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Full Length Research Paper

Effects of the age and sex of an automated assay for adenosine deaminase among a Brazilian population sample with pleural tuberculosis

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Adenosine deaminase (ADA) is a diagnostic biomarker assay for pleural tuberculosis (P-TB) using pleural fluid (P-ADA). Our aim was to establish a new reference value for P-ADA in consideration of the influence of age and sex on the cut-off value for pleural TB in a Brazilian population sample. Diagnostic accuracy study conducted in a Center for Teaching and Research in Pleurology at a hospital of a public university. An automated ADA assay was performed using a commercial kit. In the P-TB group (n=26), 11 patients were male (42%) and 15 patients were female (58%). The median ages of the patients in the P-TB, non-P-TB (n=95), and overall pleural effusion groups (n=121) were 46.0±18.98, 64.0±17.88, and 62.0±19.14 years, respectively. A correlation of the P-ADA level with sex in patients with P-TB was not found to be significant ($\rho = -0.191$, $P = 0.5739$). However, when the P-ADA levels were dichotomized by the median age (62 years) the Spearman's correlation was negative and non-significant in all patients ($r_s = -0.0061$, $P = 0.9630$), tuberculosis ($r_s = -0.0857$, $P = 0.9194$), and non-tuberculosis groups ($r_s = -0.2789$, $P = 0.0813$). For the diagnosis of pleural TB, the best cut-off value established according to the ROC curve for P-ADA was ≥ 31.5 IU/L (AUC=88.2, $P = 0.0001$). Age and sex did not have a significant influence on the clinical decision limit for the diagnosis of TB pleural effusion using the ADA automated assay. For the diagnosis of pleural TB, the cut-off value established for P-ADA was ≥ 31.5 IU/L.

Keywords: Adenosine deaminase, reference values, pleural effusion, pleural tuberculosis, tuberculous pleurisy

INTRODUCTION

Pleural effusion syndrome (PES) is the most common manifestation of pleural disease and has several causes, including tuberculosis (Gasparini and Bonifazi, 2017; Silva Junior, 2012). According to the World Health Organization, tuberculosis (TB) had an incidence of 10.4 million cases worldwide in 2015 (World Health Organization, 2017). Pleural extrapulmonary TB is the most frequent clinical presentation in human with a prevalence of 48% of all cases in Brazil, a country where there is a low level of knowledge about tuberculosis, as well as on the part of general physicians worldwide (Seiscento et al., 2009; São José et al., 2014).

The diagnosis of pleural TB is difficult to confirm because of the paucibacillary nature of the pleural fluid (Silva Junior, 2012). Symptoms, signs, and conventional tests such as microscopy and mycobacterial cultures have relatively low diagnostic parameter values for pleural TB (Silva Junior, 2012). Percutaneous or closed needle pleural biopsy (CNPB) performed blind has historically been the gold standard procedure for the diagnosis of pleural TB (Behrsin et al., 2015; Hooper et al., 2010). However, unlike thoracentesis, CNPB can lead to a number of surgical complications and is limited if performed by inexperienced individuals (Behrsin et al., 2015; Hooper et al., 2010). In response to the difficulties of clinical practice, several biomarkers are necessary to simplify the analysis, reduce costs, and increase the accuracy of the diagnosis. The Xpert mycobacterium TB (MTB)/resistance to rifampicin (RIF) assay, interferon gamma, interferon-gamma-induced protein 10 (IP-10), Fas ligand, tumor necrosis factor alpha, interleukins 18, 27, 31, and 33, and interleukin 2 soluble receptor, for example, are tests useful only for researchers (Friedrich et al., 2011; Klimiuk et al., 2015; Purohit and Mustafa, 2015). An alternative test for the diagnosis of pleural TB, reported in recent years, has been the dosage of the enzyme adenosine desaminase (ADA).

The ADA test is a diagnostic biomarker assay for TB that uses pleural fluid (P-ADA) (Behrsin et al., 2015). The total ADA assay is a test incorporating more accurate diagnostic parameters for differentiating TB from other causes of PES (Behrsin et al., 2015). Thus, we speculated that different factors and assay methods could affect the cut-off value of P-ADA for the diagnosis of tuberculous pleural effusion (Tay and Tee, 2013; Yeon et al., 2002; Abrao et al., 2014; Valdes et al., 1995). The objectives of this study were to establish a new P-ADA reference value for the diagnosis of pleural TB, where the disease activity was measured using an automated kinetic assay, and to evaluate the influence of age and sex on the cut-off value of P-ADA for pleural TB in a study population with a high incidence of TB.

MATERIALS AND ETHODS

Quality assurance and validation of the cut-off point

To prevent errors occurring in this diagnostic test study, we applied the methodological criteria recommended by the Clinical and Laboratory Standards Institute and the Standards for Reporting Diagnostic Accuracy, as well as the protocol for obtaining reference values and determining reference intervals produced by the International Federation of Clinical Chemistry (IFCC) (Henny et al., 2016; Bossuyt et al., 2015; Ceriotti, 2007; Harris and Boyd, 1990; Boyd, 2010; Lahti et al., 2004; Henny, 2009).

Study design

This was a prospective study (Thiese, 2014) conducted between March 2012 and January 2015 involving patients who underwent thoracentesis and pleural biopsy at two tertiary referral centers, Antonio Pedro University Hospital (HUAP) and Santa Teresa Hospital, both located in the State of Rio de Janeiro, Brazil. The Ethics Committee of HUAP approved this study in accordance with the recommendations in the Declaration of Helsinki under protocol number 80/02.

Inclusion and exclusion criteria

The definitions used for the diagnosis of PES were based on previously published criteria (Gasparini and Bonifazi, 2017; Hooper et al., 2010). The diagnosis of the cause of PES was confirmed with standard exams and the use of appropriate surgical procedures. The first biochemical test on pleural fluids used to aid diagnosis was the criterion of Maranhão and Silva Junior to classify pleural transudate or exudate (Maranhão et al., 2010). When the causal diagnosis of PES was unconfirmed after the thoracentesis procedure with laboratory evaluations conducted in pleural fluid, a CNPB was performed using a Cope's needle. If PES persisted and the symptoms increased, or when it was not possible to differentiate malignancy from TB, the patient was referred for video-assisted thoracoscopic surgery (Haridas et al., 2014).

Exclusion criteria included an absolute contraindication, refusal to undergo a thoracentesis or other invasive procedure, use of immunosuppressive medications, hemolysis in pleural fluids, renal failure, HIV infection, and pleural effusion of unknown cause.

Sample size and reference values according to subgroup

For this study, the minimum sample size was estimated at 120 reference individuals. This is recognized by the IFCC as a reasonable compromise for non-parametric tests (Henny, 2009). A total of 133 patients were evaluated in this study. However, 12 patients (9.0%) were diagnosed as pleural effusions of unknown cause and were excluded.

ADA assay

In brief, the ADA assay was performed using a commercial kit where the ADA irreversibly catalyzes the conversion of adenosine into inosine with hydrogen peroxide (H_2O_2) produced in the final enzymatic reaction. The assay is ready-to-use for automated chemistry analyzers via the kinetic method. The assay depends on the detection of H_2O_2 , which is more sensitive than the colorimetric method of Giusti and Galanti, and is based on the Berthelot reaction, which forms a blue colored dye produced by phenol-sodium hypochlorite that is used to analyze the concentration of ammonia in pleural fluids (Delacour et al., 2010; Giusti and Galanti, 1984; Feres et al., 2008).

Statistical approaches to P-ADA for clinical decision limits

In this study, we analyzed both descriptive and inferential statistics in all data using evaluations entered into Microsoft Office Excel 2013® and exported to the databases of GraphPad Prism version 6.0 for Windows (Graphpad Software, La Jolla, CA, USA) and MedCalc for Windows version 17.5 (MedCalc Software, Ostend, Belgium). The Shapiro-Wilk or Kolmogorov-Smirnov tests were used to assess the normality of the continuous variables. Continuous variables are reported as means, medians, and more-or-less standard deviations (SD). Categorical variables are reported as number and group percentages of the total population. P-ADA levels were measured in patients with pleural TB and in patients with pleural effusions of non-tuberculous origin as controls. The Mann-Whitney U test was performed to compare the median differences in the P-ADA levels in terms of sex and age between the two groups. Spearman's rank correlation coefficient was used to identify the strength of a relationship between the P-ADA levels and specified variables (age and sex) for the entire sample (the overall group; $N=121$) as well as for the TB and non-TB pleural groups. The Kruskal-Wallis test followed by a *post hoc* Dunn test was used to compare medians of the overall ADA results, the TB group, and the non-TB group with unpaired independent variables. For the medical decision limits, the best P-ADA cut-off value for the diagnosis of

TB pleural effusion was selected using a receiver operating characteristics (ROC) curve according to disease prevalence and the cost of false positive and negative and true positive and negative results (Eusebi, 2013; Kumar and Indrayan, 2011). The area under the ROC curve (AUC Z statistic) with 95% confidence intervals (95% CI) was calculated using a non-parametric approach (DeLong et al., 1988). The results obtained from the pleural TB and non-TB (control group) on the ROC curve for the P-ADA assay were sensitivity, specificity, predictive values, likelihood ratios, and diagnostic odds ratio. A right-sided Grubb's test was performed to check that only the largest values of the P-ADA levels had a significant outlier to influence the cut-off point on the ROC curve (Grubbs, 1969). A P-value (two-tailed) of less than 0.05 was considered to be statistically significant in order to reject the null hypothesis and define a type I error.

RESULTS

Baseline characteristics

In the TB group ($n=26$), 11 patients were male (42%) and 15 patients were female (58%). The non-TB subjects ($n=95$) included 44 (46%) males and 51 (54%) females. The median ages of patients in the TB and non-TB pleural effusion groups were 46.0 ± 18.98 (16.0–86.0) and 64.0 ± 17.88 (11.0–95.0) years, respectively. In the overall group, the median age was 62.0 ± 19.14 (11.0–95.0) years. When the median age of patients with pleural TB was compared with that of patients in the other groups (non-TB and overall) the result obtained from the Kruskal-Wallis test was 5.33 ($P=0.0569$). A normal distribution determined from the Shapiro-Wilk test was accepted for the TB group ($W=0.9723$, $P=0.6828$) and rejected for the non-TB ($W=0.9631$, $P=0.0089$) and overall ($W=0.9722$, $P=0.0132$) groups.

ADA measurements

The results revealed high levels of ADA activity in the pleural fluid of TB patients (median, 40.59 ± 34.91) compared to the control group (median, 9.12 ± 102.22), with a two-tailed p-value considered to be extremely significant by the Mann-Whitney test ($U=340.0$, $p<0.0001$), as shown in Table 1. The difference between the medians of the P-ADA levels with pleural TB and the other causes of PES was considered extremely significant (Kruskal-Wallis= 61.52 , $p<0.0001$), as shown in **Table 1**. The P-ADA test yielded a non-significant p-value (Dunn's test, $P>0.05$) for patients with lymphomas and non-tuberculous empyema, in addition to a small group of patients with other causes ($n=6$): two cases of pleural effusions after myocardial revascularization, and one case each of pulmonary thromboembolism, chylothorax,

Table 1. Pleural fluid adenosine deaminase levels evaluated by an automated method among 121 patients with various final diagnoses of pleural effusion syndrome

Study group	Patients (n)	P-ADA median±SD* (IU/L)	P-ADA range (IU/L)	P-value compared with TB
Tuberculosis†	26	40.59±34.91†	3.85-185.10	-
Non-TB†	95	9.12±102.22†	0.75-990.5	<0.0001
Transudate	26	4.02±3.21	0.75-12.21	<0.001
Adenocarcinomas	34	9.41±23.71	3.87-134.6	<0.001
Lymphomas	04	21.64±486.54	9.59-990.5	>0.05
Simple and complicated parapneumonic effusion	17	9.87±7.20	4.84-28.21	<0.05
Nontuberculous empyema	08	25.48±39.20	9.38-122.98	>0.05
Other causes	06	14.99±15.07	1.77-40.51	>0.05

Other causes: Myocardial revascularization (n=2), and one case each of pulmonary thromboembolism, chylothorax, pleural mesothelioma, and thoracobilia. *Shapiro-Wilk statistic (W=0.2263, P=0.00); †Mann-Whitney test (U = 340.0, P < 0.0001); Kruskal-Wallis test (KW = 61.52, P < 0.000), and post hoc Dunn's multiple comparisons test (P > 0.05 for lymphomas, empyema, and other; P < 0.001 for adenocarcinoma, transudates, and parapneumonic effusion). Abbreviations: P-ADA: Pleural adenosine deaminase; SD: Standard deviation; TB: Tuberculosis.

Table 2. Correlation on adenosine deaminase analysis among age and sex in 121 patients with pleural effusion syndrome

Factors	P-ADA levels, median (IU/L)±SD		
	All patients (N=121)	Tuberculosis (n=26)	Non-tuberculosis (n=95)
Male	9.80±134.22	37.80±49.25	9.50±148.44
Female	11.39±23.63	40.80±20.91	9.40±20.20
P-ADA vs. sex	rho=0.165 (P=0.2287)	rho= -0.191 (P=0.5739)	rho= 0.348 (P=0.0206)
Age < 62 years	16.70±126.92	40.85±17.60	9.41±155.61
Age ≥ 62 years	9.05±30.85	48.50±67.87	8.75±19.80
P-ADA vs. dichotomized age†	r _s = -0.0061 (P=0.9630) r ² =0.0000	r _s = -0.0857 (P=0.9194) r ² =0.0073	r _s = -0.2789 (P=0.0813) r ² =0.077

Abbreviations: P-ADA: ADA in pleural fluid; SD: Standard deviation; rho and r_s: Spearman correlation; r²: Coefficient of determination.

pleural mesothelioma, and thoracobilia, as shown in **Table 1**. However, the P-ADA analysis helped to exclude adenocarcinoma, transudates, and parapneumonic effusions with a highly significant P-value (Dunn's test, P<0.001), as shown in **Table 1**.

Table 2 lists details of the calculations used in the P-ADA analysis and correlations with age and sex among 121 PES patients. There was no significant negative correlation in the P-ADA levels between the sexes in the overall patient group (rho= -0.165, P=0.2287) and the TB group (rho= -0.191, P=0.5739), but a positive and significant correlation in non-TB group (rho=0.348, P=0.0206). **Table 2** also shows that when the ages were dichotomized, a non-significant difference was observed in all groups for P-ADA versus age. Therefore, the ROC curve for ADA levels was performed only on the overall group of patients in the population sample (**Figure 1**). A

negative correlation between age and P-ADA level was observed (r= -0.0061, P=0.9630) only in the overall group of patients, and not in the other groups (TB and non-TB). An r-value lower than 0.30 is considered to represent a negligible correlation according to the rule of thumb (Mukaka, 2012). The coefficient of determination, or r², was zero between dichotomized ages in the overall group of patients, and 0.0073 and 0.077 in the TB and non-TB groups, respectively (**Table 2**).

A right-sided Grubb's test was performed to determine whether the largest extreme value of the P-ADA level had a significant outlier to influence the cut-off point of the ROC curve (**Figure 1**). However, a P-ADA value equal to 990.5 IU/L was not removed because the Shapiro-Wilk test for normal distribution (W=0.2225) rejected the normality of data (P<0.0001) and also because no analytical error was found.

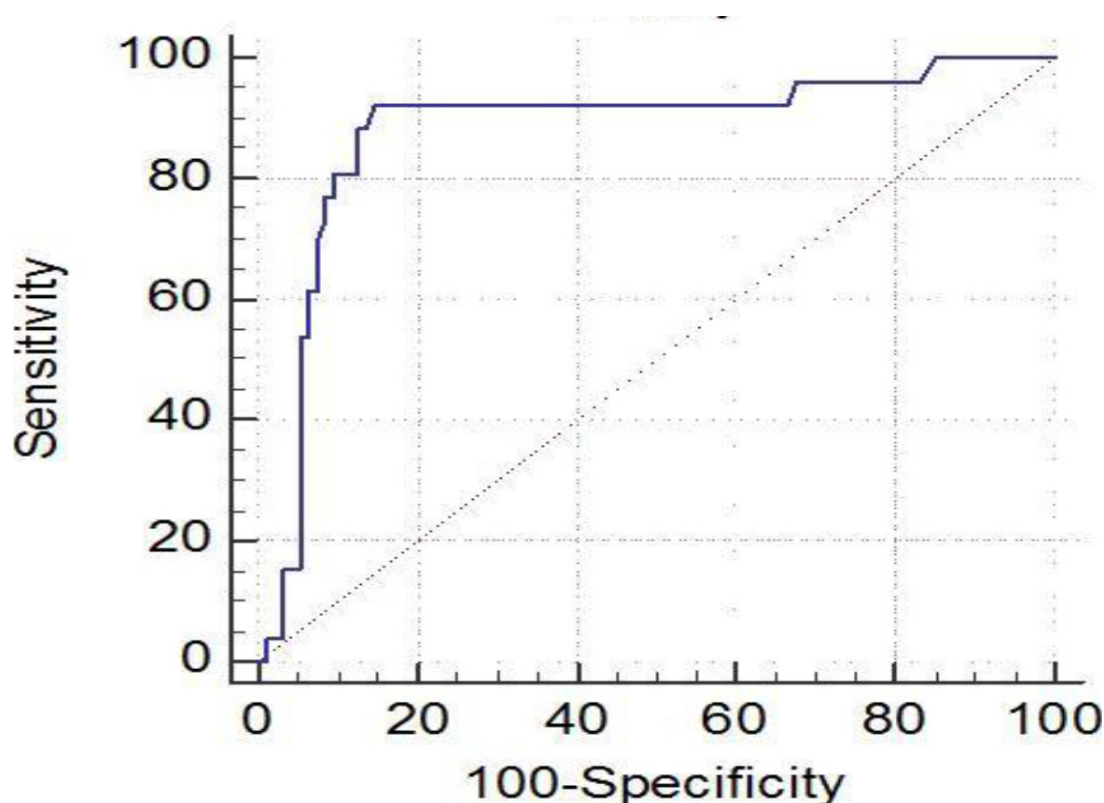


Figure 1. ROC curve for the ADA levels of pleural tuberculosis for all ages and sexes. AUC: 0.882, SE: 0.043, 95%CI: 0.812- 0.934, z statistic: 8.88, P=0.0001. Diagnostic parameters of the P-ADA biomarker for pleural tuberculosis in 121 patients for both sexes and independent of age determined by an automated ADA assay: best cut-off point ≥ 31.5 IU/L, sensitivity: 80.77 (95%CI: 60.6–93.4), specificity: 90.62 (82.9–95.6), PPV: 70.0 (50.6–85.3), NPV: 94.6 (90.5–98.6), PLR: 8.62 (4.5–16.5), NLR: 0.21 (1.10–0.50), and DOR: 41.04 (32.2–49.8). The prevalence of pleural tuberculosis was 21.30%. Abbreviations: ADA, Adenosine deaminase; AUC, Area under the ROC curve; SE, Standard error; CI, Confidence interval; PPV, Positive predictive value; NPV, Negative predictive value; PLR, Positive likelihood ratio; NLR, Negative likelihood ratio; DOR, Diagnostic odds ratio

Figure 1 presents the diagnostic performance parameters obtained from the ROC curve for the P-ADA automated assay. These parameters are important in order to establish the clinical decision limits or potential utility of the biomarker P-ADA at the cut-off point selected on the ROC curve.

DISCUSSION

The world of diagnostic testing is dynamic. Currently, there are two concepts of the thresholds that can be used by laboratories: reference intervals, defined using healthy individuals; and clinical decision limits, which are values above or below a cut-off derived from a known specific disease and controls. In medical statistics, reference intervals are defined with a high specificity for health, while clinical decision limits consider sensitivity for a particular disease (Horowitz et al., 2010; Sikaris, 2014; Boyd, 2010; Sunderman Junior, 1975; Boyd, 1997).

Although sensitivity, specificity, and predictive values have long been used as indices of test accuracy, newer mathematical tools for demonstrating the clinical usefulness of biomarkers, such as ROC curves, logistic regression analysis, likelihood ratios, and diagnostic odds ratio, are more robust indicators that are able to overcome many of the limitations of such traditional indices (Horowitz et al., 2010; Sikaris, 2014; Boyd, 2010; Sunderman Junior, 1975).

The demographic characteristics of patients with TB and non-TB pleural effusions in our study were similar to those of another Brazilian population sample studied recently (Behrsin et al., 2015).

Our results from an automated ADA assay, shown in **Table 1**, are similar to those in another paper published recently by our group (Behrsin et al., 2015). Comparison between the arithmetic means and standard deviations of the P-ADA levels between the TB and other groups in that study yielded statistically significant differences, based on an in-house ADA assay according to the Giusti

and Galanti method (Behrsin et al., 2015). This P-ADA kit also yielded non-significant P-values for patients with lymphoma and empyema and helped to exclude adenocarcinoma, transudates, and parapneumonic effusions with a high performance.

We performed a search of the literature to identify previous findings for comparison with the results shown in **Table 2**. A study by Tay and Tee found no significant difference in P-ADA levels between the sexes (Tay and Tee, 2013). However, the relationship between age and P-ADA level demonstrated in our study was similar both to their results and to those presented by Yeon et al (Yeon et al., 2002). Unlike our study, Yeon et al (Yeon et al., 2002). found that the P-ADA levels differed between the group of patients younger than 65 years (103.5 ± 36.9 IU/L) and those 65 years or older (72.2 ± 31.6 IU/L), with a P-value of less than 0.05. They found a negative correlation with age ($r = -0.384$, $P < 0.05$) in 60 patients with TB pleural effusions. Tay and Tee found that the strongest negative correlation was between the P-ADA level and the cut-off point for an age of 55 years (Tay and Tee, 2013). However, the results of our study in relation to age were similar to those of Moon et al. in Seoul (Korea) (Moon et al., 2005). In that study, the P-ADA level was 71.2 ± 27.6 IU/L in an elderly group older than 65 years and 68.5 ± 25.8 IU/L in a younger group 65 years or younger ($P = 0.690$).

The automated ADA assay is a rapid and accurate method for determining P-ADA levels in pleural fluid. However, this procedure is very expensive in Brazil. The assay has a very high positive correlation ($r^2 = 0.93$) with the Giusti and Galanti method (Delacour et al., 2010; Feres et al., 2008). The coefficient of determination, r^2 , is the percentage of variance explained by a linear model. Correlation coefficients are used to assess the strength and direction of the linear relationships between a pair of variables (Mukaka, 2012). In our model, only 0.73% of ADA levels for pleural TB diagnosis were explained by the age of subjects (**Table 2**). Another study found a significant negative correlation between P-ADA levels and an age greater than or equal to 45 years (Abrao et al., 2014).

It is recommended that each clinical laboratory establish its own cut-off point using a biomarker representative of its own patient population (Raslich et al., 2007). In our Brazilian study population, the best P-ADA cut-off value selected from the ROC curve was 31.5 IU/L (AUC=0.882, $P = 0.0001$) in all patients with TB pleural effusions (**Figure 1**). The reported cut-off values for the P-ADA assay in Brazil vary from 30.0–100.0 IU/L with the Giusti and Galanti method (Morisson and Neves, 2008). The best cut-off value for P-ADA using the same automated kit was determined to be 30.0 IU/L at the Ramathibodi Hospital, Thailand (Kawamatawong et al., 2008).

Accurate diagnostic parameters for the biochemical analysis of P-ADA can contribute to a diagnosis of pleural TB and, in some cases, render a pleural biopsy unnecessary (**Figure 1**). Other authors agree with our conclusions (Behrsin et al., 2015; Valdes et al., 1995). The AUC with a 95% CI gives an estimated diagnostic efficiency of P-ADA. A higher AUC indicates higher specificity and sensitivity among all the available cut-offs (Okeh and Ogbonna, 2013).

Smoking status, nutritional factors, a population sample with a low incidence of tuberculosis, and HIV serologic status were not evaluated in this study (Kapisyzi et al., 2011; Riantawan et al., 1999). In a population with an intermediate incidence of TB in Turkey it was concluded that tuberculous pleuritis was highly unlikely with an accuracy of 76.6% if a P-ADA cut-off value of more than 31.0 IU/L was determined via the Giusti and Galanti assay (Yildiz et al., 2011). The best cut-off point of 30.0 IU/L had a negative predictive value of 100% in a New Zealand population using an automated assay for ADA (Song, 2010). In India, Brazil, and other countries the P-ADA dosage is an accurate and useful biomarker for the diagnosis of pleural TB (Behrsin et al., 2015; Mehta et al., 2014).

Finally, in this paper we attempted to show and discuss, what does this study add to this research field? What are the limitations? What are the implications? What are the future perspectives?

CONCLUSIONS

According to the objectives from this study we concluded that age and sex are uncorrelated with P-ADA and do not influence the clinical decision limit in TB pleural effusion. However, the P-ADA automated assay may be considered useful as an adjunctive biomarker in the integrated management of pleural effusion syndrome. For the diagnosis of pleural TB, the best cut-off value established for P-ADA was ≥ 31.5 IU/L. Moreover, the assay provides complementary information to conventional evaluations. In most cases, a pleural biopsy will be unnecessary.

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