Pulmonary extracellular adenosine triphosphate in hypersensitivity pneumonitis and sarcoidosis – a preliminary study

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Abbreviation list

ATP ..... Adenosine triphosphate

BAL ..... Bronchoalveolar lavage

BALF ..... Bronchoalveolar lavage fluid

CF ..... Cystic Fibrosis

COPD ..... Chronic obstructive pulmonary disease

CHUC ..... Centro Hospitalar e Universitário de Coimbra

DLCO ..... Diffusion capacity

EBC ..... Exhaled Breath Condensate

FVC ..... Forced vital capacity

FEV1 ..... Forced expiratory volume in 1 second

HP ..... Hypersensitivity pneumonitis

ILDs ..... Interstitial lung diseases

IQR ..... Inter-quartile range

IPF ..... Idiopatic pulmonary fibrosis

Abstract

Interstitial lung diseases comprise a vast and heterogeneous group of conditions that share clinical and radiological features, creating frequent difficulties in their differential diagnosis. Purinergic signaling has been shown to be involved in several lung diseases, including idiopathic pulmonary fibrosis, chronic obstructive lung disease, and cystic fibrosis. Our purpose was to compare the extracellular levels of adenosine triphosphate in several lung diseases to gauge its usefulness for differential diagnosis. We quantified the ATP concentration in the bronchoalveolar lavage fluid of 5 patients diagnosed with hypersensitivity pneumonitis, 8 patients with sarcoidosis and 10 with other lung diseases, using the luciferin-luciferase luminescence method. Clinical data were gathered from the patient's clinical files, including pulmonary function tests. We found that the concentration of ATP was significantly lower in patients with hypersensitivity pneumonitis than in sarcoidosis. In fact, we observed that ATP was significantly correlated with total cells number and with diffusion capacity. This prompts the suggestion that the measurement of pulmonary extracellular ATP may assist in the differential diagnosis and warrens further studies to explore the signaling role of ATP in these diseases.

Keywords: ATP, Hypersensitivity pneumonitis, Sarcoidosis, BALF

Resumo

As doenças intersticiais pulmonares constituem um grupo vasto e heterogéneo de patologias que apresentam várias manifestações clínicas e imagiológicas em comum criando frequentemente dificuldades no seu diagnóstico diferencial. A sinalização purinérgica mostrou estar envolvida na patogénese de várias doenças pulmonares como a fibrose pulmonar idiopática, a doença pulmonar obstrutiva crónica e a fibrose quística. O objectivo deste estudo foi comparar os níveis de adenosina trifosfato em várias doenças pulmonares para avaliar a sua utilidade no diagnóstico diferencial. A concentração de ATP foi medida em lavados broncoalveolares de 5 doentes diagnosticados com pneumonite de hipersensibilidade, 8 com sarcoidose e 10 com outras doenças pulmonares, utilizando o método de luminescência da luciferina-luciferase. Os dados clínicos dos pacientes foram obtidos dos processos clínicos, incluindo as provas de função pulmonar. A concentração de ATP no LBA de doentes com pneumonite de hipersensibilidade foi significativamente inferior aos doentes com sarcoidose. Adicionalmente foi verificada uma correlação da concentração de ATP com o número total de células e com a capacidade de difusão. Estes resultados lançam a sugestão que a medição de ATP poderá contribuir no diagnóstico diferencial alertando para a necessidade de realizar futuros estudos para explorar o papel da sinalização de ATP nestas patologias.

Introduction

Interstitial lung diseases (ILDs) are a vast and heterogeneous group of pulmonary conditions that share clinical and radiological features, creating frequent difficulties in the differential diagnosis. (Kinder & Wells, 2009) Hypersensitivity pneumonitis (HP), also known as extrinsic allergic alveolitis, is an ILDs caused by intense and/or repeated inhalation of organic dusts and occupational antigens, with further sensitization. (Cordeiro et al, 2011; Girard et al, 2009). On bronchoalveolar lavage fluid (BALF), HP shows the highest total cell and lymphocyte counts of all ILDs, with a proportion of lymphocytes usually exceeding 50%. It is a general belief that the CD4/CD8 T lymphocyte ratio is decreased; however, recent studies have demonstrated that this ratio may vary (Bonella et al, 2010). BALF findings are also dependent on the time span from the last antigen exposure; neutrophils are elevated within 48 hours after acute exposure and return to normal within a week. When progression leads to pulmonary fibrosis, neutrophils may again be increasingly present in HP and the CD4/CD8 ratio may show mild elevation. (Cordeiro et al 2007). Pardo et al showed a positive correlation between the percentage of lung tissue polymorphonuclear leukocytes and the percentage of lung fibrosis in HP, but there was no correlation between BALF and lung neutrophils. (Pardo et al, 2000).

Sarcoidosis is a multisystem granulomatous disorder of unknown cause (ATS/ERS/WASOG, 1999). BALF shows lymphocytic alveolitis in 90% of patients at the time of diagnosis, independently of the stage of sarcoidosis and 55% show an increased CD4/CD8 ratio. Patients with active disease have a tendency to show a higher lymphocyte proportion and a particularly high CD4/CD8 ratio. In the last stage of sarcoidosis, neutrophils may also be elevated and some studies have demonstrated that an increased neutrophil count in BAL obtained from newly diagnosed patients with sarcoidosis, may indicate an unfavorable prognosis (Bonella et al, 2010).

Adenosine-5'-triphosphate (ATP) is considered a multifunctional molecule due to its several roles in metabolism, signaling and cell growth through the activation of P2 receptors (Bours et al, 2006). A role of purinergic regulation has been proposed in acute lung injury, mucociliary clearance, inflammation, wound healing, remodeling and lung fibrosis (Picher et al, 2011). In physiological situations, the extracellular concentration of ATP is kept at nanomolar concentrations through the activity of ectonucleotidases (Robson et al, 1997). Under conditions of cellular stress (such as exposure to bacterial endotoxin, reactive radicals or hypoxia), large amounts of ATP can be released into the pericellular space by many cell types, including epithelial and inflammatory lung cells and platelets. In vitro experiments have revealed that nucleotides act as chemoattractant for human neutrophils, eosinophils and dendritic cells and modulate the chemokine production and mucociliary clearance (Myrtek & Idzko, 2007).

Bronchoalveolar lavage (BAL) is a minimally invasive technique, used for collection of samples from the peripheral airway, including cells, inhaled particles, infectious organisms and solutes (Bonella et al, 2010). Several previous studies have characterized BALF from patients with cystic fibrosis (CF) (Esther et al, 2008), asthma (Idzko et al, 2007; Lázár et al, 2010), COPD (Lommatzsch et al, 2010) and idiophatic pulmonary fibrosis (IFP) (Riteau et al., 2010). In CF, the increased of ATP levels in BALF were correlated with neutrophil counts suggesting that extracellular purines are biomarkers of neutrophilic airway inflammation. In IFP, Ritteau et al (2010) identified extracellular ATP as a new danger signaling involving in the establishment of lung inflammation leading to fibrotic process.

The objectives of our study were the evaluation of ATP concentrations in the BALF supernatant of patients with HP and sarcoidosis to decide on the interest of this quantification for the differential diagnosis between these disorders.

Methods

Study Subjects

Study subjects were recruited from the population of patients referred for bronchoscopy and bronchoalveolar lavage (BAL) at Centro Hospitalar e Universitário de Coimbra – Hospitais da Universidade de Coimbra (CHUC - HUC), during a nine months period (May 2011 to January 2012), after approval by the local ethics committee. All exams were performed as clinically indicated by the patient's physician. Only patients with a definite diagnosis at the time of ATP quantification (February 2012) were included in the analysis. The exclusion criteria were clinical or laboratory signs of infection, non-representative BALF and no definite diagnosis in February 2012. The diagnosis of sarcoidosis was performed according to WASOG criteria (ATS/ERS/WASOG, 1999) and the diagnosis of hypersensitivity pneumonitis was performed according to Richerson's criteria (Richerson, 1989). Other patients (10) with lung cancer (2), pneumoconiosis (2), Wegener's granulomatosis (2), polymyositis (1), eosinophilic pneumonia (1) and leukemia (2) were also included based on the relevant diagnosis criteria and the patient's physician clinical diagnosis. For the purpose of this study, the patients were divided in three groups: sarcoidosis, hypersensitivity pneumonitis, and other diagnosis.

Clinical data and lung function

Clinical data were gathered from the patient's clinical files stored at CHUC-HUC. We collected all the data required to fulfill the inclusion criteria, namely: blood urea quantification, radiological and lung function tests, smoking habits and therapy at the time of BALF collection (with systemic or inhaled steroids). Sarcoidosis patients were staged using the Scadding criteria on chest CT (stage 0: normal; stage I: bilateral hilar lymphadenopathy

(BHL), stage III: pulmonary infiltrations (without BHL); stage IV: pulmonary fibrosis) (Rajesh, 2004), an analysis carried out by a qualified radiologist as previously reported (Costa et al., 2012). Lung function tests (FVC, FEV1, DLCO-SB) were performed using Masterscreen PFT or Masterlab body, from Jaëger (calibrated daily) by certified respiratory technicians, and predicted values were calculated using published referential equations (Quanjer, 1993). All the information was maintained anonymous during the study.

BALF collection and supernatant storage

BALF collection:

Briefly, bronchoscopy was performed with the patient in supine position and the bronchoscope was inserted through the nose or the mouth under local anesthesia with lidocaine. For BAL, the bronchoscope was wedged preferentially into a sub-segment of the right middle lobe and a total of 150 mL sterile 0.9% saline solution heated to 37ºC was instilled in three different 50 mL syringes. The BALF was recovered through gentle manual aspiration. After collection BALF was transported to the laboratory within two hours.

Sample processing and storage:

In the laboratory, BALF was filtered through sterile gauze to remove any visible particulate material and centrifuged at 380 *g*, during 10 minutes at 4°C. Approximately 1 mL of the supernatant was stored in adequately labeled Eppendorf 1.5 ml tubes. The samples were then stored at -20ºC, for a maximum of one month and then moved to -80ºC, until analysis. Total and differential cell counting was performed as routinely done in the laboratory: For the quantification of the total cell count in BALF, a particle counter (LH780) was used. The differential cell count was performed on cytospin slides after staining with May-Grünwald-Giemsa stain. Differential cell counts were made on a total count of at least 200 cells using

standard morphologic criteria. (Bonella et al, 2010). Lymphocyte subsets were determined by flow cytometry. Lymphocytes were stained by anti-CD3, anti-CD4, and anti-CD8 specific monoclonal antibodies for T lymphocytes and by anti-CD19 for B lymphocytes. (Zompi et al, 2004)

ATP quantification in BALF supernatants

ATP quantification was performed using a Sigma ATP Bioluminescent Assay Kit (Sigma®), according to the manufacturer's instructions. Briefly, 80 µL of BALF supernatant was added to 40 µL of recombinant luciferin-luciferase assay mix (Sigma®) in a new, sterile, white opaque 96 wells multiwell plate (Corning®). After one second of shaking, luminescence was detected over five seconds at a temperature of 25ºC. Both the addition and the luminescence analysis were performed by an automated multi-technology plate reader Wallac 1420 Victor 3V (Perkin Elmer®, Sweden). Samples were measured in triplicate and the mean luminescence was used in the analysis. Standard curves of ATP in 0.9% saline solution were performed at the beginning and at the end of the experiment, and the mean values were used to create a standard curve. The samples under the detection limit of $1 \times 10^{-10.5}$ mol/L of ATP were assigned to have an ATP concentration of this value. During the manipulation of the samples all efforts were made to maintain sterile conditions, including the use of sterile material and working in a flow chamber whenever possible. In previous measurements we guaranteed data reproducibility between different days (data not shown).

Dilution factor: urea measurement

Urea was measured in BALF supernatant using a commercial quantitative colorimetric assay (QuantiChrom ®). The samples were prepared following the manufacturer introductions and the optical density was read at 430 nm after 50 minutes of incubation. Using the value of

BALF urea and the serum urea obtained from the clinical files, we calculated the BALF dilution factor.

Statistical analysis

The statistical analysis was performed using IBM® SPSS® version 19. The sample characterization was done by calculating measures of location (median) and measures of spread (interquartile range) for quantitative variables and absolute and relative frequencies for qualitative variables. The normality test used was Shapiro-Wilk. The comparison of the total number of cells, neutrophils %, lymphocytes % and CD4/CD8 and pulmonary function (FVC%, FEV1%, DLCO%) between HP and sarcoidosis was performed using Mann-Whitney test. The comparisons of ATP quantifications between the HP and sarcoidosis patient groups were performed using Mann-Whitney test; when comparing the three groups Kruskal-Wallis test was used, with pairwise comparisons using Bonferroni correction. To assess the correlations between the ATP concentrations and the total cell counts, neutrophils, lymphocytes, CD4 and CD8 T lymphocyte numbers, these values were logarithmically transformed. Correlation between ATP concentration and lymphocyte, neutrophils and pulmonary function tests was performed using a Pearson correlation. To assess the correlation between CD4 or CD8 lymphocyte numbers and ATP concentrations we used a Spearman correlation test. The analysis of the differences between the time of BALF storage was performed by oneway ANOVA. All analyses were carried out establishing significance at 95% confidence.

Results

Patient population:

Out of the 83 patients who performed BALF during this period, twenty three were included. The diagnosis were: sarcoidosis (8) HP (5) lung cancer (2), pneumoconiosis (2), Wegener's granulomatosis (2), polymyositis (1), eosinophilic pneumonia (1) and leukemia (2). Subject's demographics are detailed in Table I.

None of the patients with sarcoidosis and HP was a current smoker or was under therapy with systemic or inhaled steroids, or thienopyridine class antiplatelet drugs. There was a positive smoking history in four patients with sarcoidosis and in five patients with other lung diseases' group, all former smokers. None of the patients with a positive smoking history showed evidence of COPD. Of the eight patients with sarcoidosis, two had stage I (25%), five had stage II (62.5%) and one had stage III (12.5%). Two were diagnosed with Löfgren Syndrome.

Differences of BALF characteristics and pulmonary function tests between HP and sarcoidosis group:

Patients with HP had a higher percentage of lymphocytes than patients with sarcoidosis, 65 (IQR 7) *vs* 9 (IQR 43) (p=0.04). HP patients also displayed a higher percentage of neutrophils 18 (IQR 27) *vs* 1 (IQR 4) (p= 0.014), and the CD4/CD8 ratio was higher in sarcoidosis patient's 3.17 (IQR 6.25) *vs* 0.95 (IQR 1.91) (p=0.042). No significant differences were found in the total cell count (p= 0.056) and in the percentage of eosinophils (p=0.2). BALF characteristics are show in table II and in figure 1. Regarding pulmonary function tests, which are detailed in table III and figure 2, FVC% was higher in sarcoidosis 98.1 (IQR 14.5) *vs* 76.7

(IQR 42.5) in HP; the same was observed in DLCO% (p=0.019), sarcoidosis 93.6 (IQR 29.1) *vs* HP 42.5 (IQR 39.1).

Comparison of ATP concentrations in Sarcoidosis and HP groups:

We next assessed the differences between ATP concentration in BALF supernatants of sarcoidosis and HP groups. We found that HP patients had a lower median ATP concentration in BALF $4x10^{-11}$ (IQR 4.5 $x10^{-10}$) than sarcoidosis patients 1.64 $x10^{-9}$ (IQR 6.67x10 $^{-9}$) (p=0.019).

Comparison of ATP concentrations between the three groups:

We first performed a Kruskal-Wallis test that showed significant differences (p= 0.027) between the three groups. Pairwise comparisons demonstrated that the differences are due to the difference between HP group and the other two groups. The significance levels were: Sarcoidosis and PH (p=0.17), PH and others 1.72x10-9 (IQR $1.45x10^{-9}$) (p=0.013), Sarcoidosis and others (p=1.00). The differences between the three groups are illustrated in figure 3.

Correlation of total and differential cell count composition and ATP concentration in BALF of HP and sarcoidosis patients

There was a significant correlation between logATP concentration and the total number of cells(log) (Pearson correlation, R= -0.741, p=0.004). Due to the role of CD4 and CD8 T lymphocytes in the pathogenesis of sarcoidosis and HP we investigated their possible relation of ATP. For the purpose of this analysis, we only included HP and sarcoidosis groups and the

results were as follows: correlation of LogCD4 and LogATP in sarcoidosis patients (Spearman, R=-0.429, p=0.289); correlation of LogCD4 and LogATP in HP patients (Spearman, R=-0.41, p=0.493); correlation of LogCD8 and LogATP in sarcoidosis patients (Spearman, R=-0.333, p=0.42); correlation of LogCD8 and LogATP in PH patients (Spearman, R=-0.564, p=0.322); correlation of LogCD8 and LogATP in PH and sarcoidosis patients (Spearman, R=-0.757, p=0.003); correlation of LogCD4 and LogATP in PH and sarcoidosis patients (Spearman, R=-0.702, p=0.008).

At the same we verified a correlation of the percentage of lymphocytes with Log ATP in PH and sarcoidosis (Pearson correlation R= -0.615, p=0.025); correlation of the number of lymphocytes(log) and LogATP in PH and sarcoidosis (Pearson correlation R= -0.803, p= 0.001). In opposite there was no correlation with logneutrophils and logATP (Pearson correlation R= -0.676, p= 0.066).

Possible correlation between ATP and pulmonary function in PH and sarcoidosis groups

We next investigated the correlation of lung function tests (FVC%, FEV1%, DLCO%) and the concentrations of ATP in BALF. The only parameter that showed a significant correlation was DLCO percentage (Pearson correlation, R=0.581 p= 0.048). There was found no correlation in FVC% (Pearson correlation p= 0.158) and FEV1% (Pearson correlation p=0.093).

Comparison of the time of storage in the different groups

As there have been reports of ATP degradation with time in frozen samples, to ensure that all sample had the same time of storage we tested for differences in the time of storage. We found no significant the differences between the three groups (p=0.325).

Impact of the dilution factor in the ATP concentration

The dilution factor of BALF varies between 2 and 134 in 14 samples evaluated.

Tables and images

Table I- The table shows the patients demographics data: gender, smoking habits, mean and the standard deviation of patients' age (years).

Patient characteristics	HP	Sarcoidosis	Others
Subjects (n)	5	8	10
Age (yr)	48 (13)	39.1 (8.7)	59 (14)
Sex (male/female)	0/5	2 / 6	4/6
Never smokers / ex-smokers / current smoker	5/0/0	4 /4/0	9/1/0

Table II- The table shows the median and interquartil range (IQR) of BALF characteristics: total cell count (10^6 cells/uL), neutrophils, eosinophils, lymphocytes, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes expressed in percentage (%) and CD4/CD8 ratio.

BALF characteristics	HP	Sarcoidosis	Others
Total cell count (IQR)	320 (295)	100 (58)	80 (118)
Neutrophils % (IQR)	18 (27)	1 (4)	4 (7)
Eosinophils % (IQR)	0 (1)	0	0 (8)
Lymphocytes % (IQR)	65 (47)	9 (43)	13 (15)
CD4% (IQR)	40 (36.8)	69 (29.8)	62 (36.9)
CD8 % (IQR)	30.2 (23)	22 (30.5)	21 (33.8)
CD4 / CD8 (IQR)	0.95 (1.91)	3.17 (16.25)	2.3 (3.2)

Table III-The table shows the median and interquartil range (IQR) of the lung function tests of the three patient groups (FVC %, FEV1 %, DLCO %).

Lung function tests	HP	Sarcoidosis	Others
FVC % (IQR)	84.3 (45.4)	97.3 (17.7)	111.9 (17.2)
FEV1 % (IQR)	76.7 (42.5)	98.1(14.5)	107 (23.5)
DLCO % (IQR)	42.5 (39.1)	93.6 (29.1)	91.5 (27.1)

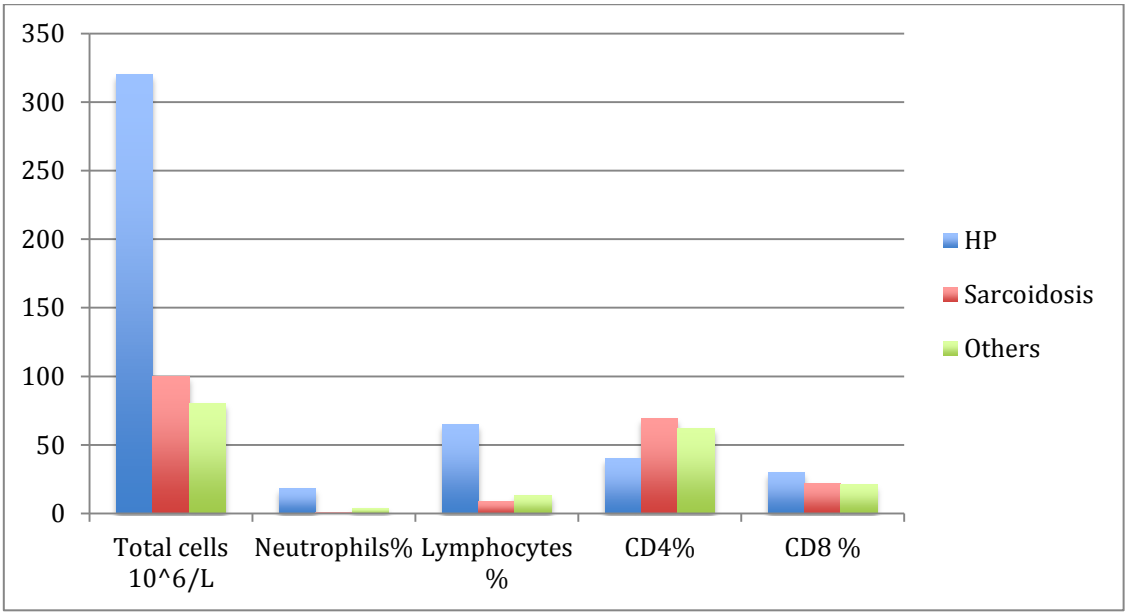

Figure 1. BALF characteristics of the three groups, HP, sarcoidosis and other diseases, including total cells(10^6 cells/L), neutrophils(%), lymphocytes(%), CD4(%) CD8(%) expressed by median. Patients with HP had higher Total cells count, lymphocytes(%), neutrophils(%) and CD8 T lymphocytes(%). In opposite, sarcoidosis patients showed a higher CD4 T lymphocyte (%).

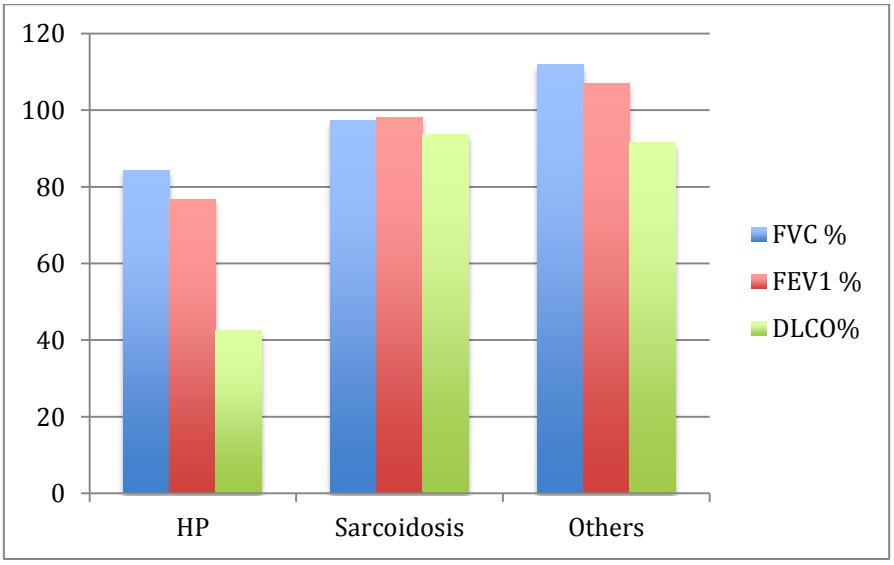

Figure 2. Lung function tests (FVC%, FEV1%, DLCO%) in HP, sarcoidosis and others groups. FVC% and DLCO% are lower in HP than in sarcoidosis and others.

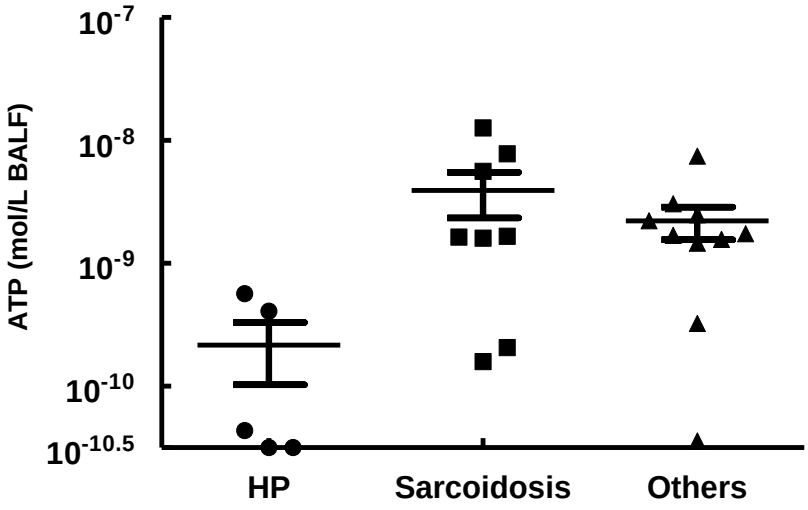

Figure 3. Comparison of ATP concentrations in BALF of the three groups (HP n=5, sarcoidosis n=8, others n=10). Significant differences were found between the three groups (p= 0.027). Two values of HP patients were below the detection limit of the technique. The vertical axis shows a logarithmic scale of ATP concentration.

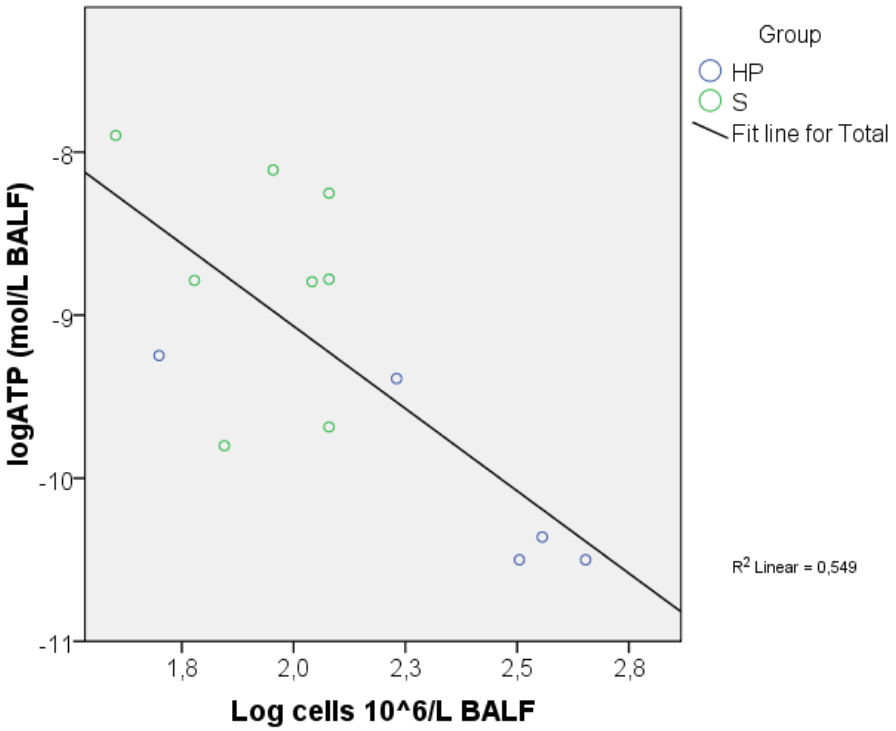

Figure 4. Correlation of logATP (mol/L BALF) and log cells (10^6) (p= 0.004) in sarcoidosis patiens (n=8) and HP patients (n=5) .

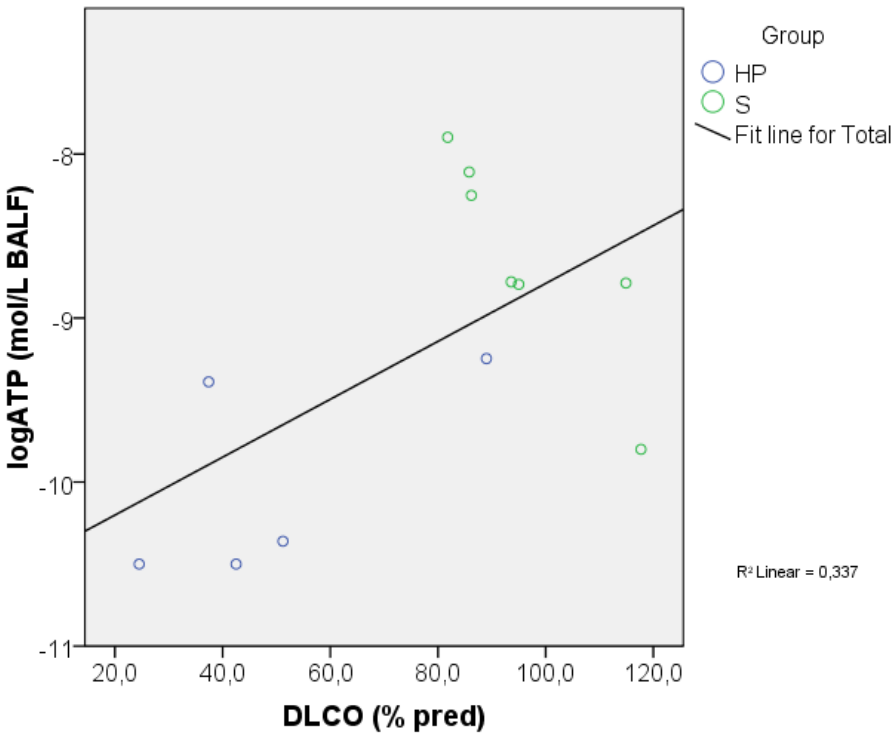

Figure 5. Correlation of logATP (mol/L BALF) and DLCO% (p= 0.048) in sarcoidosis patiens (n=8) and HP patients (n=5).

Discussion

The main finding of our study was a significant difference in extracellular ATP between sarcoidosis and HP, with HP patients displaying lower ATP concentrations in their BALFs.

As it was expected, the percentage of lymphocytes and neutrophils was higher in HP than in sarcoidosis. In fact, in both cases, it is predictable that early-stage disease BALF presents a predominance of lymphocytes, whereas in the end-stage, including fibrosis, the percentage of neutrophils can rise substantially. Furthermore, the median of CD4/CD8 ratio was 3.17 in sarcoidosis, 0.95 in HP and 2.3 in other diseases. Actually, analyzing the observed differences between lung function tests, we could assess the impact of these diseases on the subjects. FVC% and DLCO% are lower in HP than in sarcoidosis which can mean that there is a great impairment of function in HP patients compared to sarcoidosis group.

Surprisingly, we observed significant differences in ATP concentrations between HP patients (in the order of 10^{-11} mol/L) and in sarcoidoisis (in the order of 10^{-9} mol/L). In fact, extracellular ATP in the BALF of sarcoidosis patients has been described in a previous study by Lommatzsch et al. (2010), where no differences were found in concentrations of sarcoidosis patients and the healthy control group, leading the authors to conclude that sarcoidosis patients had normal ATP levels in BALF. This study included a sarcoidosis group in order to show that it is unlikely that the increase in ATP concentrations found in COPD patients reflects elevated cell numbers in BALF (Lommatzsch et al., 2010) Despite the differences in methodology, and considering the lack of a dilution analysis, we quantified levels of ATP in the BALF of patients with sarcoidosis (1.64 $x10^{-9}$ mol/L) that are similar to these described by the authors ($\pm$ 15 nmol/ml).

Our results also show a clear negative correlation between ATP concentration in both groups with the total cells in BALF. Although similar correlations were found with the differential cells percentage (lymphocytes), this needs to be carefully interpreted and integrated with the pathophysiology of the diseases, and larger studies are essential to understand the impact of each cell type in this correlation. Furthermore, the positive correlation of ATP with DLCO, suggests a possible inverse correlation between extracellular ATP concentration and the impairment of lung function.

Despite the significant correlation found between CD4 cell count and ATP levels and CD8 cell count and ATP levels when we analyzed in the two groups together, the lack of the same correlation in HP and sarcoidosis separately, is strongly limited by the size of cohort (5 and 8, respectively).

There are two hypotheses to understand ATP concentrations in BALF. On the one hand, purine concentrations in BALF may be overestimated because BALF is collected invasively and the mechanical and osmotic forces generated during lavage may trigger ATP release by airway epithelial (induced by bronchial washing), or cellular ATP leakage. On the other hand, measured ATP concentrations are probably underestimated because of rapid ATP degradation in biological fluids and because of the dilution of the liquid covering alveolar epithelium during the lavage. Enzymatic ATP degradation may also occur during sample processing. ATP concentrations in the close vicinity of cells are probably higher than measured, and are thus able to reach concentrations required for purinergic receptor activation. (Riteau et al, 2010). In fact, we cannot also exclude the possibility of ATP may have been degraded during storage and sample handling, despite our best care to prevent this during manipulation. However, all samples were similarly manipulated, namely with a similar storage period, as shown in the results.

For this study it was important to assure that the differences in ATP concentrations were not influenced by the smoking history of the patients, and natural course of the diseases by the use of systemic or inhaled therapy. The exclusion of patients with an acute infection eliminates another potential source of extracellular purines, bacteria, although inflamed airway epithelia can be significant contribution from other inflammatory cells.

Despite our initial plan to control the dilution of BALF samples using the urea assay, we faced practical problems and this could not be performed in all samples. We chose urea because of its small molecular weight, and because urea reaches complete equilibrium across the capillary-alveolar membrane, allowing us to calculate a serum urea to BALF urea ratio (Rennard *et al.* 1986). The ERS guidelines for the measurement of acellular components and standardization of BAL states that urea is widely used as an internal marker, but this report reviews evidence showing that urea has several limitations for use as an internal marker of dilution since it continues to pass rapidly from the blood and tissue spaces into the newly instilled lavage fluid (Haslam & Baughman, 1999). Regarding the problem of the unknown BAL dilution, the Task Force concludes that there is still no method to accurately determine the dilution factor and recommends efforts for the reduction of the variability of the procedure and analysis. However, the 14 samples analyzed suggest that our results were consistent with those previously reported 2- 134 (10–100-fold).

In this study, to measure the ATP concentration we used a very sensitive luminescent technique able to detect very low ATP concentration. Because of ATP's unique chemical properties, other techniques, as chromatography/mass spectrometry, cannot be easily used. (Làzar et al, 2010). We ruled out possible interactions with BALF constituents with kinetics analysis of luciferase, we tested the presence of ATP with apyrase and daily reproducibility was guaranteed (data not shown). In fact, HP concentrations were very closely to the

detection limit (2 inferior) but this fact doesn't interfere in our results and the differences may be even higher.

Our results could suggest two alternative possibilities to understand the different levels of ATP in HP and sarcoidosis: One possibility is that the ATP concentrations are related with the pathogenesis of these diseases and may reflect the differences in purinergic signaling in both diseases. The other possibility if that the differences result from a different extracellular catabolism of ATP. These differences could be due to the total cells count or to differences in BALF constituents (different types of ectonucleotidases profile in each disease). In fact, if the differences found between sarcoidosis and HP are confirmed a large scale study, extracellular ATP can have a possible role in the different diagnosis of these disorders, particularly if the same results can be transported to exhaled breath condensate (EBC), although this would probably require a technique of improved sensitivity and lower detection limit. This could actually give us an indirect evaluation of total cells counts in the lung parenchyma. EBC has been used as a non-invasive method to assess airway purine concentrations. The advantage of this method is that it only requires the subject to breathe normally through a chilled tube, which does not introduce osmotic or mechanical forces that may compromise purine concentrations (Esther et al, 2011).

In spite of the tentative conclusions derived from the present study, it would be advisable to replicate the present findings in a larger cohort of patients with sarcoidosis and HP, including patients with an advanced stage of fibrosis, as extracellular ATP is a danger signal and is involved in the establishment of lung fibrosis (Riteau, 2010). Recently, a new therapeutical target for IPF was proposed, the P2X7 receptor for which inhibitors where shown to be effective in animal models of neurological diseases. (Riteau, 2010)

Also to complement this study, additional definition of the profile of the activity of ectoendonucleotidases in BALF from patients with each disease and their correlation with BALF constituents would be of interest, specially the study of CD4 and CD8 T lymphocytes. We also tried an initial approach to the study of eosinophilic diseases as eosinophils are also related with purinergic signaling. In fact, in a patient with eosinophilic leukemia (total cells $1890x10^6$/L), we found the third lowest value of ATP. At the same time, it also could be interesting extending to certain types of tumors like leukemia and lymphoma.

Our study has some limitations. First, the small size of this cohort limits the statistical power of the comparisons and correlations attempted. Additionally, the collection of clinical information and diagnoses in a retrospective manner always limits the strength of the proposed conclusions. Finally, it is worth noting that the present study did not include a formal control group. In fact, BALF are not usually performed on healthy subjects, thus hampering the availability of this relevant group of individuals. To overcome this limitation, we included a third group of patients with different other lung diseases that mostly have an increase in the percentage and number of lymphocytes serving as a control group.

In conclusion, the present study suggests that the pulmonary extracellular ATP concentrations in BALF of patients with HP is lower than in patients with sarcoidosis and this could be related with the total cell count in BALF. It should be noted that the strength of these conclusions are limited by the size of the cohort of BALF samples analyzed, the design of the study as a retrospective collection of information and the limitations inherent to the methodology. It is hoped that future prospective studies carried out in a larger number of patients with hypersensitivity pneumonitis and sarcoidosis may confirm the presently proposed conclusions, as well as elucidate about possible correlation with the differential cell proportion. This conclusion raises the possibility for a role of the evaluation of pulmonary

extracellular ATP in the differential diagnosis of these diseases and a significant role of the purinergic signalling pathways in these diseases.

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Supplementary data

Table IV- List of cases with information on age, gender, BAL date, Sarcoidosis stage by Chest CT, Smoking habits, High Blood Pressure (HBP) and Therapeutics.

HP- Hypersensitivity pneumonitis, S- sarcoidosis, LT- Lung Tumor, Pnc – pneumoconiosis, WG- Wegener Granulomatosis, Pm – Polimiositis, EPn – eosinophilic pneumonia, EL – Eosinophilic Leukemia, CLLB – Chronic Lymphoid Leukemia B, F- female, M – male, NS – never smoker, Ex – Ex smoker, PY - Pack years, NSS – No systemic steroids

Cases	Age, yrs	Gender	BAL Date	Stage Chest CT	Smoking Habits	HBP, Therapeutics
HP1	58	F	09/08/11		NS	HBP, NSS
HP2	31	F	16/08/11		NS	NSS
HP3	57	F	30/05/11		NS	NSS
HP4	37	F	26/05/11		NS	Atorvastatin, NSS
HP5	57	F	18/07/11		NS	Simvastatin, NSS
S1	36	F	01/08/11	II	Ex 15 PY	NSS
S2	52	F	08/08/11	III	Ex 25 PY	Steroids history
S3	41	M	16/08/11	II	NS	NSS
S4	40	F	26/09/11	II Lofgren	Ex	NSS
S5	36	F	19/09/11	II	NS	NSS
S6	35	F	06/12/11	II Lofgren	NS	NSS
S7	49	F	19/10/11	I	NS	Atorvastatin, NSS
S8	24	M	24/11/11	I	Ex 5 PY	
LT1	59	M	06/12/11		Ex 100	
LT2	55	F	06/09/11		NS	

Pnc1	52	F	08/11/11		NS	NSS
Pnc2	56	M	08/11/11		NS	HBP, NSS
GW	38	F	20/06/11		NS	NSS
GW	70	F	08/09/11		NS	HBP, Symbicort®
Pm	52	F	18/07/11			Methylprednisolone
EPn	82	M	07/07/11		NS	HBP
EL	47	M	23/08/11			Prednisolone
CLLB	79	F	15/06/11		NS	HBP, Methylprednisolone

Table V –Lung Function in different patients. FVC - Forced Vital Capacity, FEV1 %, FEV1/FVC, DLCO,

Cases	FCV%	FEV1%	DLCO
HP1	59.8	61.4	24.5
HP2	43.8	48.6	37.4
HP3	88.1	84.7	42.5
HP4	84.3	76.7	51.2
HP5	106.3	110.3	89
S1	91.63	91.7	86.2
S2	108,8	109,5	85,8
S3	91.08	95.04	117.7
S4	135.5	132.8	114.9
S5	89.5	92.6	81.8

S6			
S7	97.3	98.1	93.6
S8	98.1	103.5	95
T1			
T2			
Pnc1	107.78	104.35	86.5
Pnc2	116.34	125.43	113.9
UG	111.9	107	91.5
UG	127.43	121.74	112.8
Pm	101.5	95.9	86.1
Pne			
EL			
CLLB			

Table VI– BALF cellular characteristics

Cases	Cells/ uL	Neutrophils %	Eosinophils%	Lymphocytes%	CD4/CD8
HP1	450	39	0	20	3.04
HP2	170	2	0	65	2.07
HP3	320	18	0	65	0.7
HP4	360	6	2	73	0.95
HP5	50	22	0	25	0.58
S1	120	3	0	5	3.05
S2	90	0	0	1	0.78

S3	70	4	0	18	2.21
S4	60	0	0	6	3.29
S5	40	5	0	12	1.33
S6	120	2	0	69	31
S7	120	0	0	55	22
S8	110	0	0	2	5.2
T1	60	2	0	24	0.58
T2	90	4	0	27	2.1
Pnc1	70	8	0	14	3,.8
Pnc2	160	3	0	4	0.89
UG	30	4	2	12	2.54
UG	40	9	0	22	4.22
Pm	180	6	0	40	0.56
Pne	50	16	27	10	8.75
EL	1890	0	74	8	1.33
CLLB	140	1	0	10	3.94

Table VII– ATP concentrations in BALF of the diferente patients.

Cases	ATP mol/L
HP1	3,16 x10-11*
HP2	4,08611E-10
HP3	3,16 x10-11*
HP4	4,36E-11
HP5	5,66003E-10
S1	5,60264E-09
S2	7,77911E-09
S3	1,58347E-10
S4	1,63587E-09
S5	1,26614E-08
S6	2,06174E-10
S7	1,66397E-09
S8	1,61E-09
T1	1,74681E-09
T2	2,49E-09
Pnc1	1,57341E-09
Pnc2	1,69796E-09
UG	7,49135E-09
UG	3,06459E-09
Pm	3,25223E-10
Pne	1,46324E-09
EL	3,60246E-11
CLLB	2,22104E-09

*inferior detection limit