

Inflammatory response in human lung cells stimulated with plasma from COPD patients

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ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) is a condition resulting from a persistent inflammatory state in the airways even after smoking cessation. Intriguingly, the reasons behind this persistence of the inflammatory influx without smoking exposure have not been fully unraveled. We aimed to explore the hypothesis that systemic inflammation in COPD patients influences lung cell inflammatory response.

Methods: We cultured human lung fibroblast and human airway epithelial cell lines with plasma from COPD patients (four emphysematous-COPD, four asthma-COPD overlap, four chronic bronchitis-COPD, and four bronchiectasis-COPD), and four smokers or ex-smokers without COPD as controls. Non-stimulated cells were used as controls. We measured Interleukine-8 (IL-8), C-reactive protein (CRP) and matrix metalloproteinase-9 (MMP-9) in plasma and culture supernatants by ELISA.

Results: Cells stimulated with plasma from COPD patients and non-COPD smoker subjects produced higher CRP, IL-8 and MMP-9 levels, an increase for COPD in CRP ($p=0.029$) in epithelial cells and IL-8 ($p=0.039$) in fibroblasts and decrease for MMP-9 ($p=0.039$) in fibroblasts, compared with non-stimulated cells. The response was higher in epithelial cells for IL-8 ($p=0.003$) and in fibroblasts for MMP-9 ($p=0.063$). The plasma from chronic bronchitis and bronchiectasis phenotypes induced higher IL-8 in fibroblasts.

Conclusions: Plasma from COPD patients increases the inflammatory response in lung epithelial cells and lung fibroblasts, with a different response depending on the cell type and clinical phenotype.

Key words: COPD; inflammation; MMP-9; IL-8; CRP.

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Availability of data and materials: The data that support the findings are available from the corresponding author on reasonable request.

Introduction

Chronic obstructive pulmonary disease (COPD) is associated with several extra-pulmonary manifestations [1,2] including nutritional abnormalities and weight loss, skeletal muscle dysfunction and cardiovascular, nervous system, metabolic, and osteoskeletal effects. Additionally, systemic inflammation, with an increase in serum levels of inflammatory mediators and an activation of circulating inflammatory cells [3-5], has been considered as an important systemic effect of COPD with three main implications. First, it seems to be a key link between the pulmonary disease and its related systemic manifestations [6]. Second, the persistence and intensity of this systemic inflammation have been associated with the clinical expression and prognosis of the disease [7]. Finally, this systemic inflammation has been associated with the risk of COPD [8], suggesting a potential, albeit not yet fully understood, role in the initiation of the disease.

Up to now, the relationship between respiratory and systemic inflammation has been explored in a unidirectional way. In particular, different authors have explored the lung tissues as a potential origin of this systemic inflammation, coining the spill-over hypothesis [9-11]. However, the potential relationship of systemic inflammation with respiratory inflammation has not yet been explored. When it is, this might reveal a mechanism worth studying further as a potential factor in the perpetuation of the inflammatory load after quitting exposure to risk factors (smoking) [12-14] or the initiation of a different inflammatory response leading to lung damage, as proposed by others [8].

In the present paper, we would therefore like to explore the hypothesis that the systemic inflammation response could have an influence on inflammation in the respiratory system. To test this hypothesis, we aimed to study the influence of systemic inflammation on lung epithelial cells and lung fibroblasts by culturing lung cells with plasma from COPD patients and non-COPD patients, where we measured the expression of the inflammatory biomarkers associated with COPD pathogenesis.

Materials and Methods

Subjects

From June 2016 to February 2017, plasma samples from COPD cases and non-COPD smokers were obtained. COPD cases and non-COPD patients (4:1) were recruited from our COPD-dedicated outpatient pulmonary clinic. Inclusion criteria for COPD cases were as follows: 1) male current and former smokers with a diagnosis of COPD with a post-bronchodilator forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) ratio <0.7 ; 2) a negative history of acute exacerbations in the previous three months; and 3) age 50-70. Nowadays, it is well-recognized that the clinical expression and long-term implications of gender may be influenced by gender. Therefore, as an initial evaluation of this effect, we focused on males, since in the majority of countries, COPD keeps being a predominantly males' disease (this is changing though). Now, with these results, we are planning to validate these results in patients of both sexes, and with a large sample size. The presence of a chronic or inflammatory disease (immunosuppression, chronic bronchial colonization, acute ischemic event in the previous six months, mechanical ventilation, congestive heart failure, end-stage renal failure, liver cirrhosis, autoimmune diseases, and diseases rheumatologic or active neoplastic disease), that may be associated with higher inflammatory markers, was considered as an exclusion criterion. To test for the different clinical COPD presentations, we aimed to include

samples from four clinically relevant phenotypes: 4 patients with emphysematous-predominant COPD, 4 patients with asthma-COPD overlap, 4 patients with chronic bronchitis predominant COPD and 4 patients with COPD-associated bronchiectasis. The diagnostic criteria for each one of the phenotypes were those accepted in the current national clinical guidelines [15]. We rely on GesEPOC to define a COPD patient as emphysematous [16].

Current and former smokers without COPD aged >50 years with an FEV_1/FVC ratio ≥ 0.7 were deemed eligible as controls. Samples from 4 non-COPD were selected from the smoking cessation unit at our hospital during the same period. The study protocol was approved by the Ethics Committee at Virgen del Rocio University Hospital and followed institutional and Good Clinical Practice guidelines.

Blood samples were drawn by venipuncture from each subject at rest and were centrifuged at 3000 rpm for 15 min and stored at -80°C until assayed. Written informed consent was obtained from all participants, and institutional review board approval was obtained (approval record 10/12, internal code 2012PI/244). All the samples were blinded by a numerical code, and the laboratory personnel were unaware of the COPD case-non-COPD status of each specimen.

Cell culture

Human lung fibroblast cell line (MRC-5) was obtained from the European Collection of Cell Culture (ECACC, Salisbury, UK) and human airway epithelial cell line (Nuli-1) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). MRC-5 cells were cultured as monolayers in MEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin-streptomycin and 2 mM L-glutamine (Gibco, Grand Island, NY, USA). Nuli-1 cells were cultured in airways epithelial cell basal medium supplemented with bronchial epithelial cell growth kit additives (ATCC) and cell culture plates were pre-coated with a 60 mg/ml solution of human placental type IV collagen (Sigma GmbH, Germany). MRC-5 and Nuli-1 were cultured at 5% CO_2 at 37°C and were seeded in 3.3 cm^2 wells (Nunc, Roskilde, Denmark) and co-cultured with 25% plasma from each one of the COPD patients included and from the non-COPD subjects, for 24 h. We also included one well with cells cultured without plasma, non-stimulated cells, which served as a control. The supernatants were collected, centrifuged and frozen until further measurements were taken. Interleukin (IL)-8, C-reactive protein (CRP), and matrix metalloproteinase 9 (MMP-9) were selected as markers of the different biological pathways associated with COPD pathogenesis, i.e. neutrophilic inflammation [17], systemic inflammation [18] and proteinase imbalance [19], respectively. ELISA (R&D System, Minneapolis, MN, USA) was used to measure the levels of these markers in culture supernatants and plasma from the subjects included, following the manufacturer's recommendations. To evaluate cultured cell production, the levels of cytokines in the patients' plasma were subtracted to the levels produced by the stimulated cells and to the level in the non-stimulated cells well. Data are expressed as means and standard error for continuous variables and frequencies and percentages for categorical variables. Group differences were explored using the Mann-Whitney U test (continuous variables) or the χ^2 or Fisher exact test (categorical variables). Due to the low number of experiments, statistical significance was assumed if $p < 0.1$.

Results

The characteristics of all plasma donors are summarized in Table 1. The baseline plasma levels of CRP, IL-8 and MMP9 in

COPD and non-COPD patients prior to incubation are summarized in supplementary Table 1. Neither the characteristics of the subjects, nor the results of the assays were influenced by the smoking status of the plasma donors. However, COPD patients were slightly older than non-COPD.

CRP protein expression in supernatants

The human lung fibroblasts stimulated with plasma from the COPD patients and non-COPD subjects produced higher levels of CRP than non-stimulated cells, with no difference between those stimulated with plasma from COPD and from non-COPD (Figure 1A). In the case of epithelial cells, those stimulated with plasma from COPD produced higher levels of CRP as compared to plasma from non-COPD and non-stimulated cells, with differences between those stimulated with plasma from COPD and

Table 1. Characteristics of the patients included.

	COPD (n=16)	non-COPD (n=4)	p*
Age	65.3 (5.6)	58.0 (4.8)	0.030
Body mass index	28.1 (4.2)	28.7 (7.5)	NS
Current smokers	4 (25)	2 (50)	0.003
Tobacco history (pack/year)	59.6 (21.9)	28.7 (44.6)	NS
Receiving ICS	8 (50.0)	0 (0)	NS
FVC (%)	86.8 (18.9)	119.4 (22.5)	0.038
FEV ₁ (%)	59.3 (20.5)	113.5 (7.7)	0.002

* Calculated by Mann-Whitney test or Fisher exact test.

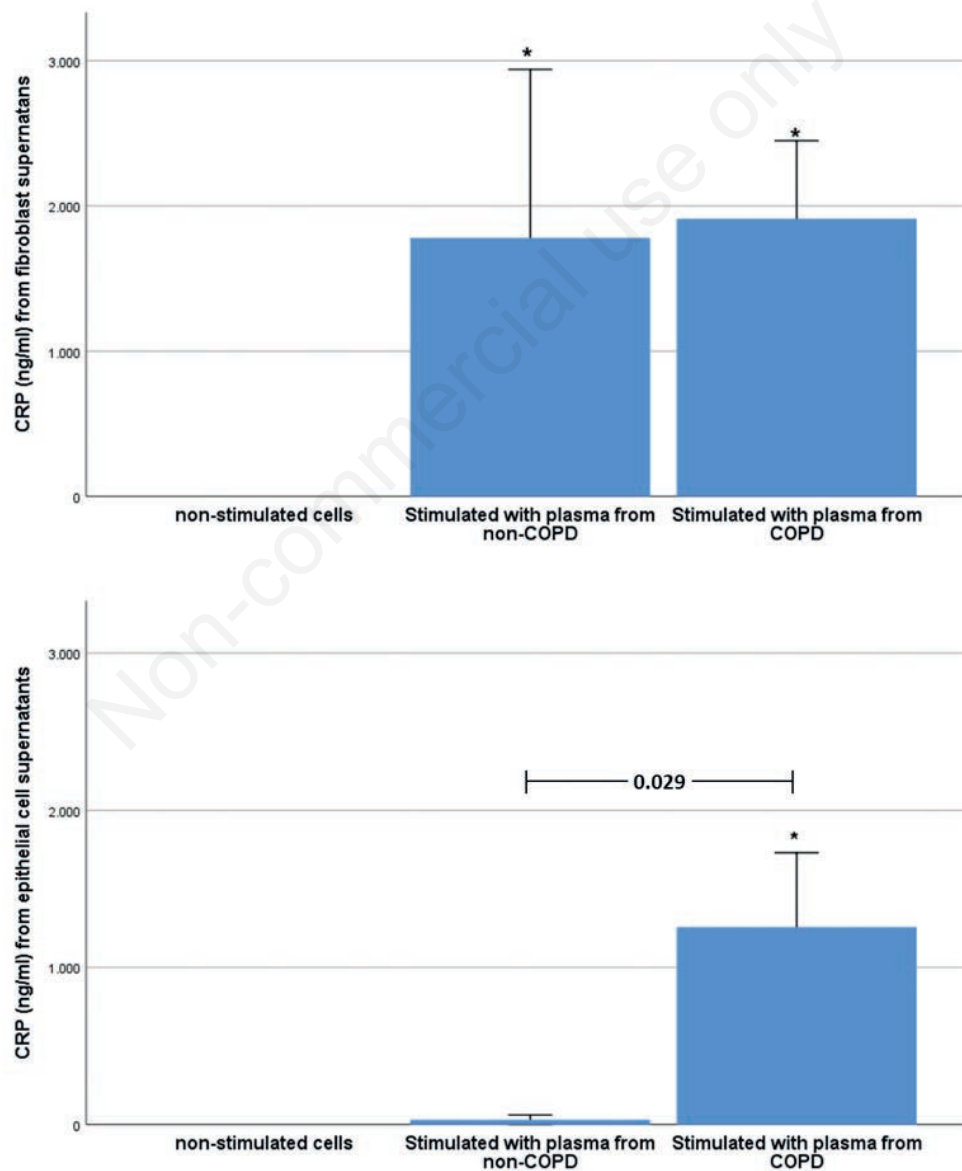


Figure 1. CRP levels in fibroblast (A) and epithelial cell (B) supernatants. * $p < 0.001$ compared to non-stimulated cells. Whiskers represent standard error.

from the non-COPD (Figure 1B). Notably, the protein expression for epithelial cells did not increase compared to fibroblasts (no significant differences). The differential expression of CRP according to the different phenotypes is shown in Figure 2. Both cell types presented a higher expression with plasma from chronic bronchitis phenotype patients. However, although there were visual differences between the different groups, only the comparison between the asthma-COPD overlap and the chronic bronchitis phenotypes reached the pre-specified significance threshold, in fibroblasts (Figure 2A). Interestingly, fibroblasts cultured with plasma from the non-COPD subjects presented increased expression in a similar way to different disease phenotypes, which did not occur for epithelial cells, although these differences did not reach the pre-specified significance threshold. No significant differences were observed between the different groups in epithelial cells (Figure 2B).

IL-8 protein expression

The human lung fibroblasts stimulated with plasma from COPD patients and non-COPD subjects produced higher levels of IL-8 than non-stimulated cells (Figure 3A), with an increased expression with plasma from COPD patients compared to the non-COPD subjects ($p=0.039$). In the case of epithelial cells, we observed that COPD patients and non-COPD subjects produced higher levels of IL-8 than non-stimulated cells, and although there were visual differences between non-COPD subjects and COPD patients, the differences did not reach the pre-specified significance threshold (Figure 3B). Notably, the protein expression for epithelial cells was higher compared to fibroblasts ($p=0.003$ for COPD and $p=0.086$ for non-COPD). The differential expression of IL-8 according to the different phenotypes is shown in Figure 4. In fibroblasts, we found that the bronchiectasis phenotype behaved differently and produced significant differences to the non-COPD, the emphysematous phenotype and the asthma-COPD overlap

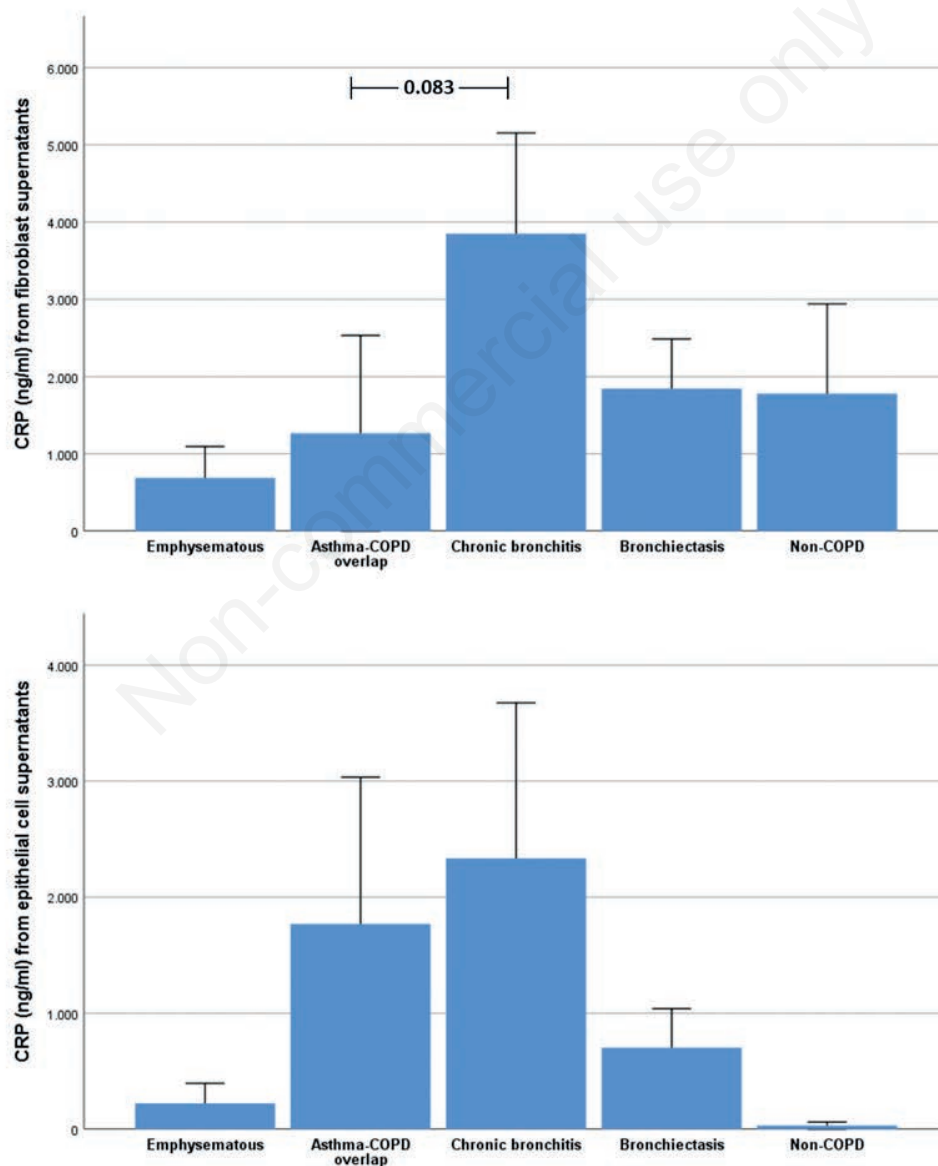


Figure 2. CRP levels from fibroblast (A) and epithelial cell (B) supernatants among different phenotypes, showing significant associations. Whiskers represent standard error.

($p=0.029$ for all comparisons) (Figure 4A). Interestingly, although there were visual differences between the non-COPD and the different phenotypes for epithelial cells, the differences did not reach the pre-specified significance threshold (Figure 4B).

MMP9 levels in plasma and supernatants

The human lung fibroblasts stimulated with plasma from COPD patients and non-COPD subjects produced higher levels of MMP-9 than non-stimulated cells with an increased expression with plasma from non-COPD subjects compared to COPD patients ($p=0.039$) (Figure 5A). In the case of epithelial cells, we observed significant differences between non-stimulated cells and two of the groups, although these differences were not statistically relevant when comparing non-COPD subjects and COPD patients (Figure 5B). Notably, the protein expression for fibroblasts was higher compared to epithelial cell only for COPD ($p=0.063$). The differential expression of MMP-9 according to the different phenotypes

is shown in Figure 6. In fibroblasts, we found various differences, with the asthma-COPD overlap producing the highest expression, followed by bronchiectasis, chronic bronchitis and, finally, the emphysematous patients (Figure 6A). As for epithelial cells, bronchiectasis produced the highest protein expression among the different phenotypes (Figure 6B).

Discussion

The present study explores the potential influence of the systemic inflammatory load on the inflammatory expression of lung cells. Our results show that i) the cells stimulated with plasma from the COPD patients and non-COPD subjects produced higher levels of CRP, IL-8 and MMP-9, with a significant increase for COPD compared with plasma from non-COPD in CRP in epithelial cells and IL-8 in fibroblasts, together with a decrease for MMP-9 levels

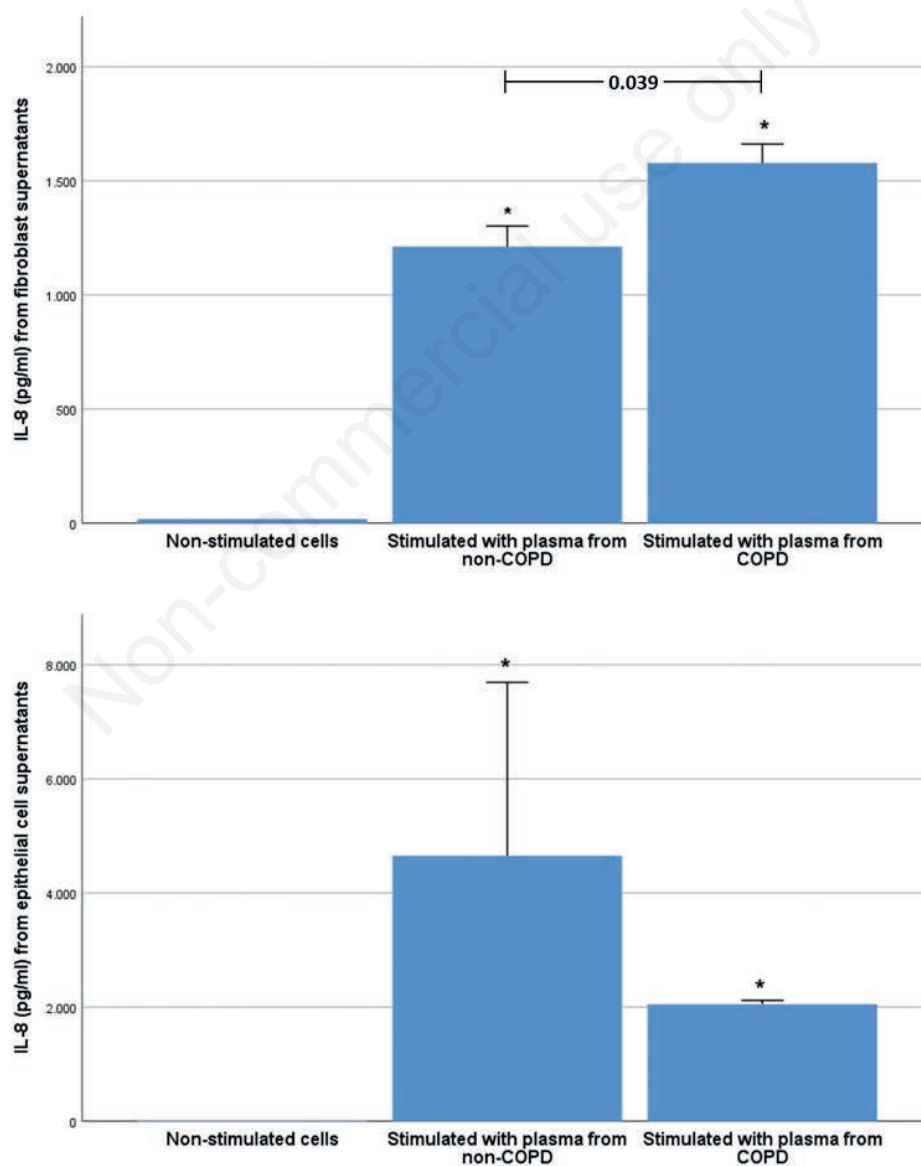


Figure 3. IL-8 levels from fibroblast (A) and epithelial cell (B) supernatants. * $p<0.001$ compared to non-stimulated cells, showing significant associations between groups. Whiskers represent standard error.

in fibroblasts; ii) comparing both cell types, the response was significantly higher in epithelial cells for IL-8 and in fibroblasts for MMP-9; iii) when exploring the different phenotypes, plasma from chronic bronchitis and bronchiectasis phenotypes exerted a higher inflammatory response in both cell types for CRP and for IL-8 in fibroblasts. Non-COPD patients were slightly older than COPD patients, however, their FVC and FEV₁ values were normal. Therefore, this age difference did not interfere with the results observed. MMP-9 did not increase in plasma from emphysematous patients. This evidence in an *in vitro* model does not necessarily demonstrate that systemic inflammation has an *in vivo* effect on inflammation of the respiratory system, since similar mediators may also be locally present in the airways. However, our results show preliminary data from a possible *in vitro* model, which will allow us to explore further the systemic inflammatory response.

The study of the persistence of the inflammatory response in

COPD is a current topic of debate [12]. To determine the degree of systemic inflammation could help assess disease severity and follow up in these patient groups. One of the main drivers of systemic inflammation is cigarette smoke, which subsequently reduces lung function. A number of studies have indicated that airway inflammation in smokers persists even after smoking cessation [12-14], with differences according to the smoking cessation time [20,21] and the type of inflammation [22]. Intriguingly, the reasons behind this persistence of the inflammatory influx without smoking exposure are not fully known, with a possible role of auto-immune phenomena [23] and immunosenescence [24] as potential explanatory mechanisms. A previous study in patients with COPD provided early insight into likely phenotypical differences between lifelong never-smokers and ever-smokers [25]. Furthermore, cigarette smoking and pro-inflammatory cytokine influence blood cell activation and release. Previous studies report-

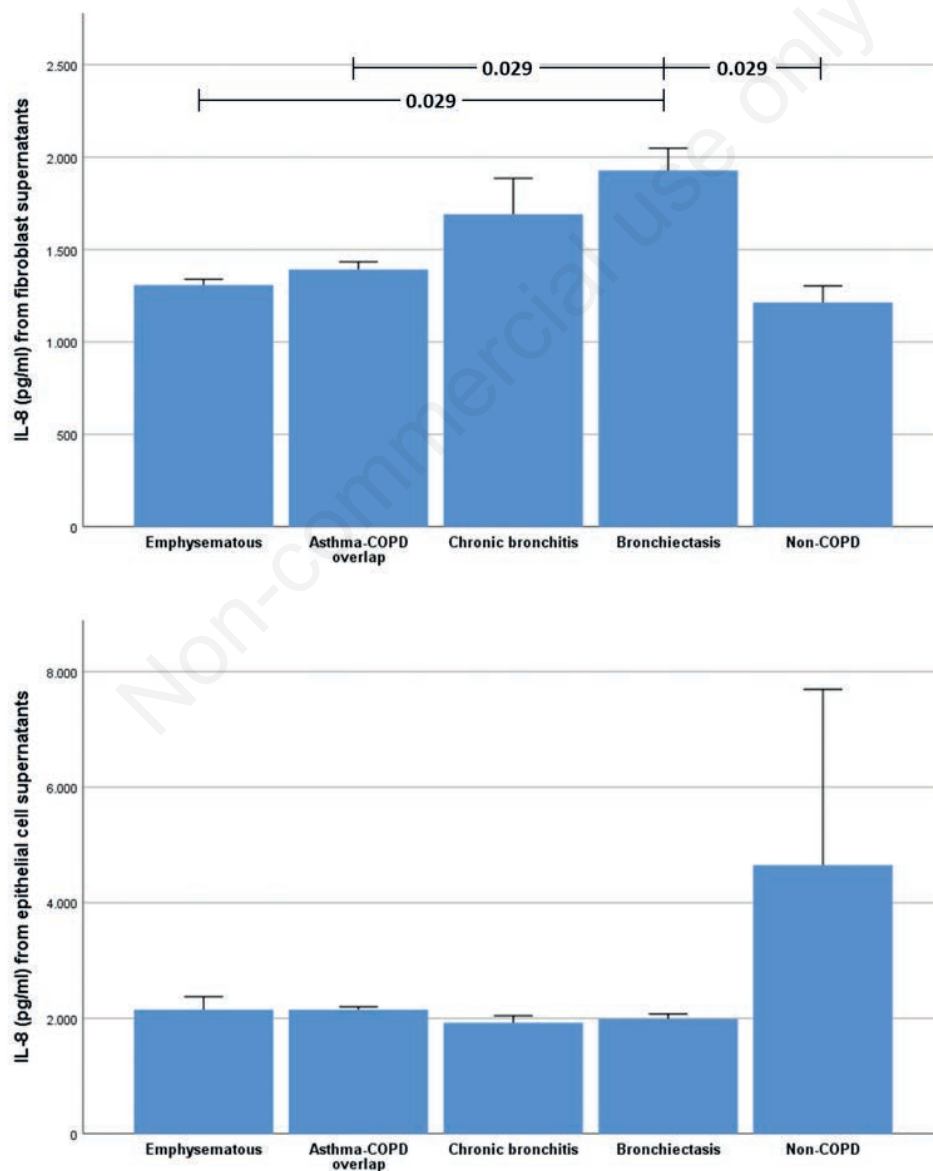


Figure 4. IL-8 levels from fibroblast (A) and epithelial cell (B) supernatants among the different phenotypes, showing significant associations between groups. Whiskers represent standard error.

ed that plasma from COPD had elevated concentrations of tissue inhibitor of metalloproteinase-1, α_1 -anti-trypsin, tumor necrosis factor- α , fibrinogen, an increased circulating neutrophil and activated neutrophils and eosinophils levels; which can affect the production of inflammatory markers [26,27]. A recent study determined the systemic inflammatory state by measuring the levels of indicators such as IL-8 and CRP, among other parameters. There is a typical pattern of inflammation with increased numbers of neutrophils and macrophages in the airway lumen of COPD patients [17]. The increase in neutrophils results in an increase in the production of chemokines, such as IL-8. Our results agree with these findings. We proposed that supernatants of fibroblast stimulated with plasma from patients with bronchiectasis, were capable of increasing the release of IL-8. The release of IL-8 observed in supernatants of epithelial cell stimulated with plasma from non-COPD could be due to the airway epithelial cells are the first cells

to encounter infections; in addition, these non-COPD are smoker, which is risk factors for inflammatory response.

Previous study observed lower CRP levels in never-smokers and increased in former and current smokers, which supports the “spill-over” theory in the development of COPD [28]. Our results agree with this theory. In particular, our results revealed that patients’ plasma with chronic bronchitis induced higher CRP levels in bronchial epithelial cells of ever-smokers, which suggests that this cell type may be involved, at least in part, in the systemic inflammation found in these patients.

Many *in vitro* studies, conducted in lung airway epithelial cells, have shown the changes in extracellular levels of various signaling molecules such as CRP. Also, in a previous study [9], our group demonstrated that the epithelial cells from COPD patients produced higher levels of CRP than fibroblasts, hence the relevance of conducting a study into the different phenotypes of

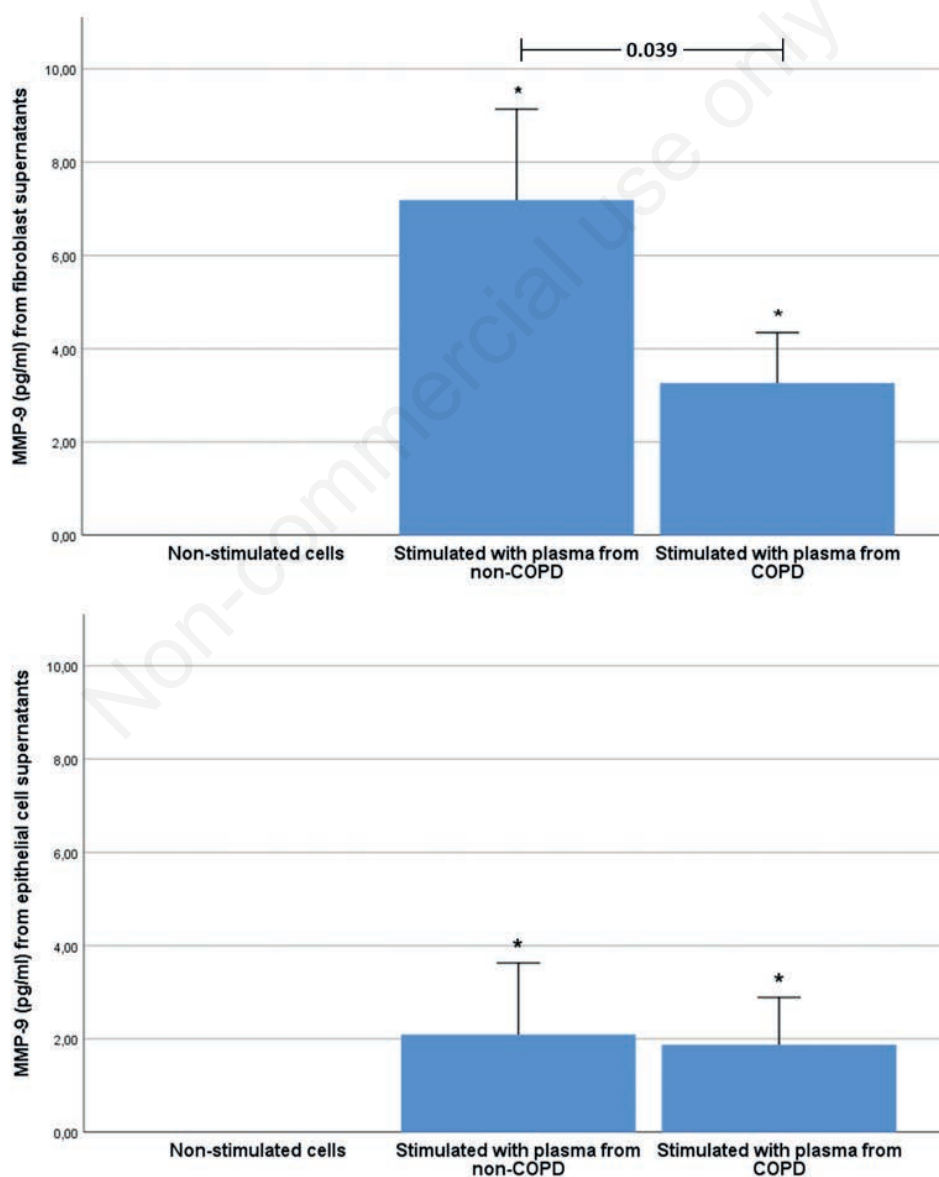


Figure 5. MMP-9 from fibroblast (A) and epithelial cell (B) supernatants. * $p < 0.001$ compared to non-stimulated cells. Whiskers represent standard error.

COPD, in order to determine the specific mechanisms involved in systemic inflammation. This finding could support that CRP levels are an indirect marker of inflammatory process that impacts on the lung function [18]. The potential implications of our findings may support the hypothesis of a possible self-perpetuated inflammatory stimulus between systemic and local inflammation. Our data represent a first approach, subsequent studies should evaluate this further in cells from COPD patients and study the potential clinical implications in patients. Although both cell types are involved in COPD pathogenesis, our results show that their behavior differs after culturing with plasma. The present results confirm the key role played by this cell type in the inflammatory load. Epithelial cells are the first barrier and are seriously affected by the remodeling that occurs in the lung tissue of patients with COPD [29]. In this regard, recent data suggest the central role of epithelial-mesenchymal transition in COPD [30]. Our study did not allow us to explore the role of epithelial-mesenchymal transition in this

inflammatory response, but it should be evaluated in future studies. Conversely, MMP-9 stimulation was higher in fibroblasts compared to epithelial cells. MMPs are zinc- and calcium-dependent endopeptidases responsible for extracellular matrix remodeling [31]. While their levels are reportedly low in normal human lungs, MMP-9 is abundant in COPD [19], with a specific participation of keratinocytes, monocytes, leukocytes, macrophages and fibroblasts as the source [32]. MMP-9 is an enzyme secreted by several cell types, including neutrophils, airway epithelial cells and macrophages. The increase in fibroblasts compared to epithelial cells may indicate that this cell type has an important role to play in MMP-9 mediated lung damage. Interestingly, the increase in MMP-9 after exposure to non-COPD plasma requires an explanation and warrants further research. Since airway inflammation has a compartmentalized importance [10], it is possible that the systemic influx interacts differently in the airways than in parenchyma participation.

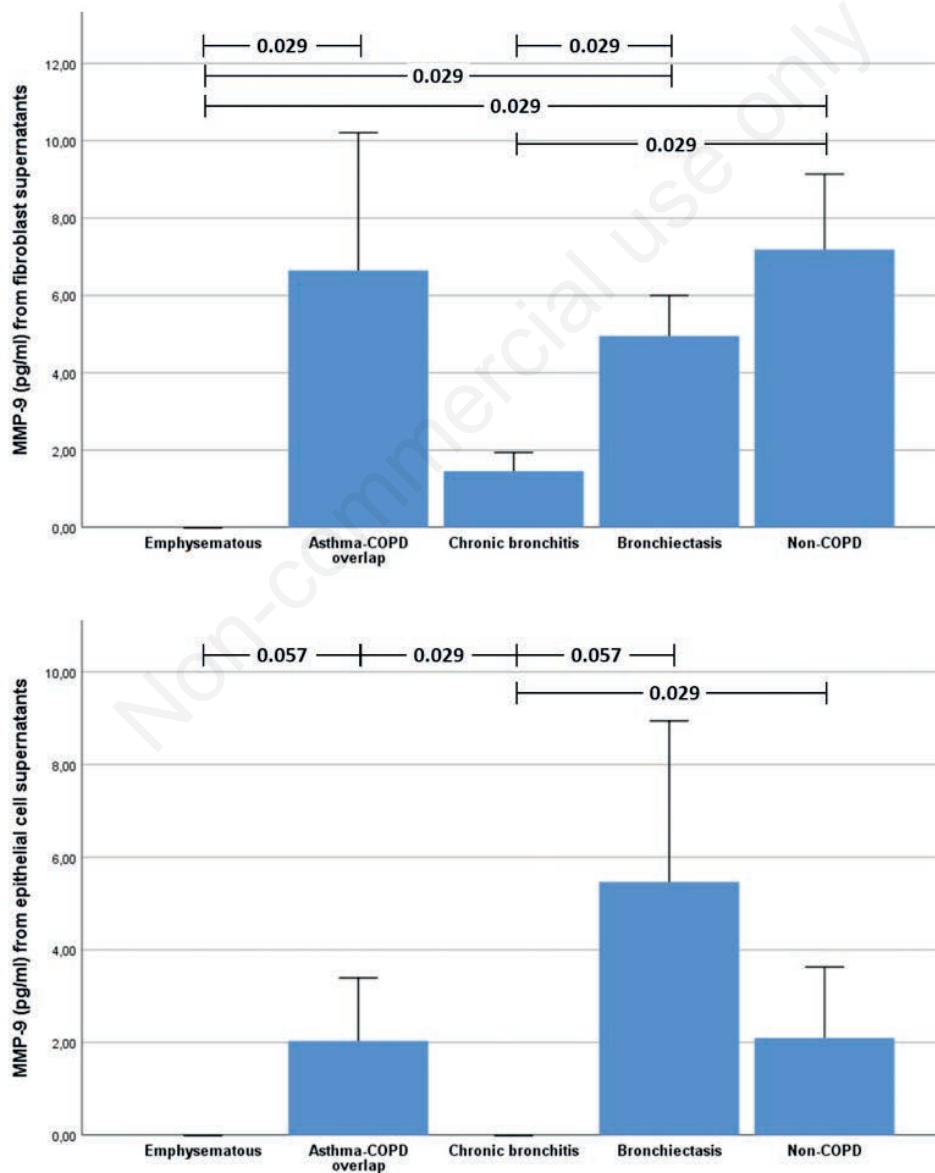


Figure 6. MMP-9 from fibroblast (A) and epithelial cell (B) supernatants among the different phenotypes. Whiskers represent standard error.

Although the different clinical expression of the disease is a characteristic feature of COPD, it was only from 2010 onwards when clinical phenotypes started to be studied more systematically, and some better characterized clinical phenotypes have been established [33]. Interestingly, the relationship between phenotypes and endotypes has not yet been studied in depth in COPD [34]. In the present study, we have been able to make an initial exploration of some differences in the inflammatory response of these clinical phenotypes. Of note is that MMP-9 does not increase after culturing with COPD plasma from emphysematous patients, as opposed to other phenotypes. This merits further study, but it may suggest that the MMP-9-dependant mechanism could be more dependent on the local than the systemic inflammatory influx. On the other hand, the pulmonary macrophages did not produce MMP in healthy lung, while, conversely, in the COPD lung microenvironment, immunocompetent cells are the main source of MMP-9 [35]. Furthermore, Koo *et al.* found a significant correlation between MMP-9 levels and severity of emphysema [36]. Consequently, the lower MMP-9 levels may be due to fact that the patient's emphysema is not so severe as to induce high levels of MMP-9 in the cells studied. Nevertheless, the role of endotypes in COPD and their relation to the clinical expression of the disease is relatively uncharted territory [37] and the data presented here should help to stimulate specific hypotheses for future work [38].

Several limitations of the present study should be taken into account. A potential major weakness is the cross-sectional design of the study, which could limit our results. On the other hand, another limitation of the present study relates to the cellular model used. Using commercial cell lines has allowed us to study cellular models and evaluate their cellular response *in vitro*. However, the actual conditions in the airway may differ considerably. Therefore, the next step in this line of research should involve the study of primary culture cell models in patients with COPD, and then move on to animal models to study this inflammatory response *in vivo*. Another stage will be to perform a stimulation experiment with a previously contrasted mediator, since this has been an exploratory proof-of-concept study to see if there was an answer. Sample size is a limitation of the study. The techniques used are complex and costly and we really aimed to do a proof-of-concept design, in which we wanted to verify whether the described effect exists. Now, with these results, we are planning to validate these results in patients of both sexes, and with a large sample size.

Conclusions

In summary, the present research uses a cellular model to explore the hypothesis that the cells of the respiratory system react with a pro-inflammatory stimulus when exposed to systemic inflammation from patients with COPD, with differences between the cell type studied and the type of COPD patient. These results open up new research questions about the relationship between local and systemic inflammation, which should be explored further and could lead to new therapeutic targets in the future.

Acknowledgments

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Abbreviations

COPD: chronic obstructive pulmonary disease;
IL-8: interleukine-8;
CRP: C-reactive protein;
MMP-9: matrix metalloproteinase-9;
FEV₁: forced expiratory volume in 1 second;
FVC: forced vital capacity;
ELISA: enzyme-linked immunosorbent assay;
MRC-5: human lung fibroblast cell line;
Nuli-1: human airway epithelial cell line;
MEM: Eagle's minimum essential medium.

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