Evaluation of pleural effusion sCD26 and DPP-IV as diagnostic biomarkers in lung disease

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In this study, we measured ADA and DPP-IV enzymatic activity and sCD26 concentration in 150 pleural effusion (PE) samples and tested for correlations between these and other cellular and biochemical measures. We found that DPP-IV in particular might improve the specificity (but not the sensitivity) of the ADA test for diagnosis of pulmonary tuberculosis, since half of the false ADA positive results in non-tuberculous PE were also DPP-IV positive. A percentage of patients with malignant PE were sCD26 or DPP-IV positive; however, some patients with benign PE also tested positive. As a pattern associated with DPP-IV (but not the CD26 protein) was observed in PE, we searched for a finding that might increase the value of these biomarkers for diagnosis of malignancy. The observed pattern was related to the presence of leukocytes, as indicated by correlations with the cell count, and to a band of 180 kDa, detected by immunoblotting.

The soluble form of CD26 (sCD26), ascribed to the DPP-4 gene and originating from shedding of the transmembrane protein, is found in many biological fluids. The physiological role of sCD26 and its relation, if any, to CD26 functions remain poorly understood. Dipeptidyl peptidase IV (DPP-IV), a serine protease belonging to type II transmembrane glycoproteins (EC 3.4.14.5), is expressed on the surface of epithelial cells of diverse tissues, on endothelial cells of blood vessels and on some immune cells such as T lymphocytes, B lymphocytes and NK cells. By cleaving dipeptides from the N-terminal end of peptides and polypeptides with proline or alanine in the second position, DPP-IV controls the activity of many bioactive molecules, including cytokines and chemokines, incretins and gastrointestinal hormones, vasoactive peptides and neuropeptides. DPP-IV is a multifunctional regulatory biomolecule, and in addition to its enzymatic activity, it interacts with many plasma membrane proteins, such as ADA and the chemokine receptor CXCR4, and with extracellular matrix components such as collagen. Thus, DPP-IV is involved in diverse biological processes apart from protein degradation in the gut, especially immune functions and inflammation, and it has also become a novel therapeutic target for inhibitors that extend endogenously produced insulin half-life in diabetics.

Pleural effusion (the pathological accumulation of fluid in the pleural cavity that surrounds the lung) may appear as the result of different benign abnormalities, which are often caused by tuberculosis (TB), or as the result of malignant disease. Tuberculous pleural effusion (TPE), which is considered a form of extrapulmonary TB, remained a diagnostic challenge for clinicians until the identification of molecular markers that yielded a rapid and accurate diagnosis of tuberculous pleuritis. Among the best-established techniques, determination of adenosine deaminase (ADA) activity and the concentration of cytokine IFN-gamma in the pleural effusion are included in the supplemental diagnostic index for pleural tuberculosis. However, none of the available tests for TPE are wholly accurate, and any biomarker that can increase their reliability in indicating whether antituberculosis therapy should be resumed or discontinued will be valuable.

Interest has recently been shown in determining sCD26 concentration and DPP-IV activity in PE, for two reasons. First, the plasma membrane CD26 was previously known as ADA complexing or binding protein (ADA-CP or ADA-BP), although the ADA of most diagnostic importance in biological fluids (including TPE) is ADA2, the isoenzyme that is produced by monocytes and that predominates in the sera of normal individuals. The other isoenzyme, ADA1, is expressed by most cells and can be found separately or connected via a dimer of...
soluble ADA-CP (sCD26). Extracellular ADA is probably involved in the control of adenosine-mediated signalling through purinergic receptors, at least in leukocytes\(^4\)

The second reason for studying sCD26/DPP-IV is that tuberculous infections generate Th1-like immune responses, such as IFN-gamma secretion. Membrane-bound expression of soluble CD26 correlated with Th1-like responses including cytokine production\(^15\)–\(^17\).

Previous studies have shown that measurement of DPP-IV activity\(^18\) and sCD26 concentration\(^19\)–\(^20\) slightly improved the already high sensitivity and diagnostic efficiency of the ADA test for tuberculous pleurisy. However, as shown in some diseases\(^2\), and even in healthy donor serum samples, enzymatic activity and enzyme concentrations are not closely correlated. We therefore determined the sCD26 concentration and DPP-IV and ADA activity in the same samples to investigate this hypothesis in relation to PE. We included PE associated with malignant pathologies (mainly lung cancer)\(^20\) in the study, partly to widen the cohort of the study and also because many recent studies have demonstrated an important role for DPPIV/CD26 (both altered cell surface CD26 expression as well as changes in serum DPP-IV activity) in the initial steps of malignant transformation, in progression of the tumour and in the metastatic process\(^2\)–\(^21\)–\(^24\).

Therefore, any information regarding the role of these molecules in PE will be important for developing diagnostic tests or treatments.

**Results**

**Basic demographic parameters of ADA and DPP-IV activity and sCD26 concentration in PE samples from patients with benign and malignant pathologies.** The ADA and DPP-IV enzymatic activities (expressed in IU L\(^{-1}\)) and soluble CD26 concentration (expressed in μg L\(^{-1}\)) were determined in PE samples from 138, 147 and 159 patients, respectively. Gender and age factors were considered in a basic demographic study as they affect DPP-IV enzymatic activity and sCD26 concentration in serum\(^2\) and also because TPE is generally diagnosed in younger patients and MPE in older patients.

The mean ADA activity of the BPE group was 63.7 ± 106.1 UL\(^{-1}\) (Tables 2 and 3, and Figure 1). The mean value for the TPE group was 75.3 ± 21.4 UL\(^{-1}\), range 11.9–905.9. The high SD associated with the mean value for the BPE group was due to cases of parapneumonic PE, some associated with high ADA values. The values were much higher than in other BPE and MPE groups (except for one lymphoma sample), as expected (Fig. 1). Almost half of the TPE patients were less than 36 years old, in contrast to the other groups (Table 2). However, although with a few cases, the ADA activity was higher in older than in young individuals only in the parapneumonic group (Table 2). Further studies are required to confirm the trend towards higher levels of activity in women.

The DPP-IV activity was lowest in the TPE group (mean 36.8 ± 12.1 UL\(^{-1}\), range 38.3–127.6; Table 2 and 3, and Figure 1), for 41.8 ± 24.0 UL\(^{-1}\) in total BPE, but only mesotheliomas (60.5 ± 37.0) and lymphomas (116.3 ± 10.7) MPE groups were significantly different (Table 2). Although not statistically significant, there was a trend towards lower DPP-IV activity in the older patients (see BPE and MPE means, Table 2).

The commercial ELISA (Bender MedSystems) used in the present study was the same as used in other studies, and therefore comparison of the serum sCD26 levels is easier\(^1\). The mean concentrations of sCD26 measured in BPE and MPE were 360.8 ± 136.1 μg L\(^{-1}\) (range 92.31–687.92 μg L\(^{-1}\)) and 392.8 ± 352.4 μg L\(^{-1}\) (range 62.6 – 256.54 μg L\(^{-1}\)) respectively (Table 1 and 2). The results were similar to those obtained for carcinomatous pleurisy in a study using the same kit\(^19\) and to those obtained with a different kit\(^20\). However, the present study included a larger number of patients, and the mean concentration of sCD26 in TPE was not only similar to that of BPE but also to those of total MPE and MPE groups (Tables 2 and 3). High values of sCD26 were only observed in paramalignant PEs in the BPE group and particularly mesotheliomas and one lymphoma in MPE. Taking age into account, we found that the concentrations of sCD26 were significant lower in older (≥56) than in younger patients (Table 3).

No differences were found for the other epidemiological factors studied, such as smoking habit, cytology and neoplastic history.

**Figure 1** Levels of (A) ADA enzymatic activity, (B) DPP-IV enzymatic activity and (C) sCD26 concentration in pleural effusion (PE) samples from patients diagnosed with the following types of PE, from left to right: benign PE (circles), including tuberculous, parapneumonic, paramalignant, non neoplasic and miscellaneous PE; malignant PE (triangles), including epithelial, mesothelioma and lymphoma PE.
non-neoplastic BPE groups from the other groups were constructed. The cut-off value of 61 UL−1 obtained for the DPP-IV activity was adjusted to 60. For the sCD26 levels, the cut-off obtained was not useful and therefore the mean value plus the standard deviation, a cut-off of 470 μg L−1, was used.

Thus, only 1 of 30 cases of TPE (in a middle-aged patient) was found to be negative for ADA (value below the cut-off). The ADA levels were lower in this age group than in the other groups (Table 3). The main problem associated with the use of ADA activity to diagnose TPE was that another 8 of 51 non-tuberculous BPE (5/21 parapneumonic PE patients) were also positive, as well as three (3/57) MPE (Table 4), i.e. this biomarker shows poorer specificity (89.8%) than sensitivity (96.7%) in clinical diagnosis.

According to these data, determination of DPP-IV activity and sCD26 concentration will not enhance the sensitivity of TPE detection since only 1 and 4 out of 30 patients were found to be positive, and the negative ADA case was also negative for both biomarkers. In fact, many more cases were found to positive for these markers, particularly DPP-IV, in the other BPE and MPE groups (Table 4). Fischer’s exact test for the contingency tables showed that all groups except the paramalignant group were statistically significantly different from the TPE group for DPP-IV (p = 0.002 for TPE compared with the other groups), whereas the sCD26 levels only differed in mesothelioma and lymphoma PEs.

However, DPP-IV and sCD26 may enhance the specificity of the ADA test for TPE detection, i.e. detection of false TPE positive results. From the group of 11 false positives, 3/5 of ADA+ parapneumonic patients were also DPP-IV+ and a fourth was close to the cut-off (the other BPE cases were negative for DPP-IV) (Table 3) and the three MPE ADA+ cases were also positive for the other two measures. Calculation of the Net Reclassification Improvement (NRI) showed that of the 12 wrongly classified cases (11 false positives and one false negative: 8.7%), 6 false positives (4.3%) could be reclassified, with an NRI of 4.4% (95% CI 2.3–6.5% and p = 0.031, see Methods). Important for the patient, the ones remaining wrongly classified have not a malignant pathology.

We used exploratory NRI with more cut-off values per biomarker (continuous NRI), to develop a model that could improve diagnosis of other PE groups, but we did not find any values of clinical interest. However, an important number of the MPE samples were ADA negative and DPP-IV (18/60) and sCD26 (13/67) positive: these were mainly from patients with lymphomas and mesotheliomas, but also epithelial MPE (14/54 for DPP-IV compared with 0/50 for ADA) (Table 4). As 11/81 BPE were also ADA- DPP-IV+, these biomarkers are not useful for MPE diagnosis in their present form. However, we did not observe any relationship between these frequencies in epithelial MPE and any other pathological condition. Also, 11/40 cases of non-small cell lung cancer (NSCLC) were positive for DPP-IV, compared with 7/42 cases for sCD26; by contrast, more sCD26+ than DPP-IV+ cases were detected in the mesothelioma group (Table 3).

Correlations between DPP-IV activity, sCD26 concentration, standard biochemical parameters and cell counts, in samples of benign and malignant PE. The DPP-IV and sCD26 biomarkers did not perform in the same way in the different groups of patients (Table 4). Also, some data were lost due to technical problems with the measurement of enzymatic activity. Thus, we tried to obtain further information that could enhance the value of these biomarkers.

Since serum DPP-IV activity and sCD26 concentration are not always correlated, the correlation values in PE were analysed for the different groups of patients. The DPP-IV activity and sCD26 values were correlated in both the BPE group (Spearman’s R = 0.34 and p = 0.001) and MPE group (Spearman’s R = 0.433 and p < 0.001) (Table 5 and Fig. 2). The DPP-IV activity and sCD26 values in the TPE sub-group and in the epithelial MPE sub-group (with higher enzymatic activity) were also correlated (Spearman R = 0.69, p < 0.001; and R = 0.44, p = 0.001, respectively). We conclude that the values of both biomarkers in PE are generally specific to the sCD26/ DPP-IV protein. Interestingly, in the other sub-groups in which the correlations could be determined, mesotheliomas in MPE and parapneumonia and miscellaneous in BPE, the correlation was totally absent, except in non-neoplastic BPE (Table 5). An additional pattern related to that protein therefore appeared in these pathological conditions.

A significant correlation between ADA and sCD26 was also detected in the MPE samples (R = 0.3, p = 0.023), probably due

| Aetiology of PE                                      | No. of Patients | No. of Men/Women | Age, years Mean (Range) |
|-----------------------------------------------------|-----------------|------------------|-------------------------|
| BPE                                                 |                 |                  |                         |
| Tuberculosis                                        | 93              | 64/29            | 55 [20–96]              |
| Parapneumonic                                       | 30              | 20/10            | 44 [20–96]              |
| Paramalignant                                       | 29              | 22/7             | 55 [26–89]              |
| Non neoplastic of unknown origin                    | 4               | 2/2              | 61 [40–81]              |
| Miscellaneous                                       | 18              | 13/5             | 65 [37–87]              |
| After surgery                                       | 12              | 7/5              | 62 [24–94]              |
| Chylothorax                                         | 3               | 2/1              | 64 [53–74]              |
| Secondary to collagen vascular diseases              | 3               | 2/1              | 73 [54–94]              |
| Secondary to drug reaction                          | 1               | 1/0              | 24                      |
| Dressler’s Syndrome                                 | 1               | 1/0              | 80                      |
| After trauma                                        | 1               | 0/1              | 83                      |
| **MPE**                                             |                 |                  |                         |
| Epithelial origin neoplasias                        | 67              | 42/25            | 67 [20–91]              |
| NSCLC                                               | 58              | 34/24            | 68 [34–91]              |
| Breast cancer                                       | 42              | 30/12            | 68 [37–91]              |
| SCLC                                                | 6               | 0/6              | 63 [34–75]              |
| Ovarian cancer                                      | 3               | 3/0              | 72 [57–81]              |
| Thymic epithelial neoplasm                          | 2               | 0/2              | 82 [79–85]              |
| Gastric cancer                                      | 2               | 0/2              | 69 [65–73]              |
| Cholangiocarcinoma                                  | 2               | 0/2              | 73 [60–83]              |
| Mesothelioma                                        | 1               | 1/0              | 61                      |
| Lymphoma                                            | 6               | 5/1              | 67 [57–87]              |
| **Lymphoma**                                        | 3               | 3/0              | 55 [20–79]              |

**Table 1** | Demographic data for the population under study

Abbreviations: MPE, malignant pleural effusion; BPE, benign pleural effusion.
to the high coefficient (Pearson’s) obtained for mesotheliomas, although no such correlation was observed in epithelial MPE samples (Table 5). No correlation between these parameters was found in BPE, as well (Table 5). A correlation between DPP-IV and ADA activity was observed in patients grouped as MPE or BPE (Pearson’s test, data not shown), but when the different sub-types were analysed separately, a correlation (Spearman’s test) was only found in patients with parapneumonic PE (Table 5). These findings confirm a biological relationship between these two proteins, although the type of relationship will depend on the aetiology of the PE.

We also compared our data with the standard values of biochemical parameters and cell counts. With respect to biochemical parameters, the most consistent finding was that LDH levels and total protein concentration were significantly correlated with sCD26, ADA and DPP-IV in MPE and BPE (with the following exceptions: LDH and sCD26 in BPE, and total protein concentration and DPP-IV or ADA in MPE [data not shown]). ADA and DPP-IV (and sCD26) significantly correlated with anemia and pH only in BPE (data not shown).

With respect to cell counts, ADA was correlated (Spearman test) with the number of MC in both BPE (R = 0.329; p = 0.003) and MPE (R = 0.264; p = 0.047)( Fig. 2C, upper dots), as well as with the number of PMN (Pearson R = 0.640; p < 0.001 in BPE, and R = 0.315; p = 0.018 in MPE), as expected. However, while DPP-IV activity was significantly correlated with the number of PMN (R = 0.351; p = 0.001) and almost correlated with the number of MC (R = 0.191; p = 0.077) in BPE, and it was correlated with the number of MC in MPE (R = 0.257; p = 0.049) (Fig. 2C, low left dot), sCD26 concentration was not correlated with the cell count (data not shown and Fig. 2C bottom right).

**Pleural effusion sCD26 immunoblot.** As mentioned before, in the mesothelioma group, unlike in epithelial MPE, we detected more sCD26+ cases than DPP-IV+ cases. Furthermore, in mesothelioma MPE and pneumatic and miscellaneous BPE, there was a total lack of correlation between CD26 and DPP-IV; finally, DPP-IV activity (but not sCD26 concentration) was significantly correlated with the number of leucocytes. Thus, we analysed samples from patients of different pathologies with disparate or uncorrelated values for sCD26 and DPP-IV by western blotting to try to obtain biochemical information to explain the above results.

The TP1/16 anti-CD26 mAb was characterized in immunoblots of cell lysates under partially denaturing conditions so that it could be compared with other known anti-CD26 mAbs, which only work under such conditions.

### Table 2 | ADA and DPP-IV activity and sCD26 concentration in PE according to gender groups in the study population (mean ± SD)

|                     | All          | Men          | Women         |
|---------------------|--------------|--------------|---------------|
| **ADA activity (UL⁻¹)** | 62.2 ± 135.9 (138) | 55.1 ± 140.1 (91) | 58.3 ± 129.0 (47) |
| **BPE**             | 63.7 ± 106.1 (81) | 50.5 ± 48.2 (57) | 91.1 ± 171.8 (24) |
| + TPE               | 75.3 ± 21.4 (30) | 69.5 ± 15.1 (20) | 86.9 ± 27.7 (10) |
| + pneumatic*        | 90.4 ± 200.1 (21) | 53.6 ± 78.8 (17) | 246.7 ± 439.9 (4) |
| + paramalignant     | 30.7 ± 41.3 (3)  | 29.0 ± 4.0 (2)  | 34.2 ± 0 (1)     |
| + non neoplastic    | 37.1 ± 42.8 (15) | 25.8 ± 9.4 (11) | 53.9 ± 66.4 (6)  |
| + miscellaneous     | 26.5 ± 14.0 (12) | 22.8 ± 6.2 (7)  | 32.8 ± 22.2 (5)  |
| **MPE**             | 46.1 ± 168.3 (57) | 62.0 ± 215.3 (34) | 21.1 ± 6.1 (23) |
| + epithelial        | 22.7 ± 7.7 (50) | 24.0 ± 7.8 (28) | 21.1 ± 6.2 (22) |
| + mesothelioma      | 34.4 ± 19.1 (5)  | 37.6 ± 20.4 (4) | 21.5 ± 0 (1)     |
| + lymphoma          | 686.8 ± 889.8 (2) | 668.8 ± 889.8 (2) | 0               |

| **DPP-IV activity (UL⁻¹)** | 64.6 ± 27.9 (140) | 47.9 ± 28.4 (89) | 42.0 ± 25.8 (51) |
| **BPE**                 | 41.9 ± 24.0 (80) | 42.9 ± 23.1 (53) | 41.0 ± 27.1 (27) |
| + TPE                   | 36.6 ± 12.1 (30) | 37.3 ± 12.9 (20) | 35.8 ± 10.9 (10) |
| + pneumatic*            | 45.3 ± 25.5 (29) | 48.2 ± 26.4 (22) | 36.1 ± 21.6 (7)  |
| + paramalignant         | 45.7 ± 17.3 (2)  | 33.5 ± 0 (1)     | 57.9 ± 0 (1)     |
| + non neoplastic        | 40.9 ± 26.7 (10) | 39.6 ± 30.8 (5)  | 45.8 ± 35.9 (5)  |
| + miscellaneous         | 49.4 ± 42.9 (9)  | 47.2 ± 34.4 (5)  | 52.1 ± 54.1 (4)  |
| **MPE**                 | 50.5 ± 30.8 (60) | 55.4 ± 33.6 (36) | 43.1 ± 24.9 (24) |
| + epithelial            | 47.3 ± 27.7 (54) | 50.0 ± 29.5 (31) | 43.6 ± 25.3 (23) |
| + mesothelioma          | 60.5 ± 37.0 (4)  | 70.1 ± 46.8 (3)  | 31.7 ± 0 (1)     |
| + lymphoma              | 116.3 ± 10.7 (2) | 116.3 ± 10.7 (2) | 0               |

| **sCD26 concentration (µg L⁻¹)** | 337.5 ± 253.1 (159) | 388.5 ± 286.4 (106) | 355.2 ± 168.5 (53) |
| **BPE**                    | 360.8 ± 136.1 (92) | 356.1 ± 128.3 (64) | 371.8 ± 154.1 (28) |
| + TPE                      | 367.1 ± 134.3 (30) | 357.5 ± 107.7 (20) | 386.4 ± 127.8 (10) |
| + pneumatic*               | 363.1 ± 143.3 (29) | 391.2 ± 119.1 (22) | 274.5 ± 184.7 (7) |
| + paramalignant            | 456.7 ± 176.1 (4)  | 422.0 ± 295.6 (2)  | 49.1 ± 27.2 (2)   |
| + non neoplastic           | 362.5 ± 151.5 (18) | 336.6 ± 142.9 (13) | 429.8 ± 168.4 (5) |
| + miscellaneous            | 300.4 ± 132.2 (11) | 258.8 ± 115.6 (7)  | 373.1 ± 143.2 (4) |
| **MPE**                    | 392.8 ± 352.4 (67) | 426.3 ± 421.9 (42) | 336.6 ± 179.4 (25) |
| + epithelial               | 322.8 ± 173.1 (58) | 328.6 ± 168.6 (34) | 338.9 ± 182.9 (24) |
| + mesothelioma             | 990.6 ± 897.6 (6)  | 1132.4 ± 925.5 (5) | 281.8 ± 0 (1)     |
| + lymphoma                 | 357.0 ± 225.3 (3)  | 357.0 ± 225.3 (3)  | 0               |

* U-Mann p < 0.001 between BPE and TPE, except pneumatic PE; * U-Mann p < 0.001 between pneumatic PE and other groups except TPE; \( t \)-Student p < 0.001 between BPE and MPE; \( \chi^2 \)-Kruskal/Wallis ANOVA p < 0.001 for MPE groups. For DPP-IV; \( \chi^2 \)-Kruskal/Wallis ANOVA p < 0.001 for MPE groups. For ADA; \( \chi^2 \)-Kruskal/Wallis ANOVA p < 0.001 for MPE groups. For ADA.
lin proteins, but not sCD26. We identified because of the potential diagnostic value, we analysed the band by Mass Spectrometry (MS/MS) in triplicate experiments. We identified of the MPE samples and absent or fainter in most of the BPE samples. The main finding (Fig. 3B) was that the 180 kDa band detected in TPE were similar to those reported by Wang et al in TPE19. However, we found that the activity of this enzyme was higher TPE19. However, we found that the activity of this enzyme was higher DPP-IV activity measured in parallel in our study. The DPP-IV levels, but this was not repeated in our data. The main finding (Fig. 3B) was that the 180 kDa band detected in serum under the partially denaturing conditions was present in most of the MPE samples and absent or fainter in most of the BPE samples. Because of the potential diagnostic value, we analysed the band by Mass Spectrometry (MS/MS) in triplicate experiments. We identified fibrinogen alpha, beta and gamma chains, and alpha-2 macroglobulin proteins, but not sCD26.

Discussion
We observed lower levels of DPP-IV enzymatic activity in TPE patients than in patients with other benign or malignant PE and similar levels of sCD26 in all types of patients. This contrasts with previous reports of significant increases in DPP-IV activity39 and sCD26 levels40,41 in TPE. Although the sCD26 levels we observed in TPE were similar to those reported by Wang et al39, these authors used a different type of control group (fewer patients and pathologies) than in the present study. We also found low levels of sCD26 in epithelial MPE (although not as low as in Wang’s study); however, many patients had high levels of sCD26 in the MPE group. We did not find any epidemiological factor or anatomopathological data that discriminated epithelial MPE sub-groups according to sCD26 levels. In comparison with a study with fewer patients, Oshikawa and Sugiyama44 found that some TPE patients had very high sCD26 levels, but this was not repeated in our data.

These results for sCD26 were confirmed by the similar data for the DPP-IV activity measured in parallel in our study. The DPP-IV activity levels were similar to those observed by Küpeli et al in TPE42. However, we found that the activity of this enzyme was higher in pneumonic BPE and MPE than in TPE. These differences may be due to the larger number of patients examined in our study.

We found that use of sCD26/DPP-IV as biomarkers did not improve the sensitivity of TPE diagnosis. However, measurement of DPP-IV may improve the specificity of TPE diagnosis because, when the ROC curve that best differentiated tuberculous plus non-TPE groups) were also DPP-IV positive. Interestingly because of the lack of biomarkers for MPE, a relatively large number of MPE were ADA negative and sCD26 positive (in particular DPP-IV). However, there were no specific clinical data that might be associated to these cases, and some BPE cases would be classified as positive for this combination of the biomarkers.

Table 3 | ADA and DPP-IV activity and sCD26 concentration in PE according to age groups in the study population (mean ± SD)

| Age Group | ADA activity (UL⁻¹) | DPP-IV activity [UL⁻¹] | sCD26 concentration [µg L⁻¹] |
|-----------|---------------------|------------------------|----------------------------|
| ≤35 years old | 131.3 ± 288.4 (19) | 43.9 ± 57.4 (32) | 389.1 ± 128.6 (21) |
| 36–55 years old | 50.2 ± 65.2 (24) | 389.1 ± 128.6 (21) | 131.3 ± 288.4 (19) |
| ≥56 years old | 70.1 ± 142.6 (39) | 389.1 ± 128.6 (21) | 131.3 ± 288.4 (19) |

In parentheses (n) number of samples. Significant differences between age groups: For DPP-IV;
\*U-Mann p = 0.007;
\#p < 0.013 and p = 0.003 respectively. For sCD26;
\$p < 0.001 and
\*$p = 0.007.
Statistical analysis of the data on these biomarkers and ADA revealed that the sCD26 concentration and ADA activity were correlated in the mesothelioma MPE group but not in the other PE groups. This was probably because of changes in the presence of ADA2 isofrom, which does not bind to sCD26 but is abundant in PE6,7,10 (we did not find the ADA1 isoform bound to sCD26 in the groups. This was probably because of changes in the presence of related proteins in the mesothelioma MPE group but not in the other PE.

The correlation between DPP-IV activity (but not sCD26) and leukocyte number (mainly MC) in both BPE and MPE groups indicates that a fraction of this activity is indeed derived from lymphocytes.

Serum DPP-IV enzymatic activity and sCD26 concentration are not closely correlated in patients of certain diseases and even in healthy donors, for at least three reasons: i) some circulating proteins other than sCD26 display DPP-IV activity; ii) hypersialylation (which is strongly enhanced in elderly individuals) of sCD26 can inhibit DPP-IV activity; and iii) the serum protein attractin may regulate the DPP-IV activity of CD26. Interestingly, the performance of sCD26/DPP-IV as biomarkers was also different in PE samples. Although DPP-IV activity and sCD26 concentration were strongly correlated, confirming that most of the data above are specific for the sCD26/DPP-IV protein, the correlation was totally absent in some PE subgroups (mesotheliomas in MPE, and pneumonic and miscellaneous in BPE), indicating that one of the above-mentioned patterns probably occurred in these pathological conditions.

Like the anti-CD26 mAb used in the present study, in samples of serum the 1F7 anti-CD26 mAb detected by western blot a band of 180 kDa which was originally described as DPPT-L, a protein originated in T cells with DPP-IV activity. The band has recently been demonstrated in the submucosal serous glands and the alveolar cells in healthy donors, for at least three reasons: i) some circulating proteins other than sCD26 display DPP-IV activity; ii) hypersialylation (which is strongly enhanced in elderly individuals) of sCD26 can inhibit DPP-IV activity; and iii) the serum protein attractin may regulate the DPP-IV activity of CD26. Interestingly, the performance of sCD26/DPP-IV as biomarkers was also different in PE samples. Although DPP-IV activity and sCD26 concentration were strongly correlated, confirming that most of the data above are specific for the sCD26/DPP-IV protein, the correlation was totally absent in some PE subgroups (mesotheliomas in MPE, and pneumonic and miscellaneous in BPE), indicating that one of the above-mentioned patterns probably occurred in these pathological conditions.

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Table 4 | Frequency of positive results for ADA and DPP-IV enzymatic activity, and sCD26 concentration in PE according to the groups under study

| Groups | Total n | +n | %   | Total n | +n | %   | Total n | +n | %   |
|--------|---------|----|-----|---------|----|-----|---------|----|-----|
| BPE    |         |    |     |         |    |     |         |    |     |
| +TPE   | 81      | 36 | 44.4| 80      | 15 | 18.8| 92      | 14 | 15.2|
| +pneumonic | 21  | 5  | 23.8| 29      | 8  | 27.6| 29      | 5  | 17.2|
| +paramalignant | 3  | 0  | 0   | 2       | 0  | 0   | 4       | 1  | 25.0|
| +non neoplastic | 15 | 2  | 13.3| 10      | 3  | 30.0| 18      | 3  | 16.7|
| +miscellaneous | 12 | 1  | 8.3 | 9       | 3  | 33.3| 11      | 1  | 9.1 |
| MPE    | 57      | 3  | 5.3 | 60      | 18 | 30.0| 67      | 13 | 19.4|
| +epithelial | 50 | 0  | 0   | 54      | 14 | 25.9| 58      | 8  | 13.8|
| +mesothelioma | 5  | 1  | 20.0| 5       | 3  | 60.0| 6       | 4  | 66.7|
| +lymphoma | 2   | 2  | 100.0| 2      | 2  | 100.0| 3       | 1  | 33.3|

Positive results for ADA, sCD26 and DPP-IV activity (+) represent all cases above the cutoff values of 40 and (60) U L\(^{-1}\) and 470 \(\mu g L^{-1}\), respectively. Frequencies shown in shaded type indicate significant differences from the TPE group, as revealed by Fisher’s exact tests done with contingency tables.

Table 5 | Correlations between ADA and DPP-IV activity and sCD26 concentration in PE according to the groups under study

| Groups | ADA vs DPP-IV | sCD26 vs DPP-IV | ADA vs sCD26 |
|--------|----------------|-----------------|-------------|
| BPE: TPE | sCD26 vs ADA | 30 | - | 0.230 | 0.222 |
|         | sCD26 vs DPP-IV | 30 | - | 0.689 | 0.000 |
|         | ADA vs DPP-IV | 30 | 0.039 | 0.839 | - |
|         | sCD26 vs ADA | 21 | - | -0.331 | 0.143 |
|         | sCD26 vs DPP-IV | 29 | - | 0.008 | 0.968 |
|         | ADA vs DPP-IV | 21 | - | 0.466 | 0.033 |
| BPE: Pneumonic | sCD26 vs ADA | 10 | - | 0.224 | 0.533 |
|         | sCD26 vs DPP-IV | 9 | - | 0.067 | 0.865 |
|         | ADA vs DPP-IV | 8 | -0.209 | 0.619 | - |
| BPE: Non neoplastic | sCD26 vs ADA | 15 | - | -0.196 | 0.483 |
|         | sCD26 vs DPP-IV | 17 | 0.796 | 0.000 | - |
|         | ADA vs DPP-IV | 14 | - | -0.323 | 0.260 |
| MPE: Epithelial | sCD26 vs ADA | 50 | 0.217 | 0.130 | - |
|         | sCD26 vs DPP-IV | 54 | - | -0.444 | 0.001 |
|         | ADA vs DPP-IV | 48 | - | 0.115 | 0.437 |
| MPE: mesothelioma | sCD26 vs ADA | 5 | 0.988 | 0.001 | - |
|         | sCD26 vs DPP-IV | 4 | - | 0.400 | 0.600 |
|         | ADA vs DPP-IV | 3 | - | 0.500 | 0.667 |

n: Number of samples analysed, with statistically significant differences shown in bold type. Pearson’s analysis was used for normally distributed samples, and Spearman’s analyses was used for non-normally distributed samples.
been identified as the protein attractin strongly bound to sCD26\textsuperscript{30,32}. In this study, the TP1/16 anti-CD26 mAb identified a 180 kDa band, which was more abundant in those pathologies in which the correlations were absent, and in which DPP-IV was associated with mononuclear cells.

However, analysis of the band by MS revealed alpha, beta and gamma chains of fibrinogen and alpha-2 macroglobulin proteins, but not sCD26. These proteins have been found in PE\textsuperscript{33,34} and may mask sCD26-attractin. However, further study is warranted by previous findings that CD26/DPP-IV acts as receptor for plasminogen type 2\textsuperscript{35,36} and that alpha-2-macroglobulin inactivates serine-proteases and also inhibits fibrinolysis by inhibition of plasmin\textsuperscript{37}.

### Methods

**Patients.** Patients (n = 159) admitted between April 2007 and December 2010 to the Unit of Interventional Bronchopleural Pathology, of the Pneumology Service of Complejo Hospitalario Universitario de Vigo (CHUVI) with a specific diagnosis for exudative PE were consecutively enrolled in this study.

All procedures followed the clinical-ethical practices of the Spanish Government; the protocol of the study was approved by the Galician Ethical Committee for Clinical Research (2007/179), and complied with the Helsinki Declaration, Oviedo Agreement, the Organic Law for Data Protection 15/1999 and Royal Decree 1720/2007. Informed consent was obtained from all subjects.

**Sample diagnosis.** As described in previous studies\textsuperscript{38,39}, initial diagnostic thoracocentesis for biochemical, microbiological and cytological studies was performed in all patients. If a diagnosis was not obtained after this test in patients with exudative PE, a second thoracocentesis and a blind percutaneous pleural biopsy with an Abrams needle were carried out. In some patients in whom PE was still not identified, thoracoscopy was conducted. The following biochemical parameters and white blood cells in pleural fluid were determined before any therapy was started: pH, protein concentration, glucose and LDH levels, and the number of leucocytes and percentage of mononuclear (MC) and polymorphonuclear (PMN) cells.

The aetiology of PE was based on accepted criteria, as described by the Spanish Society for Pneumology and Thoracic Surgery\textsuperscript{40} and the British Thoracic Society guidelines\textsuperscript{41}. Benign PE (BPE) was divided into five groups: tuberculous, parapneumonic, miscellaneous, paramalignant and non-neoplasic of unknown origin, according to recently reported diagnostic measures\textsuperscript{42}. Malignant PE (MPE) included PE in which malignant cells were demonstrated at cytological or histological examination or in a biopsy specimen. MPE was divided into three subgroups according to the aetiology of the PE: epithelial-origin neoplasia, mesothelioma and lymphoma. Patients who were not classified in any of these diagnostic groups were excluded from the study.

**Sample collection.** Approximately 10 mL of PE sample was obtained with a needle at the same time as thoracocentesis and pleural biopsy. Samples were centrifuged at 800 g for 15 minutes, and frozen in 0.5 aliquots at −20 °C until the measurements were performed.

**Enzyme assays.** Abnormal values were obtained for haemolytic and turbid samples. These values were excluded from data analysis.

The ADA was determined by an automated test carried out in a Unicel DXC 600i Syncron (Beckman Coulter). The measurement was based on the colorimetric kinetics of ADA catalytic activity where adenosine is transformed to inosine and ammonia, and the ammonia then reacts with sodium hypochlorite and phenol at 37 °C to form indophenol, which yields an intense blue colour. The indophenol was measured spectrophotometrically at 630 nm. The results are expressed in UL\textsuperscript{−1}.

DPP-IV activity was measured in 96-well culture plates with Gly-Pro-p-nitroaniline (0.2 mM, Sigma) as substrate in reaction mixtures (100 µL) containing PF samples (10 µL) and 50 mM Tris-HCl, pH 8.0\textsuperscript{1}. After incubation of the plates for 15 min, substrate hydrolysis was monitored at a wavelength of 405 nm in a BioRad Model 680 microplate reader. The results were determined by comparison with a standard curve for p-nitroaniline (Sigma data sheet) and the activity is expressed as UL\textsuperscript{−1}. All experiments were performed in duplicate, unless otherwise specified.

**Determination of the sCD26 levels.** The sCD26 concentration was measured with the sCD26 ELISA kit (Bender Medsystems; Vienna, Austria) according to the manufacturer’s instructions\textsuperscript{1,13}. Colorimetric quantification was performed with a microplate reader (model 530; Bio-Rad, USA) at 450/630 nm.

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Figure 2 | Correlations between (A) sCD26 concentration and DPP-IV activity, and (B) ADA activity and sCD26 concentration, in some types of PE. Spearman’s correlation coefficient (R) and p values are shown on the graphs; (C) Correlation between these biomarkers and the MC counts in BPE or MPE groups.
Immunoblotting of soluble CD26. First, the amount of protein in the PE was quantified by the Bradford protein assay (Biorad, CA, USA). Samples containing 20 µg of protein were then subjected to 7.5% SDS-PAGE under non-total reducing conditions so that it could be compared with the better-known anti-CD26 17F mAb, both of which perform considerably better than other antibodies used in several immune techniques. Goat anti-mouse IgG-HRP conjugated secondary antibody Fc-Specific (Sigma Aldrich) and Precision Plus Protein, Western C Standards (Bio-Rad, USA) was used for protein detection before membranes were exposed a few minutes to ECL Prime Western Blotting Reagents substrate solution (GE, Amershams, UK) under appropriate dark room conditions. Results were analysed with ChemDoc XR® (BioRad) software.

Protein identification by mass spectrometry. In parallel to the above-described immunoblot, a band of 180 kDa was excised from a replicate gel and digested with 12.5 ng/µL of trypsin (Roche Molecular Biochemicals) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37°C. The supernatant was then collected and 1 µL was spotted onto a MALDI target plate and allowed to air-dry at RT. Then, 0.4 µL of 2-cyano-4-hydroxy-cinnamic acid matrix (3 mg/mL) (Sigma) in 50% (v/v) ACN was added to the dried peptide digest spots and allowed to air-dry at RT. MALDI-TOF-MS analysis (4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems) was performed by the Proteomics Service, University of Vigo. Mass spectra were calibrated with internal peptides from trypsin autodigestion. A database search was performed with MASCOT Daemon search engine 2.1 (Matrix Science) through the Global Protein Server version 3.6 (Applied Biosystems) against SwissProt 2012_07 (536789 sequences; 190518892 residues), as described previously.

Statistical analysis. Statistical analysis was performed with the SPSS package (v16.0). Two tailed tests were carried out, and differences were considered significant at $p < 0.05$. Normal distributions and homogeneity of variances were verified by Kolmogorov-Smirnov and Mann-Whitney U tests respectively. Data from the three measures combined, alone, or grouped according to the pathological condition were calculated the NRI by testing a model with two or three cut-off values per variable (category-free or continuous-NRI) to determine whether the biomarkers could diagnose other non-TPE groups.

For ROC curve analysis of ADA, sCD26 and DPP-IV values, cut-off points were generated with MedCalc, version 11.5.1 (MedCalc Software, Belgium). We selected the cut-off values with the highest value for the combined sensitivity and specificity for TPE detection as a proxy for the optimal cut-off value. For ADA, the cut-off obtained was close to 40 UL$^{-1}$, i.e. the value used in the clinical setting. However, for sCD26, a cut-off corresponding to the mean value plus the standard deviation of the TPE group, performed better.

Net Reclassification Improvement (NRI) is an intuitive and easy to interpret measure for evaluation of potential added value of new diagnostic instruments or biomarkers in daily clinical practice. We chose this model to measure the biomarkers in combination with the ADA test for the diagnosis of TPE, using the same cut-off values obtained as explained above, i.e. category-based NRI. This enables the standard clinical values of specificity and sensitivity to be maintained. We also calculated the NRI by testing a model with two or three cut-off values per variable (category-free or continuous-NRI) to determine whether the biomarkers could diagnose other non-TPE groups.

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**Acknowledgments**

This research was partially supported by grants PS09-00405 and Research Intensification Project Fondo de Investigación Sanitaria (FIS) of the Instituto de Salud Carlos III (Spain) and funding from Xunta de Galicia and FEDER (CN 2011/024). We are grateful to the patients who participated and made the study possible. We particularly thank A. Fernández, V. Leiro, C. Represas, and M. Núñez (Pneumology Department, CHUVI) for their collaboration in acquiring the samples.

**Author contributions**

O.J.C. and M.P.d.l.C. designed the study; N.S.O. and M.I.B.R. obtained the samples; M.I.B.R. recorded the clinical data; O.J.C. and N.S.O. performed the experiments; O.J.C., N.S.O. and M.P.d.l.C. wrote and revised the manuscript; F.J.R.B. organized the group and permitted the study.

**Additional information**

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Sánchez-Otero, N., Rodríguez-Berrocal, F. J., Páez de la Cadena, M., Botana-Rial, M. l & Cordero, O. J. Evaluation of pleural effusion CD26 and DPP-IV as diagnostic biomarkers in lung disease. *Sci. Rep.* 4, 3999; DOI:10.1038/srep03999 (2014).

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