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Transplantation pulmonaire et dysfonction chronique du greffon : caractérisation de biomarqueurs diagnostiques par analyse protéomique SELDI-TOF du liquide bronchiolo-alvéolaire

Sébastien Quetant

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Transplantation pulmonaire et dysfonction chronique du greffon :

Caractérisation de biomarqueurs diagnostiques par analyse
protéomique SELDI-TOF du liquide bronchiolo-alvéolaire

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Résumé

RESUME

Objectifs : Malgré les progrès récents réalisés en transplantation pulmonaire, le pronostic à long terme est compromis par la survenue d'une dysfonction chronique du greffon dont le diagnostic est basé sur le déclin de la fonction respiratoire. Nous avons analysé les profils protéiques du liquide de lavage bronchiolo-alvéolaire (LBA) de patients transplantés pulmonaires par une technologie protéomique innovante afin de caractériser des biomarqueurs diagnostiques de dysfonction chronique du greffon.

Matériels et méthodes : Nous avons comparé de façon transversale le protéome des LBA de 13 patients avec un syndrome de bronchiolite oblitérante (groupe BOS), 21 patients avec une fonction pulmonaire stable (groupe ST) et 8 patients avec rejet aigu (groupe AR) par analyse protéomique SELDI-TOF MS (Surface Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry).

Résultats : 45 pics d'intensité statistiquement différente ont été caractérisés entre le groupe BOS et ST, et 42 entre les groupes BOS et AR. Parmi les pics les plus discriminants, des biomarqueurs correspondant aux protéines S100 (10812 Da, 10412 Da et 13335 Da, $p < 0,002$) et aux alpha-défensines 1-3 (3354 Da, 3424 Da et 3470 Da, $p < 0,01$) étaient surexprimés dans le groupe BOS. La protéine S100A8 (10812 Da) permet de différencier les patients BOS des patients stables avec une sensibilité de 92% et une spécificité de 72% (AUC=0.87) et les alpha-défensines présentent également une performance diagnostique importante.

Conclusion : L'analyse protéomique du LBA par technique SELDI-TOF MS a permis de caractériser plusieurs biomarqueurs d'intérêt pour le diagnostic de dysfonction chronique du greffon et potentiellement impliqués dans sa physiopathologie : les protéines S100A8, S100A9, S100A12 et les alpha-défensines.

Mots clés et

Abréviations

KEYWORDS

Lung transplantation, chronic allograft dysfunction, biomarkers, proteomic, SELDI-TOF MS (Surface Enhanced Laser Desorption / Ionization Time-Of-Flight Mass Spectrometry), bronchoalveolar lavage fluid, S100 proteins, alpha-defensins.

ABBREVIATIONS

AR	Acute Rejection
AUC	Area Under the Curve
BALF	BronchoAlveolar Lavage Fluid
BCA	Bicinchoninic Acid
BOS	Bronchiolitis Obliterans Syndrome
CF	Cystic Fibrosis
CsA	Cyclosporin A
Da	Dalton
FEV1	Forced Expiratory Volume in one second
ISHLT	International Society of Heart and Lung Transplantation
MALDI-TOF MS	Matrix Assisted Laser Desorption / Ionization - Time-Of-Flight Mass Spectrometry
MMF	Mycophenolate Mofetil
PBS	Phosphate Buffer Saline
PMN	PolyMorphonuclear Neutrophil
RAGE	Receptors for Advanced Glycation End products
ROC	Receiver Operator Characteristic
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SELDI-TOF MS	Surface Enhanced Laser Desorption / Ionization -Time-Of-Flight Mass Spectrometry
SP-A	Surfactant Protein A
ST	Stable
TBS	Tris Buffer Saline

Article

Characterization of chronic lung allograft dysfunction biomarkers in bronchoalveolar lavage fluid by SELDI-TOF proteomic analysis

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ABSTRACT

Objective: Despite progresses in lung transplantation, long-term prognosis is compromised by chronic allograft rejection. Its diagnosis is based on a delayed functional criterion and its physiopathology is still unclear. We investigated proteomic profiles of bronchoalveolar lavage fluid (BALF) of lung transplanted patients by an innovative proteomic technology in order to characterize diagnostic biomarkers of chronic allograft rejection.

Methods: In a cross sectional pilot study, protein profiles of 13 lung transplant recipients with bronchiolitis obliterans syndrome (BOS), 21 patients with lung function stability (ST) and 8 patients with acute rejection (AR) were studied by SELDI-TOF MS (Surface Enhanced Laser Desorption Ionization Time-Of-Flight Mass Spectrometry) analysis.

Results: Forty five differentially expressed proteins were detected in BOS group *vs.* ST population and forty two in BOS group *vs.* AR set. Among the most discriminating peaks, biomarkers corresponding to S100 proteins (10,812 Da, 10,412 Da and 13,335 Da, $p < 0.002$) and alpha-defensins (3,354 Da, 3,424 Da and 3,470 Da, $p < 0.01$) were up regulated in BOS patients compared to ST or AR patients. S100A8 protein (10,812 Da) discriminate BOS patients from ST patients with a sensitivity of 92% and a specificity of 72% (AUC=0.87), while alpha-defensins offer a slightly weaker diagnostic accuracy.

Conclusion: BALF SELDI-TOF analysis allows detection of relevant biomarkers linked to BOS diagnosis and potentially involved in its physiopathology: S100 proteins and alpha-defensins.

INTRODUCTION

Lung transplantation is currently a valuable therapeutic option for a spectrum of end-stage pulmonary diseases with no other possible effective medical treatment. Outcomes have improved over time in term of survival and quality of life (1, 2), but long-term survival remains disappointing and chronic allograft rejection has emerged as the primary obstacle to long-term outcome (3, 4). Indeed, according to the latest International Society for Heart and Lung Transplantation (ISHLT) Registry report (5), chronic allograft dysfunction, as clinically manifested by bronchiolitis obliterans syndrome (BOS), affects up to 50-60% of lung transplant recipients who survive 5 years after transplantation and accounts for the leading cause of death beyond the first year (6).

Obliterative bronchiolitis is the histologic hallmark of chronic rejection, and is characterized by submucosal fibrosis of the membranous and respiratory bronchioles, which results in luminal obliteration. Histologic confirmation of obliterative bronchiolitis is difficult because of the paucity of the bronchioles on transbronchial lung biopsies and the non-homogeneous nature of the disorder. Bronchiolitis obliterans syndrome (BOS) is the clinical feature for obliterative bronchiolitis and refers to a physiological criteria: a sustained decline of pulmonary function (decrease of 20% or more in forced expiratory volume in one second (FEV1)) in absence of other potential causes (anastomotic complications, infection or acute rejection (AR)) (7). Unfortunately this internationally accepted criterion reflects an advanced stage of the disease.

The exact pathogenesis of BOS as well as early markers of the disease are still unknown. Obliterative bronchiolitis is considered to be a multifactorial process involving both immune-mediated pathways (acute rejection, human leukocyte antigen mismatches or antibody-mediated rejection) and alloimmune-independent pathways (infection, ischemia-reperfusion injury, gastro esophageal reflux...) which leads to an aberrant tissue remodeling (8, 9).

Complex mechanisms of obliterative bronchiolitis remain unclear; however increasing body of evidence suggests that both cells of innate and adaptive immunity, as well as structural cells are involved (10-14). A number of studies have highlighted the critical role of polymorphonuclear neutrophil (PMN) and have shown that neutrophilia in bronchoalveolar lavage fluid (BALF) correlates with chronic allograft rejection (15-18). Previous studies have shown that various biomarkers such as cytokines (19, 20), chemokines (21, 22), growth factors (23, 24) or other proteins (25, 26) that are under or overexpressed in BALF were linked to BOS pathogenesis but their diagnostic value is not currently established.

Traditional research approaches that target specific compounds on the basis of previous data can not completely catch the process complexity. In contrast system-based methodologies, such as proteomics, analyze global biological changes and provide an opportunity to identify new biomarkers of diseases. Proteomic tests have been developed in the pulmonary field for several years (27-30). First 2D gel electrophoresis (31) and more recently surface enhanced laser desorption / ionization -time-of-flight mass spectrometry (SELDI-TOF MS) technology were used to analyze the proteome of BALF (32-36), blood (37, 38), induced sputum (39-41) or exhaled breath condensate (42). SELDI-TOF MS is an innovative high throughput proteomic method specifically dedicated to clinical applications (43, 44) whose relevance was emphasized in a wide range of disease such as cancer (45-47) or inflammatory diseases (48-50). In lung transplantation, only two studies have focused on BALF proteome analysis: Nelsestuen and colleagues analyzed proteome profiles of BALF samples from lung transplant recipients (LTRs) using a matrix assisted laser desorption / ionization - time-of-flight mass spectrometry (MALDI-TOF MS) technology, and characterized some biomarkers of BOS, in particular alpha-defensins (51). More recently, using gel electrophoresis and protein identification by MALDI-TOF MS (2DE-MS), Meloni and colleagues suggested that surfactant protein A (SP-A) levels in BALF may predict BOS onset in LTRs. (52).

In the present study we performed BALF proteome profiling using SELDI-TOF MS technology to characterize BOS-specific biomarkers.

PATIENTS AND METHODS

Study population

This cross-sectional study consisted of the analysis of 42 BALF samples prospectively acquired between July 2007 and September 2008 from 42 LTRs at the Grenoble University Hospital. The protocol was approved by the local ethics committee and all patients gave their informed consent to the study.

LTRs underwent regular clinical visits including functional and radiological evaluation. Spirometry was obtained routinely every month during the first postoperative year, and every 3 months after. They systematically underwent bronchoscopy with collection of BALF samples and transbronchial biopsies at 1, 3, 6 and 12 months after lung transplantation or in case of suspected infection or AR based on clinical, radiological or pulmonary function criteria. Microbiological examination was systematically performed in BALF. Infection was defined as a positive bacterial or fungal or viral culture, combined with medical findings (fever, need of antibiotics, antifungal or viral treatment), high level of C-reactive protein (CRP), and radiological examination (new radiological infiltrates). Colonization was defined as a positive culture of the investigated BALF samples without clinical findings, no increased CRP and no new radiological infiltrates.

The following groups were defined. (1) The BOS group comprised patients fulfilling the criteria of the ISHLT based on the decline of FEV1 (7). (2) The stable group comprised clinically stable patients who displayed normal and stable respiratory function ($FEV1 \geq 80\%$ of baseline) and no clinical symptom of infection or AR. (3) The AR group included LTRs with a diagnosis of AR defined and graded according to the histological analysis of

transbronchial biopsies (53). If LTRs underwent repeated BALF sampling during the study period only the first BALF sample was considered. LTRs with infection criteria were excluded but not those with colonization.

Patients received the same immunosuppressive regimen throughout the study. Anti-IL-2 monoclonal antibodies (basiliximab, Novartis, France) were given for the induction therapy on day 1 and 5. The standard maintenance treatment consisted of tacrolimus (whole blood target levels between 10 and 15 ng/ml according to the postoperative delay), mycophenolate mofetil and prednisone. Episodes of AR \geq grade A2 were treated with intravenous bolus of methylprednisolone (15 mg/kg/day on 3 consecutive days). Azithromycin (250 mg/day) was added to the immunosuppressive drug regimen as soon as the diagnosis of BOS was made.

Sample processing

BALF was performed during a flexible bronchoscopy by instilling a total volume of 120 ml of sterile isotonic saline solution (3 fractions of 40 ml) into a sub-segmental bronchus of either the lingual or the right middle lobe (54). The first recovered BALF aliquot was removed because of bronchial contamination. The next BALF samples were immediately sent to the laboratory and processed within 60 min. Cytology (total and differential cell count by cytopspin and May-Grünwald Giemsa staining) and microbiology contents were evaluated on one aliquot. The last sample was processed for the proteomic analysis. After a first centrifugation (2000g, 10 min, room temperature (RT)) to remove cells, supernatant was centrifuged a second time (14000g, 10 min, +4°C) to discard any bacteria. Then, supernatant was treated by a protease inhibitor cocktail (Roche Diagnostics, Meylan, France), aliquoted and stored at -80°C until further analysis.

SELDI-TOF MS analysis

BALF protein profiles were obtained by the SELDI-TOF-MS method (Ciphergen Biosystems, Fremont, CA), using a cation exchange array (CM10) and an anion exchange array (Q10). Protein concentration of BALF was measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA): 1 µg was loaded on each array. Samples were processed robotically on a Biomek® 2000 Automation Workstation (Beckman Coulter, Villepinte, France). First, arrays were equilibrated with 200 µl of the corresponding loading/washing buffer (20 mM Tris, 0.1% Triton X100 (v/v) pH 8 for Q10; 20 mM sodium acetate, 0.1% Triton X100 pH 4 for CM10) during 5 minutes at RT. Respective amounts of BALF diluted in 50 µl of loading/washing buffer were incubated on each chip for 60 min at RT. Spots were washed twice with 200 µl of washing buffer, twice with 200 µl of the same buffer without Triton X100 and finally with 200 µl of 5 mM Tris pH 8 (Q10) or 5 mM sodium acetate pH 4 (CM10). After drying, spots were loaded twice with 0.8 µl of saturated sinapinic acid in 0.5% trifluoroacetic acid, 50% acetonitrile and chips were analyzed on the PCS-4000 ProteinChip® reader. Two laser intensities (1,800 nJ and 3,000 nJ) were used to analyze protein profiles and mass data processing was performed with the Ciphergen Express™ software, following the classical steps of spectra calibration, normalization, peak detection and aligning. The analysis was restricted to peptide peaks above 2,500 Da.

Statistical analysis

Data were analyzed using the SPSS/PC software package (SPSS 17.0, SPSS Inc, Chicago, IL). Continuous variables were represented as median [range] and compared using the Student's t-test, the Kruskal-Wallis test or the ANOVA test. Categorical variables are described by frequencies and percentages with 95% confidence interval (CI) and compared by using the exact Fischer test. In the case of comparisons involving more than two groups, p-values were

adjusted using the Bonferroni method. A p-value below 0.05 was considered statistically significant. Mann-Whitney *U* tests were used to compare SELDI-TOF MS peak intensities. ROC analyses were performed to measure the diagnostic accuracy of each biomarker. Correlations between continuous variables were determined using the Spearman's rank test. Multivariate comparisons between two populations were performed by supervised hierarchical clustering analyses of the characterized biomarkers and results were visualized by heat maps which are a graphical representation of overexpressed (red), decreased (green) or unchanged (black) proteins previously identified as differentially expressed in the two studied populations. Heat maps and hierarchical clustering were performed automatically by the Ciphergen Express™ software.

RESULTS

Patient characteristics

We included a total of 42 BALF samples obtained from 13 LTRs who developed BOS (6 BOS grade 1, 5 BOS grade 2 and 2 BOS grade 3), 21 LTRs who still had stable graft function and 8 LTRs with an AR (3 grade A1 and 5 grade A2). Demographic parameters are summarized in Table 1.

BALF proteome profiling

An example of generated data is shown in Figure 1. Distinct protein profiles of BALF were observed on CM10 and Q10 arrays. SELDI-TOF MS spectra generated from BALF samples typically displayed proteins ranging between 2,500 and 20,000 Da. Comparison of those spectra revealed a large number of protein peaks that are differentially expressed between groups.

BOS versus stable groups

Analyses of protein profiles revealed 177 protein peaks. Statistical comparisons of all peak intensities between BOS and stable groups indicated that 45 proteins were differentially expressed in both populations (Table 2). We focused on two particularly abundant clusters of proteins that were located at approximately 3,400 Da and 10-13,000 Da. The most differentially expressed biomarkers were four proteins isolated on both arrays at $10,812 \pm 9.4$ Da, $10,737 \pm 11$ Da, $10,412 \pm 8.3$ Da and $13,335 \pm 17.5$ Da which were all overexpressed in the BOS group (Fig 2A, 2C, 2D). Their mean intensities were two to five times higher in the BOS population with significant statistical differences between $p=0.0003$ and $p=0.002$. ROC curve analyses showed that these biomarkers discriminated BOS patients from stable patients with high sensitivities and specificities (Table 3). The most discriminating biomarker was the protein of 10,812 Da that provided an accurate diagnosis of BOS with a sensitivity of 92% and a specificity of 72% (AUC=0.87; 95%IC 0.76-0.98) (Fig 2B and Table 3).

Another cluster of three peptides of approximately 3,400 Da was differentially expressed in BALF of BOS group compared to the stable population (Table 3). These biomarkers of $3,354 \pm 3.5$ Da, $3,424 \pm 3.6$ Da and $3,470 \pm 5.1$ Da, also characterized on both arrays, were statistically enhanced in the BOS group (Fig 2E). They were a little less accurate than the previous biomarkers for the discrimination of the BOS population from the stable group (Table 3).

The 45 biomarkers whose expression was significantly different in BOS and stable groups were visualized in a heat map (Fig 3). Most of them were increased in BOS samples and decreased in stable BALF. Classification of samples according to those 45 proteins did not improve the identification of BOS patients compared to the discriminating potential of individual markers: this hierarchical clustering map displayed a sensibility of 85% and a

specificity of 67% in differentiating BOS from stable samples. These results were not affected by the presence of bacterial or fungal colonization. Comparison between colonized patients and no colonized patients in the whole population showed that the expression of the previous discriminating biomarkers was not related to the colonization status (Data not shown).

BOS versus AR groups

Among 177 proteins detected in BOS and AR BALF samples, 42 were differentially expressed in both populations (Table 2). Moreover, the previously characterized 10,812 Da, 10,737 Da, 13,335 Da and 10,412 Da proteins were the most relevant to differentiate the both populations (Fig 4A, 4C, 4D). For example the average intensity of 10,812 Da biomarker was 13.7 ± 2.2 in the BOS group versus 5.4 ± 2.8 in the AR set ($p=0.003$) (Fig 4A), providing an accurate diagnosis of BOS with a sensitivity of 92% and a specificity of 87% (AUC=0.88; 95% IC 0.71-0.97) (Fig 4B).

The three proteins of approximately 3,400 Da were also characterized in the comparison of BOS and AR protein profiles with peak intensities significantly higher in the BOS population (Fig 4E). These biomarkers displayed a weaker accuracy than the above-mentioned biomarkers in discriminating BOS from AR patients (Table 4). Concerning the hierarchical clustering classification, the sensitivity of this analysis in classifying BOS patients in this group was 77%, with 3 misclassified samples. Only one AR sample was classified in the BOS pattern group, corresponding to a specificity of 87.5% (Fig 5).

AR versus stable groups

We also compared protein profiles of stable patients with those of the AR group. Among 177 peaks detected, only 4 were differentially expressed between both groups (Table 2). We did

not detect any statistical difference in the intensity of the previously described biomarkers.

Differential cell count of BALF

The differential cell count of BALF showed a significantly elevated percentage of PMN in BOS patients compared to stable patients ($p=0.003$) and AR patients ($p=0.017$). No significant difference was found between AR and stable population ($p=0.667$). The percentage of PMN in BALF discriminated BOS patients from other groups with a sensitivity of 66% and a specificity of 84% (AUC=0.75; 95%CI 0.66-0.96), which was less accurate than the biomarkers characterized in the proteomic analysis.

Characterization and correlation of the detected biomarkers

According to published data concerning the proteome of BALF we could hypothesize on the identity of the detected biomarkers. The cluster of the 3,400 Da proteins was previously identified in several SELDI-TOF MS studies as alpha-defensin-1, -2 and -3 also called human neutrophil peptides 1-3 (HNP 1-3) (35). Moreover molecular weights of the 3 biomarkers between 10 and 13 kDa were in agreement with published molecular masses of several S100 proteins (30, 32, 35, 39). The biomarkers of 10,412 Da, 10,812 Da and 13,335 Da respectively corresponded to S100A12 (calgranulin C), S100A8 (also called MRP-8 or calgranulin A) and S100A9 proteins (MRP-14 or calgranulin B). The only 10,737 Da protein was yet unidentified in BALF proteome databases. A strong correlation was found between peak intensities of this biomarker and S100A8 ($\rho=0.641$, $p<0.001$) (Fig 6E).

S100 proteins and alpha-defensins are mainly produced by PMN in inflammatory diseases. Relative peak intensities of S100A8, S100A9 and S100A12 were correlated with the percentage of PMN in BALF samples (respectively $\rho=0.613$, $p<0.001$; $\rho=0.531$, $p < 0.001$ and $\rho=0.492$, $p=0.001$) (Fig 6A, 6B, 6C). Similarly we demonstrated a statistical correlation

between peak intensities of alpha-defensins and percentage of PMN (respectively $\rho=0.531$, $p<0.001$; $\rho=0.485$, $p=0.002$; $\rho=0.366$, $p=0.02$ for biomarkers of 3,354 Da, 3,424 Da and 3,470 Da) (Fig 6D).

DISCUSSION

Chronic allograft dysfunction after lung transplantation remains a challenge. Its diagnosis is often delayed because of the poor sensitivity of functional and biological tests. The discovery of new biomarkers could improve disease diagnosis and knowledge of its pathophysiology, which may lead to the identification of new therapeutic targets. Proteomic technologies are particularly powerful tools to address these issues. Applying the SELDI-TOF MS technology to the analyses of BALF samples of LTRs, we were able to determinate a large panel of biomarkers that differentiate, with a high statistical significance, LTRs with chronic allograft dysfunction from stable patients or LTRs with acute rejection.

SELDI-TOF MS is a high throughput technic which analyze the proteome, i.e. the whole content of a biological fluid. It associates a first phase of protein fractionation on chips with distinct chromatographic surfaces (cationic, anionic...) to a second detection step of protein molecular weight by mass spectrometry. This technology has been applied to a large range of body fluids such as serum, urine and synovial fluid (55) to characterize disease specific biomarkers, but only few data are available concerning its application to BALF samples. Although the BALF procedure has been used for more than 30 years for diagnostic purposes in lung diseases, information about the protein content of BALF is still limited at least in part because of methodologic problems in sample processing (56, 57). Dilution of proteins by the lavage procedure, high salt content, and/or low concentrations of some proteins are restricting factors for many analyses and the desalting and concentration processes lead to significant loss of BALF proteins (32, 56). However these sample characteristics do not interfere with SELDI-

TOF MS analysis. Indeed, concentration and desalting of BALF samples are not required because of the femtomole sensitivity of the technique and the dilution of the sample in the specific array-type buffer. To avoid any concentration-related biases in our study, supernatants were adjusted to a total protein concentration of 1 µg/50 µl before applying on CM10 and Q10 arrays.

This analysis of BALF protein expression patterns revealed respectively 45 and 42 protein peaks that are associated with BOS phenotype in comparison with stable patients and LTRs with acute rejection. Among them, the most relevant biomarkers were three proteins of 10,412 Da, 10,812 Da and 13,335 Da significantly increased in BOS patients. Previous published data suggested that these proteins respectively corresponded to S100A12, S100A8 and S100A9 proteins (30, 32, 35, 39). These S100 proteins are calcium-binding proteins mainly produced by PMN (58) and are involved in pro-inflammatory processes associated with various inflammatory conditions (59). The S100A8 and S100A9 proteins are often complexed as a S100A8/S100A9 heterodimer called calprotectin. Calprotectin is chemotactic for PMN both *in vitro* and *in vivo* (60) and is involved in the activation of NADPH oxidase, leading to an increased release of reactive oxygen species (61). S100A12 is implicated in a novel inflammatory axis, involving Receptors for Advanced Glycation End products (RAGE) as a receptor transducing proinflammatory signals in endothelium (62), and its chemotactic properties contribute to leucocytes extravasation at sites of inflammation (63). A study of human alveolar epithelial cells showed that S100A8/S100A9 heterodimer stimulates IL-8 production in pulmonary epithelium and amplifies local neutrophilic inflammation (64). Previous clinical studies reported an up-regulation of S100A8 and/or S100A9 proteins in inflammatory lung diseases (65), particularly in cystic fibrosis (CF) (66, 67). A high level of calprotectin in serum was associated to an inflammation profile in CF (68) and more recently, using a SELDI-TOF MS approach, McMorran and colleagues demonstrated that S100A8, S100A9 and S100A12 proteins isolated in BALF were strongly associated with CF airway

inflammation (35). Interestingly, we reported here for the first time the potential involvement of these S100 proteins as diagnostic biomarkers of BOS. This result was consistent with the pivotal role of pro-inflammatory proteins in BOS pathogenesis. Indeed these S100 proteins could induce lung damages and aberrant tissue repairs in small airways by promoting neutrophil oxidative burst and leukocytes recruitment.

Another BOS biomarker was also characterized in the same molecular weight range. However the identity of this 10,737 Da protein is still unknown. The narrow difference of its molecular weight with the S100A8 mass and the strong correlation of their peak intensities suggested that this protein may be an isoform of S100A8. A truncated form of S100A8 has already been reported in BALF (35), but it is slightly shorter (10,580 Da) than this biomarker.

We also characterized a cluster of three proteins of approximately 3,400 Da overexpressed in BALF of BOS patients and that strongly discriminates the BOS population from stable or AR groups. According to the predicted molecular weight mentioned in BALF proteome databases, we speculated that these proteins corresponded to alpha-defensins (35, 51). These proteins stored in the azurophilic granules and released from neutrophils during degranulation are important antimicrobial peptides involved in the innate immune response (69-71). They also play a key role in the orchestration of subsequent adaptive immune response and repair processes (72, 73). Few publications reported the role of alpha-defensins in chronic lung allograft dysfunction. In a retrospective study, Nelsestuen and colleagues showed that alpha-defensins may serve as a biomarker predicting BOS onset (51). Moreover in our study, the overexpression of alpha-defensins in recipients with BOS was not affected by the presence of airway pathogens. Our findings are in agreement with the results of Anderson and colleagues (74) who found a higher level of alpha-defensins in BOS LTRs than in stable LTRs, whatever their colonization status. These data suggest that the increased level of alpha-defensins in BALF of BOS patients contributes to a proinflammatory microenvironment and may promote an exaggerated repair response, independently of colonization by airway pathogens.

A drawback of this study is the absence of formal identification of the biomarkers. The molecular weights of the biomarkers are closely related to their predicted molecular weight established in publications and/or protein databases. However, we cannot exclude that these protein peaks represent other proteins. Moreover the use of SELDI-TOF MS has allowed us to characterize other relevant biomarkers including proteins down-regulated in BOS patients compared to stable patients. For example a protein of 16,597 Da is highly under-expressed in BOS BALF compared to stable samples, but its identity is unknown.

Innovative bioinformatic tools presently provide an opportunity to fully apprehend the complexity of proteomic data. It would be interesting to apply bioinformatic algorithms to this data set in a decision tree that combines several biomarkers and then improves the diagnostic accuracy.

In conclusion, we underlined the usefulness of SELDI-TOF MS platform to characterize in BALF a large panel of new biomarkers that differentiate, with high statistical significances, BOS patients from other populations. To improve the strength of our results we are currently validating detected biomarkers in an independent cohort of LTRs. In addition to their diagnostic value, these biomarkers provide additional molecular details regarding BOS pathogenesis. Additional research is necessary in this area to validate prospectively these biomarkers in a larger cohort of patients and to clarify the possible link of increased levels of S100 proteins and alpha-defensins with chronic allograft dysfunction in lung transplantation. Their predictive value will be evaluated in a longitudinal proteomic analysis of LTRs followed over 5 years. Moreover, these proteins interacting with key modulators of proinflammatory pathways might be appropriate candidates for targeted intervention in BOS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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TABLES

Table 1. Patient characteristics

	Stable		AR		BOS		p-Value
Patients	n=21		n=8		n=13		
Age, years	46	[28.5-58]	41	[32.25-67]	53	[38-62]	0.547
Sex (M / F)	12/9		6/2		8/5		0.676
Transplantation							0.115
Double Ltx	20		5		10		
Single LTx	1		3		2		
Heart-Lung LTx	0		0		1		
Indication (%)							0.107
Cystic fibrosis	11	52.4%	2	25%	3	23.1%	
Emphysema	7	33.3%	5	62.5%	6	46.2%	
Fibrosis	3	14.3%	1	12.5%	1	7.7%	
PPH	0	0%	0	0%	3	23.1%	
Others	0	0%	0	0%	0	0%	
FEV1, % pred	82	[74-93]	73	[61-103]	59	[36-72]	0.002
Months after transplantation	7	[2-16]	4.5	[1.5-21.75]	61	[27.5-88.5]	<0.001
Immunosuppression							
TAC/CsA	21/0		6/2		13/0		
MMF/AZA	19/2		8/0		13/0		
Prednisone, mg	10	[8-18.75]	15	[10-20]	6	[5.5-10]	0.022
Colonization							
Presence	11	52.4%	4	50%	8	61.5%	0.724
<i>Pseudomonas aeruginosa</i>	4		1		5		
<i>Aspergillus</i>	5		3		3		
Other	2		0		0		
Biological data							
% PMN in BALF	1	[1-5]	2	[0.5-2.50]	10	[3-22.5]	0.006

Values are medians [interquartile range] or number of patients (%). AR, acute rejection; BOS, bronchiolitis obliterans syndrome; LTx, lung transplantation; PPH, primary pulmonary hypertension; FEV1, forced expiratory volume in 1 s; TAC, tacrolimus; CsA, cyclosporin A; MMF, mycophenolate mofetil; AZA, Azathioprine; PMN, Polymorphonuclear neutrophil; BALF, Bronchoalveolar lavage fluid.

Table 2. Number of biomarkers detected in BALF samples.

	Number of protein peaks detected in BALF samples		
	p<0.05	p<0.01	Total
BOS vs. ST	45	25	
BOS vs. RA	42	15	177
RA vs. ST	4	1	

BALF, Bronchoalveolar lavage fluid; BOS, bronchiolitis obliterans syndrome; AR, acute rejection; ST, stable.

Table 3. Characteristics of the biomarkers differentially expressed in BOS vs. stable groups.

	Biomarker (Da)	Array	p-value	AUC	Sensitivity (%)	Specificity (%)	Cut-off (Peak intensity)
1	16,597	CM10	0.00007	0.097			
2	10,812	Q10	0.0003	0.87	92	72	9.3
3	16,061	CM10	0.0005	0.14			
4	10,737	CM10	0.0007	0.85	85	81	24.9
5	7,999	Q10	0.0008	0.16			
6	13,335	Q10	0.0008	0.84	92	62	2.1
7	3,424	Q10	0.001	0.83	85	76	10.2
8	15,977	Q10	0.002	0.18			
9	10,412	CM10	0.002	0.82	77	67	9.3
10	11,715	CM10	0.001	0.16			
11	10,949	CM10	0.002	0.80			
12	3,354	Q10	0.002	0.81	84	72	7.2
13	13,528	Q10	0.002	0.81			
14	4,108	CM10	0.003	0.79			
15	12,744	CM10	0.007	0.77			
16	3,470	CM10	0.01	0.77	84	71	59.5

AUC, Area Under Curve.

Table 4. Characteristics of the biomarkers differentially expressed in BOS vs. AR groups.

	Biomarker (Da)	Array	p-value	AUC	Sensitivity (%)	Specificity (%)	Cut-off (Peak intensity)
1	10,812	Q10	0.003	0.88	92	87	6.5
2	10,737	CM10	0.004	0.88	92	88	7.2
3	13,335	Q10	0.005	0.87	85	88	3.5
4	10,412	CM10	0.005	0.86	70	72	9.8
5	12,665	Q10	0.005	0.85			
6	4,108	CM10	0.006	0.85			
7	3,470	CM10	0.006	0.85	84	87	51.9
8	16,074	Q10	0.007	0.15			
9	6,372	CM10	0.007	0.17			
10	13,528	Q10	0.009	0.81			
11	3,354	CM10	0.009	0.81	85	88	55.5
12	6,159	CM10	0.009	0.17			
13	13,921	Q10	0.009	0.82			
14	5,206	Q10	0.009	0.85			
15	3,424	Q10	0.01	0.81	85	75	9.8

AUC, Area Under Curve.

LEGENDS

Figure 1. Representative example of proteomic profiles of bronchiolitis obliterans syndrome (BOS) and stable (ST) patients on Q10 array (3 to 7 kDa) (A) and CM10 array (7 to 15 kDa) (B). In the range of 3 to 7 kDa a cluster of 3 peaks of about 3,400 Da was up-regulated in the BOS group (solid line) as well as a protein of 10,812 Da in the range of 7 to 15 kDa (dotted line).

Figure 2. Characterization and diagnostic value of biomarkers discriminating bronchiolitis obliterans syndrome (BOS) patients from stable (ST) patients. Relative peak intensity (A) and Receiver Operator Characteristic (ROC) curve with area under curve (AUC) (B) of the most discriminating biomarker of 10,812 Da. Relative peak intensities of 10,412 Da and 13,335 Da proteins (C); 10,737 Da (D); and of 3,354 Da, 3,424 Da and 3,470 Da biomarkers (E). Boxes represent the interquartile range, the line across the box is the median and the whiskers represent the 5th and 95th percentile.

‡ p<0.0001, ✕ p<0.005, § p<0.05.

Figure 3. Heat map visualization of the 45 biomarkers significantly differentially expressed in bronchiolitis obliterans syndrome (BOS) vs. stable (ST) bronchoalveolar lavage fluid (BALF). Enhancement of the 10,412 Da (°), 10,812 Da (°°), 13,335 Da (°°°), 3,354 Da (*), 3,424 Da (**), and 3,470 Da (***) biomarkers in patients suffering from BOS (red numbers) compared to ST patients (blue numbers).

Figure 4 Characterization and diagnostic value of biomarkers discriminating bronchiolitis obliterans syndrome (BOS) patients from acute rejection (AR) patients.

Relative peak intensity (A) and Receiver Operator Characteristic (ROC) curve with area under curve (AUC) (B) of the most discriminating biomarker of 10,812 Da (S100A8). Relative peak intensities of 10,412 Da and 13,335 Da proteins (C); 10,737 Da (D); and of 3,354 Da, 3,424 Da and 3,470 Da biomarkers (E). Boxes represent the interquartile range, the line across the box is the median and the whiskers represent the 5th and 95th percentile. ‡ p<0.0001, ✕ p<0.005, § p<0.05.

Figure 5. Heat map visualization of the 42 biomarkers significantly differentially expressed in bronchiolitis obliterans syndrome (BOS) vs. acute rejection (AR) bronchoalveolar lavage fluid (BALF). Enhancement of the 10,412 Da (°), 10,812 Da (°°), 13,335 Da (°°°), 3,354 Da (*), 3,424 Da (**), and 3,470 Da (***) biomarkers in patients suffering from BOS (red numbers) compared to AR patients (blue numbers).

Figure 6. Origin of the biomarkers. Correlation between percentage of polymorphonuclear neutrophil (PMN) in bronchoalveolar lavage fluid (BALF) and peak intensities of S100A8 (A), S100A9 (B), S100A12 (C) and alpha-defensin-1 protein (D) .Correlation between peak intensities of the biomarker of 10,737 Da and S100A8 (E).

FIGURES

Fig. 1

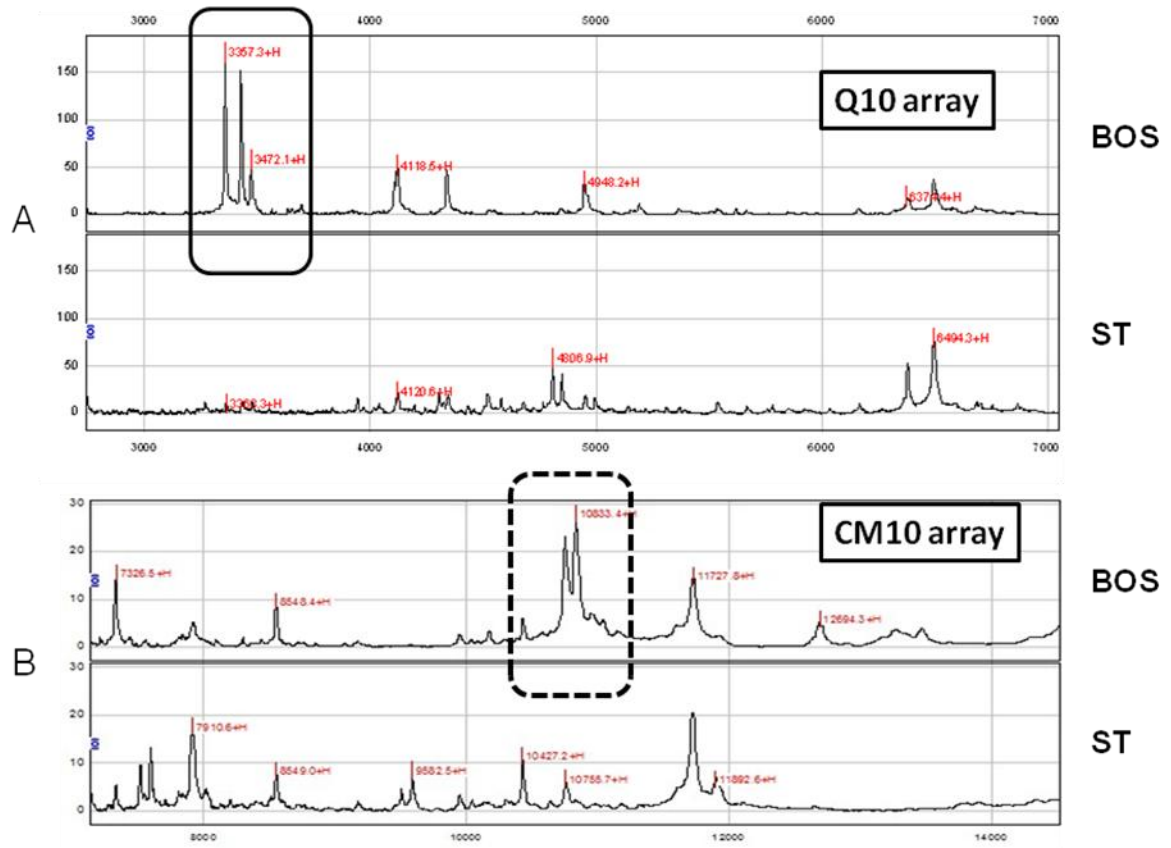


Fig. 2

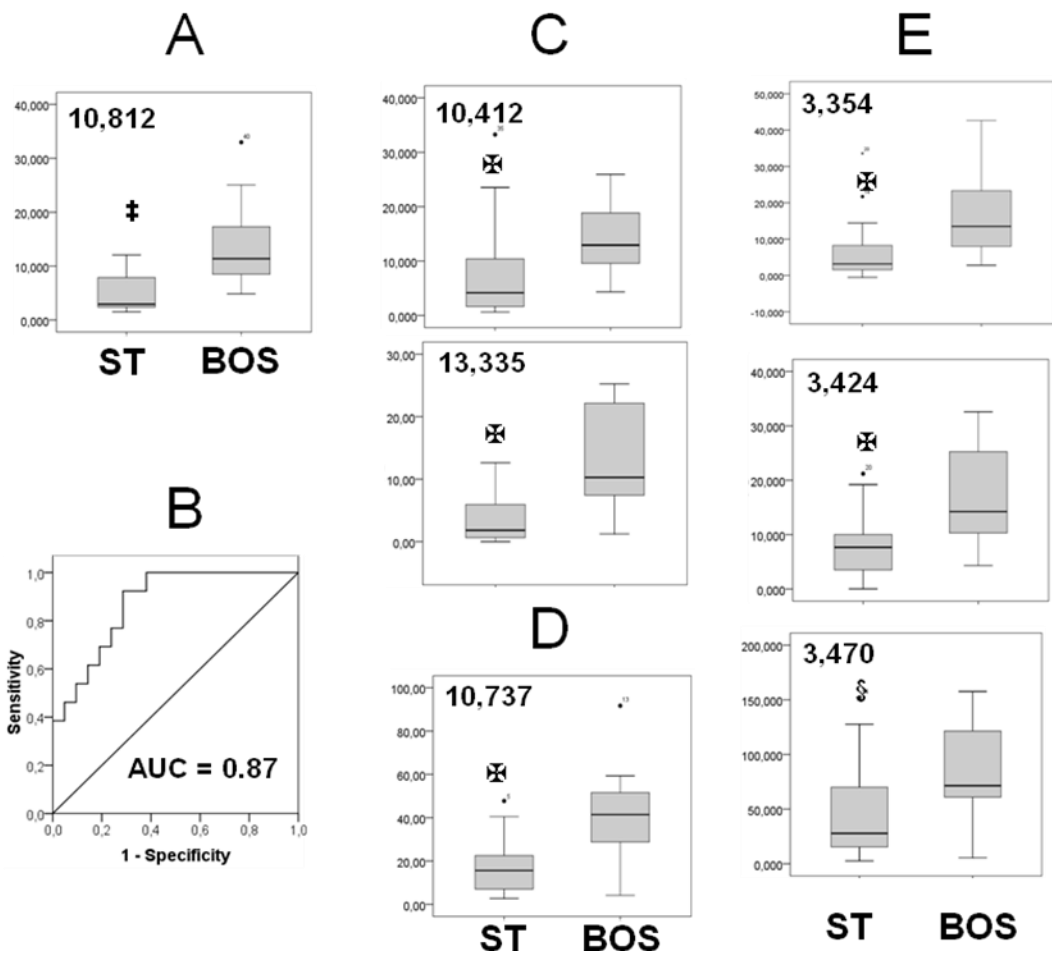


Fig. 3

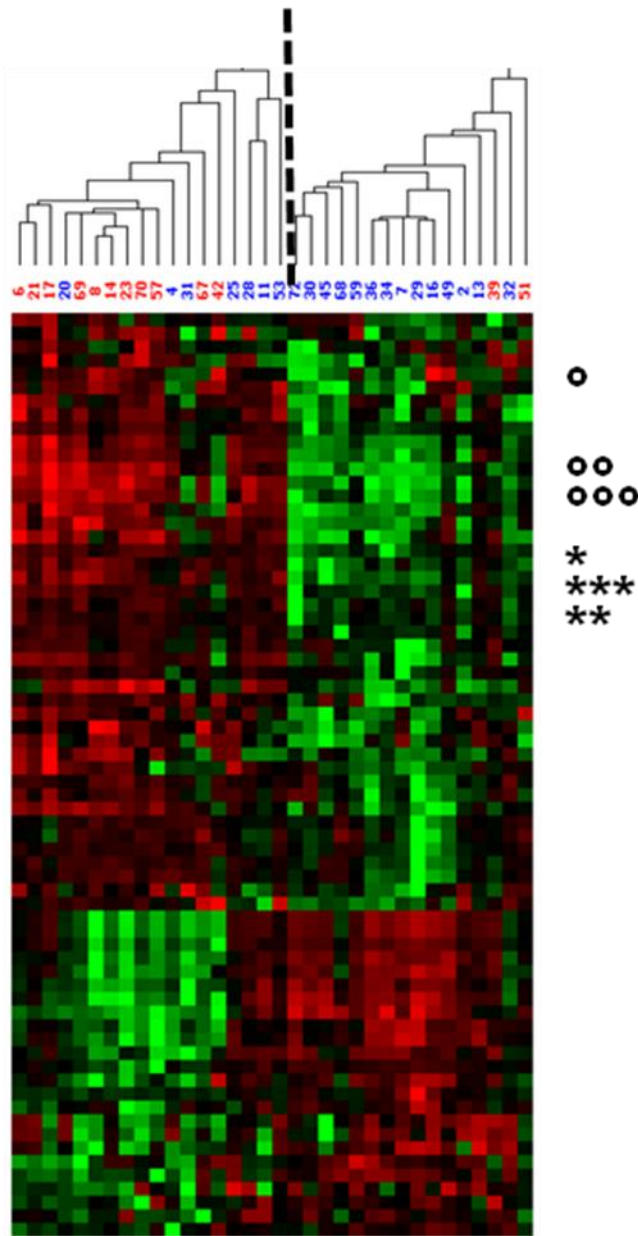


Fig. 4

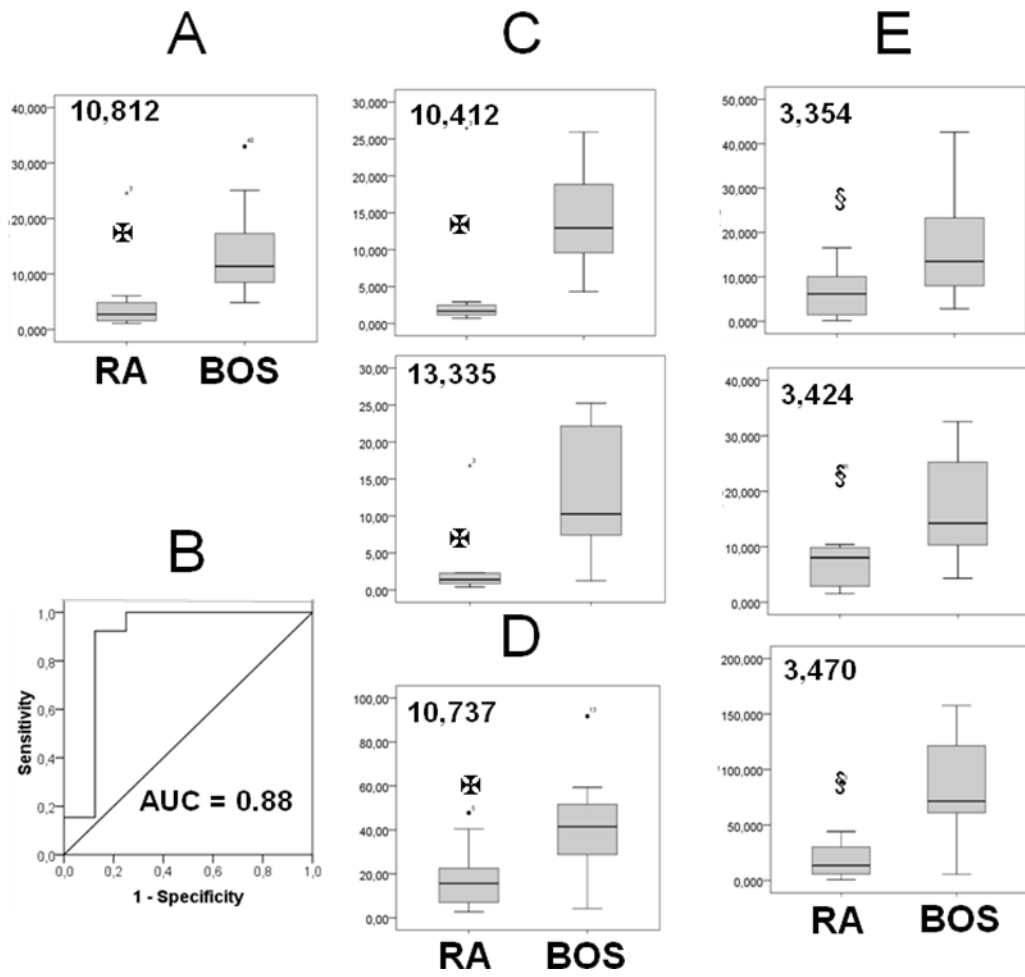
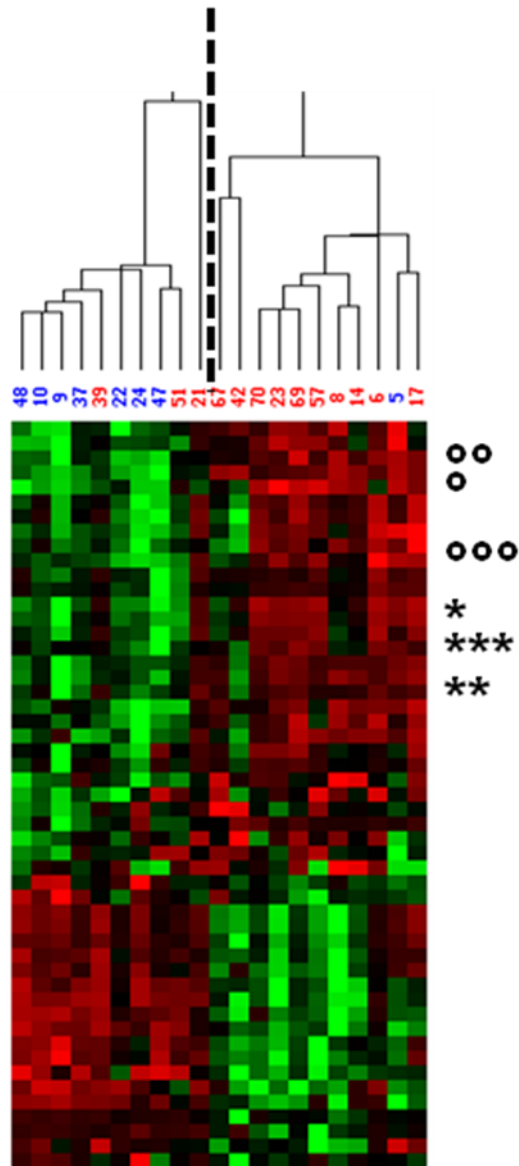


Fig. 5



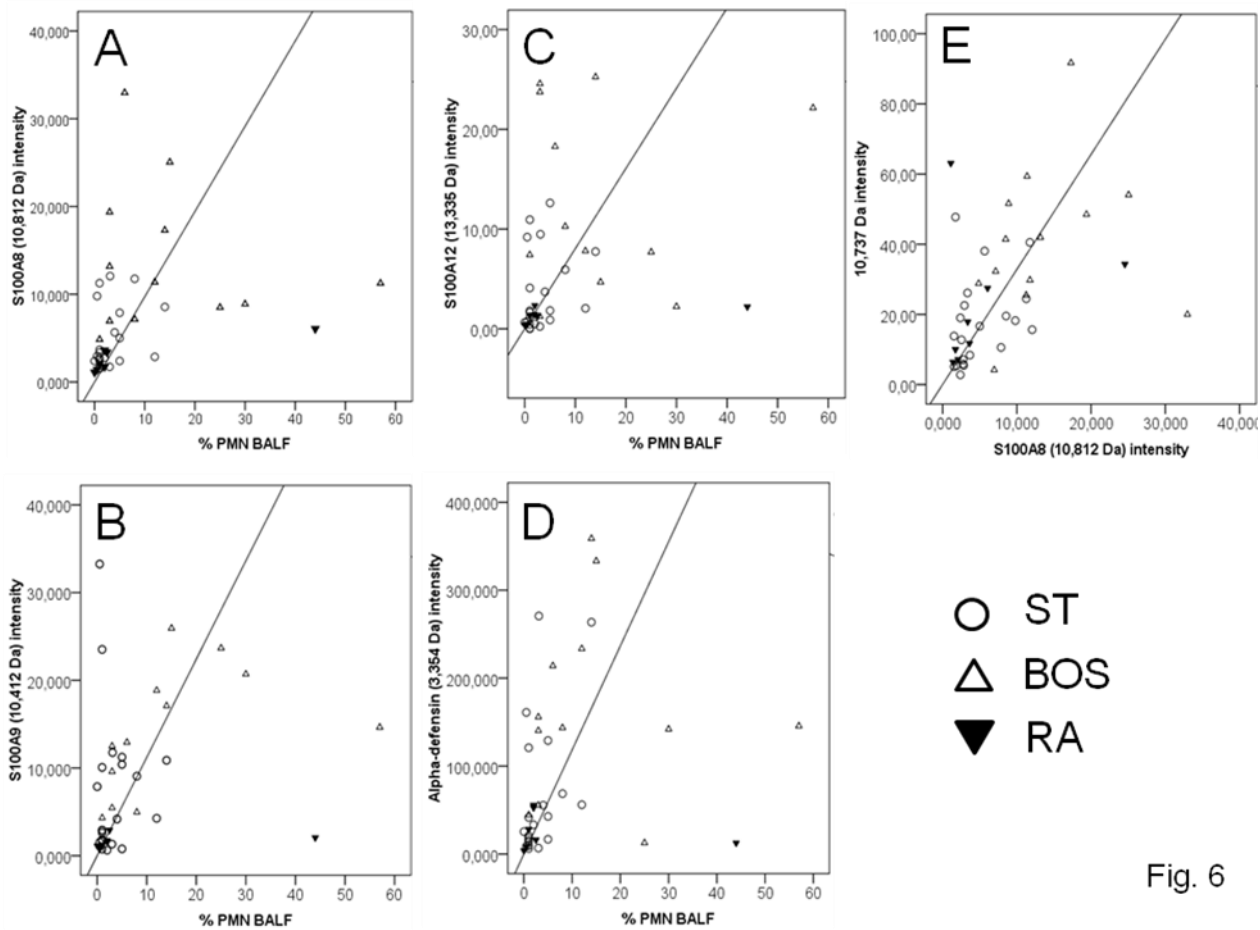


Fig. 6

Conclusion

Thèse soutenue par QUETANT Sébastien

TITRE:

Transplantation pulmonaire et dysfonction chronique du greffon : Caractérisation de biomarqueurs diagnostiques par analyse protéomique SELDI-TOF du liquide bronchiolo-alvéolaire.

CONCLUSION ET PERSPECTIVES :

La dysfonction chronique du greffon constitue un challenge pour la transplantation pulmonaire. Cette complication tardive de la greffe pulmonaire apparaît de façon insidieuse et il n'existe pas à l'heure actuelle de marqueurs non invasifs permettant son diagnostic précoce avant l'apparition d'une baisse irréversible de la fonction respiratoire. L'analyse protéomique SELDI-TOF est une technologie innovante fondée sur une approche originale : elle permet un screening haut débit de l'ensemble des protéines d'un milieu biologique avec une grande sensibilité et la recherche de marqueurs d'une pathologie n'est pas basée sur l'analyse de protéines prédéterminées *a priori*.

Nous avons donc réalisé une analyse protéomique SELDI-TOF transversale de 42 lavages bronchiolo-alvéolaires (LBA) prélevés entre juillet 2007 et septembre 2008 chez des patients transplantés pulmonaires. Trois groupes ont été déterminés selon le statut clinique des patients : un groupe de 13 patients ayant une dysfonction chronique du greffon ou bronchiolitis obliterans syndrome (BOS), un groupe de 8 patients présentant un rejet aigu (RA) et un groupe de 21 patients stables.

La comparaison des profils protéomiques des 3 groupes de patients a permis de caractériser de nombreux biomarqueurs différenciant les transplantés pulmonaires ayant une dysfonction chronique du greffon des autres patients. Parmi les biomarqueurs les plus discriminants pour le diagnostic de BOS, 3 pics à 10812, 13335 et 10412 Da correspondant probablement aux protéines S100A8, S100A9 et S100A12 permettent de différencier les patients BOS des patients stables ou RA avec une spécificité et une sensibilité élevée. Ces protéines S100 sont principalement exprimées dans les cellules de la lignée myéloïde et ont un rôle crucial dans la modulation de la réponse inflammatoire. D'autre part, nous avons mis en évidence un cluster de protéines à 3354, 3424 et 3470 Da exprimées différemment entre le groupe BOS et les deux autres groupes. Ces 3 biomarqueurs surexprimés chez les sujets BOS correspondraient aux alpha-défensines, qui sont des peptides antimicrobiens largement impliqués dans l'immunité anti-infectieuse innée et dans la régulation de la réponse immunitaire adaptative.

En plus de leur intérêt diagnostique, ces 6 biomarqueurs semblent intéressants pour la compréhension des mécanismes physiopathologiques du rejet chronique et la recherche de

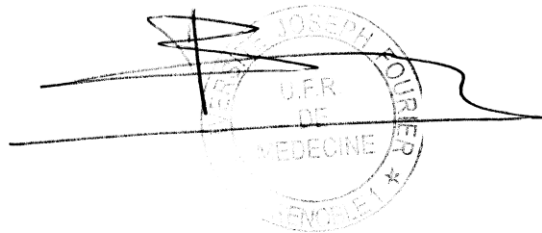
nouvelles cibles thérapeutiques. Ces résultats nous incitent à poursuivre les investigations sur le rôle des protéines S100 et des alpha-défensines dans la physiopathologie du rejet chronique. Les autres pics protéiques discriminants sont pour l'instant inconnus et nécessitent d'être identifiés. Par ailleurs, nous allons réaliser une analyse longitudinale des profils protéomiques d'une cohorte prospective de patients transplantés pulmonaires au sein d'un projet national multicentrique (COhort in Lung Transplantation – COLT) qui a pour ambition principale la recherche de biomarqueurs prédictifs de dysfonction chronique du greffon par diverses approches fondamentales (protéomique, transcriptomique, immunologique...).

VU ET PERMIS D'IMPRIMER

Grenoble, le 5 - 05 - 2005

LE DOYEN

Professeur B.SELE



LE PRESIDENT DE THESE

Professeur C.PISON

C.H.U. de GRENOBLE
HOPITAL NORD
CLINIQUE PNEUMOLOGIQUE
P. C. PISON

A large, stylized signature in black ink is written over the stamp and the text "C.H.U. de GRENOBLE HOPITAL NORD CLINIQUE PNEUMOLOGIQUE".



Qui dias memorem laudes, repetamque fideles
 Ingenij dotes, Hippocratisque decus.
 Democriti auditor Phœbea, ô, Coë propago,
 Certius an quis te tradidit artis opes?

SERMENT D'HIPPOCRATE

En présence des Maîtres de cette Faculté, de mes chers condisciples et devant l'effigie d'HIPPOCRATE,

je promets et je jure d'être fidèle aux lois de l'honneur et de la probité dans l'exercice de la Médecine.

Je donnerai mes soins gratuitement à l'indigent et n'exigerai jamais un salaire au dessus de mon travail. Je ne participerai à aucun partage clandestin d'honoraires.

Admis dans l'intimité des maisons, mes yeux n'y verront pas ce qui s'y passe ; ma langue taira les secrets qui me seront confiés et mon état ne servira pas à corrompre les mœurs, ni à favoriser le crime.

Je ne permettrai pas que des considérations de religion, de nation, de race, de parti ou de classe sociale viennent s'interposer entre mon devoir et mon patient.

Je garderai le respect absolu de la vie humaine.

Même sous la menace, je n'admettrai pas de faire usage de mes connaissances médicales contre les lois de l'humanité.

Respectueux et reconnaissant envers mes Maîtres, je rendrai à leurs enfants l'instruction que j'ai reçue de leurs pères.

Que les hommes m'accordent leur estime si je suis fidèle à mes promesses.

Que je sois couvert d'opprobre et méprisé de mes confrères si j'y manque.