established tests are not as accurate for diagnosis of childhood TB [3]. Children, especially those below 5 years old, and adolescents are thought to present relatively weaker immune responses against infection compared to adults [4]. These immune differences are likely involved in increased susceptibility to *Mycobacterium tuberculosis* (*M.tb*) infection [5]. Once infected with *M.tb*, pediatric populations who develop active TB frequently present with extrapulmonary forms of the disease [6]. Recent studies estimated that TB is one of the main causes of childhood

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morbidity and mortality, leading to approximately 1 million new infections (10% of all cases) and almost 210,000 deaths per year [7].

The lack of reliable TB diagnostic tests to be used in pediatric populations poses an important challenge to the disease control. Indeed, both tuberculin skin test (TST) and blood-based gamma interferon (IFN-γ) release assays (IGRA) fail to differentiate latent from active TB [2]. Furthermore, collection of sputum smear samples for microbiological diagnosis is difficult in children, resulting in a low sensitivity (< 15%) despite the use of optimized methods [8]. Sputum culture, which is the gold standard for diagnosis of TB in adults, is less accurate in pediatric patients because the latter usually present with paucibacillary infection [9]. Of note, the diagnostic performance of molecular tests such as Xpert MTB/RIF is also described to be lower in children [10–12]. With regard to extrapulmonary TB (EPTB), confirmatory diagnosis is complicated by the need to access a diverse range of disease sites. This scenario results in high number treatment implementation following presumptive TB diagnosis which are not microbiologically confirmed [12]. Thus, finding a reliable biomarker with reasonable diagnostic performance and which is not dependent on tissue or sputum sampling is desirable to optimize clinical management of pediatric TB patients.

India is a highly endemic country for *M.tb* infection. In this country, BCG vaccination has been systematically performed by the TB control program for the last 5 decades [13]. This high TB prevalence is generally attributable to low quality of monitoring and treatment of the patients [13]. It is possible that the TB burden in India may reduce with increased diagnostic performance of tests for pediatric TB. Therefore, a point-of-care, non-sputum based, diagnostic tool which can be used in such vulnerable population is extremely necessary. Immune-based assays can bring light to this problem, even though only a few assays for diagnosis of childhood TB have been investigated [9]. Since previous studies have already depicted immunological differences related to sex [14,15], and that circulating concentrations of sexual hormones change during adolescence, it is important to investigate whether such changes may impact TB diagnosis based on immunoassays. In the present study, we tested if plasma concentrations of different biomarkers of acute inflammation, immune activation and tissue remodeling are capable of discriminating different clinical presentations of microbiologically confirmed cases of active TB in children from India. We also performed an exploratory examination of the influence of sex on the diagnostic performance.

2. Materials and methods

2.1. Ethics statement

Written informed consent was obtained from all participants or their legally responsible guardians, and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The project was approved by the institutional review board of the Institutional Review Board of the National Institute for Research in Tuberculosis, Chennai, India.

2.2. Study design

The present study is a retrospective assessment of a databank containing epidemiological, immunological, and clinical data from 55 children between 2011 and 2012 from the Childs Trust Hospital, located in Chennai, India. The details of the recruitment, diagnosis and clinical definitions were published previously [9]. The study population was composed by 14 (25.4%) PTB patients, 22 (40%) with EPTB and 19 (34.5%) uninfected healthy controls (HC). All TB patients were recruited at the time of active disease presentation and before antitubercular treatment implementation. PTB cases were microbiologically confirmed by sputum microscopy and *M.tb* solid culture (Lowenstein-Jensen medium). All participants were positive for both acid-fast bacilli (AFB) identification in sputum smears and cultures.

Children with EPTB were diagnosed based on clinical symptomatology, physical evaluation and biopsies according to the clinical manifestation, such as fine-needle aspiration for the cases of TB lymphadenitis or cerebrospinal fluid analysis for TB meningitis. Out of the 22 patients with EPTB, 14 (63.6%) presented with spinal TB, 6 (27.3%) with TB lymphadenitis and 2 (9%) had abdominal TB, which included peritonitis or tuberculomas. Participants included in the HC group were asymptomatic children who went to the hospital for routine vaccinations. Prior to study enrollment, children were evaluated for presence of major comorbidities such as bacterial or viral coinfection, including HIV, the present study included only individuals who screened negative for such conditions. Peripheral venous blood was collected in all study participants for evaluation of exclusion criteria which included HIV infection status as well as for latent TB infection (LTBI) investigation in the HC. LTBI screening was performed by QuantiFERON TB Gold-in-Tube enzyme-linked immunosorbent assay (ELISA) (Cellestis).

2.3. Immunoassays

Data on several mediators were selected in order to assess the overall inflammatory profile; and all the biomarkers contained in the databank were analyzed. In the original study, biomarkers were measured in EDTA-treated plasma samples. Biomarkers included were cytokines, acute-phase proteins and tissue remodeling proteins. Bio-Plex multiplex ELISA cytokine assay system (R&D Systems) was employed to measure the cytokines analyzed. The list of cytokines included interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IL-20, IL-21, IL-22, IL-23, interferon (IFN)-γ, tumor necrosis factor α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) and transforming growth factor (TGF)-β. Moreover, IFN-α and IFN-β levels were quantified using the VeriKine serum ELISA kit (PBL Interferon Source). Plasma levels of fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), platelet-derived growth factor AA (PDGF-AA) and granulocyte colony-stimulating factor (G-CSF) were measured using the Milliplex map kit system by Merck Millipore.

Levels of C-reactive protein (CRP), haptoglobin, serum amyloid A (SAA), and α-2 macroglobulin (*α-2-M)* (Bio-Rad) were quantified by Bio-Plex multiplex ELISA system according to manufacturer issued instructions. Concentration of extracellular matrix metalloproteinases (MMPs)-1, 7, 8 and 9, and tissue inhibitors of metalloproteinases (TIMPs)-1, 2, 3 and 4 were measured using Luminex technology (R&D Systems), according to the manufacturer's protocols. *Limulus* amebocyte lysate assay were performed to measure Lipopolysaccharide (LPS) levels (Cell Sciences Hycult Biotech) following the manufacturer's protocol. Plasmatic Hemoxygenase 1 (HO-1) was measured by ELISA (Assay Designs). ELISA kits were used to quantify plasmatic levels of IgG endotoxin core antibodies (EndoCAb) (Cell Sciences Hycult Biotech), lipid-binding protein (LBP) and soluble CD14 (sCD14) from R &D Systems. The sensitivity of each biomarker assay is listed in Supplementary Table 1.

2.4. Data analysis

The median values with interquartile ranges (IQR) were used as measures of central tendency and dispersion, respectively. Pearson's chi-square test was used to compare frequencies between the study groups. Continuous variables were compared between the study groups using the Mann-Whitney *U* test (2 group comparisons), or the Kruskall-Wallis test with Dunn's multiple comparisons ad hoc test (between 3 or more groups). Hierarchical cluster analyzes were performed using the Ward's method with 100× bootstrap. Venn diagrams were used to display the differentially expressed markers between the indicated study groups. A machine-learning based decision tree including the values of biomarkers which were differentially expressed between the study groups was designed the Weka 3 software [16]. Receiver Operator Characteristics (ROC) curve analysis was employed to test the

accuracy of the differentiation of indicated clinical groups using different combinations of plasma biomarkers. P-values were adjusted for multiple measurements using the Holm-Bonferroni method. A P-value below 0.05 was considered statistically significant. The analyzes were performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA) and JMP 11 (SAS Institute Inc., Cary, North Carolina).

3. Results

3.1. Characteristics of the study participants

Patients with PTB or EPTB as well as controls were similar with regard to age (median age in PTB: 9 years, IQR: 5–13 vs. median in EPTB: 7 years, IQR: 8–13 vs. median in HC: 10, IQR: 5–12; p = 0.607) and sex (male in PTB: 6 [42.9%] vs. male in EPTB: 13 [59.1%] vs. male is HC: 13 [68.4%]; p = 0.337). Moreover, the groups differed in terms of BCG vaccination status (p = 0.012), with the majority of PTB patients being BCG vaccinated (n = 11; 78.6%), whereas vaccination was lower in EPTB (n = 11; 50%) and lowest in HC (n = 5; 26.3%).

3.2. Inflammatory profile of pediatric TB

Median plasma concentration values of each mediator of inflammation per study group are shown in Supplementary Table 2. Data were log-transformed and z-score normalized. These values were then used in model of hierarchical cluster analysis to test whether there are distinct expression profiles that could characterize the different study groups. This approach revealed 4 clusters of markers which exhibited distinct expression profiles among the clinical groups (Fig. 1A). The cluster 1 included most of the acute phase proteins together with MMP-1, MMP-8 and HO-1, which had increased expression in both PTB and EPTB patients compared to controls (Fig. 1A) but with relatively higher values found in EPTB.

The second cluster was composed by a heterogeneous mix of markers, with presence of molecules involved in tissue remodeling and immune activation at the highest levels detected in PTB (Fig. 1A). Markers which had the lowest values detected in PTB patients and highest values in EPTB (EndoCAb was statistically significant) were part of the third cluster (Fig. 1A). Lastly, the fourth cluster underlined the markers which exhibited the highest relative concentration values in the healthy uninfected children compared to the disease groups, although only LBP was statistically significant between EPTB and HC groups (Fig. 1A).

We next performed paired comparisons between the study groups to examine statistically significant log fold-differences of the circulating concentrations of each molecule (Fig. 1B, raw values are shown in Supplementary Table 3). Compared to HC, PTB patients had on average substantially higher levels of MMP-1, MMP-7, MMP-8, TIMP-1, TIMP-3, CRP, α-2-M, TGF-β, haptoglobin, HO-1, IL-21 and IL-23 whereas those with EPTB exhibited higher values of MMP-1, HO-1, TIMP-3, IL-21 and EndoCAb and lower levels of LBP (Figs. 1B and 2A). When the subgroups of TB patients were compared, PTB was associated with lower concentrations of EndoCAb and IL-6 and higher levels of TIMP-3, MMP-7, TGF-β, IL-20, LPS and LBP compared to extrapulmonary disease (Figs. 1B and 2A).

We used a Venn Diagram to summarize the profiles of differentially expressed markers between PTB and EPTB compared to controls. This approach revealed that IL-21, HO-1, MMP-1 and TIMP-3 were all found at higher values in both PTB and EPTB patients compared to controls, whereas no marker was found to exhibit lower concentrations related to controls (Fig. 2A). Of note, PTB was associated with specific changes in biomarker levels, hallmarked by heightened values of α-2-m, CRP,

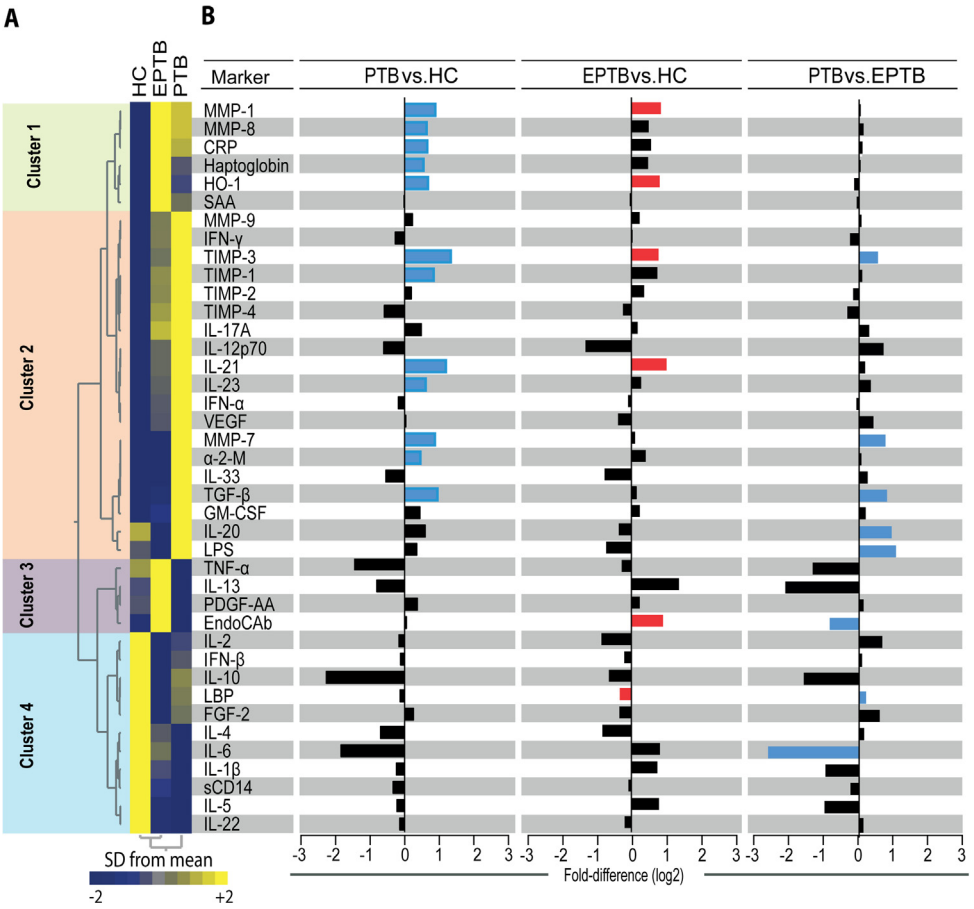

Fig. 1. Children with either pulmonary or extrapulmonary Tuberculosis exhibit a distinct inflammatory profile of plasma markers. Plasma levels of cytokines, chemokines and soluble receptors from uninfected individuals (n = 19) and patients with pulmonary TB (n = 14) or extrapulmonary TB (n = 22). Data were log10 transformed and z-score normalized. (A) A hierarchical cluster analysis (Ward's method) with 100X bootstrap was performed to test if the study groups could be separated based on the overall expression profile of the biomarkers. Dendrograms represent Euclidean distance. Using this approach, 4 clusters of biomarkers were detected. (B) Bars represent fold-differences in biomarkers levels between indicated study groups. Comparisons were calculated using Kruskall-Wallis test with Dunn's multiple comparisons (adjusted P-value ≤ 0.05). Values that were statistically significance are represented in colored bars.

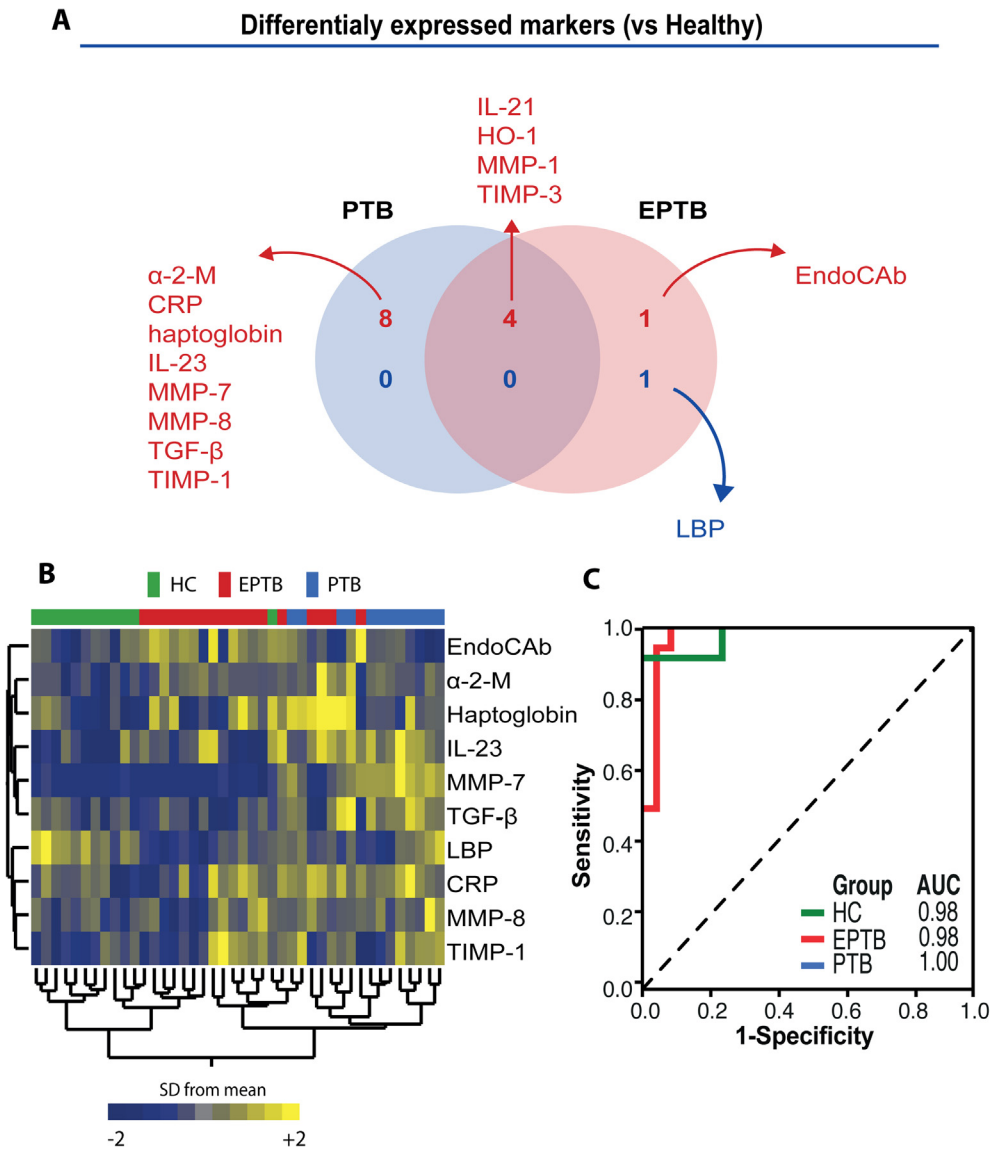

Fig. 2. A biosignature composed of 10 molecules is capable of differentiating PTB from EPTB or healthy controls. (A) Venn Diagram describes the markers which values were statistically different between disease groups (PTB or EPTB) and the control group (healthy) as shown in Fig. 1A. Plasma concentrations of markers in red were higher whereas in blue were lower in disease groups compared to healthy controls. (B) A hierarchical cluster analysis was made to analyze if the combination of biomarkers that were found to be statistically different exclusively in PTB or EPTB in univariate analysis could separate the groups according to individual levels of each subject. Data were log10 transformed and z-score normalized. (C) Receiver Operator Characteristics (ROC) curve analysis was employed to test the accuracy of the discrimination of the different study groups when using the plasma levels of molecules represented in (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

haptoglobin, IL-23, MMP-7, MMP-8, TGF-β and TIMP-1 (Fig. 2A). On the converse, EPTB was linked to exclusive augmented levels of EndoCAb and dampened concentrations of LBP (Fig. 2A).

An important aim of the present study was to find a biomarker signature that could distinguish different clinical forms of TB from healthy controls in a pediatric population. To address this question, we performed 2 approaches of discriminant analyses using the combination of markers which were uniquely expressed by either PTB or EPTB patients. First, hierarchical cluster analysis of z-score normalized values demonstrated that the different disease groups could be clustered separately with only very few misclassifications (Fig. 2B). Second, we used a multiparametric Receiver Operator Characteristics (ROC) curve analysis of the combined markers. The results confirmed the cluster analysis and demonstrated a relatively high accuracy of the biomarker combination (LBP, α-2-M, CRP, haptoglobin, IL-23, MMP-7, MMP-8, TGF-β, TIMP-1 and EndoCAb), assessed by area under the curves (AUC), in distinguishing the study groups (Fig. 2C).

3.3. MMP-7, LBP and CRP are the biomarkers with strongest power to discriminate pulmonary from extrapulmonary TB in children

We next employed a machine learning-based decision tree model to select the best biomarkers able to discriminate PTB, EPTB and controls, out of the 10 differentially expressed molecules identified. This analysis revealed that only 3 biomarkers (MMP-7, LBP and CRP) appeared as essential to differentiate the clinical conditions, with a step-wise approach that resulted in an accuracy of 87.5% ($p < 0.0001$) to distinguish all clinical groups (Fig. 3A). Furthermore, we used a discriminant algorithm using ROC curves [17] and found a very high accuracy of the combined biomarkers, MMP-7, LBP and CRP, in distinguishing the clinical groups (Fig. 3B). Sub-analyses were performed stratifying the study participants in males and females and found that the discriminant power of the combined markers was independent on sex (Fig. 3C).

3.4. Sex influences the inflammatory profile of pediatric TB

The results described above suggested that children with pulmonary or extrapulmonary TB exhibit a distinct inflammatory profile. During childhood, individuals are exposed to changes in sex hormones that peak in adolescence [18]. Aside from their role in reproduction and sexual differentiation, sex hormones have also been described to affect immune responses [18]. Here, we performed an exploratory investigation to depict the systemic inflammatory profile associated with sex in our study population. A hierarchical cluster analysis of the overall expression levels uncovered that male and female TB patients exhibit some distinctions in their biomarker profiles. There were no

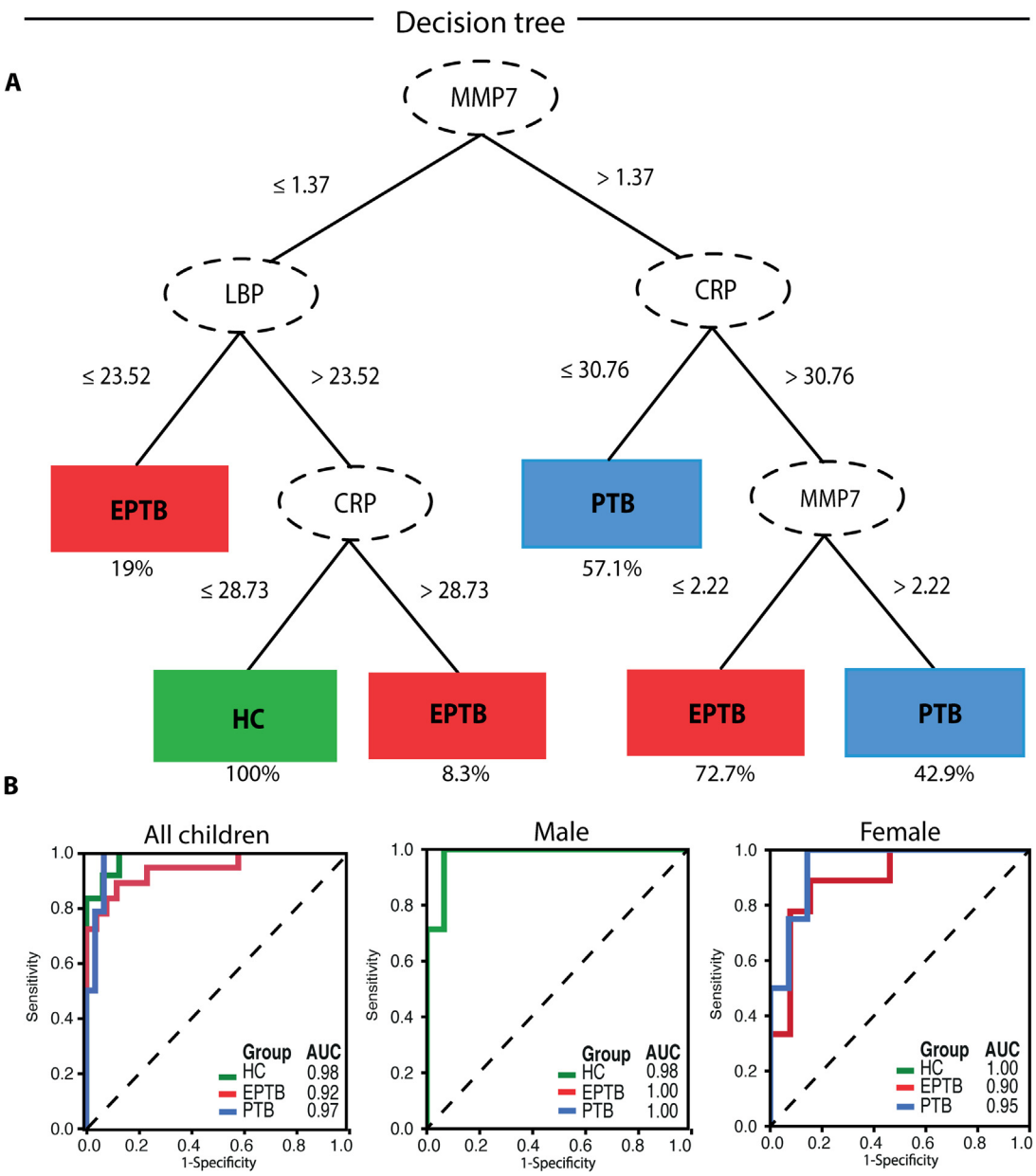

Fig. 3. Machine learning analysis identifies the combination of markers that more accurately discriminates PTB from EPTB and HC in children. (A) A Decision tree with an accuracy 87.5% was performed to analyze, among the differentially expressed biomarkers, the minimum of targets and their quantification that was capable of separate PTB, EPTB and HC. The levels of MMP7 and CRP are in ng/mL and levels of LBP in μg/mL. Below each rectangle, frequencies of individuals that are identified in a given study group at each step of the decision tree are shown. (B) Receiver Operator Characteristics (ROC) curve analysis was employed to validate how well the biomarkers included in the decision tree separate all patients as well as in individuals stratified by sex; all the ROC analyses resulted in p-values < 0.0001.

significant differences in biomarker levels between subgroups of uninfected controls stratified by sex (Fig. 4A and data not shown). Furthermore, this method demonstrated differences in biomarker expression patterns that resulted in 5 large clusters (Fig. 4A).

Each disease group stratified by sex was compared with HC with regard to plasma concentrations of the biomarkers. The statistical differences between the groups and their respective p-values are shown in the Supplementary Tables 4 and 5. Fold-difference analyses indicated that in male children, PTB was associated with higher levels of CRP, MMP-7, TIMP-1, IL-21, TIMP-3, haptoglobin, α-2-M, TGF-β and lower quantifications of MMP-9 in plasma compared to HC (Fig. 4B). In EPTB patients, CRP, TIMP-1, TIMP-3, HO-1 and α-2-M values were augmented, whereas IL-21, IL-22 and LBP levels were lower than that in controls (Fig. 4B). With regard to female participants, PTB patients had lower expression values of IL-21 and higher levels of MMP-1, MMP-7, TIMP-3 and TGF-β whereas EPTB exhibited augmented concentrations of MMP-1 and VEGF and diminished concentrations of IL-4 and LBP than healthy controls (Fig. 4B). Again, Venn Diagrams were used to summarize the differences in biomarker expression levels between the study subgroups (Fig. 4C).

In male children, both PTB and EPTB were associated with upregulation of CRP, TIMP-1, TIMP-3, IL-21 and α-2-M levels compared to uninfected individuals (Fig. 4C). Strikingly, in female patients, MMP-1 was the only parameter which was a common differentially expressed marker between PTB and EPTB, presenting with higher levels compared with controls (Fig. 4A). We also compared groups of males vs. female patients with either PTB or EPTB to sex-matched controls to delineate differences in biomarker profiles directly associated with sex. We found

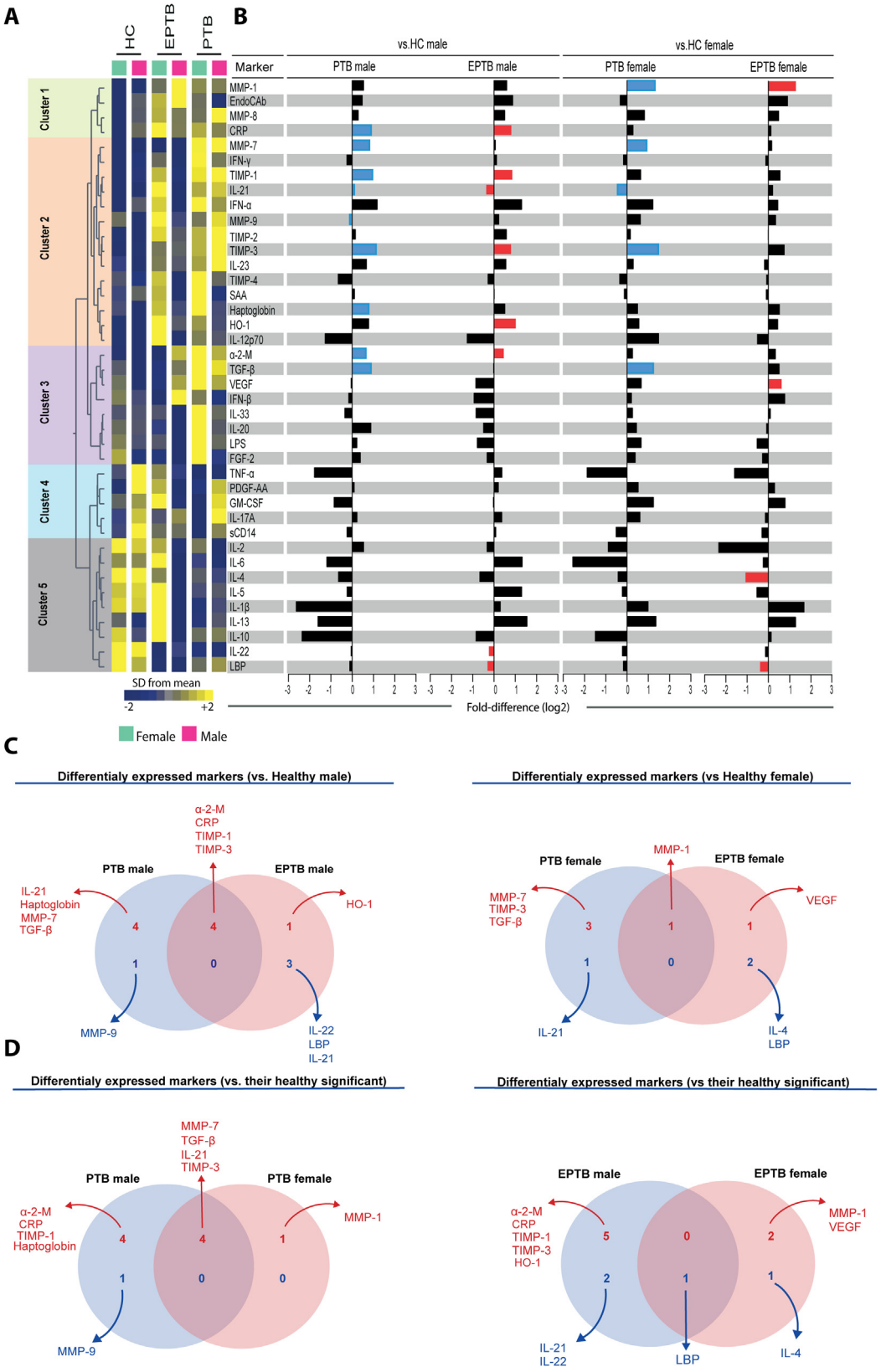

Fig. 4. Biomarker profiles of systemic inflammation are distinct in pediatric TB patients stratified by sex. (A) A hierarchical cluster analysis (Ward's method) was performed to test if the expression profile of biomarkers groups could be separated based on sex. Dendrograms represent Euclidean distance. Using this approach, 5 clusters of biomarkers were detected. (B) Statistical differences based on multiple comparisons as described in Methods (adjusted P-value ≤ 0.05) in biomarkers levels for male and female children with PTB and EPTB compared to healthy subjects were calculated. Values that presented statistical significance are represented in colored bars. (C) Venn Diagrams summarize the markers which were statistically different between PTB or EPTB and HC groups, with separation of each group according to sex. Markers in red were higher whereas in blue were lower in disease groups compared to healthy controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

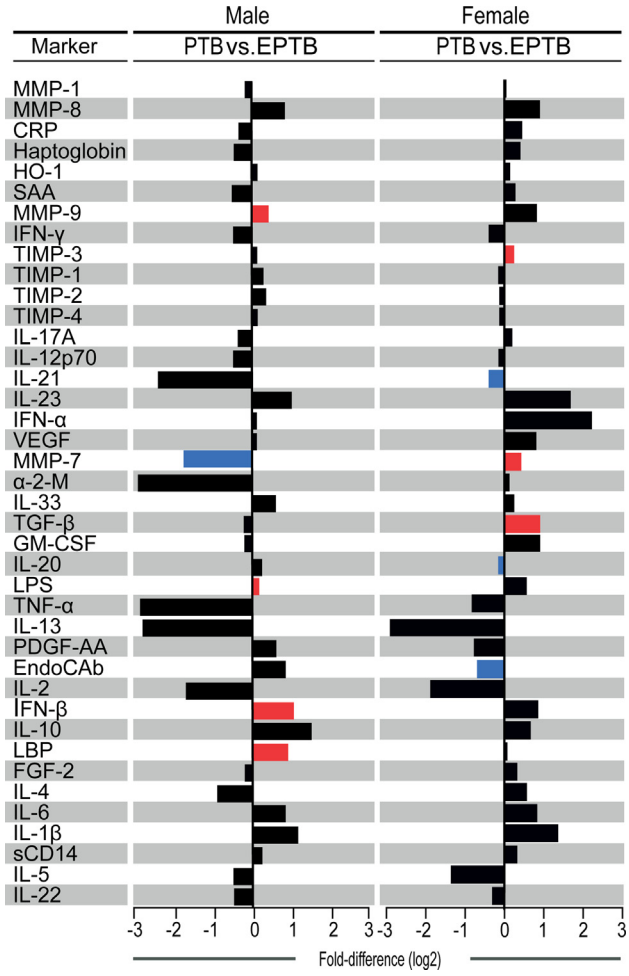

Fig. 5. Males and females have different patterns of expression molecules in PTB or EPTB. Statistical differences based on multiple comparisons testing as described in Methods (adjusted P-value ≤ 0.05) and fold-difference analysis in biomarkers levels between the clinical conditions in unhealthy males and females were calculated. Values substantially higher in PTB are shown in red whereas those which were higher in EPTB are shown in blue. Black bars were not statistically significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that MMP-1 was uniquely at higher levels in female PTB patients compared to controls whereas haptoglobin, TIMP-1, α-2-M and CRP were higher and MMP-9 and IL-21 concentrations were lower only in male PTB patients (Fig. 4D). Among EPTB patients, both male and female participants had lower levels of LBP compared to healthy controls. Nevertheless, female sex was associated with uniquely higher levels of MMP-1 and VEGF and lower IL-4 expression than controls whereas male sex exhibited higher expression of α-2-M, TIMP-1, TIMP-3, CRP and HO-1 and lower levels of IL-22.

Finally, we compared the biomarkers levels between PTB and EPTB within the subgroups of male or female participants (Fig. 5). In the subgroup of male patients, PTB was associated with lower levels of MMP-7 and higher concentrations MMP-9, LPS, IFN-β and LBP than those detected in EPTB (Fig. 5). Moreover, Female patients with PTB exhibited increased levels of TIMP-3, MMP-7 and TGF-β and lower values of IL-20, IL-21 and EndoCAb than those with EPTB (Fig. 5). These exploratory findings describe the differences in plasma biomarker concentration profiles in PTB and EPTB and the contribution of biological sex in such phenomenon.

4. Discussion

Pathogenesis of TB have been extensively studied over the years [19]. However, data related to the diagnosis or progression of TB in infants are still scarce and only recently this topic has started being more systematically investigated [20,21]. Seeking to fill important gaps in the presentation of TB in children, a population with immature immune system and at high risk of mortality linked to TB [22], we performed a characterization of systemic inflammation, immune activation and tissue remodeling activity in children with distinct clinical forms of TB. The present study contributes to the current knowledge in the field as it identifies a combination of plasma markers (MMP-7, CRP and LBP) that can reliably distinguish between different PTB and EPTB in a pediatric population from a highly endemic area. Such blood-based markers could potentially be used in a point-of-care assay without depending on complex laboratory structure [23]. Sex stratification of individuals was also performed, since the relevance of this characteristic, even in prepubescents, has been indicated in previous data on immunology and infectious diseases [18].

Overall, we observed that patients with pulmonary TB presented greater changes in biomarker levels compared to the control group than patients with extrapulmonary TB. Host MMPs drive extensive lung damage which hallmarks human TB [24]. In the present study we found higher levels of MMP-1 in both PTB and EPTB when compared to uninfected individuals whereas MMP-7 and 8 were higher only in PTB. MMP-1, is commonly high expressed by wounded pulmonary epithelial cells and is a protagonist in the immunopathology of TB [25]. Previous data suggest that MMP-7 secretion, towards chronic lung infections, also become upregulated and possibly contributes to pulmonary immunity [26], stimulating neutrophil influx, activating other MMPs [27], as well as degrading elastin, fibronectin, and proteoglycans [28]. Augmented levels of MMP-8 were associated with lung function decrease [29]. Higher circulating levels of both MMP-1 and 8 where reported previously in TB and may be useful to predict the pulmonary involvement and progression of treatment [30].

The activity of the MMPs is controlled by tissue inhibitors of metalloproteinases (TIMPs), and the disruption of the proteolytic balance of MMPs and their TIMPs was demonstrated in several pathological processes [31,32]. In TB, the balance between MMPs and TIMPs has been described previously [33]. Here, PTB patients exhibited higher levels of TIMP-1, which has been already reported [34], and this marker has been described as a potential biomarker for the diagnosis of pulmonary infection in adult population [35]. Whether MMPs and TIMPs play important role in the pathogenesis of TB disease progression in pediatric populations deserves further investigation.

Acute-phase response is a systemic reaction that follows inflammatory processes and tissue injury [36]. The acute-phase proteins have been shown to act in variety of host immune-defense mechanisms [37]. Three of those proteins (CRP, haptoglobin and α-2-M), were significantly augmented in PTB patients after comparisons between the other study groups indicating that children with pulmonary *M.tb* infection are potentially more inflamed. CRP is highly expressed in response to most of inflammations, infections, and tissue damage, being considered as marker of nonspecific response [38]. The utility of CRP for diagnosis and screening, especially for smear-negative active TB, has been highlighted previously [39], but the lack of specificity for TB is a drawback.

The biosignature associated with EPTB included only few markers which were also differentially expressed in PTB compared to controls, such as MMP-1, HO-1, TIMP-3 and IL-21. Of note, children with EPTB had significantly reduced levels of LBP in relation to both HC and PTB, despite being associated with upregulation as part of the acute-phase response [40] Plasma LBP enhances the macrophages response by binding pathogen associated molecular patterns (PAMPs), such as Lipoarabinomannan (LAM), a major mycobacterial cell wall component [41] and transferring these microbial products to the cell surface

receptor CD14 [42]. LBP can be produced by respiratory type II epithelial cells [41] suggesting that extrahepatic formation could have an important role in microbial in situ contention [43].

Some studies relate the higher expression of PAMPs (e.g. LPS), LBP and endotoxin core IgG antibody (EndoCAb) as a common feature of microbial translocation (MT) in different types of infections [44,45]. The fact that LBP levels were lower in EPTB whereas EndoCAb concentrations were higher may indicate a failure to respond to bacterial spread and related to increased disease severity [46]. In contrast, levels of LPS were statistically higher in PTB only when compared to EPTB, indicating potential greater activation of inflammatory response.

HO-1 is a relevant marker of oxidative stress response and is highly expressed in the lungs [47], being an important mediator of cytoprotection [48]. Both diseased groups of children exhibited augmented levels of HO-1 than uninfected controls. The upregulation of HO-1 towards infection by *M.tb* have already been reported to hallmark TB disease, capable of distinguish active infection from latent TB or healthy individuals in an adult population [49].

In response to TB, TGF-β is related to deactivation of macrophages and suppression of T-cell leading to an anergic response [50], and its activity is found commonly in PTB [51]. We also found TFG-β with higher expression in PTB than in HC or in EPTB children. Moreover, IL-23 levels were elevated only in PTB when compared to HC. IL-23 is thought to provide a moderate level of protection and immune regulation in setting characterized by absence of IL-12p70 and IL12-p40 production [52].

Our analysis indicated, among all the biomarkers examine, only 3 (MMP-7, CRP and LBP) were sufficient to distinguish between PTB, EPTB and HC in children. As far as their capacity of being good biomarkers, MMP-7 is one of the most described markers in idiopathic pulmonary fibrosis (IPF), considered as a predictor of lung function decline and disease progression [53]. Another study reported the overexpression of MMP-7 alongside with MMP-1 plasma concentrations as a biomarker combination capable of distinguish IPF from other chronic lung diseases [54]. Interestingly, in the comparisons between PTB and HC, we identified these 2 molecules with high expression in pulmonary disease. A recent study highlighted that macrophages of active TB patients expressed MMP-1 and MMP-7 around areas with tissue destruction, but only MMP-1 secretion was considered to be relatively specific to *M. tuberculosis* [55].

C-reactive protein (CRP) is a marker related to inflammation [56] and its characterization as a biomarker appears in a large number of distinct conditions, such as cardiovascular disease risk [57], obesity [58], diabetes [59], chronic obstructive pulmonary disease (COPD) [60] and TB [61]. The fact that CRP is related to general inflammation does may argue against the idea of being used as screening approach to diagnose TB. Nevertheless, the combination of CRP with MMP-7 and LBP improved the overall performance and demonstrated high diagnostic accuracy. LBP levels were found to be increased in patients with active TB compared with controls in a previous study [62], However, further studies indicated that LBP is a non-specific inflammatory response marker [63,64], much like CRP.

Associations between the increase in the concentrations of LBP and CRP, both acute phase proteins, have been reported in infectious endocarditis [65], acute diverticulitis [66] and pleural fluid levels of patients with infectious effusions [67]. In serum biomarkers associated with treatment of pulmonary TB, CRP and LBP are among the molecules that had their expressions diminished after 8 weeks of therapy [68]. Nevertheless, no direct relations with MMP-7 expressions were found. Our results made us speculate that these 3 markers may be employed as a diagnostic tool in children who may screen negative for AFB identification in sputum smears.

Finally, we sub-stratified each group (HC, PTB and EPTB) according to sex, a biological variable that affects the functions of the immune system along with age [69]. A transitory activation of the hypothalamus-pituitary-gonadal axis starts in the first months of life, in a process called “mini-puberty” [70]. Progesterone and testosterone leads to an overall immunosuppression, potentially increasing severity of some infections, including TB [71,72]. Estrogen effects can enhance Th1 immunity at smaller levels and also increase Th2 at higher concentrations [73]. Since Females tend to have stronger immune modulation [74], they are also more vulnerable to increased immunopathology in certain infectious diseases [75]. In our study groups, when compared to HC, male children had a greater quantification of markers related to inflammation, such as the acute phase proteins, especially in males with PTB. This group was also the one that exhibited high levels of haptoglobin, as well as of IL-21. Male PTB patients showed lower levels of MMP-9, probably due to the high expression of their specific inhibitor, TIMP-1 [76]. Males with EPTB showed increased levels of HO-1 and decreased expression of IL-22, potentially highlighting factors that are associated with mycobacterial dissemination.

In females it was interesting to note that both PTB and EPTB had elevated levels of MMP-1, but these levels did not appear to be altered in males, suggesting that females, although less inflamed, suffer more from tissue damage related to the infection. Extrapulmonary TB in females exhibited increased expression of VEGF and curiously decreased IL-4. These findings may suggest dampened capacity to modulate Th1 immune responses. A study in mice described that *M.tb* infected macrophages secreted VEGF and that was followed by increased angiogenesis in patients with TB [78], emphasizing the principle mycobacteria subverts macrophage function via induction of VEGF and formation of new vessels in order to disseminate [79].

This study is a retrospective analysis and presents some limitations as the absence of patients with latent TB and the small number of subjects. However, the topic is relevant and scarce data on biomarkers are available in children, giving the challenges to study such population. The molecules presented were quantified once with no follow up data. Some molecules taken into consideration herein could be increased in plasma in a non-specific way. Therefore, further studies are needed to detail pediatric TB and improve the validation of blood-based biomarkers.

5. Conclusion

We identified host biomarkers that were able to differentiate between children with active TB from healthy (MMP-1, HO-1, TIMP-3 and IL-21), distinguish PTB, EPTB and HC (MMP-7, CRP and LBP) and includes some markers previously described in adults. We also noticed that HO-1 and MMP-1 are more expressed in active TB irrespective of the disease site. Results on sex related characteristics in children demonstrated different expression of systemic inflammatory biomarkers, acute phase proteins and tissue remodeling molecules between pulmonary and extrapulmonary TB. These findings improve comprehension about the immunopathology of both clinical forms of TB in pediatric patients. The combination of biomarkers identified here could help developing point-of-care diagnostic or prognostic tools.

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Author contributions

NK, SB, BA, PS, KF, MA and VA performed experiments. PM, KF, BA and VA designed experiments. NK, SB, BA, PM, KF and VA provided materials and infrastructural support. BV, MA, PM, VA and BA wrote the manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of financial and commercial interests, distancing themselves and this research from any conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154773.

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