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From cigarette smoking to Chronic Obstructive Pulmonary Disease or Idiopathic Pulmonary Fibrosis. Why?

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ABSTRACT

Chronic Obstructive Pulmonary Disease (COPD) and Idiopathic Pulmonary Fibrosis (IPF) are two chronic lung diseases with distinct clinical, pathological and epidemiologic features. In both disorders cigarette smoking represents a major risk factor; indeed, it causes a repetitive alveolar damage that elicit inflammatory response and may enhance the establishment of the disease. During my PhD, we investigated the role of innate and adaptive immune response in COPD and IPF, and how it may be related to different clinical outcomes.

In COPD, the role of inflammation is consolidated; alveolar macrophages (AM), which are one of the main actors of innate immune response, were firstly recognized to play a key role in the pathogenesis of the disease, by releasing protease that damage lung parenchyma. In our studies, we investigated how AM may be involved in the development of COPD with alternative mechanisms; for example, we analyzed AM polarization, classic (M1) and alternative (M2), in lung tissue and how it may be influenced by smoking and disease severity. The model of COPD as a disease caused only by an antiprotease-protease imbalance has been overtaken; indeed, not all subjects with α1-Antitrypsin (AAT) deficiency (AATD) develop disease, meaning that other immune regulatory factors are involved. In fact, we investigated α1AT polymerization in AM as a pro-inflammatory trigger, due to its abnormal accumulation in AM endoplasmatic reticulum. Moreover, we performed a genome wide analysis in siblings with AATD, with extreme phenotypes of the disease (emphysema/no emphysema), searching for genes variants that may modulate or promote inflammatory response, possibly explain these opposite manifestations.

Since the study of biomarkers is becoming of great interest in the field, we focused our research on adaptive immune cells in the blood, examining blood eosinophils (BE) and lymphocytes (BL), to investigate whether they can be predictor of clinical outcomes, and if they reflect what is happening in the lung. In addition, we have started to unravel the possible role of the peripheral BL in the development or prevention of cancer. Then we moved from peripheral blood to the lung tissue, examining how lymphocyte and eosinophil counts in the lung may change after the introduction of an anti-inflammatory treatment with Roflumilast, adding some clues on its mechanisms of action. Indeed, the study of lung infiltrate is mandatory to better understand the pathogenesis of the disease, and we are currently developing new techniques to deeply analyze the immune cells in COPD lung, and the presence of markers of immunomodulation.

In IPF, the role of inflammation is more debated than COPD. Our idea that an inflammatory reaction could be an important part of IPF has lead us to investigate the possible role of innate and adaptive immune response in the pathogenesis of the disease, not only in explanted lungs of end-stage IPF, but also in the surgical lung biopsies of patients with mild disease. The pathological evidences we found were then correlated to clinical outcomes, as disease progression, to study if they may account for the variability in lung function decline. Moreover, we also explored whether different lung tissue inflammatory profiles may impact on the response to Pirfenidone treatment, which exerts also an anti-inflammatory effect. Finally, we investigated HRCT as a possible non-invasive predictor of disease behavior and treatment response in IPF.

The results of the studies we have conducted so far, highlights how inflammatory response, and especially the immune regulation, is pivotal in COPD and IPF, adding novel findings to previous knowledge in the areas of innate and adaptive inflammation, and putting the basis for future research in these smoking-induced diseases.

RIASSUNTO

La Broncopneumopatia Cronica Ostruttiva (BPCO) e la Fibrosi Polmonare Idiopatica (IPF) sono due malattie croniche del polmone con caratteristiche cliniche e patologiche ben distinte. In entrambe le malattie, il fumo di sigaretta rappresenta un importante fattore di rischio in grado di determinare un danno alveolare cronico che stimola la risposta infiammatoria tissutale, predisponendo la possibile insorgenza della malattia. Durante il mio dottorato, abbiamo studiato il ruolo dell'immunità innata e acquisita nella patogenesi della BPCO e dell'IPF e la loro possibile correlazione con outcomes clinici. Nella BPCO, il ruolo dell'infiammazione è noto; i macrofagi alveolari (MA), tra i principali attori della risposta infiammatoria innata, erano stati inizialmente considerati come fondamentali nella patogenesi della malattia, in quanto rilasciano proteasi che danneggiano il parenchima polmonare. Nei nostri studi, abbiamo indagato come i MA possono essere coinvolti nello sviluppo della BPCO con meccanismi alternativi, analizzando ad esempio come la polarizzazione dei MA, classica (M1) e alternativa (M2), sia influenzata dalla storia di fumo e dalla severità di malattia. La BPCO non può essere considerata solo come una malattia da squilibrio proteasi/antiproteasi, infatti non tutti i soggetti con deficit di α1-Antitripsina (AAT) sviluppano la malattia, suggerendo la presenza di altri fattori che influiscono sulla risposta immune. Tra questi abbiamo studiato la polimerizzazione dell'AAT nei MA come possibile trigger pro-infiammatorio, e le differenze nell'espressione di geni coinvolti nella riduzione/stimolazione della risposta infiammatoria con una analisi genetica tra fratelli con deficit di AAT, che presentavano o meno enfisema. Ci siamo poi focalizzati sulle cellule dell'immunità innata nel sangue periferico, esaminando gli eosinofili (E) ed i linfociti (L), indagando se questi possano essere dei predittori di andamento clinico e rispecchiare la risposta infiammatoria presente nel tessuto polmonare. Inoltre, abbiamo anche studiato il ruolo dei L su sangue nello sviluppo/prevenzione dei tumori. Successivamente, ci siamo focalizzati sul tessuto polmonare, analizzando come il numero di L ed E nel tessuto possa variare a seguito dell'introduzione di un trattamento con un farmaco antinfiammatorio, il Roflumilast, evidenziandone anche un possibile meccanismo d'azione. Lo studio dell'infiltrato infiammatorio polmonare è fondamentale per cercare di comprendere meglio la patogenesi della BPCO, ed attualmente stiamo pertanto sviluppando nuove tecniche come la digestione polmonare, per indagare meglio la risposta immunitaria e l'eventuale presenza di markers di immumodulazione nel polmone.

Nell'IPF, il ruolo dell'infiammazione è più dibattuto rispetto alla BPCO. L'idea che la reazione infiammatoria possa essere un importante fattore causale anche nell'IPF ci ha portato ad esaminare il possibile ruolo della risposta innata e adattiva nella patogenesi della malattia, non solo in polmoni espiantati, ma anche in biopsie chirurgiche in IPF precoci. Le evidenze che abbiamo trovato a livello istologico sono state poi correlate con gli outcomes clinici come la progressione di malattia, per capire se queste possano spiegare la variabilità del declino funzionale. In aggiunta, abbiamo anche esplorato se un diverso profilo infiammatorio tissutale potesse determinare una diversa risposta al Pirfenidone, un farmaco anti-infiammatorio usato nel trattamento dell'IPF. Infine, abbiamo studiato se la TC potesse avere un ruolo non solo nella diagnosi, ma anche nel predire l'andamento clinico e la risposta al trattamento.
I risultati di questi studi ad oggi hanno evidenziato come la risposta infiammatoria, e soprattutto la regolazione immunologica, siano fondamentali per COPD e IPF, aggiungendo nuove conoscenze a quelle già presenti nel campo dell'immunità innata e acquisita, e gettando le basi per la ricerca futura sulle malattie fumo-correlate.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) and Idiopathic Pulmonary Fibrosis (IPF) are two severe, chronic and disabling lung diseases characterized by quite distinct pathological, clinical and epidemiologic features.

Indeed, principal features of COPD are the presence of an inflammatory cellular infiltrate of the small airways that leads to airway walls thickening and narrowing, and the destruction of alveoli (emphysema); these abnormalities cause a progressive and not fully reversible airflow limitation. Whereas in IPF, the exaggerated deposition of extracellular matrix in the interstitium determines a reduction of the lung volumes, causing a restrictive pattern.

Patients with COPD report chronic productive cough and a progressive shortness of breath that, over decades, ultimately leads to respiratory failure[1]. Conversely, IPF is an invariably fatal disease characterized by a relentlessly decline in lung function over years, and carries a worse life expectancy than that of many cancers[2]. Similarly, dyspnea is the most disabling symptom reported, associated to a typically dry cough.

The incidence and prevalence of these diseases are also quite different, since COPD affects more than 10% of the world's population over the age of 40 years and it is the 3rd cause of death worldwide[1]. Conversely, IPF, even if it is the most common of the idiopathic interstitial pneumonias, is considered a rare disease with a prevalence ranging from 8 to 50 persons per 100000 cases, varying according to geographical area and age[3].

Nevertheless, COPD and IPF share some common features, and one of the most important is indeed the risk factor of cigarette smoking.

Each puff of a cigarette contains more than 2000 xenobiotic compounds and 10^{14} free radicals that injure lung epithelial cells. Chronic exposure to all these pathogenic noxae causes a repetitive alveolar damage, which induce the productions of inflammatory mediators.

Therefore, cigarette smoking elicits the inflammatory response in the lung, and we believe that the key to better understand the pathogenesis of both diseases is to study the immune system and how its activation is modulated after the epithelial damage.

Indeed, in my PhD project I investigated the role of innate and adaptive immune response in both COPD and IPF, and how it may be related to different clinical outcomes.

Starting with COPD, the classic pathogenetic paradigms of protease/antiprotease imbalance was derived almost 50 years ago by the description that a deficiency of α1-Antitrypsin (AAT) was associated with emphysema[4], and few years later, that

neutrophil elastase was the target of AAT[5]. AAT is the archetypal member of the serine protease inhibitor superfamily, and the discovery of its anti-elastase function recognized the imbalance between proteases and anti-proteases as the hypothesis for the pathogenesis of emphysema due to AAT deficiency (AATD) and, eventually, also in 'common' COPD with normal AAT levels[6]. In addition, the observation of increased numbers of neutrophils and macrophages in the lungs of smokers, pointed to a connection between neutrophil elastase and macrophage proteinases as the primary effectors of lung destruction in COPD, further supporting this idea[7].

However, it has become increasingly evident that many individuals with AATD, even if they smoke, do evade the development of emphysema, indicating that other susceptibility modifiers, likely linked to the control of immune tolerance, are needed for the disease to develop, and that the mechanism of emphysema production in AATD, but probably in "common" COPD, goes far beyond the antiprotease deficiency.

AATD is a rare disease with a prevalence of approximately 0.06% of individuals of northern European, and about 95% of the significant clinical deficiency is caused by the Z variant of the protein[8,9]. The substitution of a glutamic acid by a lysine at position 342 in Z-AAT results in abnormal protein folding within the endoplasmic reticulum (ER), protein polymerization, and intracellular retention with consequent low AAT serum levels[8,9]. Thus, the effect of the Z mutation is not a failure of synthesis, indeed Z-AAT is processed normally until it reaches the final stage of the hepatocyte ER pathway, but a failure in folding and secretion. About 85% of the Z-AAT is removed by ER-associated degradation or aggregates to form polymers, while only 15% is secreted in the serum[10]. Polymerization of Z-AAT in the liver causes a "toxic gain of function" within hepatocytes[8], with ER stress and activation of NF-kB[11], triggering an inflammatory reaction in response to protein misfolding and polymerization that predisposes to neonatal hepatitis and liver cirrhosis[12].

Alveolar macrophages develop from fetal liver under the control of granulocyte-macrophage colony-stimulating factor in the first days of life[13], and they produce threefold higher AAT than blood monocytes[14] suggesting that alveolar macrophages are preprogramed by their liver origin or that, once in the lung milieu, they up-regulate AAT gene expression. It would seem that the normal production of AAT by alveolar macrophages, potentially increased under the modulation of inflammatory mediators and elastase, could well polymerize in the ER of alveolar macrophages in PiZZ individuals, not only contributing to loss of AAT function due to diminished secretion in the alveoli, but also, as in the liver, to "toxic gain of function" with all its complex and detrimental consequences.

Alveolar macrophage, except from the of AAT production, exerts others essential roles as in coordinating immunological functions, contributing to the maintenance of homeostasis in the lung. As an example, when the host is challenged by infection or injury, they play a critical part in the coordination of host defense and eventually tissue repair[15,16]. Upon encountering pathogens or danger signals, macrophages express a strong pro-inflammatory phenotype including cytokines and reactive oxygen and nitrogen species, which is recognized as the "classically activated" or M1, and can be produced in vitro in response to inflammatory stimuli like Lipopolysaccharides (LPS) or Interferon (IFN)-γ. By contrast, homeostatic signals, as well as Th-2 cytokines in vitro, induce macrophages to adopt phenotypes linked with tissue remodeling and repair; this phenotype is generally recognized as "alternatively activated" or M2[17,18]. Although it is clear that the M1/M2 classification might be simplistic[19] since a spectrum of macrophage phenotypes has been observed in in vitro experiments[18], the M1/M2 nomenclature[20] is still widely used and is still the basis for the description of macrophage behavior in human diseases as evidenced in recent publications[21,22].

Innate inflammation has unquestionably an important role in the first immune response towards epithelial damage inducted by smoking, however, by itself it could not fully explain the pathogenic mechanism of COPD. Indeed, after the description by Saetta and Cosio that the main inflammatory cellular component of the lungs in COPD were the $CD8^+$ and $CD4^+$ lymphocytes, and B lymphocytes, which persists for years after cessation of smoking, an adaptive immune response has been identified as a key component in the pathogenesis of COPD[23–26].

Tissue lymphocyte infiltration in COPD has been widely investigated, but less is known about blood lymphocyte count, which could result an important and underestimated asset for subsequent biological and clinical investigations in COPD.

Already in 1971[27], it was described that cigarette smoking was associated with an increase in leukocyte counts in the blood, which was related to amount smoked. The eventual awareness of the important role of the lymphocytes in the pathogenesis of COPD prompted numerous studies assessing lymphocytes in peripheral blood and the possible associations with smoking and COPD.

Recently, in a large study[28], systemic markers of innate and adaptive immunity, including absolute blood cell counts, were correlated with COPD severity and progression.

These findings suggest a critical role for immune response in the development of the disease, and are evocative that lymphocyte numbers in peripheral blood might reflect pathological events in the lung; however, this knowledge is still incomplete.

More evidences than that on blood lymphocytes concern blood eosinophils, which became a "hot topic" in the field especially as biomarkers of clinical outcomes. Indeed, there is a growing number of publications about the possible role of blood eosinophils, as a reflection of tissue eosinophils, in both the prediction and treatment of COPD exacerbations.

About 20% of patients with COPD is reported to have an eosinophilic airway inflammation measured in bronchial biopsies and induced sputum[29,30], especially during exacerbations associated to viral infections or with concomitant chronic bronchitis[31,32].

Currently, an increased number of eosinophils in blood has been used as a treatable trait in the management of COPD exacerbations, accepted as an echo of eosinophilic airway inflammation[30,33]. However, the potential role of blood eosinophils as biomarker in COPD patients is based on data mainly derived from post-hoc and retrospective analyses of several large COPD clinical trials, where history of asthma was not systematically excluded. These studies showed an association between an increased blood eosinophil count and risk of exacerbations, as well as mitigation of this risk with inhaled corticosteroids (ICS)[34–37]. Patients with high blood eosinophils may have a distinct inflammatory profile, making them more susceptible to develop severe events and suggesting that eosinophils may have a deleterious effect in COPD.

However, the role of eosinophils as a biomarker to guide the response to inhaled steroids in COPD patients has been recently challenged. Indeed, there are other studies that showed a better effect of ICS on the exacerbation rate in the COPD with lower eosinophils[38], or a less efficacy of inhaled steroids than inhaled bronchodilator in preventing COPD exacerbations, regardless the level of blood eosinophils[39–41].

These controversial results might be due in part to the inclusion of patients with a history of asthma in many of these trials[34,36,42] which emphasizes the need for prospective long-term studies on the possible role of blood eosinophils in the clinical outcomes of patients with pure COPD.

Immune inflammation in peripheral blood is fascinating and essential to explore, because it possibly reflects the systemic response to an insult or a disease. However, is by studying directly what happens in the lung tissue that we may have better clues on the pathogenesis of COPD.

One of the main goal of our research group since years is to understand why nearly 80% of smokers do not develop, or "evade", COPD[43]. We believe that, if we could better understand the mechanisms of "evasion" of COPD, we would be closer to comprehend why in some smokers this mechanism fails, leading to the disease.

Our research group have previously proposed a likely mechanism to explain how the T-cell–mediated inflammation could lead to COPD and also possibly explain the evasion from COPD in the majority of smokers[7].
Epithelial cells injured by cigarette smoke would release "danger signals" triggering an innate inflammation which may harm the extracellular matrix, promoting tissue breakdown and the production of antigenic substances (collagen, elastin and fibronectine peptides). Antigens could be picked up by dendritic cells which will mature and migrate to local lymph organs where, in a propitious milieu for antigen presentation, T cell activation may result with proliferation into effector $CD4^+$ and $CD8^+$ T cells. Immune regulation or tolerance mechanisms will determine at this stage the degree of proliferation of T cell effectors, homing, and eventually, disease severity. An absence of tolerance would be associated with most severe disease while full tolerance would be associated with evasion from the disease.
The adaptive immune system has evolved to eliminate virtually any threat from the organism. However, the immune system must do so while sparing healthy cells and maintaining self-tolerance. This task is accomplished through multiple checks and balances on immune responses that function in lymphoid organs and in the periphery, as described by the Nobel prize winners Allison and Honjo[44,45]. It is likely that the maintenance of tolerance to self-antigens could be an important strategy for the evasion from COPD in smokers. Along these lines we have shown that smokers who evade COPD or have mild disease, are at higher risk for the development of lung cancer than smokers with severe COPD[46]. Plausibly this may happen because in severe COPD the fully engaged immune reaction in the lung contributes to prevent the development of lung cancer, while in smokers without COPD the immune tolerance that prevents COPD might favor lung cancer growth.
Therefore, a different modulation of these immune checkpoints as CTLA-4, PD-1 and PDL-1 may be involved also in the immune pathogenic mechanisms of COPD.

The pathogenesis of IPF, compared to COPD, is still poorly understood. Current evidences suggest that after repetitive alveolar damage, IPF develops in genetically susceptible individuals with dysfunctional alveolar epithelial repair mechanisms, resulting in an abnormal wound-healing response of epithelial cells and deposition of extracellular matrix and collagen[47].
However, a chronic inflammatory process of the lung had long been considered the main mechanism underlying IPF[48,49]. The conceptual transition in IPF pathogenesis from an inflammatory driven process to a primarily fibrotic one, was due to the histologic description of mild inflammation, consisting of a patchy interstitial infiltrate of lymphocytes and plasma cells[50,52], which was not considered an important

component of IPF pathology, or neither a factor contributing to pathogenesis of the disease. In addition, the lack of efficacy of anti-inflammatory treatment (e.g. systemic steroids) in IPF clinical trials[51] was considered an important factor confirming this idea.

Nevertheless, in the meantime, observations in keeping with an important role of immune inflammation in the pathogenesis of IPF were growing, as well as evidences of a worse outcome related to higher expression of pro-inflammatory factors[52], as well as chemokines involved in B cell regulation[53,54] or even the presence of autoantibodies[55].

IPF is a deadly disease, and the rate of decline and progression is highly variable, with the majority of patients remaining relatively stable over time or progressing slowly, and others experiencing a more rapid decline[56]. A reliable, valid and reproducible measure of disease progression in these patients is forced vital capacity (FVC)[57], and especially its change in percentage predicted (FVC% pred.) over time, which is a well-established predictor of mortality. It has been shown that patients who experience a decrease in FVC% pred. greater than 10% over a 12-month period have a significantly lower 5-year survival compared to patients whose FVC% pred. declines of 10% or less during the same period of time[58]. While there is no universally agreed upon definition for these two clinical phenotypes, they are commonly referred to as “rapid” and “slow” progressors, respectively. Notably, the observation that rapidly progressive and relatively stable/slowly progressive patients with IPF display distinct gene expression[59,60] supports the notion that the mechanisms underlying these distinct clinical phenotypes may also be different.

IPF patients not only have an unpredictable disease behavior, but also a highly variable response to treatment, which differs within each subject.

Pirfenidone, a pyridone derivative with anti-inflammatory, anti-fibrotic and anti-oxidant properties, is approved worldwide for the treatment of IPF based on its ability to slow down functional decline and disease progression as shown in three phase III clinical trials since few years[61,62]. Clinical trials, however, are usually performed in highly selected patient populations and in clinical settings that reflect only partially real-life clinical practice. In addition, IPF population are always considered as a single entity, and no differentiation between the diverse functional decline, rapid or slow, was contemplated to evaluate a possible distinct response to treatment.

Predicting disease behavior is actually a major challenge for all clinicians’ expert in the field, especially at the moment of diagnosis. A useful tool that may help in forecasting the disease course is High Resolution Computed Tomography (HRCT), which already plays a central role in the diagnosis of IPF, avoiding the need of lung biopsies in a high proportion of cases[63]. HRCT-derived scores for fibrosis extent have

been widely shown to correlate with degree of physiological impairment and may be more sensitive to subtle changes in disease status than physiological metrics[64,65].
To predict the variable and poorly defined natural history of IPF, composite scoring systems are increasingly being developed. The Gender, Age, Physiology (GAP) index, which is based only on clinical and functional variables, was able to predict one-year mortality in a cohort of patients with IPF[66]. Moreover, integrating CT scores to the GAP model increased the accuracy of mortality prediction[67] indicating a potential role for the HRCT in the prediction schemes. However, these scoring systems, even if useful, are still neither able to foresee prospectively the highly heterogeneous and unpredictable disease behavior, nor able to guide treatment response.

Thus, COPD and IPF, even though they appear so diverse, they share a common risk factor as the chronic exposure to cigarette smoking, which determines repetitive epithelial injury eliciting immune inflammation. We believe, and currently is even emerging from literature, that they may have also in common the pivotal role played by the immune system in modulating the inflammatory response in the development of both diseases.

AIM OF THE THESIS

I have focused my PhD research on the study of:
1) the role of smoking-triggered different inflammatory cells in the pathogenesis of the COPD and how this might compare to the possible and yet unknown smoking-triggered inflammation in IPF;
2) the relation of the inflammatory findings with clinical outcomes in these two diseases.

In COPD, we focused on the pattern of alveolar macrophage (AM) polarization, classic (M1) or alternative (M2), directly in human lung tissue and examined whether and to what extent this pattern may changes from the normal to COPD lung, giving some clues on the role of macrophage in the pathogenesis of the disease.
Then, we used the model of COPD with alfa-1 Antitrypsin (AAT) deficiency (AATD) to investigate whether AAT in these patients polymerizes in alveolar macrophages, and if polymerization could be related to inflammation within the lung and the development of COPD.
Furthermore, we prospectively studied blood eosinophil and lymphocyte count in a group of smokers with and without COPD followed long term, to evaluate whether they were predictors of clinical outcomes. Additionally, in a number of these patients who underwent lung resection, we also investigated the possible relationship within blood cell counts and lung tissue infiltrate (lymphocytes and eosinophils).
To obtain more information's regarding the role of inflammation in the pathogenesis of COPD, we also performed a study in collaborations to other European Centers, that was built-up to investigate the anti-inflammatory effects of the phosphodiesterase-4 inhibitor (Roflumilast) on airway tissue inflammation, directly investigating the change of immune cell count (lymphocyte, eosinophils) in the bronchial biopsy specimens from patients with moderate-to-severe COPD with chronic bronchitis.
Finally, to identify modifier genes that might potentially account for the variability in penetrance and expressivity of pulmonary involvement in subjects with AATD, we conducted a whole exome sequencing on families from AATD Italian registry.

Regarding IPF instead, we studied the inflammatory immune profile of the lung, taking advantage from the unique opportunity of having the explanted lungs of these patients, and we correlate this findings to clinical outcomes.
Firstly, we hypothesized that in patients with IPF, the different clinical behavior,

slow or rapid progressors, may be accounted for, at least partially, the different lung inflammation of the lung.
Then, we performed a longitudinal study, with the aim of assessing whether long-term (24 months) response to pirfenidone treatment was different according to the different disease behavior before starting treatment. This was based on the hypothesis that the two clinical phenotypes of rapid and slow progressors have different amounts of inflammatory infiltrate in the lung, and therefore they may respond differently to the anti-inflammatory effect of Pirfenidone.
Moreover, to validate the idea of a strong immune component in IPF, we also measured lymphoid follicles in the lung tissue, and compared to that in COPD.
Ultimately, we used the High Resolution Computed Tomography (HRCT) of the chest available for each IPF patients to evaluate whether HRCT at diagnosis may predict disease behavior. Moreover, in another population, we assessed HRCT change over time after 1 year of treatment and whether these changes were correlated with functional decline. In addition, when histologic data were available, we also correlated these findings to the radiological findings, to investigate if radiological patterns mirror what is happening in the lung.

METHODS AND RESULTS

Studies in COPD

*1. Bazzan E, ..., **Biondini D**, et al. **Dual polarization of human alveolar macrophages progressively increases with smoking and COPD severity**. Respir Res. 2017; 18: 40. doi: 10.1186/s12931-017-0522-0*

Subject characteristics

Fifty-three lungs from subjects undergoing lung surgery were studied. Eleven were smokers with severe COPD who had lung volume reduction surgery and no lung tumor; 25 were smokers who had surgery for peripheral malignant nodules of which 12 had moderate COPD and 13 normal lung function; 17 were non-smokers of which 11 had surgery for lung tumor (5 malignant and 6 benign) and 6 died of accidental death (donors). Except for donors, pulmonary function tests were performed shortly before surgery, and to define COPD the post- bronchodilator ratio of forced expiratory volume in one second over forced vital capacity (FEV1/FVC) <70% was used. None of the patients had a history of exacerbations or pulmonary infections in the month prior to surgery or history of atopy or asthma.

Immunohistochemical and confocal analysis

Lungs were fixed in 4% formaldehyde and tissue blocks were taken from the subpleural areas of the lung as far as possible from the tumor, and embedded in paraffin[68]. Sections 5 μm thick were cut and processed for immunohistochemical analysis. For the identification of the AM M1 phenotype we used anti-iNOS (inducible isoform nitric oxide synthases)[69,70] and confirmed the results by using anti-HLA-DR (Human Leukocyte Antigen - antigen D Related)[71]. CD206 expression was used for the identification of the AM M2 phenotype[70,72]. Additionally, in a subgroup of patients, the expression of Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-4 and IL-13 in AM was investigated by immunohistochemical analysis as indexes of M1 (TNF-α) and M2 (IL-4 and IL-13) polarization[70].

Positive alveolar macrophages, defined as mononuclear cells with a well-represented cytoplasm, present in the alveolar spaces, were quantified in at least 20 non-consecutive high-power fields inside the alveolar spaces in each subject. Results were expressed as percentage of positive macrophages over the total number of macrophages visualized. The quantification of iNOS+ (M1) and CD206+ (M2) AM was performed on two consecutive sections. In two cases from each group, confocal

microscopy was also performed to study the possible co-expression of iNOS and CD206. Sections were coded and the measurements made without knowledge of clinical and functional data.

Statistical analysis

Group differences were evaluated by analysis of variance (ANOVA) and unpaired Student t test for clinical data, and by Kruskal–Wallis test and Mann–Whitney U test for morphological data. Correlation coefficients were calculated by the Spearman rank method.

The aim of this study was to investigate directly in human lung tissue the pattern of alveolar macrophage (AM) polarization, classic (M1) or alternative (M2), and examine how this pattern changes from the normal lung to that in COPD.

Table 1. Clinical characteristics of subjects in the Study Cohort

	Severe COPD	Moderate COPD	Smokers w/o COPD	Non Smokers	Donors
Subjects examined (nM/nF)	9 M/2 F	11 M/1 F	13 M	6 M/5 F	4 M/2 F
Age, yrs	62 ± 9	66 ± 8	63 ± 8	62 ± 14	56 ± 6
Smoking history, pk-yrs	46 ± 28	50 ± 19	44 ± 23	-	-
FEV_1, % pred	33 ± 9 †‡	68 ± 9‡	100 ± 10	106 ± 17	-
FEV_1/FVC (%)	36 ± 11 †‡	64 ± 5‡	77 ± 7	79 ± 4	-
PaO_2, mmHg	65 ± 14 †‡	81 ± 6	87 ± 8	82 ± 4	-
$PaCO_2$, mmHg	40 ± 6	41 ± 4	40 ± 11	38 ± 3	-

Values are expressed as mean ± SD
†Significantly different from patients with moderate COPD ($p < 0.005$)
‡Significantly different from smokers without (w/o) COPD and non-smokers ($p < 0.0001$)

In this study, we included a population of COPD, severe and moderate, smokers without COPD, non smokers and donors.

Table 1 shows the clinical characteristics of these groups, where no demographic differences were observed. The smoking history was similar in the groups of severe COPD, moderate COPD and smokers without COPD.

The percentage of $iNOS^+$ AM (M1 polarization) increased progressively with smoking and disease severity, from 26% in donors to 84% in severe COPD (Fig. 1).

Notably, two of the donors that were ventilated for more than 24 hours, showed a twofold increase in the percentage of M1 AM (identified as

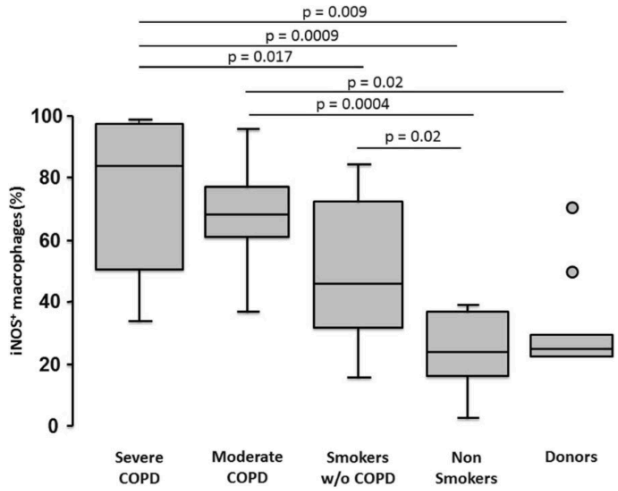


Figure 1. Analyses of M1 polarized alveolar macrophages (iNOS+) in the study cohort. The percentage of M1 (iNOS+) alveolar macrophages was low in donors and non-smokers, and increased progressively with smoking and COPD severity. Two ventilated subjects in the donors group are identified as outliers (circles). Bottom and top of each box plot, 25th and 75th percentiles; solid line, median; brackets, 10th and 90th percentiles

outliers in the figure) compared with the non-ventilated donors (Fig. 1).
Indeed, this procedure is known to induce lung inflammation and it may explain this finding[97]. The percentage of AM expressing CD206 (M2) also increased with smoking and disease severity, from 7% in donors to 78% in severe COPD (Fig. 2).

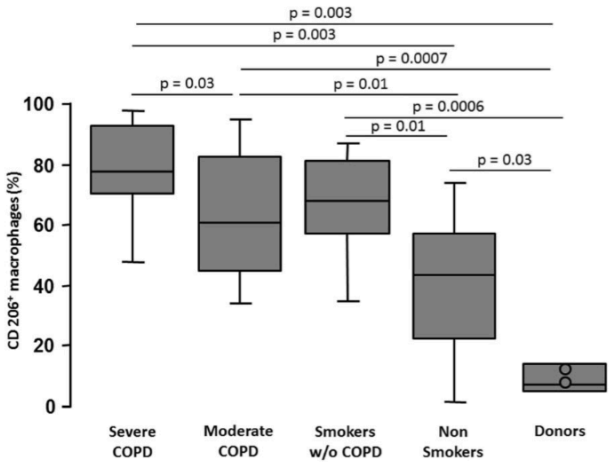


Figure 2. Analyses of M2 polarized alveolar macrophages (CD206+) in the study cohort. The percentage of M2 (CD206+) alveolar macrophages was low in donors, and increased with smoking and COPD severity. Two ventilated subjects in the donors group are identified (circles). Bottom and top of each box plot, 25th and 75th percentiles; solid line, median; brackets, 10th and 90th percentiles

Interestingly, the percentage of M2 AM was higher in non-smokers compared to donors, possibly because of the presence of lung tumors in non-smokers surgical lungs. Indeed, when non-smokers were divided according to the type of tumor, CD206 expression was only increased in subjects with malignant tumor, but not in those with benign tumor, suggesting that malignancy could influence the expression of M2 in the lung parenchyma.
However, it should be noted that patients with severe COPD (Fig. 2), whose lungs were obtained by lung volume reduction surgery and had no lung cancer, had the highest percentage of M2, indicating that most of the increase of M2 is secondary to smoking and COPD severity rather than to the presence of tumor itself.
In healthy lungs of donors, more than 80% of AM did not express any polarization marker and this percentage decreased progressively with smoking and disease severity to 20% non-polarized AM in severe COPD (Fig. 3).
Of interest, observing the values of both iNOS+ (84%) and CD206+ (78%) AM in severe COPD (Fig. 1, 2), the combined value reached more than 100%, indicating that some macrophages were expressing both markers. The staining of serial sections confirmed that, in severe COPD, iNOS and CD206 immunoreactivity could be present simultaneously in the same alveolar macrophage (Fig. 4). This finding was further confirmed by confocal microscopy (Fig. 5).

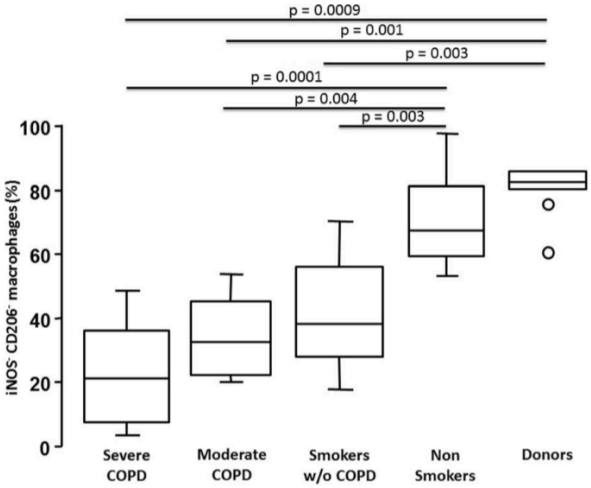


Figure 3. Analyses of non-polarized alveolar macrophages (iNOS− and CD206−) in the study cohort. The percentage of non-polarized alveolar macrophages (iNOS−CD206−) was high in donors and non-smokers and decreased progressively with smoking and COPD severity. Bottom and top of each box plot, 25th and 75th percentiles; solid line, median; brackets, 10th and 90th percentiles

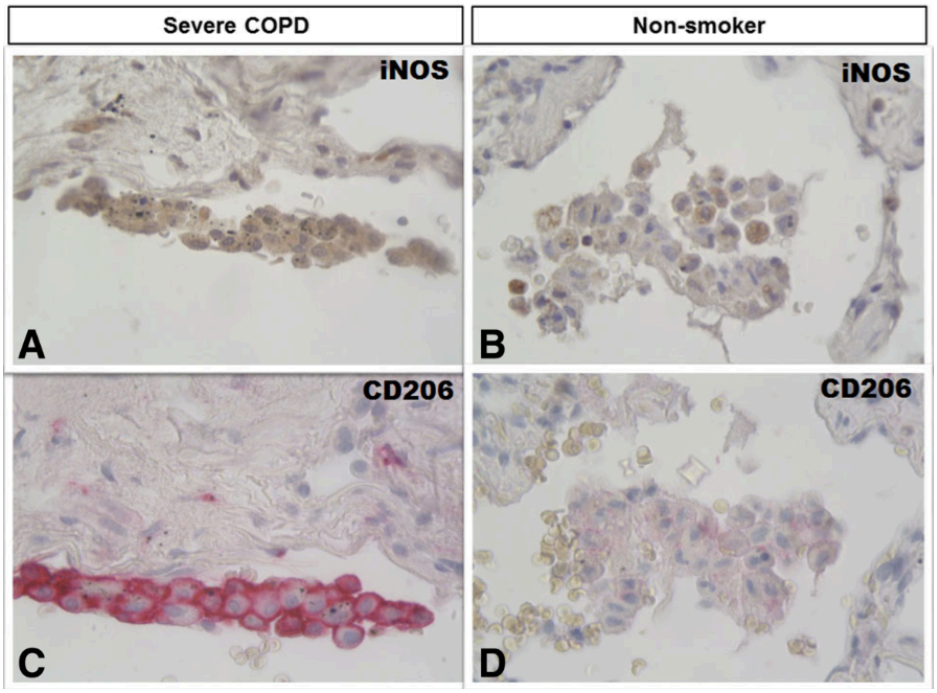


Figure 4. Immunohistochemistry of M1 and M2 alveolar macrophages in lung tissue. M1 (iNOS+) and M2 (CD206+) expression in clusters of alveolar macrophages in consecutive lung sections from a patient with severe COPD (panels a and c) and from a non-smoker (panels b and d). iNOS immunoreactivity appears as a brown diffuse cytoplasmic granular pattern (panel a), while CD206 immunoreactivity appears as a red linear pattern around the cellular membrane (panel c). In the smoker with severe COPD both M1 (iNOS+) (panel a) and M2 (CD206+) (panel c) immunoreactivity was present in the same cluster of alveolar macrophages. The alveolar macrophages in the non-smoking subject (panels B and D) were mostly negative for either stains. Immunostaining with anti-iNOS (in brown) and anti-CD206 (in red). Original magnification: X 400

Indeed, in two cases per group, we quantified the percentage of AM co-expressing both M1 and M2 markers using confocal microscopic images. We found that the percentage of AM expressing both M1 and M2 markers was reduced with disease severity and smoking, from 95% (range 89–100) in severe COPD, to 63% (range 50–76) in moderate COPD, 35% (range 20–50) in smokers without COPD and 0 (range 0–0) in donors. Confocal analysis shows that there are also macrophages showing only M1 polarization or only M2 polarization.

Of interest, cessation of smoking significantly decreased the expression of iNOS but not of CD206. Indeed, when all smokers (with and without COPD) were considered together, ex-smokers had a lower percentage of iNOS+ AM compared to current smokers (p=0.028).

However, when analyzed separately in the different subject groups, this difference remained

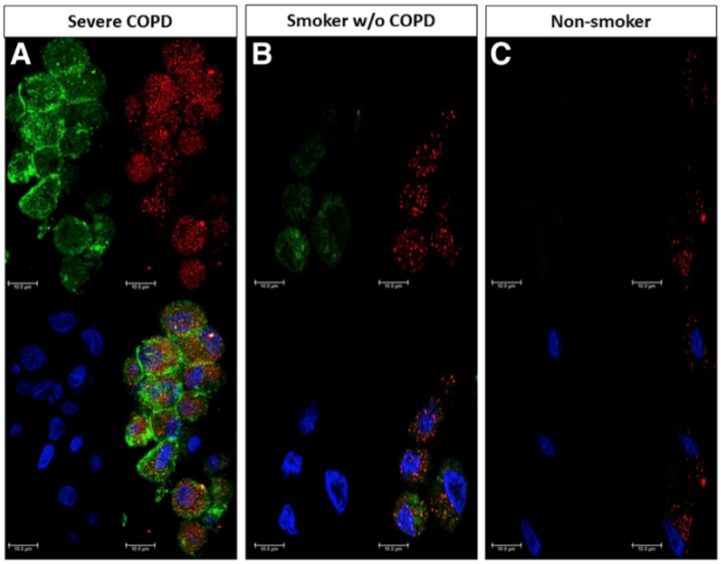


Figure 5. Confocal microscopy of M1 and M2 alveolar macrophages in lung tissue. iNOS (M1) and CD206 (M2) expression in clusters of alveolar macrophages in lung sections from a patient with severe COPD (panel a), a smoker without COPD (panel b) and a non-smoking subject (panel c). iNOS immunoreactivity appears as a red diffuse cytoplasmic granular pattern (panel a), while CD206 immunoreactivity appears as a green linear pattern around the cellular membrane. In the smoker with severe COPD, M1 and M2 markers were co-expressed in the same cluster of alveolar macrophages, while in the smoker without COPD only a reduced co-expression can be observed (panel b). The alveolar macrophages in the non-smoking subject were mostly negative for both M1 and M2 markers (panel c). Alveolar macrophages were stained with anti-iNOS (red) and anti-CD206 (green). Nuclei were stained with DRAQ5 (blue). Bars: 10 µm

statistically significant only in moderate COPD ($p<0.05$) and in smokers without COPD ($p<0.01$), but not in severe COPD, where the disease is fully established.
The expression of iNOS with smoking exposure and disease progression increased in parallel with the expression of TNF-α ($r=0.52$; $p=0.002$). Similarly, the percentage of CD206+ AM was paralleled by increased percentage of AM expressing IL-4 ($r=0.64$; $p=0.004$) and IL-13 ($r=0.55$; $p=0.012$), indicating that both M1 and M2 AM were likely active in their functions.

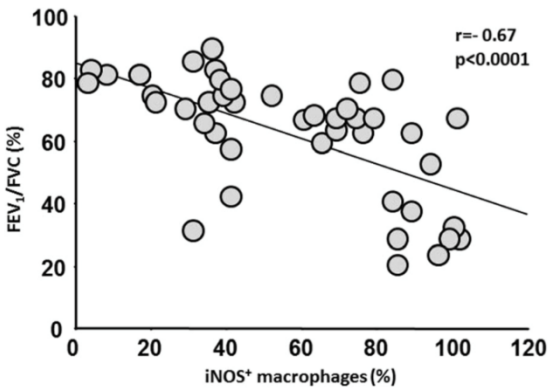


Figure 6. Relationship between M1 alveolar macrophages and lung function. Correlation between percentage of M1 (iNOS+) alveolar macrophages and FEV1/FVC (%) in all the subjects of the study, excluding donors who did not have lung function. Spearman rank correlation $r = -0.67$ and $p < 0.0001$

When we examined the relationship between AM polarization and lung function, we found that the percentage of AM expressing iNOS was correlated with the severity of airflow obstruction measured by the FEV1/ FVC% ($r=-0.67$, $p<0.0001$) (Fig. 6). A similar correlation was observed for CD206+ AM ($r=-0.44$, $p=0.003$).

In conclusion, resident AM in normal lungs do not show either M1 or M2 polarization, while both M1 and M2 expressions increase with exposure to cigarette smoking and disease severity. In addition, we showed how in humans AM polarization with M1 or M2 does not exclude each other, but often coexists.

*2. Bazzan E, Tinè M, **Biondini D**, et al. .... **α1-Antitrypsin polymerizes in alveolar macrophages of smokers with and without α1-Antitrypsin deficiency.** Chest. 2018; 154:607-616. doi: 10.1016/j.chest.2018.04.039. Epub 2018 May 12*

Subject characteristics

We studied tissues from the lungs of 33 patients undergoing lung transplantation for severe COPD: nine had PiZZ a1-antitrypsin deficiency (COPD with AATD) and 24 had normal levels of AAT (usual COPD). AATD was confirmed by serum levels, together with genotyping/phenotyping in all cases. Sections from the lungs of 11 smokers with normal lung function and 13 nonsmoking subjects,
who had lung resection for solitary nodules, were included for comparison. All 57 subjects underwent pulmonary function tests prior to surgery and provided informed

written consent. The study conformed to the Declaration of Helsinki. All aspects of this study were approved by the local ethics committee (reference No. 0006045).

Histochemistry, immunohistochemistry and morphometric analysis

Lung tissue preparation, histochemistry, and immunohistochemistry were performed as previously described[73,74].

The lung tissue specimens were fixed in formalin, embedded in paraffin wax, and cut. At least three lung sections per case were stained with periodic acid–Schiff (PAS) and immunostained according to the standard peroxidase-antiperoxidase method with a commercial polyclonal anti-AAT antibody recognizing total AAT (both native and polymerized) (IR505; Dako) and with the specific monoclonal antibody 2C1 that recognizes intracellular AAT polymers but not native (monomeric), reactive loop–cleaved, or latent AAT[75]. Negative control subjects for nonspecific binding were processed, either omitting the primary antibody or using isotype IgG, and revealed no signal.

To quantify AAT-positive alveolar macrophages, PAS-positive inclusions in alveolar macrophages, and AAT polymer–positive alveolar macrophages at least 20 to 40 nonconsecutive high-power fields and at least 100 macrophages inside the alveolar spaces were evaluated for each subject. Results were expressed as the percentage of positive macrophages relative to the total number of macrophages examined[73,74]. Alveolar macrophages were defined as mononuclear cells with a well-represented cytoplasm, present in the alveolar spaces.

As a positive control for AAT polymer staining we examined six liver samples from PiZZ patients who underwent liver transplantation related to AATD. Sections (5 mm) were stained with PAS and the specific monoclonal antibody 2C1 to detect AAT polymerization, following the same protocol used for pulmonary tissue.

Neutrophils, macrophages, CD4+ T lymphocytes, CD8+ T lymphocytes, and B lymphocytes were identified by immunohistochemistry and counted in the alveolar walls in order to evaluate a possible correlation between AAT (native and polymerized) and the degree of lung inflammation[73,74].

Using the semiquantitative method described by us[76] we assessed the small airways disease score (inflammation, muscle, wall thickness) in all airways less than 2 mm in diameter. Each bronchiole 2 mm or less in diameter was examined separately for the presence of inflammatory cell infiltrate, smooth muscle hypertrophy, and wall thickness. For each airway, a score from 0 (normal) to 3+ (most abnormal) was assigned for each pathologic feature. Scores for individual features were summed and expressed as the percentage of the maximal possible score[76].

A macroscopic quantification of emphysema was performed in all explanted lungs, using the method of Heard[77]. Because lungs were not fixed in inflation at a constant

pressure, we were not able to use the mean linear intercept for the microscopic quantification of emphysema (air space size). We instead undertook a semiquantitative score of the extent of microscopic emphysema (0, 1, 2, 3+) in every slide available in all cases and expressed this as the percentage of the maximal possible score[76].

The possible relationship between AAT polymerization and inflammatory response was also examined in liver tissue. From each liver surgical sample, two consecutive 5-mm-thick sections were cut and stained with 2C1 antibody to identify polymers in one section (following the same protocol used for pulmonary tissue) and with CD45 antibody to identify total leukocytes in the other consecutive section. An intensity score from 0 to 3 for the extent of polymerization and of CD45-positive cells was graded in 50 fields for each slide pair.

All analyses were performed with a Leica light microscope and video recorder linked to a computerized image analysis system (Leica LAS w3.8).

Statistical analysis

Group differences were evaluated by analysis of variance (ANOVA) and unpaired Student t test for clinical data, and by Kruskal-Wallis test and Mann-Whitney U test for morphologic data. Correlation coefficients were calculated by the Spearman rank method. P values of .05 or less were considered to indicate statistical significance.

The aim of this study was to assess whether alveolar macrophages, in the lung tissue from individuals with PiZZ α-1 Antitrypsin (AAT) deficiency, formed AAT polymers and whether polymerization could be related to inflammation within the lung.

Table 2. Clinical characteristics of subjects in the Study Cohort

	COPD With AATD	"Usual" COPD	Smokers Without COPD	Nonsmokers
Subjects, No.	9	24	11	13
Age, y	53 ± 3	57 ± 1	62 ± 2	56 ± 6
Smoking history, pack-years	34 ± 8	41 ± 7	48 ± 7	...
Current/ex-smokers, No./No.	0/9	2/22	4/7	...
FEV_1, % predicted	19 ± 2[a]	20 ± 2[a]	98 ± 3	108 ± 5
FEV_1/FVC, %	35 ± 5[a]	37 ± 3[a]	77 ± 2	85 ± 4

Values are expressed as means ± SD. AATD = α_1-antitrypsin deficiency; "usual" COPD = COPD with normal AAT levels.
[a]Significantly different from smokers without COPD and from nonsmokers ($P < .0001$).

We evaluated a population of nine patients transplanted for severe COPD due to AAT deficiency, confirmed by either low serum AAT and genotyping/phenotyping (eight ZZ; one ZI). All other patients with usual COPD (n=24), smokers without COPD (n=11), and nonsmokers (n=13) had a normal α-1 band on protein electrophoresis.

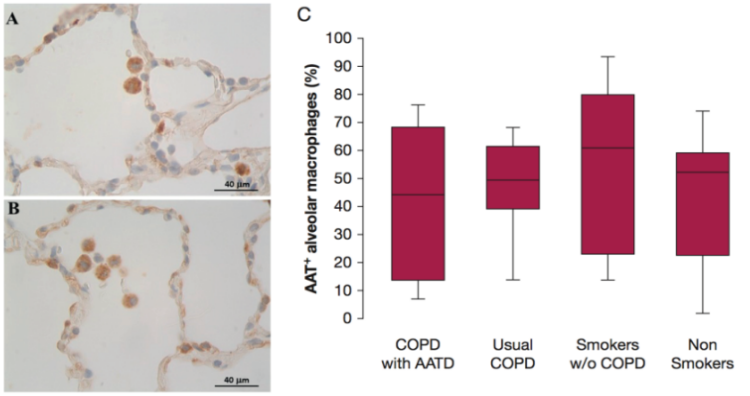


Figure 7. Total (native and polymerized) a1-antitrypsin (AAT) immunostaining in alveolar macrophages. Quantification of AAT expression in alveolar macrophages of patients with COPD and a1-antitrypsin deficiency (COPD with AATD), "usual" COPD (COPD with normal AAT levels), smokers without COPD, and nonsmokers. A and B, Representative examples of AAT expression in the lung of a patient with COPD with AATD (A), and in the lung of a nonsmoker (B). Positive staining (in brown) was observed mainly in alveolar macrophages and occasionally in the alveolar wall. Immunostaining with polyclonal anti-AAT antibody IR505 (A and B). Scale bars, 40 mm. C, The percentage of alveolar macrophages positive for AAT was not significantly different among the four groups of subjects examined. Horizontal bars represent median values

The clinical characteristics of the subjects in this study are shown in Table 2. There were no differences in age and amount smoked overall, with 14% current smokers and 86% recent ex-smokers.

Positive staining with anti-AAT antibody IR505, which stains both native and polymerized AAT, was observed mainly in alveolar macrophages (AMs) and occasionally in the alveolar walls (Fig. 7A, 7B). There was no significant difference in the percentage of alveolar macrophages positive for total (native and polymerized) AAT between COPD with AATD, usual COPD, smokers without COPD, and nonsmokers (Fig. 7C).

Then, we evaluated the percentage of PAS-positive AMs, and found that it was increased not only in COPD with AATD, but also in smokers with or without COPD and normal AAT levels compared with nonsmokers, where no PAS-positive intracellular inclusions were seen (Fig. 8). Furthermore, the percentage of PAS-positive AMs was also increased in smokers with usual COPD compared with smokers without COPD (Fig. 8). The PAS inclusions were similar to those seen in the liver from individuals with PiZZ AATD (Fig. 9A, 9B).

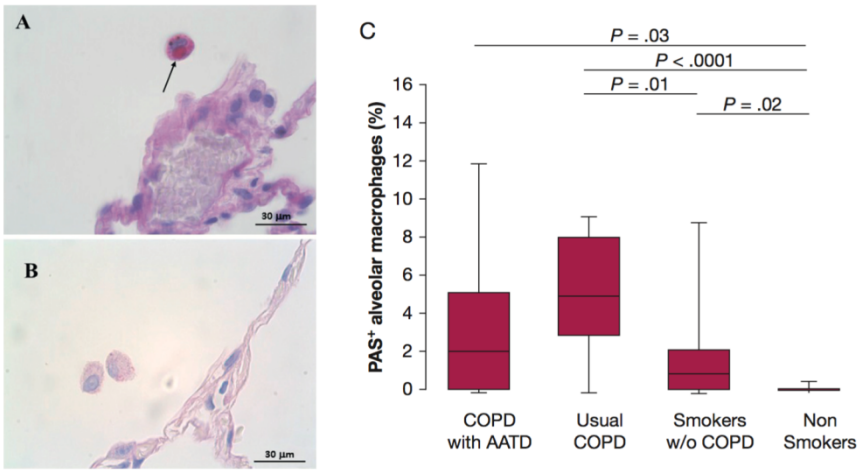


Figure 8. Periodic acid–Schiff (PAS) staining in alveolar macrophages. Shown is the quantification of PAS expression in alveolar macrophages of patients with COPD and a1-antitrypsin deficiency (COPD with AATD), "usual" COPD (COPD with normal AAT levels), smokers without COPD, and nonsmokers. A and B, Representative examples of PAS expression in the lung of a patient with COPD with AATD (A) and in the lung of a nonsmoker (B). Positive staining (in violet) was observed mainly in alveolar macrophages; arrow indicates PAS-positive inclusion. Scale bars, 30 mm. C, The percentage of PAS-positive alveolar macrophages was increased in patients with AATD, and in smokers with and without COPD compared with nonsmokers. Furthermore, the percentage of alveolar macrophages positive for PAS was increased in usual COPD compared with smokers without COPD. P values in the figure represent the results of Mann-Whitney U tests. Kruskal-Wallis test: P < .0001. Horizontal bars represent median values

To have a further confirmation that PAS+ inclusions were referred to AAT polymers, we used a monoclonal antibody that recognizing specific intracellular AAT polymers. With this method, we found that the percentage of AMs stained positive for polymers was increased not only in individuals with AATD, but also in smokers with or without COPD and normal AAT levels compared with nonsmokers, where no polymerization was seen (Fig. 10). As for PAS inclusions, the use of the polymer-specific 2C1 monoclonal antibody showed a similar pattern for polymerization in AMs and in liver sections of PiZZ AAT individuals (Fig. 9C, 9D).

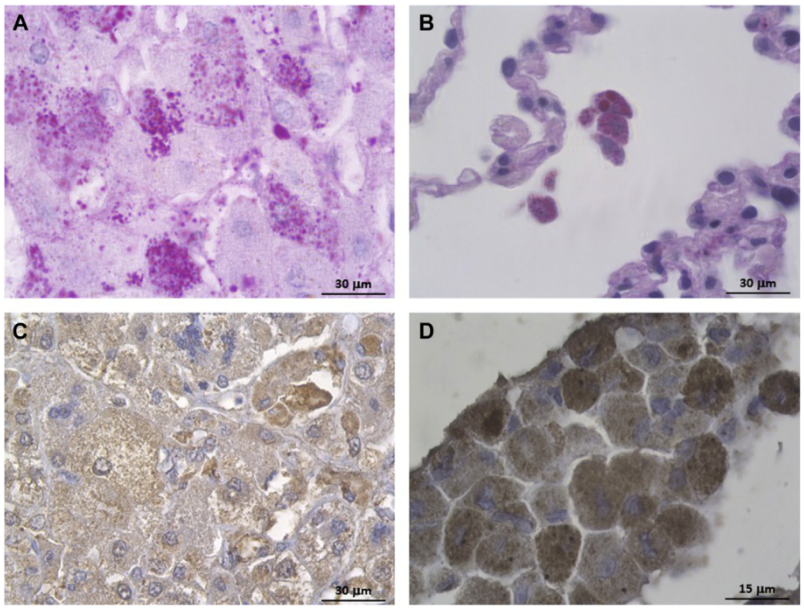


Figure 9. PAS staining and immunostaining for AAT polymers in liver and lung sections of patients with AATD. A and B, Representative examples of PAS expression in the liver of a patient with AATD (A) and in the lung of a patient with COPD with AATD (B). Positive PAS staining in violet. C and D, Representative examples of AAT polymer expression in the liver of a patient with AATD (C) and in the lung of a patient with COPD with AATD (D). Positive immunostaining with monoclonal antibody 2C1 specific for AAT polymers in brown (C and D). Scale bars: A-C, 30 mm; D, 15 mm

Then, to magnify the impact of cigarette smoke on alveolar polarization, when we considered all subjects together, the cumulative exposure to cigarette smoke (pack-years) was positively correlated with the percentage of macrophages showing PAS$^+$ inclusions (r=0.41; p=0.003) and those positive for AAT polymers (r=0.53; p=0.0001).

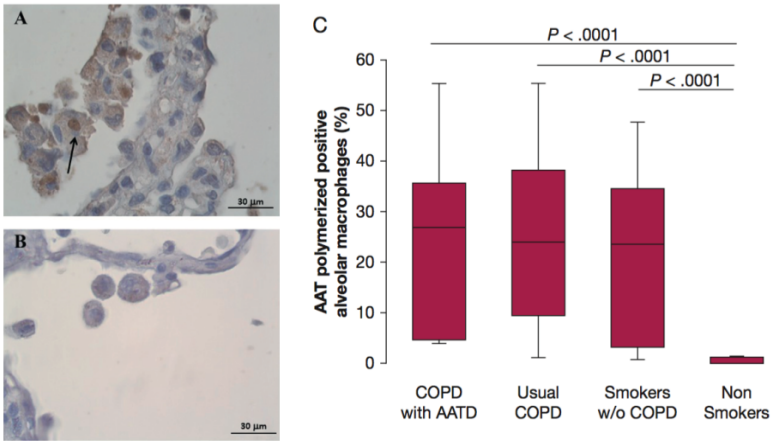


Figure 10. AAT polymers in alveolar macrophages. Shown is the quantification of AAT polymer expression in alveolar macrophages of patients with COPD and a1-antitrypsin deficiency (COPD with AATD), "usual" COPD (COPD with normal AAT levels), smokers without COPD, and nonsmokers. A and B, Representative examples of AAT polymer expression in the lung of a patient with COPD with AATD (A) and in the lung of a nonsmoker (B). Positive staining (in brown) was observed mainly in alveolar macrophages; arrows indicate AAT-positive polymers. Immunostaining with monoclonal antibody 2C1 specific for polymerized AAT (A and B). Scale bars, 30 mm. C, The percentage of alveolar macrophages positive for polymerized AAT was increased in patients with COPD with AATD, in usual COPD, and in smokers without COPD compared with nonsmokers. P values in the figure represent the results of Mann-Whitney U tests. Kruskal-Wallis test: P < .0001. Horizontal bars represent median values. See Figure 1 legend for expansion of abbreviations

Moreover, we confirmed the role of polymerized AAT in eliciting inflammation by showing that the percentage of polymerized alveolar macrophages correlated significantly with the emphysema score (r=0.55; p=0.002), the small airways disease score (r=0.44;

p=0.004), the number of neutrophils (r=0.31; p=0.05) and $CD8^+$ T lymphocytes (r=0.51; p=0.002) in the alveolar walls. Furthermore, the percentage of polymerized AMs was inversely correlated with pulmonary function (FEV1: r=0.44, p=0.002; and FEV1/FVC: r=0.41, p=0.005).

Table 3. Quantification of Lung Pathology and Inflammation

	COPD With AATD	"Usual" COPD	Smokers Without COPD	Nonsmokers
Small airways disease, score %	78 (43-92)[a]	67 (33-100)[a]	26 (0-63)	17 (0-50)
Emphysema, score %	83 (67-100)[a]	84 (33-100)[a]	0 (0-17)	0 (0-0)
Lymphoid follicles/cm^2	4.6 (0.7-16.5)[a,b]	1.5 (0-6.1)[a]	0 (0-2.5)	0 (0.0-0.8)
B cells/mm of alveolar wall	2.1 (0-4.4)[a]	0.9 (0-5.0)[a]	0.2 (0-0.63)	0.3 (0-0.9)
$CD4^+$ T cells/mm of alveolar wall	5.5 (0.9-10.8)[c]	6.1 (1.6-11.9)[c]	2.26 (0.2-4)	2.1 (0-5.4)
$CD8^+$ T cells/mm of alveolar wall	3.4 (0.6-6.8)[d]	4.1 (3.0-6.8)[c]	3.4 (0.6-5.1)	2.1 (0-5.2)
Neutrophils/mm of alveolar wall	6.3 (1.2-15.9)	9.4 (4.5-13.9)[d]	6.8 (2.5-9.5)	3.8 (0-15.1)

Values are expressed as median (range). See Table 1 legend for expansion of abbreviations.
[a]Significantly different from smokers without COPD and nonsmokers ($P < .01$).
[b]Significantly different from usual COPD ($P < .05$).
[c]Significantly different from smokers without COPD and nonsmokers ($P < .05$).
[d]Significantly different from nonsmokers ($P < .05$).

Another interesting finding in this study involves the number of lymphoid follicles/cm^2 in subjects with COPD, with and without AATD, that was significantly higher than in smokers without COPD and in nonsmokers, as were the number of B, $CD4^+$ T, and $CD8^+$ T lymphocytes in the alveolar wall (Table 3), highlighting once again how the immune system is pivotal in the disease.

In conclusion, in this study we found that polymerization of AAT in alveolar macrophages occurs in the lungs of individuals with AATD, but also in smokers with normal AAT levels with or without COPD.

3. Turato G, ..., ***Biondini D,*** *et al.* ***Blood eosinophilia neither reflects tissue eosinophils nor worsens clinical outcomes in Chronic Obstructive Pulmonary Disease.*** *Am J Respir Crit Care Med. 2018; 197:1216-1219. doi: 10.1164/rccm.201708-1684LE.*

4. ***Biondini D,*** *et al.* ***Reassessing the role of eosinophils as a biomarker in Chronic Obstructive Pulmonary Disease.*** *J Clin Med. 2019 2; 8. pii: E962. doi: 10.3390/jcm8070962.*

Patient population

The population studied were part of a cohort of smokers initially free of major comorbidities recruited among smokers attending the Pulmonary Clinic at the Hospital Universitario Miguel Servet in Zaragoza (Spain) between January 1997 and

December 2009. Eligible subjects were current or former smokers (≥20 pack-years) older than 40 years. COPD was defined by FEV1/FVC <0.70 post-bronchodilator and severity was assessed using the spirometry criteria of the GOLD guidelines. Subjects with history of asthma, bronchiectasis and interstitial lung disease or the coexistence of malignancy were excluded.

At baseline, all subjects underwent clinical examination, spirometry and complete blood cell count. The annual frequency and type of exacerbations were collected. Exacerbations, and acute respiratory events in patients without established COPD, were defined as acute respiratory symptoms requiring antibiotics or systemic steroids by medical prescription; severe events were defined by the need for hospitalization. The incidence of comorbidities was recorded. Mortality and cause of death was recorded up to May 2016.

For the purpose of this report, a total of 512 smokers, 303 with COPD and 209 without, who had blood eosinophils measured were studied, and the distribution and median eosinophil numbers calculated. Following previous publications and recent recommendations[34,78] we used the absolute value of 150/μL, rather than the equivalent value of 2% eosinophils, as the cut-off to categorize the population as persistently <150/μL, persistently ≥150/μL or variable. This characterization was used to investigate the relationship between blood eosinophils levels and clinical outcomes.

The study was approved by the human-research review board and all patients provided informed written consent.

Blood eosinophils and lung tissue eosinophils

We examined surgical lung specimens obtained from 51 smokers (34 with COPD and 17 without COPD) undergoing lung resection surgery. Using optical microscopy eosinophils were counted in central and peripheral airways and lung parenchyma and expressed as number of eosinophils/mm2 of tissue[26,32]. In 36 cases blood eosinophil obtained at the time of surgery were correlated to tissue eosinophils.

Statistical analysis

Differences between groups were evaluated with Kruskal-Wallis test and Mann-Whitney U tests. The association between categorical variables was investigated by $\chi 2$ or Fisher exact probability test. Analyses of overall survival and event-free survival were performed using Kaplan-Meier survival curves. Correlation coefficients were evaluated using the nonparametric Spearman rank method.

The aims of these studies were to clarify the claimed role of blood eosinophils in the clinical features of COPD, and to investigate their consistency over time, the

possible relation with lung tissue eosinophil counts and their potential effect in clinical outcomes in smokers with and without COPD followed long term.

Table 4. Clinical characteristics of subjects in the Study Cohort. Data are presented as number (%) or mean ± SD, p value refers to Kruskal-Wallis test or χ2 test

	All subjects	**Non COPD**	**COPD**	**p**
Number of subjects, n (%)	512	209 (41%)	303 (59%)	-
Age (years)	58 ± 10	52 ± 11	62 ± 8	**<0.01**
Smoking history (pack years)	43 ± 24	35 ± 19	49 ± 25	**<0.01**
FEV_1 post-bronchodilator (l)	2.33 ± 0.85	2.88 ± 0.78	1.95 ± 0.67	**<0.01**
FEV_1 post-bronchodilator (% predicted)	79 ± 22	95 ± 15	68 ± 19	**<0.01**
FEV_1/FVC post-bronchodilator (%)	64 ± 15	78 ± 5	54 ± 11	**<0.01**
GOLD 1, n (%)	-	-	81 (27%)	-
GOLD 2, n (%)	-	-	169 (56%)	-
GOLD 3-4, n (%)	-	-	53 (17%)	-
Bronchodilator response (% FEV_1 pre-bronchodilator)	6.33 ± 9.99	2.58 ± 6.97	8.88 ± 10.91	**<0.01**
Decline of FEV_1 per year (ml/year)	32 ± 46	33 ± 37	31 ± 51	0.54
Subjects with at least one exacerbation, n (%)	235 (46%)	73 (35%)	162 (53%)	**<0.01**
Number of total exacerbations per year	0.79 ± 1.54	0.49 ± 1.05	1.00 ± 1.78	**<0.01**
Subjects with chronic bronchitis, n (%)	193 (38%)	56 (27%)	137 (45%)	**<0.01**
Subjects with mMRC ≥ 2, n (%)	215 (42%)	71 (34%)	144 (48%)	**<0.01**
Subjects with CAT ≥ 10, n (%)	195 (38%)	70 (34%)	125 (41%)	0.06
Subjects with comorbidities, n (%)	391 (77%)	148 (71%)	243 (81%)	**<0.01**
Distance at 6-minute walking test (m)	419 ± 122	481 ± 114	376 ± 109	**<0.01**

We investigated a population of 512 smokers, 303 with COPD and 209 without COPD. The mean follow-up time was 9.6±5.8 years. At recruitment, among those smokers with COPD, disease severity was classified as GOLD 1 in 81, GOLD 2 in 169, and GOLD 3-4 in 53. Subjects with COPD were older, had a greater smoking history (table 4). The annual FEV1 decline was not different between subjects with and without COPD. During follow-up, 35% of subjects without COPD and 53% of those with COPD had at least one moderate or severe exacerbation ($p<0.01$). The number of exacerbation per year was higher in subjects with COPD compared to those without ($p<0.01$). The percentage of subjects with symptoms of chronic bronchitis and of those with dyspnea (measured by an mMRC ≥2) were higher in COPD subjects compared to non COPD ($p<0.01$). Seventy one percent of subjects

without COPD and 81% of those with COPD developed at least one comorbidity during the observation period ($p<0.01$) (Table 4).

The median number and interquartile ranges of blood eosinophils measured upon recruitment were similar between smokers with and without COPD (156, 101–250 cells/ml vs. 160, 107–256 cells/ml), and no different from the values reported in a normal population[37].

*Table 5. Clinical characteristics of subjects in the Study Cohort. Data are presented as number (%) or mean ± SD, p value refers to Kruskal-Wallis test or χ^2 test. * p <0.05 compared to eosinophils < 150 cells/μL; † p <0.05 compared to variable.*

	Eosinophils < 150 cells/μL	**Eosinophils ≥ 150 cells/μL**	**Variable**
Number of subjects, n (%)	73 (15%)	104 (22%)	295 (63%)
Age (years)	60 ± 9	57 ± 10*†	59 ± 10
Smoking history (pack years)	44 ± 23	42 ± 26	45 ± 24
FEV_1 post-bronchodilator (l)	2.21 ± 0.78	2.41 ± 0.81	2.24 ± 0.79
FEV_1 post-bronchodilator (% predicted)	78 ± 23	82 ± 22	77 ± 22
FEV_1/FVC post-bronchodilator (%)	62 ± 15	66 ± 14	63 ± 15
Bronchodilator response (% FEV_1 pre-bronchodilator)	6.62 ± 11.00	6.33 ± 11.28	6.36 ± 9.59
Decline of FEV_1 per year (ml/year)	23 ± 38	32 ± 50	34 ± 47
Subjects with at least one exacerbation, n (%)	41 (56%)	43 (41%)	136 (46%)
Number of total exacerbations per year	1.02 ± 1.69	0.76 ± 1.66	0.80 ± 1.54
Subjects with chronic bronchitis, n (%)	28 (38%)	36 (35%)	114 (39%)
Subjects with mMRC ≥ 2, n (%)	31 (43%)	34 (33%)	138 (47%)
Subjects with CAT ≥ 10, n (%)	23 (32%)	39 (38%)	122 (41%)
Subjects with comorbidities, n (%)	51 (70%)	83 (80%)	236 (80%)
Distance at 6-minute walking test (m)	405 ± 120	435 ± 119 *†	403 ± 120

In the consecutive blood samples taken, eosinophils were not always stable over time. Therefore, using the three to five available eosinophil counts, we classified the patients as having persistently high (>150/ml), persistently low (<150/ml), or variable (oscillating above and below 150/ml) eosinophil counts[30,78]. The percentage of subjects with persistently low, high, and variable eosinophil counts was similar among the different severity groups according to the Global Initiative for Obstructive Lung Disease grading system. The clinical characteristics of the population divided according to eosinophils levels are shown in Table 5.

The percentage of subjects with persistently ≥150 eosinophils/μL was higher in smokers without COPD than in those with COPD (29% vs 18%, p=0.03).

The annual FEV1 decline, the bronchodilator response, the prevalence of chronic bronchitis, the dyspnea index (mMRC ≥2) and the COPD assessment test (CAT) were not different between subjects with persistently <150/μL, ≥150/μL or variable eosinophils. These results apply to both the COPD and non COPD. Subjects with ≥150 eosinophils/μL performed better in the 6-minute walking test than those with <150 eosinophils/μL and variable (Table 5).

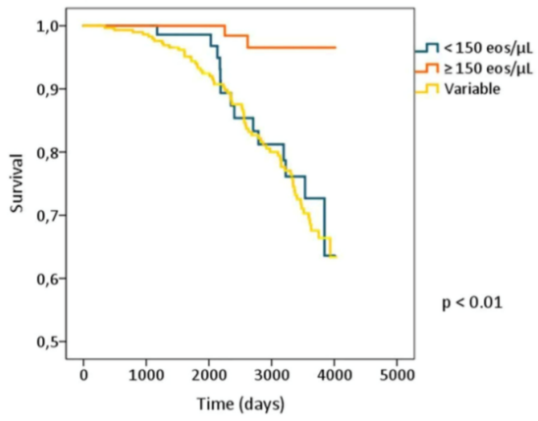


Figure 11. Survival in relation to blood eosinophil. Kaplan-Meier lots showing survival in smokers with blood eosinophils persistently <150 cells/μL, persistently ≥150 cells/μL and variable over the 5 yearly blood samples. P-value < 0.01

The percentage of subjects with COPD and at least one exacerbation, either moderate or severe, was similar among the persistently low (57%), persistently high (49%), and variable groups (54%). Even subjects with more than 300 eosinophils/μL did not have excessive severe exacerbations. The number of total exacerbations per year (moderate and severe) was similar in smokers with persistently <150/μL, ≥150/μL and variable eosinophils in all subjects. Treatment with inhaled corticosteroids was similar among the three eosinophil-count groups.

Eighty-six subjects (80 with and 6 without COPD) died during the study, 33% from respiratory causes (COPD exacerbations, respiratory failure and pneumonia), 21% from lung cancer and 11% from cardiovascular diseases. Kaplan-Meier analysis showed that subjects with persistently ≥150/μL had a better survival than those with <150/μL or variable (p<0.01) (Fig. 11).

There was no difference in the median number of eosinophils/mm^2 in central airways (15, 4–39 vs. 25, 6–114), peripheral airways (10, 1–20 vs. 0, 0–13), and lung parenchyma (1, 0–3 vs. 2, 0–9) in smokers with and without COPD, and tissue eosinophil counts did not change with disease severity. Eosinophil numbers in lung parenchyma correlated with those in central airways (r=0.65; p=0.002). In 36 subjects with blood eosinophils measured at the time of lung surgery, the relationship between blood eosinophils and tissue eosinophils was examined. No correlation was found for any of the three lung compartments (central airways: r=0.22, p=0.42; peripheral airways: r=0.20, p=0.31; lung parenchyma: r=0.26, p=0.12).

In conclusion, high numbers of blood eosinophils are not associated with worse outcomes and might be even beneficial, as we showed better survival compared to

low eosinophils. Moreover, blood eosinophils are not related to higher risk of exacerbations, and cannot be assumed to reflect lung tissue eosinophils.

5. Semenzato U, ..., ***Biondini D,*** *et al.* ***Consistently low blood lymphocytes in smokers are associated with worse clinical outcomes.*** *Eur Respir J 2018 52: PA947. doi: 10.1183/13993003.congress-20188.PA947.*

Patient population

The population is the same of our previous studies on eosinophils. Following previous publications smokers were divided in two group using the cut-off of 1800 cells/μL[79].

The study was approved by human-research review board and all patients provided informed written consent.

Blood lymphocytes and lung tissue lymphocytes

We examined surgical lung specimens obtained from 43 smokers (29 with COPD and 14 without COPD) undergoing lung resection surgery. Using optical microscopy lymphocytes were counted in peripheral airways and expressed as number of lymphocytes/mm^2 of tissue. In 24 cases blood lymphocytes obtained at the time of surgery were correlated to tissue lymphocytes.

Statistical analysis

Differences between groups were evaluated with Kruskal-Wallis test and Mann-Whitney U tests. The association between categorical variables was investigated by $\chi 2$ or Fisher exact probability test. Analyses of overall survival and event-free survival were performed using Kaplan-Meier survival curves. Correlation coefficients were evaluated using the nonparametric Spearman rank method.

The aim of this study was to clarify the possible role of blood lymphocyte in the clinical features of COPD, and to investigate their consistency over time, the possible relation with lung tissue lymphocyte counts and their potential effect in clinical outcomes in smokers with and without COPD followed long term.

In the same population of 512 smokers (Table 4), all patients underwent a yearly blood lymphocyte (BL) measurement besides eosinophils count. As we can see in Figure 12, the number of lymphocytes in each blood samples obtained were significantly lower in smokers with COPD than in those without COPD. Moreover, because there was no significant variation in BL counts in the five samples neither in COPD nor in non-COPD, the mean value of the five sample was used for all the statistical analysis. Figure 13 shows the distribution of BL in COPD (mean value 1880 cells/µL) and non-COPD subjects (mean value 2300 cells/µL) ($p<0.01$). BL numbers were well within the normal range in most subjects; only 4 patients (0.7%) (COPD n=1, non-COPD n=3) had ≥4800 cells/µL while 28 patients (5.4%) (COPD n=22, non-COPD n=6) had <1100 cells/µL.

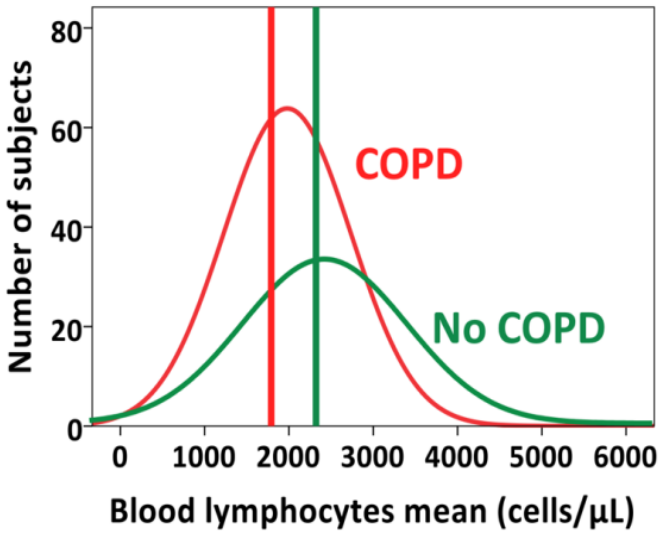


*Figure 12. Blood lymphocyte count at each blood samples. Mean blood lymphocyte count at each of the five blood samples in patients with and without COPD. No variability was found between the 5 blood samples in the smokers without COPD and in smokers with COPD. At each blood sample, smokers with COPD had a significative lower amount of lymphocyte compared to non-COPD smokers. *p<0.05*

When smokers were divided in low (<1800 cells/µL) and high (>1800 cells/µL) blood lymphocyte count, 23% (n=48) in non COPD and 43% in COPD (n=130) had low lymphocyte counts, while 77% (n=161) of non-COPD and 57% with COPD (n=172) had high lymphocyte counts.

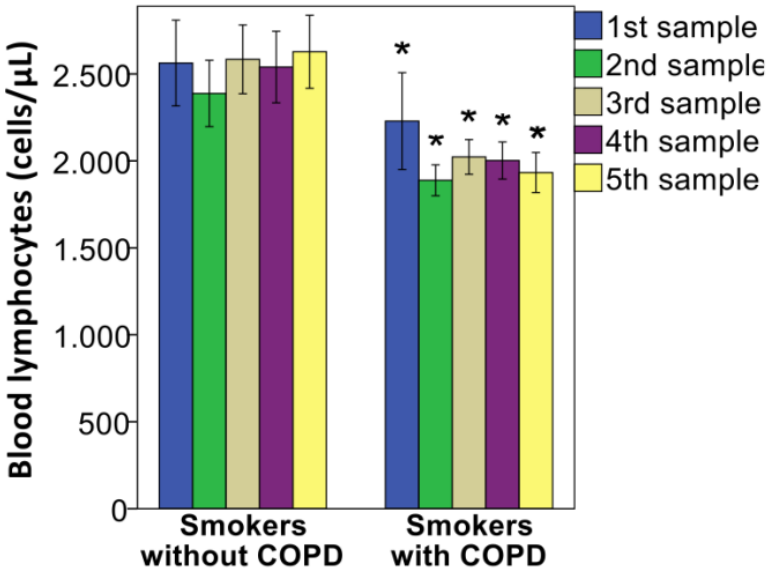


Figure 13. Mean blood lymphocytes distributions in all subjects divided by COPD (red lines) and non COPD (green lines) (1880 vs. 2300 cells/µL; p<0.01)

The lymphocyte numbers in peripheral blood were similar in active smokers and ex-smokers in the whole population and in the COPD and non-COPD groups indicating that in a population of smokers, lymphocyte numbers are not dependent of the smoking activity.

These results qualify previous knowledge stating that blood lymphocytes are increased in smokers, showing that there is an important variation of the number of

blood lymphocytes in smokers independent of the smoking activity and of the total number of white blood cell count in peripheral blood.

Table 6. Clinical characteristics of subjects in the Study Cohort. Smokers with and without COPD were divided in low blood lymphocytes (< 1800/μL) and high blood lymphocytes count (≥ 1800/μL). Data are presented as number (%) or mean ± SD, p value refers to Mann-Whitney test or χ2test

	No COPD			**COPD**		
	BL<1800/μL	**BL≥1800/μL**	**p**	**BL<1800/μL**	**BL≥1800/μL**	**p**
Subjects, n (%)	48 (23%)	161 (77%)	-	130 (43%)	173 (57%)	-
Age (years)	57 ± 11	50 ± 10	**<0.01**	64 ± 7	60 ± 8	**<0.01**
Smoking history (PY)	40 ± 25	33 ± 16	0.12	50 ± 24	49 ± 26	0.57
FEV_1 post-bd (l)	2.83 ± 0.82	2.90 ± 0.76	0.69	1.90 ± 0.67	2.01 ± 0.67	0.11
FEV_1 post-bd (% pred.)	97 ± 18	95 ± 14	0.48	67 ± 20	69 ± 19	0.36
FEV_1/FVC post-bd (%)	77 ± 4	79 ± 5	0.06	53± 12	55 ± 11	0.11
FEV_1 decline (ml/year)	43 ± 41	30 ± 35	0.05	35 ± 49	28 ± 53	0.46
N° of total exacerbations per year	0.56 ± 1.08	0.47 ± 1.05	0.26	1.27 ± 2.25	0.80 ± 1.29	**0.03**
N° of severe exacerbations per year	0.04 ± 0.11	0.03 ± 0.78	0.24	0.08 ± 0.21	0.04 ± 0.11	**0.05**
Subjects with mMRC ≥2, n (%)	20 (42%)	51 (32%)	0.07	78 (60%)	65 (38%)	**<0.01**
Subjects with comorbidities, n (%)	38 (79%)	122 (76%)	0.39	109 (84%)	149 (87%)	0.30
6-minute walking test distance (m)	468 ± 123	485 ± 112	0.54	344 ± 125	399 ± 89	**<0.01**

The clinical and demographic characteristics of smokers without and with COPD and <1800/µL or ≥1800/µL BL are shown in table 6. Smokers with no COPD and low BL were slightly older, tended to be more dyspneic and had a significantly higher yearly decline in FEV1 when compared to no COPD smokers with high BL.

Smokers with COPD with low BL, for similar values of FEV1 had a significantly higher percentage of patients with mMRC>2 (60% vs. 38%; p<0.01), a worse CAT score (11.5 vs. 9.26; p<0.01) and performed significantly worse in the 6 minutes walking test (344 vs. 399; p<0.05) compared to COPD with high BL (Table 6). From these analysis, it could be said that smokers with COPD have a much higher proportion of subjects with low BL and it is unclear whether they are cause or consequence of the development of COPD. There is a subtle effect of the low BL in smokers without COPD which tended to be more dyspneic and loose more function yearly than those with high BL. Smokers with COPD with low BL were significantly more dyspneic and limited to exercise, in spite of similar airflow limitation, than smokers with COPD and high BL.

There was no difference in the number of BL in the patients in different GOLD stage, although there was a large individual variation specially in the more severe GOLD stages.

Smokers with COPD had significantly more exacerbations per year than smokers without COPD (1.00 ± 1.78 vs 0.49 ± 1.05; p<0.01). Fifty-three percent (n=161) of the smokers with COPD developed at least one exacerbation during the follow-up. There was a significantly higher rate of total and severe exacerbation in COPD patients with low BL (1.27 vs. 0.80, p=0.03; 0.08 vs. 0.04, p=0.05 respectively) compared to COPD with high BL (Table 6).

Out of 511, 134 subjects (26%) in our cohort died during follow up. 114 (85%) were smokers with COPD and 20 (15%) smokers without COPD; 41% died from respiratory causes (COPD exacerbations, respiratory failure and pneumonia), 19% from lung cancer and 12% from cardiovascular diseases.

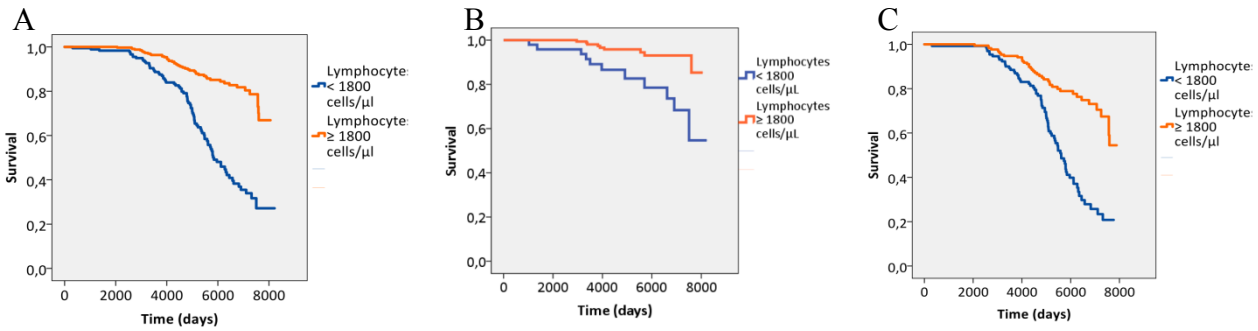


Figure 14. Survival in smokers with and without COPD. Survival of all population according to lymphocyte > or < 1800 cell/ µL (A), smokers without COPD according to lymphocyte > or < 1800 cell/µL (B), smokers with COPD according to lymphocyte > or < 1800 cell/µL (C). Smokers with low lymphocytes have a significantly lower survival in the overall population and in smokers with and without COPD.

In the whole cohort Kaplan-Meier analysis showed that subjects with lymphocytes >1800 /µL had a better survival than those with <1800/µL ($p<0.01$) (Figure 14A). The same findings were confirmed when considering smokers without and with COPD separately (Fig. 14B, 14C).

In a subgroup of subjects that had peripheral blood sample, we have then identified and quantified the specific lymphocyte populations.
Using the FACS analysis, we measured the percentage of T lymphocyte (CD4 and CD8), B cells and NK cells in each subject. There were no statistical differences between the percentage of these cells in COPD and no COPD with high and low BL count, probably due to the low number of patients analyzed. However, of interest, subjects with COPD and low lymphocytes had a lower numbers of B cells, higher amount of NK cells and lower CD4/CD8 ratio compared to COPD with high lymphocytes and also to subjects without COPD (Fig. 15).

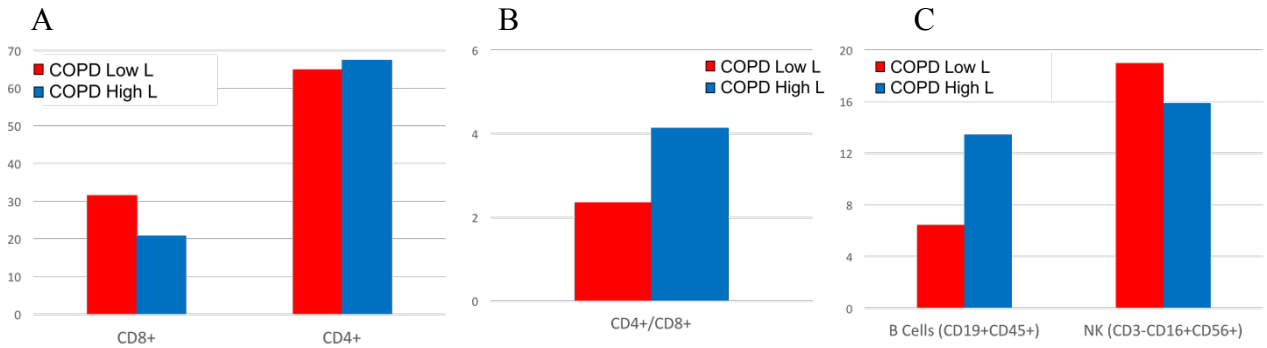


Figure 15. Lymphocyte populations in patients with COPD, divided in subjects with high and low BL. Percentage of CD8+ and CD4+ cells in all CD3+CD45+ (A), CD4/CD8 ratio (B) and percentage of B cells and NK cells on CD45+ cells (C)

In conclusion, in this study we showed how BL are significantly lower in COPD compared to no COPD subjects, and that in both groups individuals with low lymphocytes have worse outcome as exacerbations, dyspnea and survival.

6. ***Biondini D,*** *et al.* ***Lymphopenia is linked to an increased incidence of cancer in smokers without COPD.*** *Eur Resp J 2019 54: PA2586. doi: 10.1183/13993003.congress-2019.PA2586*

Patient population and the statistical analysis is similar to the previous study.

The aim of this study was to investigate in this population, which was free from any cancer at the beginning of the study, the incidence of cancer in smokers and whether it is influenced by blood lymphocyte count.

In the smoker population, 115 patients (22.5%) developed any type of cancer during the follow-up. The incidence of all types of cancer was significantly higher in smokers with COPD compared to smokers without COPD (27% vs 17%, p<0.01). The incidence of the different types of cancers in COPD and non-COPD smokers are shown in Figure 16.

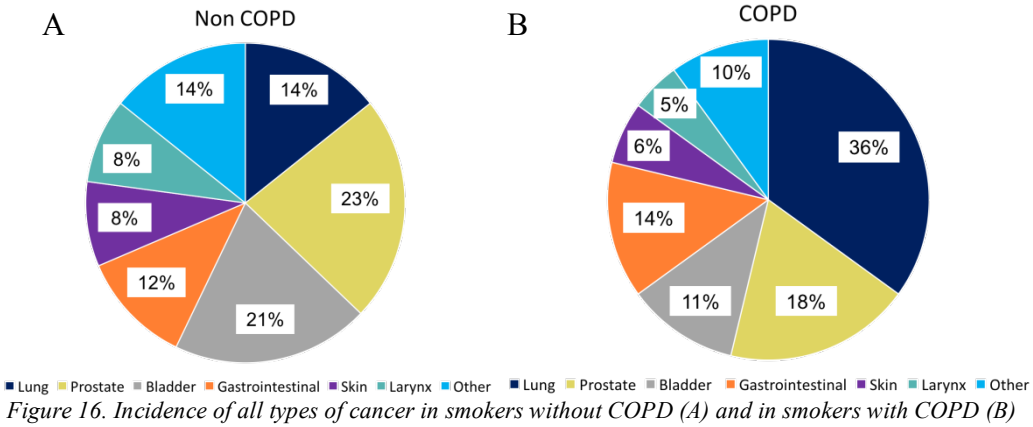


Figure 16. Incidence of all types of cancer in smokers without COPD (A) and in smokers with COPD (B)

In COPD, lung, prostate and GI cancers were the most frequent (36%, 18% and 14%) (Fig. 16B), while in non-COPD were prostate, bladder and lung (23%, 21% and 14%) (Fig. 16A).

In the whole cohort, there was a significant higher incidence of cancer in the subjects with low BL group than those with high BL (55/178, 31% vs 60/333, 18%, respectively; p=0.001). The incidence of lung cancer showed a trend towards the same direction (16/178, 9% vs 17/333, 5%, respectively; p=0.067) (Fig. 17A).

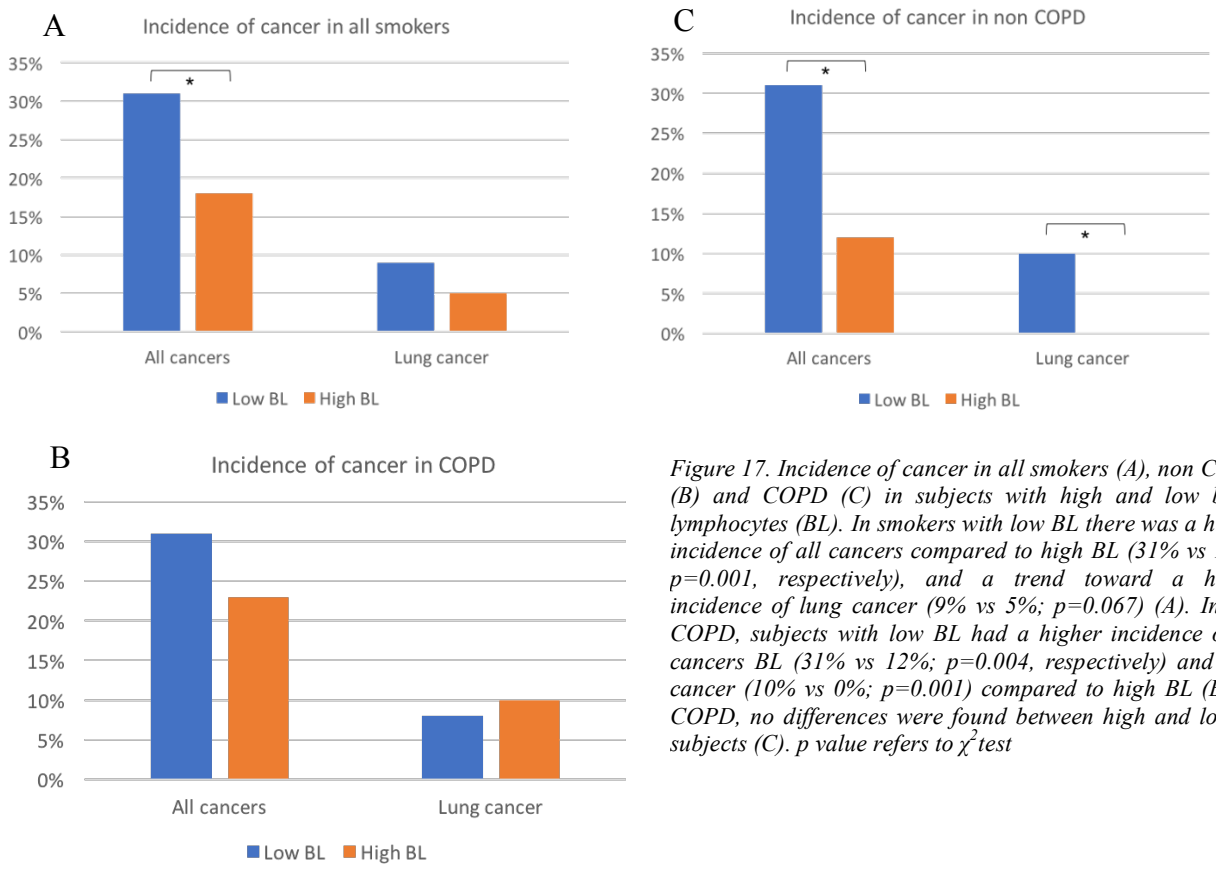


Figure 17. Incidence of cancer in all smokers (A), non COPD (B) and COPD (C) in subjects with high and low blood lymphocytes (BL). In smokers with low BL there was a higher incidence of all cancers compared to high BL (31% vs 18%; p=0.001, respectively), and a trend toward a higher incidence of lung cancer (9% vs 5%; p=0.067) (A). In non COPD, subjects with low BL had a higher incidence of all cancers BL (31% vs 12%; p=0.004, respectively) and lung cancer (10% vs 0%; p=0.001) compared to high BL (B). In COPD, no differences were found between high and lob BL subjects (C). p value refers to χ^2 test

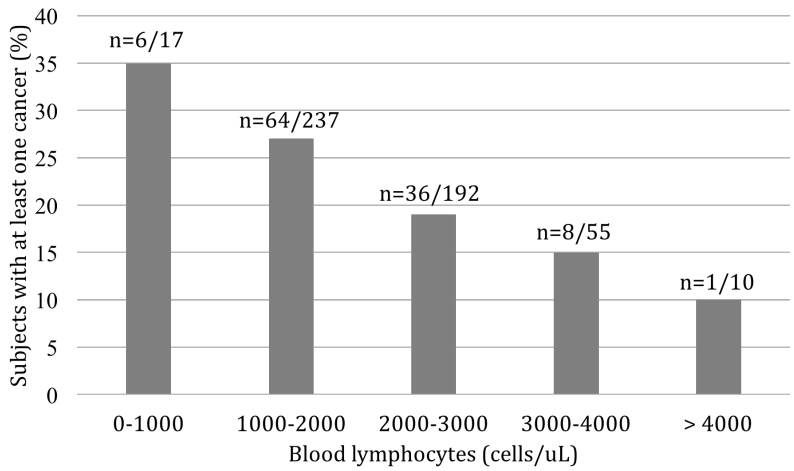


Figure 18. Incidence of cancer in the all smokers population, according to the different ranges of blood lymphocytes count

In smokers with COPD, similar incidence was found between high or low BL group regarding any type of cancer (40/130, 31% vs 40/172, 23%; p=0.15) or lung cancer (11/130, 8% vs 17/172, 10%; p=0.84) (Fig. 17B).

Interestingly, smokers without COPD with low BL had a higher incidence of any type of cancer (15/48, 31% vs 20/161, 12%; p=0.004) and lung cancer (5/48, 10% vs 0/161, 0%; p=0.001) when compared with smokers without COPD and high BL counts (Fig. 17C). Moreover, in the smokers population, there was a higher incidence of cancer in subjects with the lower interval of BL (0-1000 cells/ μL), that decreased as the BL count interval get higher (Fig. 18).

In conclusion, lymphopenia is associated to a higher incidence of all types of cancer and lung cancer especially in smokers without COPD.

7. Rabe KF, ..., ***Biondini D,*** *et al.* ***Anti-inflammatory effects of Roflumilast in Chronic Obstructive Pulmonary Disease (ROBERT): a 16-week, randomised, placebo-controlled trial.*** *Lancet Respir Med. 2018; 6:827-836. doi: 10.1016/S2213-2600(18)30331-X. Epub 2018 Sep 14*

Study design and participants

ROBERT was a double-blind, randomised, parallel-group, phase 3 biopsy study done at 18 centres specialising in lung diseases in five countries (Denmark, Germany, Poland, Sweden, and the UK). The design and rationale of ROBERT have been published previously[80]. Eligible patients were current or former smokers (smoking history of ≥20 pack years) aged 40–80 years who had a history of COPD diagnosed at least 12 months before the baseline visit, with chronic productive cough for at least 3 months in each of the 2 years before randomisation. Patients also had to have a post- bronchodilator predicted FEV_1 30–80% and a post- bronchodilator FEV_1/forced vital capacity (FVC) ratio of 70% or less. Patients who had a moderate or severe COPD exacerbation in the preceding 6 months, a respiratory tract infection

in the previous 4 weeks, a diagnosis of asthma, or known α-1-antitrypsin deficiency, were excluded. Concomitant glucocorticoids (including inhaled corticosteroids), theophylline, lipoxygenase inhibitors, antiplatelet therapy, and leukotriene antagonists were not permitted. The study was conducted in accordance with the Declaration of Helsinki, the International Council for Harmonisation Harmonised Tripartite Guidelines for Good Clinical Practice, and all applicable local or regional regulatory requirements. The protocol was approved by the ethics committee at each participating centre. All patients provided written informed consent to participate.

Randomisation and masking

Participants were enrolled by investigators. All patients completed a 6-week single-blind run-in of once daily placebo administration to assess compliance, during which inhaled corticosteroids and other drugs that were not permitted during the study were stopped. Patients who complied with the run-in regimen were randomly assigned (1:1) to 500 μg Roflumilast once daily or placebo for 16 weeks. Patients could also continue standard inhaled bronchodilator therapy. Patients who did not successfully complete the screening period or chose to drop out of the study before randomisation had the option to be re-enrolled 4 weeks later (allowed once per patient). Patients who discontinued the study at randomisation because of an exacerbation could be re-enrolled after resolution of the exacerbation at the discretion of the treating physician, but further exacerbations led to permanent exclusion from the study. A new case report form was completed for each re-enrolled patient, specifying re-enrolment.

Investigators used a computerised central randomisation system, stratified by concomitant use of long- acting β agonists, to randomly assign participants to groups. Both participants and investigators were masked to group assignment. Roflumilast and placebo were supplied as identical yellow, triangular tablets in wallet cards containing 60 tablets. The investigational drug blinding was maintained by using an interactive voice- response system and interactive web-response system.

Procedures

Bronchial biopsy specimens, induced sputum, and peripheral blood were collected and analyzed as previously described[80]. Bronchoscopies were done at baseline (ie, at randomisation) and week 16 to quantify the number of CD8, CD68, CD4, and CD45 cells, neutrophils, and eosinophils per mm^2 in the submucosa. CD8 and CD68 cells were also quantified in the bronchial epithelium. Absolute (cells per mL) and differential (%) counts of neutrophils, macrophages, eosinophil, and lymphocytes were also done in induced sputum, via nebulised saline inhalation 2 weeks before randomisation and at weeks 6 and 14. Basophils, eosinophils, erythrocytes, leucocytes, lymphocytes, monocytes, and neutrophils in peripheral blood were quantified in blood samples drawn at weeks –2, 6, and 16. FEV_1, FVC, and the

FEV_1:FVC ratio were measured at baseline and week 16.

Biopsy samples and sputum samples were analyzed centrally (one central laboratory for biopsies and one for sputum samples). All participating centres had to undergo training and continuous quality control before and during the study[80]. Bronchial biopsy samples were taken from each lobar and sub-segmental carina. To take into account inter-airway and intra-airway variability, two or three samples were taken from the lobar bronchus and from the subsegmental airway at each bronchoscopy session. Samples were taken from the same lung during each session, and from the other lung at the next session. Biopsy samples had to be 0.1 mm^2 or thicker and contain at least 1 mm basement membrane and at least 100 μm of submucosa[32]. Samples of adequate quality were selected on the basis of morphological criteria (presence of intact submucosa, artifacts not crushed, and without blood clots). The minimal area was judged not to have been achieved if the area occupied by the submucosa was insufficient- eg, the biopsies were mainly occupied by other airway wall components such as epithelium (too superficial) or airway smooth muscle or glands (either too deep or not cut perpendicularly), or because the submucosa was not intact (crushed or bloody). After each of the biopsy samples was confirmed as being of adequate quality, the number of positively stained cells in the submucosal area up to 100 μm below the basement membrane was counted. All adjacent non-overlapping fields were counted until all the subepithelial area had been covered. Positively stained cells were expressed as the number of cells per area of tissue examined in each biopsy from each patient.

Outcomes

The primary endpoint was the change in the number of CD8 inflammatory cells in bronchial biopsy samples (submucosa) between the randomisation visit and week 16. Secondary endpoints included changes in the number of inflammatory cells measured in the submucosa (CD68 cells, neutrophils, eosinophils, CD4 cells, and CD45 cells), bronchial epithelium (CD8 and CD68 cells), induced sputum (neutrophils, macrophages, eosinophils, and lymphocytes), and blood at week 16, and pulmonary function changes (ie, FEV_1, FVC, and the FEV_1:FVC ratio). Safety endpoints included adverse events, and changes in laboratory parameters, vital signs (including blood pressure and heart rate), physical examination findings, and bodyweight and body-mass index.

Statistical analysis

We estimated that, to detect a 30% reduction in CD8 cell count with Roflumilast compared with placebo with 90% power, with an assumed significance level of 5% and a CD8 cell count of 200 and 285 cells per mm^2 with Roflumilast and placebo, respectively, we would need to enroll 158 patients. The primary endpoint was

analyzed with Poisson regression, with CD8 cell count at week 16 as the dependent variable, and treatment and the dependent variable at baseline as covariates. The secondary endpoints related to bronchial biopsy samples were analyzed with the same Poisson regression model. All other statistical comparisons were based on a two-sided null hypothesis test assessed at the 5% (alfa=0.05) significance level, unless stated otherwise[80]. The full analysis set included all randomly assigned patients who received at least one dose of Roflumilast or placebo. All primary and secondary analyses were done in the intention-to-treat population.

We did exploratory analyses of the primary endpoint and a key secondary endpoint (CD68 cell count in bronchial submucosa) by ANCOVA in prespecified subgroups (total, percentage, and differential cell counts in induced sputum and concentration of inflammatory biomarkers in induced sputum and blood serum) as mentioned in our statistical analysis plan. These subgroup analyses were exploratory, and thus no adjustment for multiplicity was planned. No interaction test was done for the treatment subgroup analyses. A two-sided p-value testing treatment difference, and a point estimate of the least-squares mean treatment difference with SEs and corresponding 95% CIs were derived from the ANCOVA model. The estimated least-squares mean, its SE, and 95% CI were summarized for each treatment. The association between eosinophil counts in each of the three compartments (sputum, mucosa, and blood) was assessed by cross tabulations and $\chi 2$ tests.

Sensitivity analyses were done at week 16 to establish the robustness of the assumptions of the primary analysis, especially with regard to missing data (namely, biopsy samples that did not reach the minimal area requested per protocol). Additional information about sensitivity analyses is in the appendix. All statistical analyses were done in SAS for Windows (version 9.2). This study is registered with ClinicalTrials.gov, identifier NCT01509677.

The aim of this study was to investigate the anti-inflammatory effects of Roflumilast on airway tissue inflammation after 16 weeks, assessed in bronchial biopsy specimens from patients with moderate-to-severe COPD with chronic bronchitis.

158 COPD patients were randomized, 79 assigned to the Roflumilast group and 79 to the placebo group. Overall, nine (6%) randomly assigned participants permanently discontinued treatment. Baseline demographics and disease characteristics were similar between treatment groups (Table 7). Patients had a mean age of 63.2 years, 77% were male, and 78% had Global Initiative for Chronic Obstructive Lung Disease stage II disease (Table 7).

Table 7: Clinical characteristics of subjects in the Study Cohort

	Roflumilast (n=79)	Placebo (n=79)
Age (years)		
Mean	64·0 (8·2)	62·5 (8·4)
≤65	37 (47%)	46 (58%)
>65	42 (53%)	33 (42%)
Sex		
Male	60 (76%)	61 (77%)
Female	19 (24%)	18 (23%)
Body-mass index, kg/m²	28·5 (5·4)	27·3 (5·1)
Smoking status		
Former	40 (51%)	32 (41%)
Current	39 (49%)	47 (59%)
Cigarette pack year history	51·0 (21·0)	48·8 (21·5)
Chronic obstructive pulmonary disease stage		
I	2 (3%)	2 (3%)
II	62 (78%)	61 (77%)
III	14 (18%)	15 (19%)
IV	0 (0%)	1 (1%)
Concomitant use of long-acting β agonist	48 (61%)	48 (61%)
Former use of inhaled corticosteroids	38 (48%)	35 (44%)
Post-bronchodilator FEV_1, L	1·8 (0·5)	1·8 (0·5)
Post-bronchodilator FEV_1, % predicted	59·9 (12·1)	59·2 (12·9)
Post-bronchodilator FVC, L	3·6 (0·9)	3·5 (1·0)
Post-bronchodilator FEV_1:FVC ratio, %	50·5 (9·9)	51·8 (11·2)

Data are n (%) or mean (SD). FVC=forced vital capacity.

At week 16, biopsy samples were available from 148 patients (94%); 76 (96%) in the Roflumilast group and 72 (91%) in the placebo group.

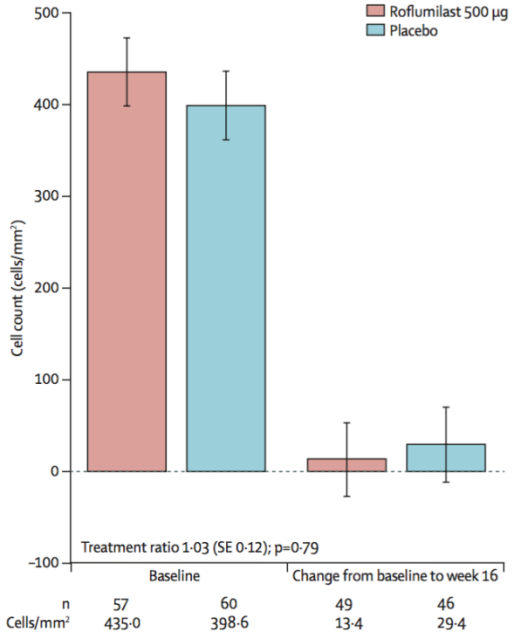


Figure 19. Baseline CD8 cell count and mean change from baseline to week 16 in CD8 cell count in bronchial biopsy submucosa. Error bars show the standard error of the mean

The analysis on lymphocytes change after treatment was the primary outcome. When it was compared the mean change from baseline to week 16 in CD8 cell counts in the bronchial submucosa, no significant differences were found between groups (treatment ratio 1.03, SE: 0.12; p=0.79; Fig. 19). The same analysis in CD8 and CD68 cell counts in the bronchial epithelium were similarly not significant (p=0.07 and p=0.36, respectively).

Then, eosinophils (secondary outcome) were evaluated, and at 16 weeks, the decrease in submucosal eosinophils was significantly greater in the Roflumilast group than in the placebo group (treatment ratio 0.53, 95% CI 0.34–0.82; p=0.0046; Fig. 20A).

Roflumilast treatment was also associated with significant reduction of eosinophils in induced sputum at week 14 in both absolute (p=0.0042) and differential cell counts (p=0.0086) compared with placebo (Fig. 20B, 20C). Conversely, the mean peripheral blood eosinophil counts did not change from baseline to week 16 in either the Roflumilast or placebo groups (Fig. 20D).

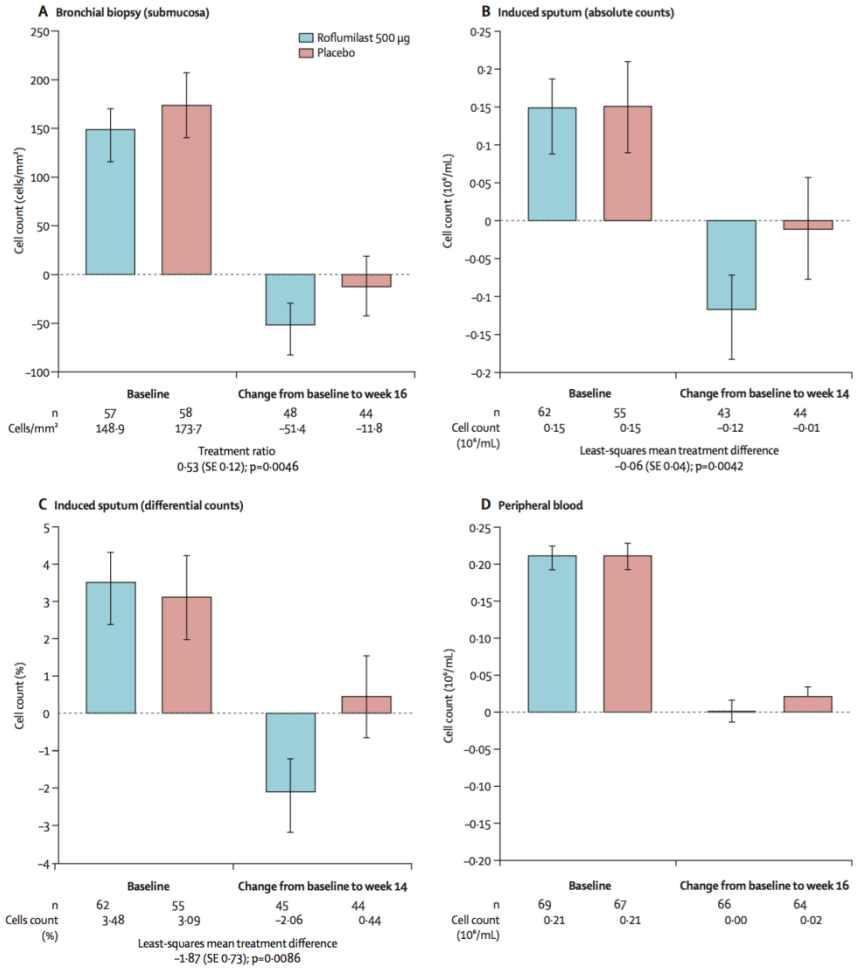


Figure 20. Baseline eosinophil count and mean change from baseline in eosinophil cell count in bronchial biopsy (submucosa) (A), absolute (B) and differential (C) induced sputum, and peripheral blood (D) in patients receiving roflumilast or placebo

The availability of tissue, sputum and either blood eosinophils in this study allowed a very noteworthy analysis of the relationship between each of these compartments. In detail, although a high eosinophil cell count in the submucosa was associated with a high eosinophil cell count in induced sputum (p<0.0001), we noted no association between eosinophil counts in peripheral blood neither in sputum (p=0.12) nor in the submucosa (p=0.10), confirming our previous finding.

In this study, the effect of 16-week treatment with Roflumilast on lung function was explored, showing a 0.063 L improvement in FEV1 compared with placebo (least-squares mean difference 0.063, SE: 0.03; p=0.04), not replicated on FVC (0.064, 0.06; p=0.25).

Overall, 69 (87%) patients treated with Roflumilast and 57 (72%) treated with placebo experienced at least one adverse event during the treatment period. The most common in the Roflumilast group were nasopharyngitis (16%), diarrhea (14%), and cough (13%). Eight patients in the Roflumilast group and five in the placebo group reported a serious adverse event, but no deaths were recorded during the study.

In conclusion, although Roflumilast was not associated with a reduction in CD8 cells in bronchial submucosa at 16 weeks, in exploratory analyses we noted a

significant reduction in eosinophil cell counts in bronchial biopsy samples and induced sputum. Moreover, blood eosinophil counts were not associated with either lung biopsy or sputum eosinophil counts. These data generate the hypothesis that the anti-inflammatory effect of Roflumilast in COPD could be mediated by an effect on lung eosinophils.

8. Rigobello C, ..., ***Biondini D****, et al.* ***Exome sequencing reveals immune genes as susceptibility modifiers in individuals with α_1-Antitrypsin deficiency.*** *Sci Rep. 2019 Sep 11; 9: 13088. doi: 10.1038/s41598-019-49409-1*

Study design

The study included 9 individuals from 4 different families in the Italian Registry for AATD. The probands selected from the registry were aged ≥18 years with the presence of severe AAT deficiency, defined by the carriage of the PI*ZZ genotype, or null genotypic variants. Within the same family, we compared siblings who were concordant for genotype, but discordant for clinical presentation (i.e. emphysema or no emphysema). e presence of emphysema in the affected sibling (and its absence in non-affected ones) was determined by chest HRCT. To confirm that non-affected subjects were free of any respiratory disease we also required them to have a preserved lung function with normal di using capacity for carbon monoxide (DLCO) and normal blood gases. To minimize the potential confounding effect of smoking, we have selected among the families in the Italian registry those whose smoking history was unremarkable (either nonsmokers or former smokers who had quit by >10 years and had <20 pack-years cumulative exposure). e study was approved by the Institutional Review Board (Policlinico San Matteo, Pavia) and complied with the principles set out in the Declaration of Helsinky. Informed consent was obtained from each participant regarding storage of biological samples and genetic sequencing. e clinical results are presented here in a fully anonymous form.

DNA was extracted from whole blood and genotyping performed as previously described[81]. Exome libraries were prepared using the Ion Proton Targeted Sequencing Library (Ion AmpliSeqTM Exome RDY Kit) with a mini- mum coverage of 80X. Data were processed with standard.

To filter and prioritize the identified variants, we used QueryOR[82], a new online pipeline developed by the bioinformatics unit of our university. We included only high confidence variants and variants that were non-synonymous, and excluded false positive variants in the reference genome. We did not apply any further filtering criteria, including both novel and annotated variants.

We analyzed affected and non-affected subjects considering different inheritance models: (a) recessive model (shared homozygosity in QueryOR); (b) dominant model (shared variants in QueryOR). e analysis was restricted to variants confirmed at least in three families out of four.

ANNOVAR[83] and dbNSFP database (v.2.9)[84] were used to integrate annotation, linking variants to genes, transcripts, proteins and biological ontologies. To further estimate the impact of variants on protein structure and function, we used scores that consider the functional impact on evolutionary conserved domains: CADD[85] (*Kircher, M. et al. A general framework for estimating the relative pathogenicity of human genetic variants. Nat. Genet. 46, 310–315 (2014)*), DANN[86] and PROVEAN[87]. Novel variants were compared across multiple specie in Ensembl[88], to verify conservation. To determine if our gene lists were enriched in any functional categories or metabolic pathways, we per- formed analyses using DAVID[89]. Reactome[90] and KEGG[91]. Finally, Enrichment map app[92] was used to visualize the enriched gene-set as a network in Cytoscape3 platform[93]. We used simulations with other families (without AATD), to assess the power of this approach under different levels of genetic heterogeneity (data not shown).

The aim of this study was to identify modifier genes that might potentially account for the variability in penetrance and expressivity of the pulmonary disease induced by the AATD.

Table 8. Clinical characteristics of subjects in the Study Cohort

Family ID	Subjects ID	Sex	Age (yrs)	AAT level (mg/dL)	Genotype	FEV_1 (% pred)	Smoking History
3	AWI	F	67	18	ZZ	94	Non Smoker
	AWJ	M	65	43	ZZ	106	Non Smoker
	AWK*	**M**	**60**	**22**	**ZZ**	**25**	**Ex Smoker**
185	**AWL**	**F**	**73**	**37**	**ZZ**	**57**	**Non Smoker**
	AWM	F	71	36	ZZ	85	Non smoker
237	**BIR**	**F**	**53**	**28**	**ZZ**	**102†**	**Non Smoker**
	BQ0	F	43	35	ZZ	100	Non Smoker
114	**BIP**	**M**	**50**	**23.3**	**Z/Q0**	**32**	**Ex Smoker**
	BIQ	F	47	20	Z/Q0	99	Non Smoker

In this study, we analyzed 4 families from the AATD Italian Registry with siblings concordant for genotype, but with discordant phenotypes (emphysema/no emphysema).

Clinical characteristics are reported in Table 8. All siblings had severe AATD, defined by the carriage of the PI*ZZ (families 3, 185, 237) or PI*Z/$Q0_{brescia}$ genotypes (family 114). Whole Exome Sequencing confirmed the genotype in all individuals. The particularity of this population was that each family had at least one affected sibling with emphysema and a non-affected sibling, free of disease. The population studied comprised 9 subjects, 4 affected and 5 non-affected, with a predominance of females (n=6; 66.7%) and a mean age of 56.5±15.5 years old.

We performed a whole Exome Sequencing, and from a total of 77204 variants at the beginning, we performed a filtering process and we finally analyzed 16319 variants. As the number of potential segregating variants is large, we have limited the number of candidate genes to those confirmed in at least three families out of four, and we have taken into consideration recessive and dominant models. In affected siblings, 14 genes with 15 variants in the recessive model (57% immune-related) and 21 genes with 23 variants in the dominant model (29% immune-related) were found. Figure 21A shows genes with recognized immune function identified in the affected subjects, none of which were present in the non-affected subjects. All these genes have been described to exert a pro-inflammatory function, affecting key steps involved in the immune response, such as: coagulation, complement and innate immunity (KNG1, THSD7B, IFIH1/MDA5), DC activation/maturation (IFIH1/MDA5, KNG1), ubiquitination antigen processing and presentation (MIB2, KLHL3), T cell function (AKNA, MS4A14, THSD7B, DNTTIP2) and B cell function (AKNA, DNTTIP2, MS4A14, PIK3AP1).

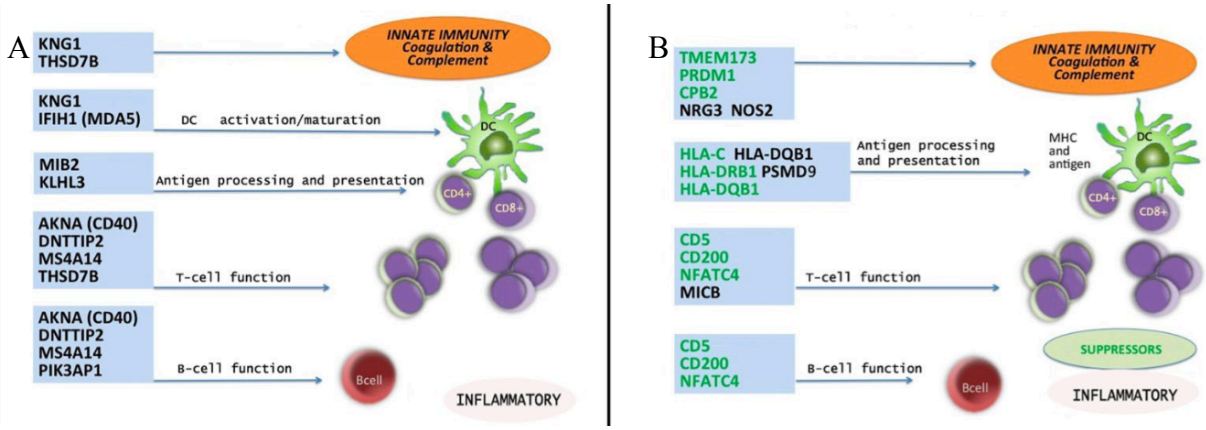


Figure 21. The discriminating genes with immune functions and their targets. Panel A shows variants with recognized immune function in the affected subjects. All these genes have been described to exert a pro-inflammatory function (pro-inflammatory genes in black font). Panel B shows variants with recognized immune function identified in the non-affected subjects. Most of these genes exert an immune suppressor function (suppressor genes in green font, pro-inflammatory genes in black font)

In non-affected subjects, 21 genes with 21 variants in the recessive model (43% were immune-related genes), and 50 genes with 62 variants in the dominant model (24% were immune-related genes) were found. Figure 21B shows genes with a recognized immune function identified in the non-affected subjects, none of which was present in the affected subjects. Contrary to what was found in the affected subjects, a significant number of the gene expressed in the non-affected group have been described to exert a suppressor function at the different steps of the immune cascade: complement and innate immune response (CPB2, PRDM1, TMEM173), antigen presentation (HLA alleles, mainly DRB1 DQB1), T and B cell functions (CD5, CD200, NFATC4).

Using Reactome and KEGG we investigated the possible functional significance, network interactions and biological pathways of the whole number of genetic variants,

differentially found in affected or non-affected individuals.

*9. **Lung digestion: Development of the technic for new and future approaches to the study of lung tissue inflammation***

We have developed the lung digestion technic following the methods used by Prof. Brusselle laboratory.
This technique is very important, because it give us the unique opportunity to evaluate directly in the lung tissue, all different components of inflammatory cells. With this purpose, in the last year of my PhD we have build-up in our laboratory the "lung digestion", with the idea of evaluating lung inflammatory infiltrate in a different way.

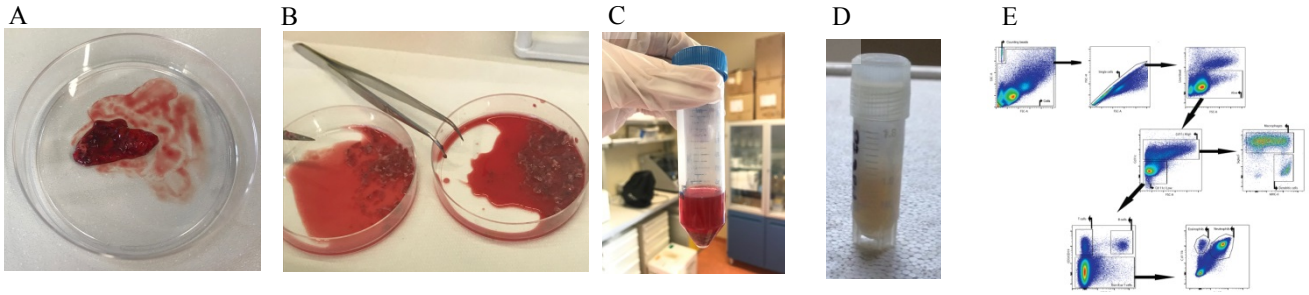


Figure 22. Lung digestion procedure consisting in taking a slice from a lung resection (A), cut the slice into very small pieces (B), then use DNAse and Collagenase to digest the tissue (C), to obtain a final single cell suspension (D) that will be further analyzed with FACS (E)

Starting with a lung specimen taken from a lung resection for peripheral lung nodule (Fig. 22A), we cut it into very small pieces (Fig. 22B) and then we digest the tissue slices with DNAse and Collagenase (Fig. 22C) After several centrifugations, we obtain a single cell suspension (Fig. 22D) that will be later analyzed by FACS (Fig. 22E). The aim of the FACS analysis is to quantify the number of inflammatory cells, and especially to evaluate its immunosuppressive state using markers as CTLA-4, PD-1, PDL-1.
At the moment, we have collected 18 patients, equally divided in smokers with COPD (severe and mild), smokers without COPD and non smokers. We have stored the single cell suspensions and we do not yet proceed with the FACS analysis, because we are still working on the setting procedure.

Studies in IPF

*1. Balestro E, ..., **Biondini D**, et al. **Immune inflammation and disease progression in idiopathic pulmonary fibrosis.** PLos One 2016; 11: e0154516. doi: 10.1371/journal.pone.0154516*

Patient population

In this study, we defined the clinical and functional progression in a group of 73 IPF patients referred for possible lung transplantation to our center in Padova between 2000 and 2014. In the 41 patients from this group who underwent lung transplantation we performed a quantitative pathological study of the native lung and compared the findings in lung pathology with the clinical progression.

Clinical analysis

Seventy-three patients with IPF referred to our center for possible lung transplantation were included in the study. IPF was diagnosed according to the ATS/ERS or the ATS/ERS/JRS/ ALAT Guidelines (according to whether they were referred before or after the publication of the 2011 guidelines). Information collected retrospectively was integrated with a longitudinal follow-up to determine the clinical progression of IPF (slow or rapid) from the beginning of symptoms to lung transplantation, death or end of follow-up (up to December 2014).

Medical records were reviewed and data on serial lung function tests, high-resolution computed tomography (HRCT) of the chest, right heart catheterization and/or echocardiography were collected for all patients. None of the subjects had a clear history of occupational or environmental exposure to fibrogenic agents, nor clinical features of hypersensitivity pneumonitis or connective tissue disease. All patients had negative autoimmune serologic testing including antinuclear antibodies (ANA), anti-double strand (anti-ds) DNA antibodies, anti-extractable nuclear antigens (ENA), antineutrophil cytoplasm antibodies (ANCAs) and cyclic citrullinated peptide (CCP). The study population included 2 brothers with familial IPF.

During follow-up, spirometry was serially performed every 6 to 12 months. The fall in % predicted FVC per year was used to characterize the disease progression as "rapid" (fall in % predicted FVC >10% per year) and "slow" (fall in % predicted FVC <10% per year) as previously reported[59].

The study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee for Clinical Experimentation of Padova. Written consent was obtained from all subjects. None of the transplant donors were from a vulnerable population and all donors or next of kin provided written informed consent that was

freely given.

Pathological analysis

Forty one of the 73 patients underwent lung transplantation. The native lungs were fixed in formalin by airway perfusion and samples from upper and lower lobes were obtained and embedded in paraffin. Sections 5 μm-thick were cut and stained for histological and immunohistochemical analysis.

In all transplanted cases the diagnosis of IPF was confirmed by our expert pathologist by the presence of an usual interstitial pneumonia (UIP) pattern[50]. Fibroblastic foci and lymphoid follicles were counted in sections stained with hematoxylin–eosin and antiCD20 respectively and expressed as number per square cm of area examined. Diffuse alveolar damage (DAD), defined as stratified hyaline membranes, was assessed on sections stained with haematoxylin-eosin and considered as "present" when detected in at least 30% of each lung section. Results were expressed as % of patients with presence of DAD over total number of patients.

Cellular inflammatory infiltrate comprising total leukocytes ($CD45^+$), neutrophils, macro- phages ($CD68^+$), $CD4^+$ and $CD8^+$ T lymphocytes as well as B lymphocytes ($CD20^+$) was identified by immunohistochemistry as previously described[80]. Each inflammatory cell type was quantified in 20 non-overlapping high-power fields per slide and expressed as cells/mm^2 of area examined. Morphometric analysis was performed by an experienced researcher (GT) and the intraobserver and interobserver reproducibility were than assessed.

Statistical analysis

Differences between groups were analyzed using Mann-Whitney U test and Fisher Exact test, as appropriate. The relationship between different outcomes was evaluated using Spearman's rank correlation. Multivariate regression analysis was performed to investigate if any of the clinical data available at diagnosis was associated with the rapid or slow rate of decline.

The aim of this study, first in the series, was to characterize the type of disease progression, slow or rapid, in a group of patients with IPF followed for long time before undergoing lung transplantation, and correlate it with a detailed quantitative study of the pathology of the explanted lung. Indeed, we hypothesized that the different clinical behavior may be accounted for, at least partially, by the different lung pathologies.

Table 9. Clinical characteristics of subjects in the study cohort

	Slow (n = 48, 66%)	Rapid (n = 25, 34%)	p value
Age at diagnosis–years	54(36–64)	54(33–69)	
Male sex–n. %	37(77%)	20(80%)	
Smokers–n. %	35(73%)	18(72%)	
Smoking history (only smokers)–pack-years	25(0.1–120)	24(3–93)	
Duration of symptom before diagnosis–months	31(2–96)	6(1–58)	0.0031
FVC at diagnosis–% predicted	61(20–94)	70(46–108)	0.022
FEV_1 at diagnosis—%predicted	64(26–97)	69(45–118)	
FEV_1/FVC at diagnosis—%	84(71–111)	80(73–89)	
DLco at diagnosis–% predicted	40 (3–100)	38(10–85)	
FVC at end follow up–% predicted	52(21–87)	38(22–91)	0.0025
Follow up (from diagnosis to end study*)–months	36(7–158)	24(12–60)	0.015
6MW distance–mt	270(40–437)	230(48–390)	
mPAP–mmHg	20(10–51)	20(13–45)	
FVC decline/year -% predicted	2.8(0.0–9.1)	14.2(10.2–28.0)	<0.0001
FVC decline/year—ml	98(0–416)	480(297–1499)	<0.0001
AE–n %	11(23%)	3(12%)	

Values are expressed as numbers and (%) or medians and (ranges)
* transplant (n = 41), death (n = 14) or end follow-up (n = 18)

Seventy-three patients with IPF were included, and as previously explicated, 48 (66%) had a slow FVC decline while 25 (34%) had a rapid decline.

Demographics and clinical characteristics of slow and rapid progressors are summarized in Table 9.

Age, sex and smoking habits were similar in the two groups. The duration of symptoms before diagnosis was significantly shorter in rapid as compared to slow progressors. At diagnosis, functional characteristics, including diffusing capacity of the lung for carbon monoxide (DLco) and FEV_1, were similar in the two groups. However, against expectations, the percent predicted FVC at diagnosis was significantly lower in slow than in rapid progressors. By contrast, and as expected, at the end of follow-up the percent-predicted FVC was significantly lower in rapid compared to slow progressors (Table 9).

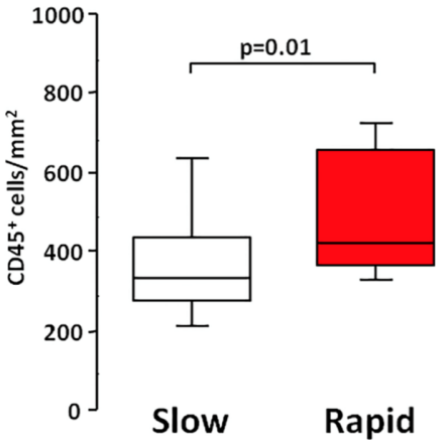


Figure 23. Total inflammatory cells in slow and rapid progressors. Number of total leukocytes (CD45+/mm2) in the lungs of slow and rapid progressors. Horizontal bars represent median values; bottom and top of each box plot 25th and 75th, brackets 10th and 90th percentiles. Slow: white; rapid: red.

The annual decline in FVC in slow progressors was 98 ml/year, while in rapid progressors was nearly 5 times higher (480 ml/year) (Table 9). There were no differences in treatment regimens, based on prednisone with or without azathioprine according to existing guidelines, between slow and rapid progressors.

Multivariable analysis showed that a lower FVC at diagnosis (OR: 0.22, 95% CI: 0.06–0.71, p=0.012) and a long duration of symptoms before diagnosis (OR: 6.56, 95% CI: 1.66–25.59, p=0.023) were the only clinical variables associated with a slow FVC decline during the follow up. The combination of these two variables had an additive predictive effect.

Lung pathology was examined in those patients who underwent lung transplantation

(n=41), 27 slow progressors and 14 rapid progressors. The clinical characteristics of these 41 transplanted patients were comparable to the 73 patients in the whole group.

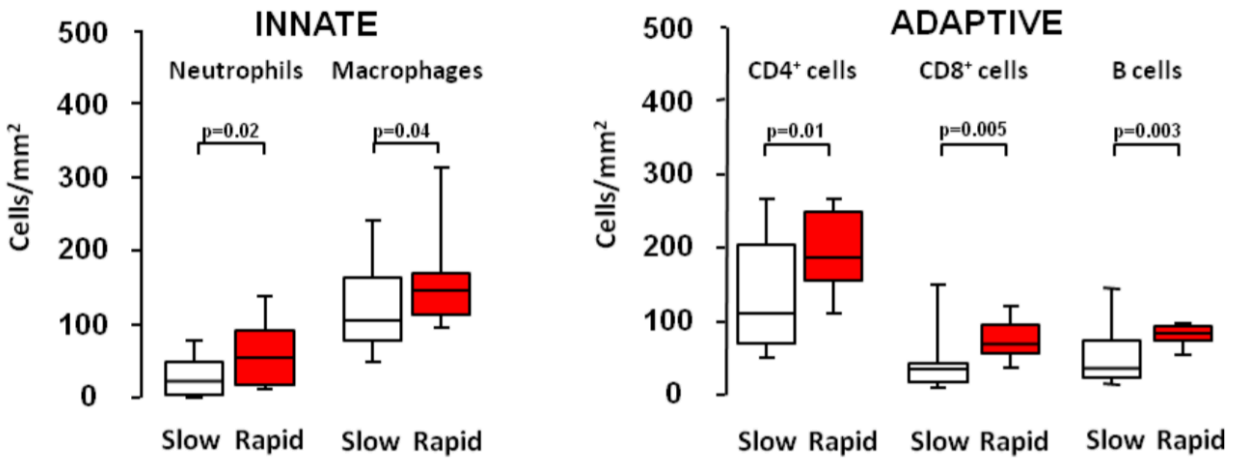


Figure 24. Differential Inflammatory cells in slow and rapid progressors. Number of innate inflammatory cells (neutrophils and macrophages) and adaptive inflammatory cells (CD4+, CD8+, and B lymphocyte) in the lungs of slow and rapid progressors. Horizontal bars represent median values, bottom and top of each box plot 25th and 75th, brackets 10th and 90th percentiles. Slow: white; rapid: red

Morphometric analysis of the explanted lung showed a prominent cellular inflammatory infiltrate with the number of total leukocytes/mm^2 (CD45+) being significantly higher in rapid than in slow progressors (p=0.01) (Fig. 23). In addition, both innate (neutrophils and macrophages) and adaptive (CD4+, CD8+ and B lymphocytes) inflammatory cell numbers were significantly higher in the rapid progressor group compared to the slow one (Fig. 24).

In conclusion, in a cohort of IPF patients referred for lung transplantation, an innate and adaptive inflammatory process of the lung is a significant feature and appears to be an important determinant of the rate of disease progression.

2. Cocconcelli E, Bazzan E, ***Biondini D****, et al.* ***Lymphoid follicles are a prominent feature in Idiopathic Pulmonary Fibrosis lung****. Eur Respir J 2019 54: PA1322; doi: 10.1183/13993003.congress-2019.PA1322*

Patient population

In this study 15 early IPF patients undergoing surgical lung biopsy for diagnosis, 42 end-stage IPF patients undergoing lung transplantation, 24 severe COPD patients undergoing lung transplant and 17 control smokers undergoing lung resection for nodules were studied.

Each IPF patient included in the study were referred to our center for diagnosis of unknown interstitial lung disease or possible lung transplantation. IPF was diagnosed according to the ATS/ERS or the ATS/ERS/JRS/ ALAT Guidelines (according to

whether they were referred before or after the publication of the 2011 guidelines). Information were collected retrospectively and patients were divided in slow or rapid progressors as previously reported.

Pathological analysis

All patient's sections from upper and lower lobes were taken and fixed in 10% phosphate-buffered formalin, and 5 μm-thick sections were stained for histological and immunohistochemical analysis.

Each inflammatory cell type (CD20, CD8 and CD4) was quantified in 20 non-consecutive high-power fields per slide as previously described All sections were immunostained for B lymphocytes (CD20+) to identify lymphoid follicles (LF), as aggregates containing more than 50 contiguous mononuclear cells that demonstrated the characteristic topographical arrangement of B cells, that were then counted (20x) in sections stained with anti-CD20 and expressed as number of LF/cm^2 and LF area/mm^2. T lymphocytes (CD4+ and CD8+) were also stained, counted (63x) and results expressed as numbers of cells/mm^2 of follicle area.

Statistical analysis

Differences between groups were analyzed using Mann-Whitney U test and Fisher Exact test, as appropriate. The relationship between different outcomes was evaluated using Spearman's rank correlation. Multivariate regression analysis was performed to investigate if any of the clinical data available at diagnosis was associated with the rapid or slow rate of decline.

The aim of this study, which is currently still ongoing, was to quantify lymphoid follicles in lungs of patients with IPF and compare them with those in severe COPD and healthy smokers. Then, we investigated whether number and size of lymphoid follicles in the lung of patients with IPF are related to the annual rate of FVC decline (slow or rapid).

Table 10. Clinical characteristics of entire population, slow and rapid progressors in "end stage" IPF (left part table), and in "early" IPF (right part table). Data are presented as number (%) or mean ± SD, p value refers to Mann-Whitney test or χ2test

	Entire population (n=42)	Slow progressors (n=30)	Rapid progressors (n=12)	p	Entire population (n=15)	Slow progressors (n=10)	Rapid progressors (n=5)	p
Male – *n (%)*	32 (76)	22 (73)	10 (83)	0.77	13 (87)	9 (90)	4 (80)	0.6
Age at dignosis - *yr*	53 (33-64)	54 (40-64)	53 (33-64)	0.54	58 (54-61)	58 (54-61)	58 (55-61)	0.93
Smoking history – *p/y*	15 (0-120)	14 (0-120)	18 (0-92)	0.93	4 (0-30)	0 (0-30)	8 (0-15)	0.99
FVC at dignosis - *L*	2.24 (0.7-3.7)	1.8 (0.7-3.6)	2.9 (2-3.7)	**0.0008**	3.6 (2.9-4.06)	3.2 (2.9-4.06)	3.9 (3.5-4)	0.70
FVC diagnosis - *% pred.*	61 (20-93)	58 (23-93)	71 (56-86)	**0.0078**	75 (40-109)	75 (40-109)	86 (50-107)	0.72
DLCO diagnosis - *pred.*	36 (10-85)	37 (15-79)	36 (10-85)	0.53	39 (16-74)	39 (18-65)	38 (16-74)	0.93
FVC decline - *%pred/yr*	3 (0-28)	1 (0-9)	12 (10-28)	**< 0.0001**	9 (0-16)	3.9 (0-8.6)	15.8 (10-16)	**0.05**

In the study, forty-two patients with "end stage" IPF were included, 30 had a slow

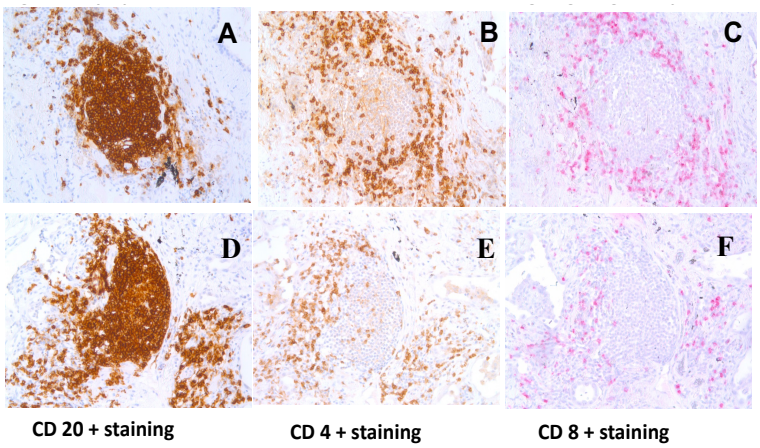


Figure 25. Lymphoid follicle (LF) identification in end-stage IPF, three consecutive staining of the same LF in rapid progressor patient with CD20+ (A), CD4+ (B) and CD8+ (C) and slow progressor patient with CD20+ (D), CD4+ (E) and CD8+ (F)

FVC decline while 12 had a rapid decline, and 15 with "early" IPF, 10 had a slow FVC decline while 5 had a rapid decline. Demographics and clinical characteristics of slow and rapid progressors in both groups are summarized in Table 10.

We identified and quantified LF area and number in lungs of patients with IPF and compared to severe COPD (n=24) and control smokers (n=17) by using CD20+ staining (Fig. 25A, 25D).

Interestingly, we found that LF area was smaller in "early" IPF compared to either "end stage" IPF, severe COPD and controls (Fig. 26A). However, when we counted the numbers of LF in "early IPF", they were consistently higher than "end stage" IPF (Fig. 26B). To note, both IPF subgroups had a higher number of LF than severe COPD. Conversely, controls had the lower numbers of LF/cm^2 compared to all groups.

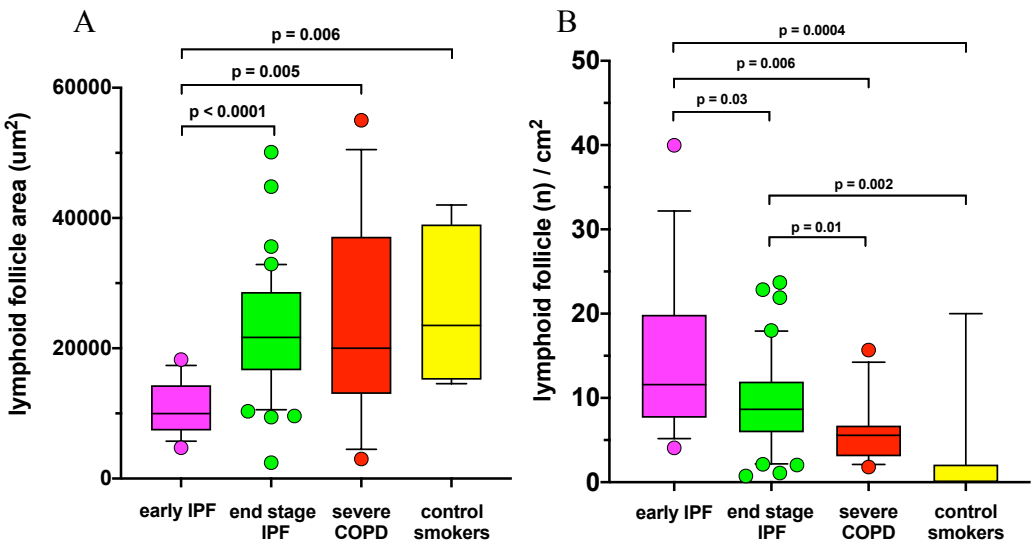


Figure 26. Lymphoid follicles area (A) and number (B) comparison between IPF, early and end stage, severe COPD and control smokers. LF area in early IPF was significantly lower than end-stage IPF, severe COPD and control smokers (A). Conversely, LF numbers in early IPF are significantly higher than end-stage IPF, severe COPD and controls. End-stage IPF have consistently higher LF number than severe COPD (B)

Then, we are currently studying CD4+ and CD8+ in LF of transplanted lungs of "end stage" IPF patients (Fig. 25B, 25C, 25E, 25F) and preliminary results showed that CD4+ cells were significantly higher in "end stage" IPF compared to severe COPD (Fig. 27A) and to controls. Conversely, CD8+ cells did not differ between these groups (Fig. 27B).

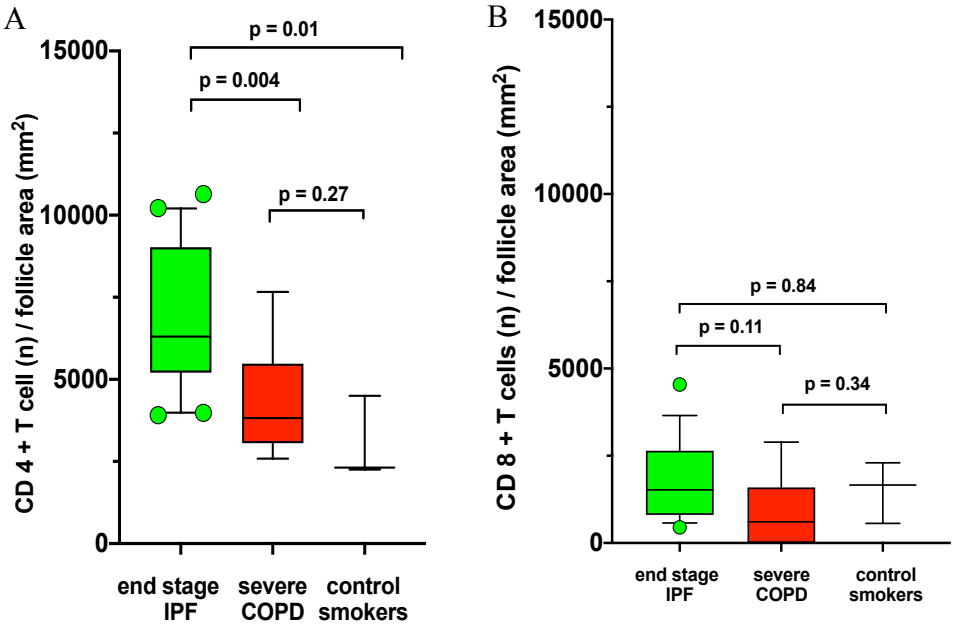


Figure 27. CD4+ and CD8+ T cells/ follicle area in end-stage IPF, early IPF and control smokers. End stage IPF has significantly higher CD4+ Tcells than severe COPD and controls (A), while CD8+ cells were not different between the three groups (B)

In conclusion, based on these findings, we found that well-formed lymphoid follicles are a prominent feature of both early and end stage IPF, supporting our previous study that inflammation in IPF, pointing toward an important immune/inflammatory scenario in IPF.

3. ***Biondini D****, et al.* ***Pretreatment rate of decay in forced vital capacity predicts long-term response to pirfenidone in patients with idiopathic pulmonary fibrosis****. Sci Rep. 2018; 8:5961. doi: 10.1038/s41598-018-24303-4*

Patients and study design

Informed consent was obtained for all study participants. This was a prospective, longitudinal, multicenter study, in which we analyzed a unique and well-characterized cohort of patients with IPF, with a long clinical and functional follow-up before and after the initiation of pirfenidone treatment. Fifty-six patients were selected from four Italian Interstitial Lung Disease (ILD) centers (e.g., University Hospital of Padua, n=27; University Hospital of Foggia, n=14; University Hospital of Modena, n=10; and General Hospital of Udine, n=5). For all patients, the diagnosis of IPF was made in accordance with current guidelines[2].

e peculiarity of this study was to include only patients for whom lung function data

were available for at least one year before (*pretreatment period*) starting pirfenidone treatment. Based on their annual rate of decline in FVC% pred. in the pretreatment period, patients were classified as either "rapid" (decline in FVC% pred. >10%) or "slow" (decline in FVC% pred. ≤10%) progressors. They were then followed-up during pirfenidone treatment every 6 months up to 24 months (*follow-up period*). At the 12-month follow-up, functional data were available for the entire patient population, whereas at 24 months functional data were available for 38/56 (68%) patients.

Negative values of annual FVC decline during the follow-up indicated amelioration. e study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the University Hospital of Padua (4280/AO/17).

Additional important clinical and functional parameters, including symptoms, di using capacity of the lung for carbon monoxide (DL_{CO}) and 6-minute walking test, were also collected but the amount of missing data did not allow for a meaningful statistical analysis to be performed.

Statistical analysis

Categorical variables were described as absolute (n) and relative values (%) and continuous variable were described as median and range. To compare demographic data and baseline clinical characteristics between rapid and slow progressors Chi-square test for categorical variables and Mann-Whitney test for the continuous variables were used.

In the entire population, as well as in the rapid and slow progressor subgroups, we performed the repeated measurements analysis of variance (ANOVA) at all time points to evaluate the difference in FVC decline between pretreatment and the follow-up period. To evaluate the difference between the pretreatment FVC decline and the first time point available (6 months) in the follow-up period in the entire population, in the rapid progressors and in the slow progressors we performed a paired t-test analysis. Finally, in order to evaluate whether this potential difference was maintained in the follow-up period (6, 12, 18 and 24 months), we performed the repeated measurements analysis of variance (ANOVA) between these time points in the entire population, and in the rapid and slow progressor subgroups.

All data were analyzed using SPSS So ware version 22.0 (IBM USA). p-values < 0.05 were considered statistically significant.

This study aimed to assess long-term (24 months) response to pirfenidone treatment in a well-characterized cohort of patients with IPF in a real-life setting, stratified in rapid and slow progressors.

A total of 56 patients were included in the study. Baseline patient demographics and clinical characteristics are summarized in Table 11.

Table 11. Clinical characteristics of subjects in the Study Cohort. Values are expressed as numbers and (%) or median and (ranges)

	Entire population (n=56)	Slow progressors (n=39)	Rapid progressors (n=17)	p value
Male - *n (%)*	44 (78%)	31 (79%)	13 (76%)	0.8
Age at diagnosis - *years*	67 (37–78)	67 (37–78)	67 (54–77)	0.8
Former smokers - *n (%)*	40 (71%)	28 (72%)	12 (71%)	0.9
Smoking history - *Pack-Years*	10 (0–60)	8.5 (0–60)	15 (0–60)	0.4
Radiologic diagnosis - *n (%)*	37 (66%)	24 (62%)	13 (76%)	0.3
FVC at diagnosis - *L*	2.66 (1.19–4.72)	2.72 (1.19–4.72)	2.66 (1.69–3.79)	0.5
FVC at diagnosis - *%pred.*	80 (35–116)	74 (35–116)	83 (61–105)	0.1
DL_{CO} at diagnosis - *%pred.*	54 (28–114)	52 (28–114)	61 (48–75)	0.1
Transplanted patients - *n*	3	3	0	0.5
Deaths - *n*	6	5	1	0.4

Most patients were males (78%) and ex-smokers (71%), with a median age at diagnosis of 67 years (range 37–78). Importantly, these patients were not on antifibrotic treatment at the beginning of the study, permitting us to clearly compare if there were a change in lung function decline before and during Pirfenidone treatment.

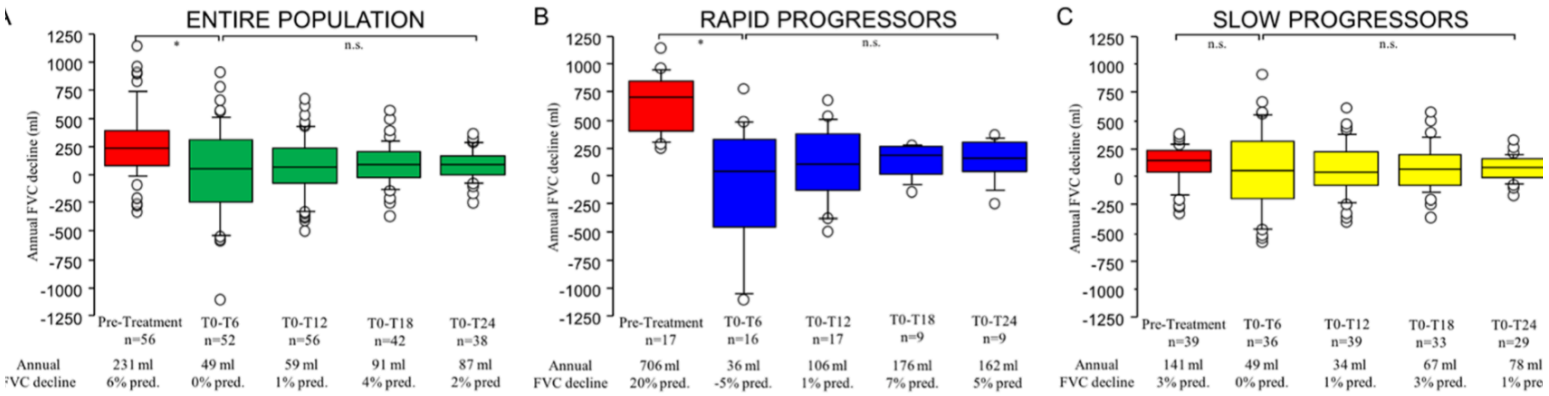


*Figure 28. Panel (A) Annual FVC decline in the entire population (n56) before and after pirfenidone treatment at 6 (T0-T6), 12 (T0-T12), 18 (T0-T18) and 2 (T0-T24) months. Overall comparison between all time-points was performed using the repeated measurements analysis of variance (ANOVA) (p=0.03) Pirfenidone reduced significantly the annual decline in FVC already at 6 months (paired t-test, p = 0.003) and this reduction was maintained at 12-, 18- and 24 month follow-up (repeated measures analysis of variance at all time points, p=n.s.). Panel (B) Annual FVC decline in the rapid progressors (n=17) before an after pirfenidone treatment at 6 (T0-T6), 12 (T0-T12), 18 (T0-T18) and 24 (T0-T24) months. Overall comparison between all time-points was performed usin the repeated measurements analysis of variance (ANOVA) (p < 0.001). Pirfenidone reduced significantly the annual decline in FVC already at 6 months (paire t-test, p < 0.01) and this reduction was maintained at 12-, 18- and 24-month follow-up (repeated measures analysis of variance at all time points, p=n.s.). Pane (C) Annual FVC decline in the slow progressors (n=39) before and after pirfenidone treatment at 6 (T0-T6), 12 (T0-T12), 18 (T0-T18) and 24 (T0-T24) months Overall comparison between all time-points was performed using the repeated measurements analysis of variance (ANOVA) (p = 0.1). Pirfenidone did no significantly reduce the annual decline in FVC at any of the time points examined (paired t-test, p=n.s.; repeated measures analysis of variance at all tim points, p=n.s.). Negative values mean improvement of FVC. Horizontal bars represent median values, bottom and top of each box plot represents 25th and 75t percentiles, brackets 10th and 90th percentiles, while circles represent outliers. *p value < 0.01, n.s. non significant*

Based on the decline in the pretreatment period, 39 patients were classified as slow (FVC% pred.≤10%) and 17 as rapid progressors (FVC% pred.>10%). Gender, age at diagnosis, smoking history and functional impairment (as assessed by FVC and DLCO) were similar in the two groups. Notably, a trend towards a higher FVC% pred. at diagnosis was seen in rapid progressors, consistent with previous publications from our group. Pulmonary function data were available for all patients at the 12-month follow-up, and for most of them at 24 months (38 out of 56, 68%).
In the pretreatment period, the median rate of annual FVC decline was 231 ml (range −323 to 1140 ml) in the entire population, 141 ml (range −323 to 375 ml) in slow progressors and 706 ml (range 242 to 1141 ml) in rapid progressors (Fig. 28A-28C).
We first analyzed the IPF population as a whole (n=56), confirming that pirfenidone treatment reduced significantly the rate of annual FVC decline from 231 ml/year (corresponding to 6% pred./year) in the pretreatment period to 49 ml/year at 6 months (T6) (0% pred., p=0.003), and this reduction persisted at 12- (59 ml/year, 1% pred.), 18- (91 ml/year, 4% pred.) and 24-month follow-up (87 ml/year, 2% pred.) (p value for trend from T6 to T24 n.s.) (Fig. 28A).

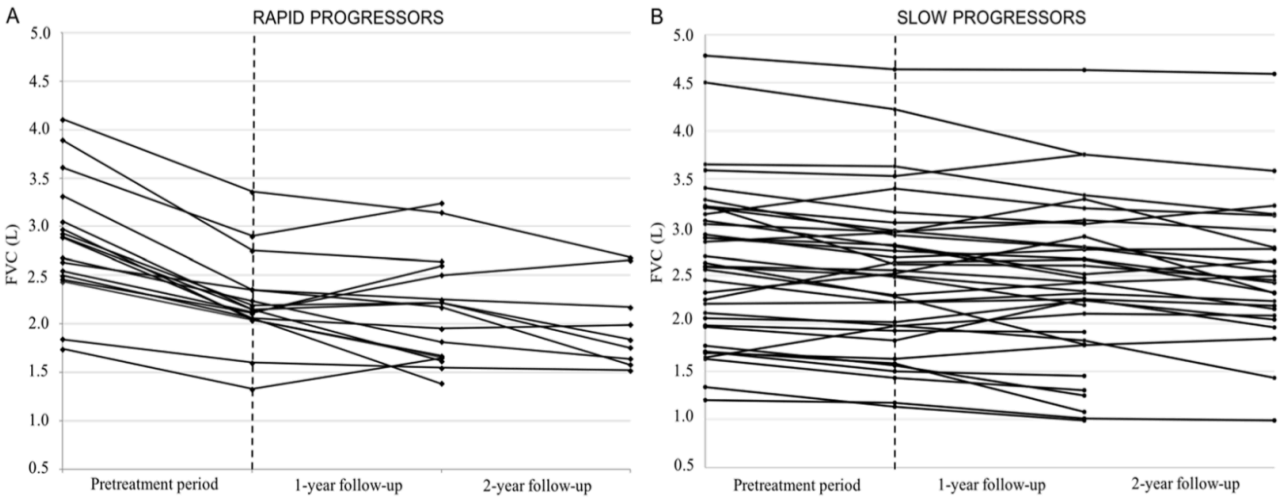


*Figure 29. Panel (A) Individual FVC trajectories in rapid progressors from pretreatment period through 2-year follow-up. Overall comparison between all time-points was performed using the repeated measurements analysis of variance (ANOVA) (p<0.001). In the pretreatment period, the FVC trajectory of the rapid progressors is depicted by a line with a steep slope. Whereas, after the institution of pirfenidone (vertical dotted line), FVC trajectory is depicted by a line with a significantly flatter slope compared to the pretreatment period (paired t-test, p<0.01). The FVC values during treatment were stable in the 2-year follow-up (repeated measures analysis of variance at 1-year and 2-year time points, p=n.s.). (B) Individual FVC trajectories in slow progressors from pretreatment period through 2-year follow-up. Overall comparison between all time-points was performed using the repeated measurements analysis of variance (ANOVA) (p=n.s.). In the pretreatment period, the FVC trajectory of the slow progressors is depicted by a line with a flat slope. After the institution of pirfenidone (vertical dotted line), FVC trajectory is depicted by a line with a slope as flat as the pretreatment period (paired t-test, n.s.). The FVC values during treatment were stable in the 2-year follow-up (repeated measures analysis of variance at 1-year and 2-year time points, p=n.s.). FVC (on the y axis) is expressed as litres. *p value<0.01, n.s. non significant*

Then, we investigated the effect of pirfenidone treatment in rapid and slow progressors separately. Among rapid progressors (n=17), the beneficial effect of pirfenidone on FVC decline was already evident at 6 months (T6), (from 706 ml/year, 20% pred. in the pretreatment period to 36 ml/year, −5% pred., p=0.002), and maintained at the 12- (106 ml/year, 1% pred.), 18- (176 ml/year, 7% pred.) and 24-month follow-up (162 ml/year, 5% pred.) (p value for trend from T6 to T24 n.s.) (Fig. 28B, 29A).
On the other side, in slow progressors, the reduction of the annual FVC decline did not reach statistical significance neither at 6 months (T6) (49 ml/year, 0% pred. vs. 141 ml/year, 3% pred. pretreatment, p = 0.3), nor in any of the other follow-up time points (34 ml/year, 1% pred. at 12 months; 67 ml/year, 3% pred. at 18 months; 78 ml/year, 1% pred. at 24 months) (p value for trend from T6 to T24 n.s.) (Fig. 28C, 29B).

In conclusion, this study confirms that Pirfenidone treatment reduces significantly the rate of FVC decline in patients with IPF, and the effect is significantly more pronounced in patients with rapidly progressive disease.

4. Cocconcelli E, Balestro E, ***Biondini D****, et al.* ***High-Resolution Computed Tomography (HRCT) reflects disease progression in patients with Idiopathic Pulmonary Fibrosis (IPF): relationship with lung pathology****. J Clin Med. 2019 22; 8. pii: E399. doi: 10.3390/jcm8030399*

Patient Population
In this longitudinal study, we analyzed a well-characterized cohort of Idiopathic Pulmonary Fibrosis (IPF) patients, with a long clinical functional and radiological follow up, referred between 2011 and 2014, naïve of antifibrotics.
All patients in our study, whether from our center or referred to our center, were offered antifibrotic therapy as soon as it became available, provided they met the Forced Vital Capacity (FVC), DL_{CO} and age criteria for treatment and they had no clear contraindications to it. However, given that the aim of our study was to look at a population of patients off treatment, we considered only radiological and functional data before antifibrotic therapy was instituted. In addition, a minority of our patients belonged to an historical cohort from the pre-antifibrotic therapy era (before 2014) and they had no access to antifibrotic therapy.

Forty-nine patients from two Interstitial Lung Disease Centres in Italy (University of Padova, Italy, n=43 and University of Foggia, Italy n=6) were included.
The diagnosis of IPF was made in accordance with the latest guidelines[2,63]. Clinical and functional data were collected at the time of diagnosis.
Based on their annual rate of decline in forced vital capacity percent (FVC%) predicted, patients were categorized as slow (<10%) or rapid progressors (≥10%).
The study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital of Padova (4280/AO/17). Informed consent was obtained for all study participants.

Study Design and Radiological Analysis

A HRCT was obtained at diagnosis ($HRCT_1$) in all patients. Twenty-one patients had a second HRCT ($HRCT_2$), after a median of 17 months of follow-up. The clinical and functional data of this subgroup are shown in Table S1. $HRCT_1$ and $HRCT_2$ were scored blindly and independently by two expert thoracic radiologists by using a quantitative scale, as previously described[65]. Briefly, this score consists of the assessment of ground glass opacities (GGO) (alveolar score, AS%) and fibrotic extent (interstitial score, IS%) for each lung lobe. After each individual lobe was scored for both IS and AS, the final result was expressed as mean value of the five lobes for the whole lung and in different lung regions (upper and lower). The inter-observer agreement between the two radiologists was good (Cohen's kappa 0.7), a value similar to that reported in previous studies[94].
In the twenty-one IPF patients in whom a second HRCT was available, we studied the correlation between radiological changes and FVC% decline by calculating the change of Alveolar Score (ΔAS/month), the change of Interstitial Score (ΔIS/month) and the change in FVC (ΔFVC mL/month) in the period from $HRCT_1$ to $HRCT_2$. We expressed the radiological changes per month to normalize the differences in timing between $HRCT_1$ and $HRCT_2$ in the slow and rapid progressors.

Pathological Analysis

Thirteen of the 49 patients underwent lung transplantation during the follow up (for clinical-functional data, see Table S2). In all cases, the presence of UIP pattern was histologically confirmed by our expert pathologist (FC)[2]. The native lungs were fixed in formalin by airway perfusion and samples from upper and lower lobes were obtained and embedded in paraffin. Sections with a thickness of 5 μm were cut and stained for histological and immunohistochemical analysis, as previously described.
Fibroblastic foci were counted in sections stained with hematoxylin–eosin and expressed as number of fibroblastic foci/mm^2 of area examined. Cellular infiltrate including total leukocytes (CD45+), neutrophils, macrophages (CD68+), and total

lymphocytes calculated as sum of CD4+, CD8+ T lymphocytes as well as B lymphocytes (CD20+) was identified by immunohistochemistry as previously described[95]. Each inflammatory cell type was quantified in 20 non-overlapping high-power fields per slide and expressed as cells/mm^2 of area examined.
In the thirteen IPF patients in whom the histological tissue and a HRCT performed at time close to the transplantation were available, we studied the correlation between the radiological changes and the cellular inflammatory infiltrate and between the radiological changes and the fibroblastic foci count.
Statistical analysis
Statistical analyses were performed as previously described. To compare clinical and pathological data between rapids and slows, Chi square test or Fischer's exact test and Mann-Whitney U test were used when appropriate. To evaluate the difference between HRCT1 and HRCT2, Wilcoxon analysis was performed.
Correlation coefficients between radiological, functional and pathological findings were calculated using nonparametric Spearman's rank method. Adjusted p-values for multiple comparison were calculated using the Holm method. The inter-observer agreement between the two radiologists was evaluated by kappa statistic measure. All data were analyzed using SPSS Software version 25.0 (New York, NY, US: IBM Corp. USA) p-values < 0.05 were considered statistically significant.

In this study, we assessed whether HRCT pattern at diagnosis may predict disease behavior (slow or rapid progressors), and whether it may have a pathological basis. Moreover, we investigated if changes of the HRCT pattern over time are linked to functional decline, without the confounding factor of treatment.

In this study we included 49 patients, of them 30 patients were slow and 19 rapid progressors. The clinical and radiologic characteristics at baseline are shown in Table 12.
Most patients were males and former smokers. None of the patients was treated with antifibrotics and 60% (equally distributed between the two groups) were treated with low dose prednisone with or (equally distributed between the two groups) were treated with low dose prednisone with or without azathioprine according to previous guidelines[50].
In the HRCT at diagnosis (HRCT1), AS was significantly greater in rapid in rapid than in slow progressors ($p=0.008$), while IS was similar in the two groups, either in the entire lung entire lung (Fig. 30) or in different lung regions, upper and lower zones.

Table 12. Clinical characteristics of subjects in the Study Cohort

	Entire Population (n = 49)	***Slow Progressors* (n = 30)**	***Rapid Progressors* (n = 19)**	***p* Value**
Male – n (%)	42 (86)	24 (80)	18 (94)	0.22
Age at diagnosis – years	58 (33–74)	58 (46–74)	60 (33–69)	0.75
Smoking history – pack years	20 (0–93)	15 (0–60)	21 (0–93)	0.24
• Current – n (%)	2 (4)	1 (3)	1 (5)	1
• Former – n (%)	40 (82)	23 (77)	17 (89)	0.45
• Non smokers – n (%)	7 (14)	6 (20)	1 (5)	0.22
Symptoms duration at diagnosis – months	20 (0–240)	20 (0–240)	18 (0–120)	0.58
Radiological diagnosis – n (%)	28 (57)	20 (67)	8 (42)	0.13
FVC at diagnosis – L	2.34 (1.19–4.06)	2.18 (1.19–4.06)	2.51 (1.75–4)	0.38
FVC at diagnosis – %pred.	67 (36–109)	66 (36–109)	76 (46–107)	0.52
DL_{CO} at diagnosis – %pred.	47 (10–97)	45 (25–97)	50 (10–82)	0.73
FVC decline per year – mL	275 (−330–1498)	130 (−330–380)	689 (331–1498)	<0.0001
FVC decline per year – %pred.	9 (−30–35)	4 (−30–9)	16 (11–35)	<0.0001
Patients undergoing transplant – n (%)	13 (27)	6 (20)	7 (37)	0.31
Patients who died – n (%)	28 (57)	15 (50)	13 (68)	0.2

Values are expressed as numbers and percent or median and ranges. Negative values mean improvement of FVC. *p*-values refers to comparison between slow and rapid progressors.

To corroborate the findings observed in previous analyses, we obtained a ROC curve on Alveolar Score data in rapid and slow progressors. We found that the area under the curve was 0.72 (95% Confidence Interval 0.57–0.87; p=0.008). On the other hand, in ROC curve for Interstitial Score, we did not observe any statistically significant results (95% Confidence Interval 0.35–0.67; p=0.88).

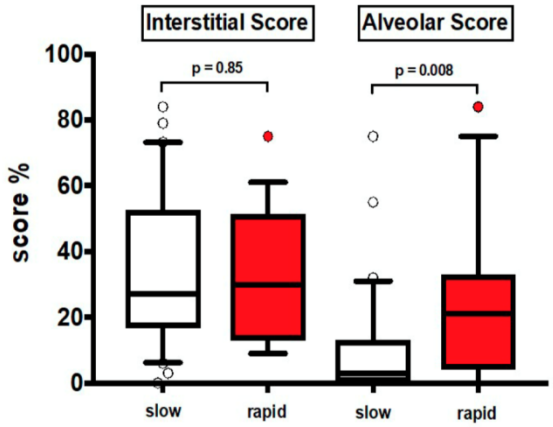


Figure 30. Values of HRCT1 Interstitial Score and Alveolar Score at baseline in slow progressors (slow) and rapid progressors (rapid). Horizontal bars represent median values; bottom and top of each box plot 25th and 75th; brackets show 10th and 90th percentiles; and circles represent outliers. White boxes t 25th and 75th; brackets show 10th and 90th percentiles; and circles represent outliers. White boxes indicate slow progressors and red boxes rapid progressor.

In our population, we had the unique opportunity to evaluate 13 lung specimens of patients who were transplanted (Table 13), and we quantified the lung pathology.

The number of CD20+ B lymphocytes, CD4+ and CD8+ T lymphocytes (considered both individually or all together as total lymphocytes) was significantly increased in rapids than in slows (Table 13). No significant difference in the number of CD45+, neutrophils, macrophages and fibroblastic foci was found between rapid and slow progressors.

Table 13. Inflammatory cells numbers of the entire population with lung pathology (n = 13), including six slow and seven rapid progressors

	Entire Population (n = 13)	***Slow Progressors* (n = 6)**	***Rapid Progressors* (n = 7)**	***p* Value**
Total leukocytes $CD45^+$ -, cells/mm^2	352 (149–732)	284 (149–383)	379 (333–732)	0.7
Macrophages, cells/mm^2	136 (63–308)	132 (63–308)	136 (71–303)	0.9
Neutrophils, cells/mm^2	51 (2–138)	6 (2–62)	51 (4–138)	0.1
Total lymphocytes, *cells/mm^2*	273 (74–414)	152 (74–273)	353 (256–414)	0.002
• CD 20^+ B lymphocytes	42 (25–115)	36 (27–115)	62 (25–115)	0.008
• CD 4^+ T lymphocytes	138 (20–284)	87 (20–138)	194 (115–284)	0.002
• CD 8^+ T lymphocytes	44 (12–120)	33 (12–45)	66 (26–120)	0.001
Fibroblastic foci, n/mm^2	2.7 (1–7)	2.8 (2–7)	2 (1–4.6)	0.09

Values are expressed as median and ranges. *p* values refers to comparison between slow and rapid progressors.

Then, we performed a correlation between pathological and radiological feature, and found that the total number of lymphocytes/mm^2 was positively correlated with the HRCT AS in the whole population (Fig. 31).

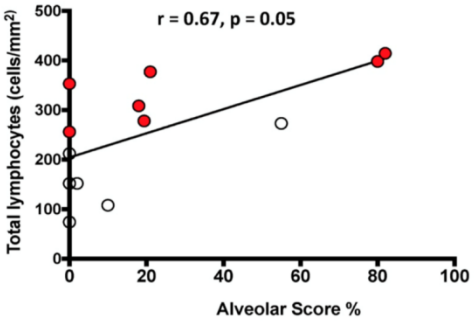


Figure 31. Relationship between the number of total lymphocytes infiltrating the lung tissue and the Figure 3. Relationship between the number of total lymphocytes infiltrating the lung tissue and the HRCT Alveolar Score. The black line represents the correlation in the entire population. White circles HRCT Alveolar Score. The black line represents the correlation in the entire population. White circles indicate slow progressors and red circles rapid progressors. Spearman's rank correlation: r = 0.67, indicate slow progressors and red circles rapid progressors. Spearman's rank correlation: r = 0.67, p = p = 0.01 in the entire population; r = 0.33, p = 0.48 in slow progressors alone; r = 0.81, p = 0.03 in rapid 0.01 in the entire population; r = 0.33, p = 0.48 in slow progressors alone; r = 0.81, p = 0.03 in rapid progressors alone

The number of FF/mm^2 did not correlate with the HRCT IS in rapid progressors, slow progressors, or when considering the entire population.

Then, in 21 patients who had a follow up HRCT2, we found that both AS and IS increased significantly over time in both groups together. When the patients were divided by rate of decline, IS increased over time in both slows and rapids, while AS increased significantly only in rapids (Fig. 32). When we compared functional and radiologic data, we found a significant correlation between the functional decline, defined as ΔFVC mL/month, and the radiological changes in IS, defined as ΔIS/month, but not with

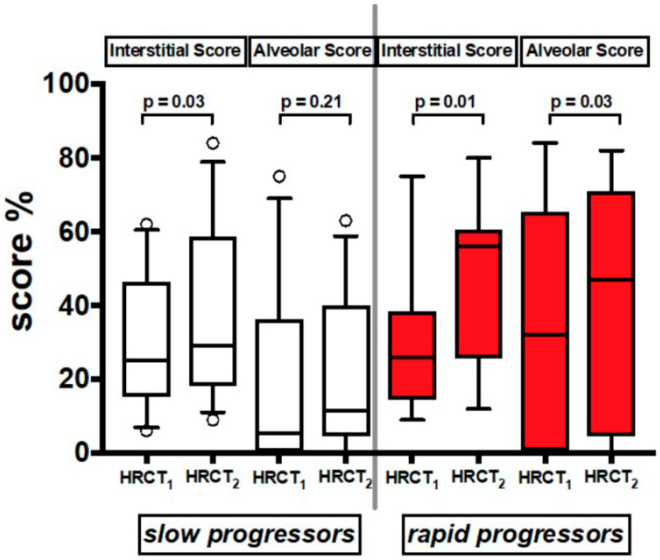


Figure 32. Values of Interstitial Score and Alveolar Score of the tw serial HRCT scans (HRCT1 at Figure 4. Values of Interstitial Scor and Alveolar Score of the two serial HRCT scans (HRCT1 at baselin and HRCT2 at follow up). Horizontal bars represent median values bottom and top of each baseline and HRCT2 at follow up). Horizonta bars represent median values; bottom and top of each box plot 25t and 75th; brackets show 10th and 90th percentiles; and circle represent outliers. White box plot 25th and 75th; brackets show 10t and 90th percentiles; and circles represent outliers. White boxe indicate slow progressors and red boxes rapid progressors

ΔAS/month. However, when stratified by the rate of decline, the correlation between ΔFVC mL/month and ΔIS/month was no longer significant in the rapid or slow decliners (Fig. 33).

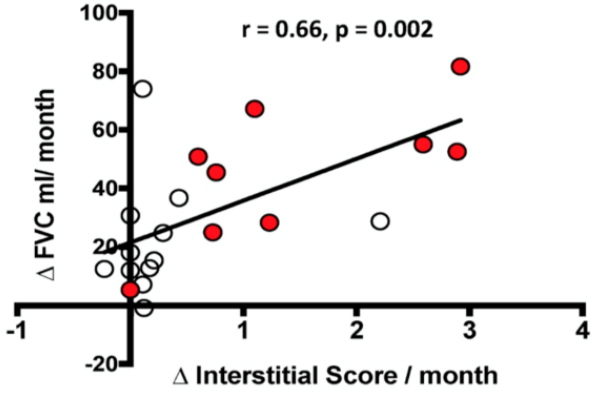


Figure 33. Relationship between the change over time in FVC mL (ΔFVC mL/month) and the change over time in Interstitial Score (ΔInterstitial Score/month). The black line represents the correlation in the entire population. White circles indicate slow progressors and red circles represent rapid progressors. Spearman's rank correlation: r=0.66, p=0.002 in the entire population; r=0.31, p=0.6 in slow progressors alone; r=0.73, p=0.06 in rapid progressors alone

In conclusion, quantitative estimation of High-Resolution Computed Tomography alveolar (AS) and interstitial (IS) scores reflects the distinct clinical and pathological behavior of IPF slow and rapid decliners. Furthermore, the alveolar score, which reflects the immune/inflammatory infiltrate found in lung tissue, could be a useful tool to differentiate rapid from slow progressors at presentation.

5. Balestro E, ..., ***Biondini D****, et al.* ***High-Resolution CT change over time in patients with Idiopathic Pulmonary Fibrosis on antifibrotic treatment****. J Clin Med. 2019 15; 8. pii: E1469. doi: 10.3390/jcm8091469*

Patient Population and Study Design

In this restrospective longitudinal study, we analyzed a cohort of phenotypically well characterized patients with IPF referred to our center between April 2014 and April 2018 and followed clinically, functionally (FVC, forced vital capacity in one second, FEV_1, forced expiratory volume in one second and diffusing capacity of the lung for carbon monoxide (DL_{CO})) and radiologically for at least one year after initiation of anti-fibrotic treatment (either pirfenidone or nintedanib).

Sixty-eight patients were included from two ILD centers in Italy (University Hospital of Padova, n = 59 and University Hospital of Foggia, n = 9). For all patients, the diagnosis of IPF was made in accordance with the ATS/ERS/JRS/ALAT guidelines[2,63]. Thirty-three cases required a histological confirmation of the diagnosis of IPF, whereas, in the remaining cases (n=35), the diagnosis was made based on clinical and radiological data only. Patients with a clear history of environmental or

occupational exposure and those with clinical features or serological data suggestive of an underlying connective tissue disease were excluded.
For all patients, clinical and lung function data were collected at the time of treatment initiation and at regular time intervals (every three months) for up to 12 months while HRCT was performed at treatment initiation and after 12 months. Based on their annual rate of decline in absolute FVC% pred., patients were classified as progressors (absolute FVC% pred. decline/year >5%, *n*=20)
or stable (absolute FVC% pred. decline/year ≤5%, *n*=48). Improvement of FVC (%pred. and mL) was expressed as a negative value.
The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the University Hospital of Padova (4280/AO/17). Informed consent was obtained for all study participants.

Radiological and Functional Analysis

For each patient, an HRCT was available at treatment (either pirfenidone or nintedanib) initiation (HRCT1) and at the 12-month follow-up (HRCT2). The HRCTs were performed by a 64 slice Siemens Somatom Sensation (Siemens Healthcare, Erlangen, Germany), applying a slice thickness ≤1.5 mm.
Two expert thoracic radiologists, who were blind to clinical and functional data and timing of HRCT, scored HRCT1 and HRCT2 images independently using a semi-quantitative scale. Specifically, the radiologic features considered in this study were ground glass opacities (GGO) (alveolar score, AS), reticulation (interstitial score, IS) and honeycombing (HC) (honeycombing score, HC). For each lung lobe, the two radiologists assessed the extent of AS, IS and HC using a scale from 0–100 and estimated the extent to the nearest 5%. After each individual lobe was scored, the result was expressed as the mean value of the five lobes in AS, IS and HC. Finally, the IS and HC were pooled (IS + HC) to analyze the amount of fibrotic abnormalities. The level of interobserver agreement was obtained for each patient as a mean of 5 lobes and for each radiologic abnormality (i.e., IS, AS and HC) and expressed as Cohen's k value. Disagreement between radiologists was resolved by consensus. The correlation between radiological change and FVC decline was calculated as the change in AS (ΔAS/month), IS (ΔIS/month), HC (ΔHC/month), pooled IS and HC (ΔIS + HC/month) and the change in FVC milliliters (mL) per month (ΔFVC mL/month) and FVC% pred. per month (ΔFVC% pred./month) between HRCT1 and HRCT2[96].

Statistical Analysis

Categorical variables are described as absolute (n) and relative values (%), whereas continuous variables are described as median and range. To compare demographic data and baseline clinical characteristics between stable patients and progressors, a

Chi square test and Fisher's exact test for categorical variables and a Mann–Whitney U test for continuous variables were used as appropriate.
Wilcoxon signed rank test was performed to compare HRCT1 and HRCT2 for the grading scores of different variables (AS, IS, HC and IS + HC) in the entire population, in stable patients and progressors. Correlation coefficients between radiological and functional data were calculated using the nonparametric Spearman's rank method. The level of interobserver agreement between the two radiologists was evaluated by kappa statistic measure.
The overall survival was calculated from diagnosis to death or lung transplantation with data censured at 1 June 2019. The cumulative survival rate was calculated using a Kaplan–Meier method and clinical characteristics and radiological scores were evaluated to determine their relationship with disease progression in a univariate and multivariate analysis of Cox proportional hazards regression testing (Supplementary Materials).
All data were analyzed using SPSS Software version 25.0 (New York, NY, USA: IBM Corp. USA). *p*-values <0.05 were considered statistically significant.

The aim of this study was to evaluate whether and to what extent HRCT abnormalities change after 1 year of antifibrotic treatment and how these changes correlate with different functional disease trajectories (i.e., stable patients vs. progressors) in patients with IPF.

We included 68 patients, and stratified them in stable (n=48) and progressors (n=20) according on whether, or not, they had a decline in FVC>5% pred/year. The clinical and radiologic characteristics at baseline are shown in Table 14.
Most patients were males (81%) and former smokers (59%) with a median age at diagnosis of 66 years. Compared to the previous study, these patients were all evaluated radiologically during antifibrotic (Pirfenidone and Nintedanib) treatment.
At treatment initiation, progressors tended to be younger and with significantly more preserved FVC and DLCO as compared to stable patients. There were no between-group differences in the HRCT score (Table 14); the radiologic features considered in this study were ground glass opacities (alveolar score AS), reticulation (interstitial score IS) and honeycombing (HC).

Table 14. Clinical characteristics of subjects in the Study Cohort

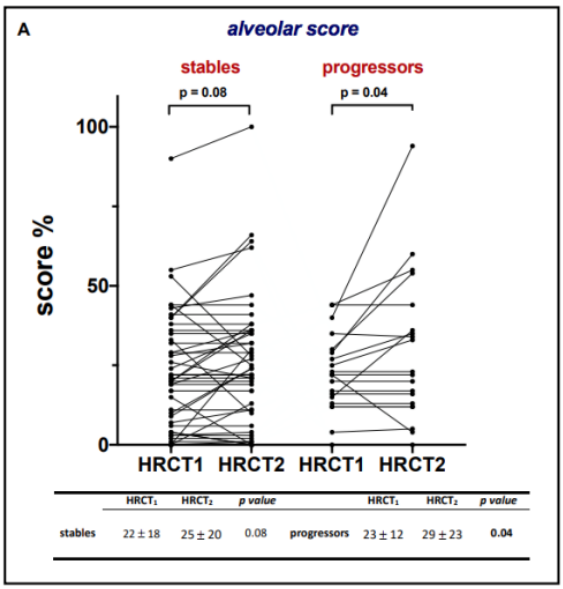
	Entire Population ($n = 68$)	Stables ($n = 48$)	Progressors ($n = 20$)	*p*-Value
Male—*n* (%)	55 (81)	37 (77)	18 (90)	0.31
Female—*n* (%)	13 (19)	11 (23)	2 (10)	0.31
Age at diagnosis—*years*	66 (44–78)	68 (46–78)	61 (44–78)	0.07
Smoking history—*pack years*	15 (0–80)	15 (0–80)	15 (0–55)	0.31
Current—*n* (%)	9 (13)	7 (15)	2 (10)	1.00
Former—*n* (%)	40 (59)	29 (60)	11 (55)	1.00
Nonsmokers—*n* (%)	19 (28)	12 (25)	7 (35)	0.55
Clinical-radiological diagnosis—*n* (%)	35 (51)	27 (56)	8 (40)	0.29
Histological diagnosis—*n* (%)	33 (49)	21 (44)	12 (60)	0.29
FVC at diagnosis—*L*	2.76 (1.19–5.68)	2.6 (1.19–5.29)	2.97 (1.68–5.68)	0.04
FVC at diagnosis—% *pred.*	78 (44–120)	78 (44–120)	78 (50–107)	0.40
FEV1 at diagnosis—*L*	2.21 (1.02-4.45)	2.19 (1.02–4.45)	2.50 (1.40–3.70)	0.06
FEV1 at diagnosis—% *pred.*	83 (40–127)	83 (40–127)	86 (49–122)	0.27
DL_{CO} at diagnosis—% *pred.*	57 (34–114)	53 (34–114)	65 (37–97)	0.02
6MWT at diagnosis—mt	400 (125–600)	400 (125–600)	408 (250–540)	0.50
FVC decline per year—*mL*	86 (−1381–1155)	37 (−1381–371)	413 (135–1155)	<0.0001
FVC decline per year—% *pred.*	2 (−25–29)	0 (−25–4.7)	9 (5–29)	<0.0001
Deaths—*n* (%)	16 (23)	8 (17)	8 (40)	0.05
Alveolar score in HRCT1—%	21 (0–90)	21 (0–90)	22 (0–44)	0.68
Honeycombing in HRCT1—%	7 (0–70)	6 (0–70)	9 (0–50)	0.32
Interstitial score in HRCT1—%	26 (0–100)	26 (0–100)	28 (0–52)	0.92
Pooled interstitial score and honeycombing—%	40 (8–100)	38 (17–100)	43 (8–70)	0.52

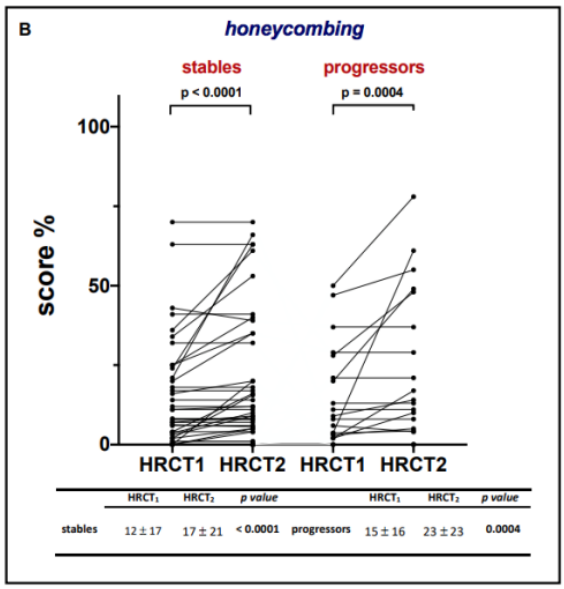
Values are expressed as numbers and (%) or median and ranges as appropriate. Negative values mean improvement of FVC (Forced Vital Capacity). To compare demographic data and baseline clinical characteristics between stable and progressors, Chi square test and Fisher t test ($n < 5$) for categorical variables and Mann–Whitney t test for continuous variables were used.

Forty-seven patients were treated with pirfenidone and twenty-one with nintedanib during the study period.

In the entire study population, AS and HC increased significantly between HRCT1 and HRCT2 from 22%±17% to 26%±21% (p=0.008) and from 13%±16% to 19%±22% (p<0.0001), respectively.

When the study population was stratified by the rate of functional decline, in stable patients, HC increased significantly between HRCT1 and HRCT2 from 12%±17% to 17%±21% (p<0.0001) (Fig. 34B), whereas AS and IS did not (Fig. 34A, 34C). Conversely, among progressors, both AS and HC increased significantly from 23%±12% to 29%±23% (p=0.04) and from 15%±16% to 23%±23% (p=0.0004), respectively (Fig. 34A, 34B), whereas IS did not (Fig. 34C). Moreover, the pooled IS and HC in both stable and progressors significantly increased from HRCT1 and HRCT2 (p<0.001) (Fig. 34D)





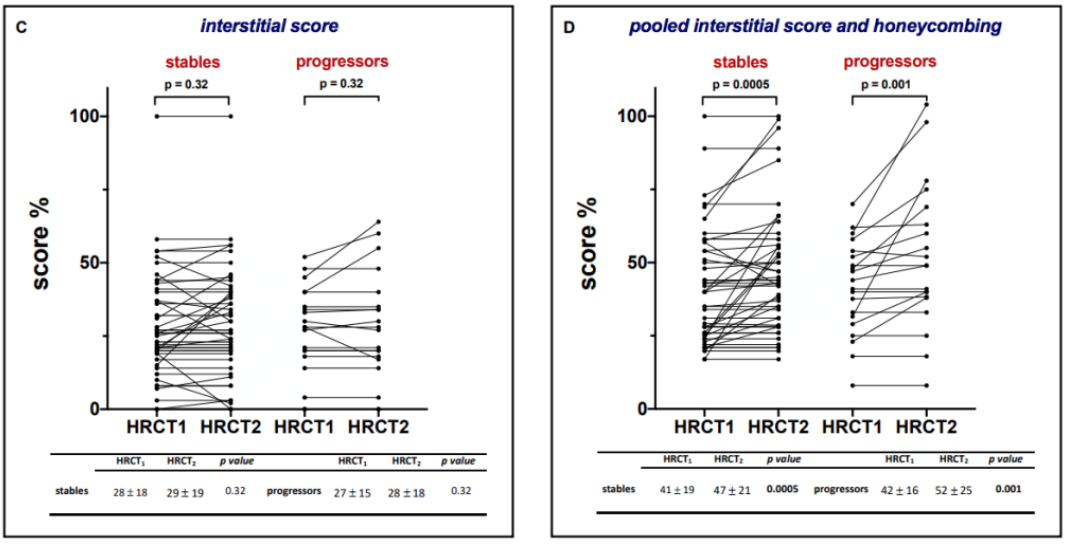


Figure 34. Change in alveolar score (A), honeycombing (B), interstitial score (C) and pooled interstitial and honeycombing (D) between HRCT1 (at treatment initiation) and HRCT2 (after one year of treatment) in stable and progressors patients. Values in the table below are expressed as mean and standard deviation. p values refer to comparison between HRCT1 and HRCT2

In the entire study population, we observed a positive correlation between the change in FVC (ΔFVC) mL/month and the combined change of IC and HC (ΔIS+HC)/month (r=0.24, p=0.04) (Fig. 35), while none of the correlations between ΔFVC mL/month and ΔAS, ΔIS and ΔHC were significant (r=0.10, p=0.40; r=−0.04, p=0.60 and r=−0.07, p=0.50, respectively).

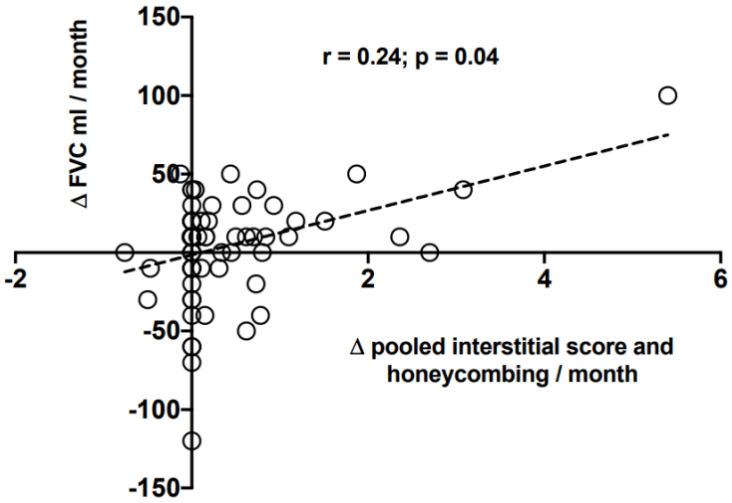


Figure 35. Correlation between change over time in FVC mL (ΔFVC mL/month) and change over time in the pooled Interstitial Score and Honeycombing (Δ pooled Interstitial Score and Honeycombing) in the entire study population. Negative values mean improvement of FVC

In conclusion, in patients with IPF on antifibrotic treatment, the extent of honeycombing increases over time both in patients experiencing functional decline and in those who remain functionally stable over 12 months, suggesting that CT is able to capture subtle subclinical disease progression.

DISCUSSION

In the studies performed during my PhD, we analyzed the role of immune inflammation in response to a chronic epithelial injury induced by cigarette smoke in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) and Idiopathic Pulmonary Fibrosis (IPF), and its relationship with clinical outcomes.

We started our investigation in COPD by studying the evolving role of the alveolar macrophage as the severity of the disease progresses, since the macrophage is probably the essential cell preserving homeostasis in the alveolar space and first responding to lung insults. As such we were interested in studying the M1 pro-inflammatory, and M2 anti-inflammatory phenotypes to evaluate their possible role in the pathogenesis of the disease.

We used the traditional M1/M2 classification, which is still widely used in the description of human macrophage polarization in the lung and other human tissues[21,22,98–100], in order to define, in broad terms, the response of alveolar macrophages to smoking and to the development of COPD.

In healthy lungs, a higher proportion of alveolar macrophages did not express any markers of either M1 or M2 activation, a finding that might have been expected in view of the tight control of the state of AM activation exerted by the alveolar epithelium, which limits unwanted inflammatory responses[99].

During inflammation, the loss of ligands after epithelial damage is believed to bring the balance towards a pro-inflammatory M1 phenotype activation of alveolar macrophage. Accordingly, our data show that an acute inflammatory trigger, like mechanical ventilation[97], was able to promote M1, but not M2 polarization, confirming that acute tissue injury induces a prompt pro-inflammatory M1 phenotype response. The proportion of pro-inflammatory M1 AM increases also with the introduction of chronic stimulus as cigarette smoke and the development of COPD, increasing with the severity of the disease.

Interestingly, in favor of the importance of smoking as trigger for M1 polarization is the finding that, upon smoking cessation, the percentage of AM expressing M1 decreased significantly. However, this decrease in M1 polarization is only found in smokers without COPD and smokers with mild COPD, but not in smokers with severe COPD, indicating the importance of chronic inflammatory response.

While pathogens or tissue injury will induce the M1 phenotype, homeostatic signals from the local environment will induce macrophages to adopt M2 phenotypes linked with tissue remodeling and repair[101,102]. In donors' normal lungs, M2 polarized AM

were minimally present, but increased with exposure to cigarette smoking and disease severity.
Interestingly, the group of non smokers who had surgery for lung tumors, showed an increase in the percentage of M2 polarization, but only if the tumor was malignant, suggesting that malignancy might influence M2 polarization[21,103]. However, severe COPD, who did not have lung cancer, had the highest percentage of M2 AM, suggesting that most of the increase of M2 is secondary to smoking and COPD severity, as previously reported[98,100].
In addition, in smokers with COPD, the combined percentage of M1 and M2 AM reached more than 100% in the confocal microscopy, indicating that some macrophages were expressing both markers.
The expression of TNF-α or IL-4/IL-13 as indexes of M1 and M2 activity increased along with the percentage of M1 and M2 AM respectively, indicating that both phenotypes in our study were likely active in their functions. These data confirm in humans the fact that rather than distinct, macrophage populations with M1 and M2 signatures do not necessarily exclude each other, but often coexist as it has been shown in animal models[104].
The importance of this study was to examine for the first time in human lung tissue, the M1 and M2 AM polarization, and how the balancing between these phenotypes is influenced by smoke and disease severity, playing an important role in the pathogenesis of COPD.

We continued our investigation in the alveolar macrophage in COPD secondary to α1-Antitrypsin (AAT) by going beyond the protease-antiprotease paradigm, which no longer should be considered the only pathogenic cause of COPD, especially after the evidences that subjects with AAT deficiency (AATD) may not develop emphysema. In this study, we wanted to better investigate whether the polymerization that takes place in liver cells could also occur in human alveolar macrophages (AM) in patients with AATD. Our results confirmed the hypothesis that AAT polymers are also present in AM in the lungs of individuals with PiZZ AAT deficiency (COPD with AATD). However, to our surprise, we also found AAT polymers in AM of smokers with COPD and normal AAT levels ("usual" COPD) and in smokers without COPD, but not in nonsmokers.
The presence of significant polymerization of AAT in AM directly in human lung tissue had never been previously reported, and this added importance to our study.
It has been clearly demonstrated that under stimulation PiMM and PiZZ AM produce similar AAT mRNA levels[14]; however, PiZZ AM produced 10 times less AAT protein than PiMM AM. This suggests that the defect is at the secretory level due to

protein misfolding and polymerization, and it happens also in AM, as seen in the liver.
Unexpected was the finding of AAT polymers in the AM of smokers with COPD and normal AAT levels ("usual" COPD) and also in smokers without COPD, but not in nonsmokers. The association between cigarette smoking and polymerization is in line with the evidence that cigarette smoke can greatly accelerate PiZ-AAT polymerization and oxidize PiM-AAT in mouse and human plasma[105]. In addition, this may explain our novel finding of AAT polymers present in AM from smokers with normal levels of AAT.
AAT polymerization not only determine a reduction of the circulating levels of AAT, but could also contribute to inflammation by triggering important pro-inflammatory effects. Indeed, the additional "toxic gain of function" originating from the accumulation of misfolded and aggregated AAT in the ER of AM, could induce "ER stress" and the consequent unfolded protein response (UPR).
Therefore, it may happen that cigarette smoke and inflammatory stimuli increase the production of AAT in AM, which could misfold and polymerize in the endoplasmic reticulum, causing endoplasmic reticulum stress and activation of the UPR. UPR, in turns, increases the production of pro-inflammatory cytokines and chemokines, increasing the inflammation that will induce further AAT production and additional misfolding and retention in the ER of macrophages, thus perpetuating ER stress as in a vicious circle.
The findings described with this study emphasize the complex role that could be played by the molecular abnormalities of AAT in the development of COPD and emphysema, and highlight another important and potentially damaging effect of cigarette smoking. Our findings also highlight the similarities, even more evident, in the pathophysiology of COPD in smokers with and without AAT deficiency and add another potentially important step to the complex mechanism underlying the disease.
Immune inflammation is fundamental in the development of COPD, and AM are the first barrier toward external insults responsible for the tissue homeostasis, as well as the most important actor of the innate inflammatory response.

Another clue of the importance of immune regulation in the development of COPD, was highlighted in the study on the genetic susceptibility factors for emphysema in siblings with AATD, where at least one was affected with emphysema and one was non-affected. Notably, our findings suggested that gene variants mainly involved in the regulation and the maintenance of self-tolerance could contribute to the development or suppression of the disease.

After these studies, which evaluated innate inflammation in the lung tissue, we decided to focus the research project on the blood immune cell counts, in particular eosinophils and eventually lymphocytes, and to investigate whether they could be reliable biomarkers of clinical outcome, and if they may represent a consistent reflection of the inflammation present in the lung.

The blood eosinophil, at the time of the initiation of our study, was considered a faithful indicator of the tissue eosinophil, a fact never proved before. Furthermore, because the literature using post hoc data analysis emphasized the important role of the eosinophil counts in blood in COPD exacerbations, we wanted to see if such data could be confirmed in a real-life population of COPD in which presence of asthma was discarded from the time of recruitment.

As such in these studies we analyzed a "real life" population, that presented voluntarily in respiratory clinics for diagnosis and care without previous selection, and had periodic follow-up of at least 5 years, conversely to the usually selected cohorts for large research trials that represent fewer than 5% of patients in the routine care[106]. The long follow up permitted us to have a more reliable evaluation of blood eosinophils and lymphocytes over time.

Interestingly, the median of eosinophil numbers in our population (157 cells/μL) was similar to that reported in normal populations[37], and only 3.3% of smokers had blood eosinophil counts above 500 cells/μl (the accepted upper limit of normality), which is very different from the 27–33% reported in asthmatics[107]. Interestingly, 96% of smokers with COPD in our study have blood eosinophil counts within normal limits, suggesting that patients diagnosed of COPD with blood eosinophil greater than 500 cells/μL at baseline conditions, might be asthmatics and possibly should be excluded from COPD trials, as done in recent publications[41].

Blood eosinophil counts were very variable over time with only about 40% remaining consistently below or consistently above 150/μL in the five blood samples and about 60% varying form above to below the 150/μL value. Variability in blood eosinophil counts has been shown before in normal and COPD populations[30,108], but was not taken in consideration in the possible consequences in disease outcomes.

Thus, the recommendation of treating COPD patients with inhaled steroids based on one value of blood eosinophils above 100 cells/μL, as recently proposed by GOLD[109], does not take in consideration variability over time and would practically mean that the majority of COPD patients (about 75%) would be considered for treatment.

In order to assess the possible effects of blood eosinophil numbers in disease outcomes, we characterized our subjects according to their cell numbers in the five blood samples: persistently <150/μL, persistently ≥150/μL, and variable.

The use of blood eosinophils as a biomarker in COPD is based on the premise that they reflect and correlate with tissue eosinophilic inflammation in pulmonary airways and parenchyma. We were able to study large sections of lung obtained during surgery, which included large and small airways and lung parenchyma, allowing for an extensive evaluation of the lung tissue. Notably, we showed that tissue eosinophilia did not differ between smokers with and without COPD, and, more importantly, it was not related to blood eosinophilia.

Tissue eosinophils are unquestionably part of the immune component of COPD inflammation. Indeed, they are found in large numbers in exacerbated COPD and in smaller numbers in stable COPD. However, eosinophils are associated to an increase in RANTES (regulated upon activation normal T-cell expressed and secreted), a chemokine that recruits all inflammatory cells to sites of inflammation, but not by an increase in IL-5[32,110], as in asthma. Therefore, they likely have different functions and recruiting mechanisms than eosinophils in asthma. It is possible that eosinophils in exacerbated COPD are a response to viral infections, as supported by the reported relationship between sputum eosinophilia and viral loads during exacerbations[111]..

In our population, eosinophil numbers did not impact on the degree of dyspnea, distance walked, prevalence of chronic bronchitis, or severity of COPD. Moreover, in about half of the subjects with COPD that developed at least one exacerbation during follow-up, no difference in the number of eosinophils compared non-exacerbators was found.

Based on the reported effectiveness of inhaled steroids in exacerbations reduction, it was hypothesized that diminishing the numbers of blood eosinophils using biological medications might have been beneficial[112–115]. Treatment with IL-5 monoclonal antibody mepolizumab showed minimal efficacy in one of the two treated groups of eosinophilic COPD patients but not in the other[115]. Moreover, another targeted anti-eosinophil therapy, the anti-IL-5 receptor-α benralizumab, failed to reduce exacerbations in COPD patients with high blood eosinophils (>300 cells/μL) and showed a tendency to increase exacerbations in patients with low eosinophils[112], a result confirmed by the recent GALATHEA and TERRANOVA trials. Both these trials reported a substantial depletion of blood and sputum eosinophils that, unlike the results observed in benralizumab-treated asthma[116,117], did not reduce the annualized exacerbation rate among COPD patients[113]. Thus, reducing blood eosinophil counts, which is considered a treatable trait in COPD[33], does not improve patient's outcome, which underlines the complexity of the disease and the multifactorial mechanisms of COPD exacerbations[78]. It is of interest that, in contrast with the studies that used biologicals, the use of inhaled steroids might reduce exacerbations in COPD subjects with high eosinophil counts, but will not reduce the blood eosinophil numbers[118],

which indicates that much has to be learned about the reasons for the high eosinophil counts, their variations and their controversial effects on the fate of COPD patients.
In addition, we found that persistently high eosinophil counts, even if within the normal range, had a beneficial effect on survival. This finding is likely the result of the multiple beneficial effects observed in subjects with COPD and relatively high numbers of eosinophils as better FEV1, fewer symptoms with lower St. George's Respiratory Questionnaire and modified Medical Research Council Dyspnea Scale scores, lower BODE (body mass index, obstruction, dyspnea and exercise capacity) index, less emphysema[119], and fewer comorbidities[113] and pneumonias[34], which together would allow for better survival. This effect is biologically plausible because eosinophils constitute a major component of the innate immune response against infections by way of their microbicidal function, antigen presentation, and amplification of the T-helper cell type 1 response[120]. Moreover, new biological treatments pointed at eosinophil elimination should be reconsidered, not only for the lack of efficacy, but especially because they may be even harmful.

After these findings on blood lymphocytes and eosinophils in smokers, we performed another study with other European Centers, that aimed at evaluating how these tissue cells count may change after the introduction of an anti-inflammatory treatment as Roflumilast in COPD patients.
Roflumilast is a selective inhibitor of phosphodiesterase 4 (PDE4), it acts by increasing intracellular concentrations of cAMP in epithelial airway cells and inflammatory cells[121], reducing airway inflammation. It is a drug that reduces exacerbations when given as add-on to inhaled therapy compared with inhaled therapy alone in patients with severe or very severe COPD associated with chronic bronchitis and a history of exacerbations[121–123].
Although there is a substantial amount of evidence about the clinical effects of Roflumilast[121–124] the anti-inflammatory mechanisms underlying the drug's clinical efficacy have been investigated in few studies, and it was one of the main aims of this study called ROBERT. To our knowledge, this was the first biopsy study to assess the anti-inflammatory properties of Roflumilast directly in the lungs of patients with moderate-to-severe COPD and chronic productive cough.
The primary endpoint was to evaluate the reduction in the number of inflammatory CD8 cells in bronchial submucosa with treatment compared to placebo, and no differences were found. However, we noted a significant reduction of eosinophils count (secondary endpoint) in both bronchial biopsy specimens and induced sputum in the Roflumilast group compared with the placebo group. Moreover, blood samples

that were available in a subgroup of patients, showed no relation with sputum or tissue eosinophils, in line with the results of our previous study.
The association between sputum and lung eosinophil counts noted in our study is supported by an analysis from the SPIROMICS cohort[125], in which sputum eosinophils were a significant biomarker (p=0.002) for the identification of patients with an increased risk of COPD exacerbations, but blood eosinophils were not (p=0.35).
Our patient population had less severe and more stable disease than the populations included in most clinical studies of Roflumilast so far, because bronchial biopsy and sputum collection would not be appropriate in patients who are not in a sufficiently stable condition. Patients with a history of a recent exacerbation were excluded from the study for the same reason.
Although the primary endpoint was not met in this large biopsy study, findings for the secondary endpoints of eosinophil counts in bronchial biopsy samples and induced sputum generate the hypothesis that Roflumilast might reduce lung eosinophils in patients with COPD. Even though, in view of the exploratory nature of these analyses, the results need to be interpreted carefully. The precise mechanism behind this reduction in eosinophils, and any potential effects of this eosinophil reduction on clinical outcomes, merit further investigation.

Regarding blood lymphocytes (BL), we showed how the counts of each sample were stable over time contrary to blood eosinophils, allowing us to use the median value of the 5 samples for the statistical analysis. Moreover, the stability of blood lymphocyte may suggest that one analysis could be sufficient to categorize these patients as having high or low BL already at the first visit, conversely to what happens with blood eosinophils which are more fluctuating over time.
Our results essentially showed that smokers with COPD have significantly lower BL numbers than smokers without COPD, and that lower numbers of BL (μL) were associated with significantly worse outcomes like exacerbations, cancer incidence and survival.
It is well known that in normal populations lymphocyte numbers in peripheral blood (median 2100, range 1100-4800) exhibit a great degree of inter individual variation, and it is unclear whether these differences are due to individual's characteristics that remain stable with aging or possible environmental factors. Smoking has been shown in a few small studies to increase the numbers of BL when compared to non smokers, but the distribution of BL numbers and the presence of COPD in these populations were not documented[126].

To our knowledge, this is the first study comparing BL numbers in smokers with and without COPD, an important assessment due to the large difference in the tissue inflammation and lymphocyte infiltration between them. Our results show that smokers with COPD had a systematic reduction in lymphocyte numbers in peripheral blood (median 1800 cells/μL) when compared with smokers without COPD (median 2300 cells/μL) that remained unchanged over the time of follow up.

The numbers of BL in smokers without COPD in our study are similar, and within the normal range, to previously reported numbers, but contrarily to previously reported data[126], BL in our population were not different between active smokers and ex-smokers, indicating that the differences found in our population is not due to actual smoking at the timing of collection.

Such changes may reflect a combination of reduced production of naïve lymphocytes and the accumulation of memory lymphocytes as the results of the reduced overall production of lymphocytes, and of the host-environment interaction over time. Despite the overall trend of age-associated changes, striking variations in the numbers of lymphocytes exist between individuals, and it is currently unknown whether the observed variations are due to stable decreases that are maintained over time or to different rates of reduction that change with aging.

Some of the alterations in lymphocyte composition are considered biomarkers of immunosenescence (ratio of CD4/CD8, increase of CD28- T cells, and increase of NK cells) because they are associated with mortality in elderly[127]. The role of the BL count as a predictor of clinical outcome in COPD has never been assessed before. Nevertheless, it has never been assumed the role of blood lymphocyte in COPD and if it is a faithful representation of tissue lymphocyte.

In COPD patients, in our study the level of blood lymphocytes considerably influences clinical outcomes as symptoms, exacerbation rate and survival. Interestingly, these findings are strengthened by the fact that age, presence of chronic bronchitis, smoking exposure and even lung function (FEV1) were comparable between high or low BL group.

Indeed, COPD patients with low BL had a significantly lower distance walked at 6-minute walking test compared to COPD with high BL. Regarding dyspnea scores, COPD patients with low BL are significantly more symptomatic than COPD with high BL. Interestingly, COPD with high BL have similar dyspnea score than smokers without COPD with low and high BL. This is a quite astonishing finding that smokers without COPD reports similar symptoms scores than patients with COPD, even if they have a marked difference in pulmonary functions test. This leaves opens questions regarding the effective value of these scores to evaluate dyspnea in COPD,

or regarding the multiple determinants that takes part in the pathogenesis of dyspnea, other than lung function alone.

It was previously proposed that a higher amount of lymphocyte in the peripheral airways was related to worse lung function[25]. However, the relationship with blood lymphocyte count has never been assessed before. In our group of COPD who undergone lung surgery for lung cancer, we found no relationship between blood and peripheral airways lymphocyte.

COPD patients with low BL have also a higher incidence of total and severe acute exacerbations compared to high BL, meaning that BL could be used a potential predictive biomarker and deserves further investigations.

In our population of smokers, during follow-up about one fifth of them developed cancer, which is a very high percentage.

The higher incidence of cancer in smokers with low BL group may be related to the older age and to the higher exposure to cigarette smoke.

However, when we selected smokers without COPD, a higher incidence of any type of cancer in low BL was influenced not only by age, but also by lymphocytes in a multivariate analysis.

A remarkable data was the absence of lung cancer in smokers without COPD and high blood lymphocyte. This may be linked to the recent discoveries in the oncologic field[44,45], where the suppression of lymphocyte function is one of the main mechanism that allow tumor growth, and drugs that block these immune checkpoints and "awake" suppressed lymphocyte showed efficacy in cancer control.

Another clue of the key role of lymphocyte in the pathogenic role in COPD is the profound impact on survival in the overall smoker population, and also in the two subgroups of COPD and non COPD. The link between higher lymphocyte and better survival is the first evidence in literature and emphasize the importance that may also have blood lymphocyte in COPD.

This lead us to the analysis of lymphocyte population in a subgroup of smokers, and even without any statistical signal probably due to the small samples analyzed, a trend toward a higher $CD8^+$ and NK cells and lower B cells in COPD with low BL was found. Whether this may be the reason of a worse survival in COPD with low BL need further confirmation, and our future research will focus on this aspect.

Then, the idea is to extend similar analysis on blood cell to the lung inflammatory infiltrate, collected using the lung digestion. This ambitious project will allow us to deeply investigate the inflammatory milieu of the lung in smokers with and without COPD, especially if they express markers of immune suppression (immune check points), and how these findings will be reflected in the blood.

Cigarette smoking is a potent risk factor for both COPD and IPF. It seems that epithelial endothelial injury is the initial step triggering an inflammatory reaction in both diseases. However, while an abnormal inflammatory response in COPD is an accepted component in the mechanism of disease, in IPF the old inflammatory mechanism possibility has been totally rejected. In our research in IPF we wanted to revisit the possibility of an inflammatory component that might participate in the pathogenesis of this disease reasoning that the predominant role of immune inflammation highlighted in these studies conducted on COPD patients may also be a predominant feature that characterize also the pathogenesis of IPF.

Using the criteria proposed by Boon and colleagues[59], based on the fall in percent predicted FVC/year, we differentiated our IPF patients into slow (66%) and rapid (34%) progressors. On average, the absolute yearly fall in FVC was 98 ml/year in the "slow" decliners and 480 ml/ year in the "rapid" ones.

The importance of these categorization is due to the evidence that the decrease of >10% in FVC% pred. over a 12-month period (rapid progressors) is related to a significantly lower 5-year survival than declines of ≤10% (slow progressors)[60].

The possibility of distinguishing between slow and rapid progressors early in the disease course is clinically relevant, especially in view of the current availability of medical therapies that are effective in slowing functional decline and disease progression in the early stage of the disease[61,128].

Once we had defined the different clinical course in our population, we hypothesized that different progression might be associated with distinct underlying pathological findings. Indeed, important differences in lung pathology were observed between slow and rapid progressors, consisting mainly on the presence of an extensive degree of innate and adaptive immune inflammation in the rapid group, more prominent than in the slow progressors. The results of the detailed quantitative pathological analysis in IPF lungs appear to support a contribution of inflammation in determining disease behavior in IPF.

Despite the increased evidences that immune system might play a role in IPF[52,129–132], inflammation is not considered an important component of UIP or a factor contributing to the progression or pathogenesis of the disease. Furthermore, previous pathological descriptions, performed in lung biopsies from IPF patients at the time of diagnosis, reported no differences in pathology between the slow and rapid decliners[60]. Unfortunately, we could not examine the lung pathology at the time of diagnosis in our patients, so we do not know if some differences were already present at that stage.

The presence of an exuberant immune inflammatory infiltrate, found predominantly in the rapid progressors, is consistent with the gene expression profile reported by

Boon and colleagues[59], who indeed described the activation of important pro-inflammatory pathways that may potentially play a role in the immune activation and disease progression of the rapid decliners[59]. Our findings are also in keeping with previous observations showing that lymphocyte density in IPF lung is associated with FVC decline[133] and poor survival[130]. Moreover, in observance with an important role of immune activation and inflammation, recent publications suggest that increased expression of pro-inflammatory factors may predispose to worse outcomes in IPF[52,129–132]. For instance, the presence of B cell aggregates[53,55,132] and highly differentiated circulating B cells in patients with IPF[55], findings usually observed in autoimmune syndromes. In this context, it is of interest to note that autoantibodies to heat shock protein 70[52] and periplakin[131], which have been identified in serum and bronchoalveolar lavage of patients with IPF, have been reported to be associated with a more severe disease, further supporting the presence of an autoimmune response in the pathogenesis of IPF. In this regard, our finding of an increased number of B lymphocytes in the lungs of rapid progressors supports a potential role for these cells, along with T lymphocytes, in the development of an adaptive immune response, possibly contributing to the course of the disease.
We are well aware that by studying the pathology of the explanted lung we are looking at the "terminal", or at least advanced, stages of the disease. In other words, we may be looking at the consequence rather than the cause of the pathologic process. Yet, the clear differences in pathology found between slow and rapid progressors, both at a terminal stage of their disease, validate the significance of our findings and indicate that the "end stage" itself cannot explain the enhanced immune inflammatory process observed in rapid progressors.

Moreover, in favor of the strong immune feature of IPF was further highlighted by the presence of numerous well formed lymphoid follicles (LF), which are tertiary lymphoid structures characteristically seen in tissues with chronic inflammatory/immune reactions such as autoimmune diseases, that are found in IPF even in higher amounts than COPD. Lymphoid follicles were even more numerous in the lungs of diagnostic lung biopsies of early IPF than in late IPF, indicating that the adaptive immune reaction is present in this disease from the early stages and possibly plays a role in its progression.

After the evidence of a strong inflammatory component in IPF lung tissue, which was more marked in rapid progressors, we investigated whether these differences may determine a diverse response to Pirfenidone treatment. Pirfenidone is approved for the treatment of IPF based on its ability to slow down functional decline and disease

progression due to its antifibrotic effect[134]. Pirfenidone is also known to have important anti-inflammatory properties.

The IPF patients studied had been followed for a prolonged time before starting antifibrotic treatment, during which we had the opportunity to categorize them as rapid or slow progressors based FVC decline in the pretreatment period, as previously explained.

In the overall patient population, the annual rate of FVC decline in the pretreatment period was 231 ml/year (6% pred.) similar to what has been reported in the placebo arms of other treatment trials[61]. After treatment, the rate of FVC decline in the whole population was variably but significantly reduced already after 6 months of treatment, an improvement that was maintained during the entire 24-month study duration. Overall, after one year of pirfenidone treatment, 7 (12%) patients experienced a FVC decline >10% compared with 17 patients (30%) in the pretreatment period (OR 3.0, p=0.02), corresponding to a relative reduction of 59%, while the absolute fall of FVC decreased from a pretreatment value of 231 ml/year to 59 ml/year ($p<0.05$), consistent with a previous study in Japanese patients[135]. Our data provide, we believe, a reliable picture of the effect of pirfenidone treatment on the rate of FVC change between the pretreatment and follow-up period, since each patient served as its own control, which clearly strengthens our findings.

The effect of Pirfenidone differed significantly between slow and rapid progressors. Among rapid progressors, the median decline of FVC prior to treatment was 706 ml/year, which was significantly reduced to 36 ml/year after 6 months of treatment, and maintained throughout the two-year study period. Pirfenidone was also beneficial for patients with slow pretreatment decline since, although the rate of FVC decline did not change significantly, it seemed to stabilize the disease.

Our results are in line with those of previous retrospective analyses of lung function changes in patients with mild to moderate IPF treated with Pirfenidone[136,137]. These studies showed that patients with progressive disease (FVC% pred. decline >10%/year) may benefit substantially from Pirfenidone treatment, which may even result in improvement of FVC, as shown by Loeh et al.[136], while patients with slowly progressive disease tend to experience disease stability under treatment.

The assessment of disease progression and treatment response in patients with IPF is complicated by its variable clinical course[56]. In a recent post-hoc analysis of patients from the placebo arms of the CAPACITY and ASCEND trials[138], Nathan and colleagues observed a weak negative correlation between changes in FVC% pred. during two consecutives 6-month intervals, that the authors interpreted as a reflection of the variability in both the magnitude and direction of change. An important source of variability in that study could have been the combined analysis of slow and rapid

progressors together, particularly since the follow-up period was relatively short (i.e., 6 months). In our study, the median pretreatment observation period was 15 months, which minimizes the possibility that the observed rate of disease progression was confounded by the inherent intra-individual variability in longitudinal change in FVC.

The mechanisms through which rapid progressors display a particularly favorable response to pirfenidone treatment is not known but may be related to differences we previously described in the lung pathology of slow and rapid IPF patients. Possibly, extracellular matrix deposition and removal may be much more rapid, thus amenable to antifibrotic therapy, in the rapid progressive than in relatively stable IPF[136,139]. Although speculative and based on pathological description of the disease, we believe this possibility deserve some attention since it might explain, at least in part, the different response to pirfenidone treatment.

Our long-term prospective study, even if relatively small, provides further evidence of the efficacy of pirfenidone in patients with IPF up to 24 months of treatment and shows that response to therapy is influenced by the rate of decline, slow or rapid, in the pretreatment period. Strengths of our study include the careful patient characterization and the availability of long-term pre- and post-treatment data. Owing to the progressive nature of the disease and the availability worldwide of two efficacious antifibrotic drugs (pirfenidone and nintedanib), studies on IPF patients off-treatment will become progressively less common, if at all possible and ethical. This makes our data regarding the pretreatment period particularly relevant.

As we have previously said, IPF has an unpredictable clinical course, and HRCT may have a potential usefulness as a tool to predict future disease behavior at the time of diagnosis[140]. Our findings in explanted lungs, showing the possible role of inflammation in the different patterns of progression (slow and rapid), led us to search other ways of assessing inflammatory and immune mechanisms, other than biopsies, as for example with radiology.

Indeed, we investigated the possible role of HRCT made at diagnosis in predicting disease behavior, and whether there was any correlation with pathological abnormalities.

A group of IPF patients were followed long term, prior to available anti-fibrotic treatment, and fibrotic abnormalities and ground glass opacities in HRCT at diagnosis were quantified. Our results showed that, in the HRCT performed at diagnosis, patients who had experienced a rapid functional decline, rapid progressors, had a higher alveolar score (AS) than slow progressors, while the extent of fibrosis

(Interstitial Score, IS) was similar in the two groups. Furthermore, in a second HRCT at follow up, changes in IS over time was correlated with functional decline.
Previous studies assessing the value of HRCT in the prediction of IPF behavior did not differentiate their patients by the predetermined rate of progression, rapid or slow, a factor of crucial significance for the understanding of disease prognosis. Moreover, differently from previous studies[141,142], we evaluated not only the degree of HRCT interstitial score (IS) but also the degree of ground glass opacities, alveolar score (AS). Although pure ground glass opacity was not considered a feature of UIP, many patients with fibrotic lung disease have ground glass opacity admixed with reticular abnormality and/or traction bronchiectasis. In this context, the ground glass opacity should be regarded as part of the fibrotic process, as indicated by the recent Fleischner Society white paper[143] and, as such, we believe it needed to be assessed[63].
The HRCT findings in our study, showing that at baseline rapid progressors had an AS significantly greater (almost double) than slow progressors, is of high interest since it might help to identify, early in the course of the disease, the more aggressive phenotype with worse prognosis.
The significance of ground glass opacities in IPF is not clear, but it might be related to parenchymal inflammatory/exudative infiltrates, probably more evident in cases with more aggressive disease and rapid progression. In support of this possibility are our previous findings showing more prominent cellular immune infiltrate, both innate and adaptive, in rapid than slow progressors.
The possibility that AS might represent an alveolar inflammatory/exudative infiltrate is supported by the correlation observed in the present study between AS and the total number of lymphocytes in the 13 explanted lungs in which HRCT was available shortly prior to transplantation. In the 21 patients with two consecutive HRCTs, we calculated the changes over time in IS, AS, and FVC and expressed them as change/per month. The change in FVC/month in the whole group (rapids and slows together) correlated significantly with IS change/month a result in agreement with previous analysis[144]. Fitting with our previous findings, the HRCT AS, plausibly representing a cellular/exudative inflammatory response, increased significantly at follow up in rapid progressors, while it remained stable in slow progressors. The exact explanation of this feature needs to be elucidated, however we can speculate that the AS signals a more exudative and thus unstable disease, rich in fibroblast foci, and more likely to rapidly progress towards fibrotic changes and consequently rapid functional worsening.
These findings seem in line with our previous evidence on lung pathology that showed the presence of an intense lung immune infiltrate in the rapid progressors, but not in the slow progressors. These results would support a role of inflammation and

of adaptive immune response in determining disease behavior[145] and might account, at least in part, for the different responses to antifibrotic drugs among IPF patients. Furthermore, the differential presence of AS in HRCT of rapid progressors might be used to possibly phenotype the disease and its progression at the time of diagnosis.
This clear between-group difference in response to Pirfenidone treatment, led us to investigated whether and to what extent the assessment and quantification of HRCT patterns reflects different responses to antifibrotic treatment, and if it may identify disease progression.
At the start of treatment, HRCT scores were similar in both stable and progressors. Among progressors, both AS and HC increased significantly after 1 year of treatment, whereas IS did not. On the other hand, in stable patients, HC increased significantly, while AS and IS did not. The observation that HC tends to progress in both stable patients and progressors confirms previous findings in untreated patients and demonstrates that the overall extent of lung fibrosis on CT (combination of reticulation and honeycombing) is a proxy of disease severity, as well as representing a strong independent predictor of mortality in patients with IPF[64]. Moreover, it may suggest that CT is able to capture subtle subclinical disease progression. Our study shows that progressors displayed a significant increase of AS over time despite treatment, whereas stable patients did not. This is an interesting finding, although the significance of alveolar opacity or ground glass attenuation remains debated. The term "ground-glass attenuation" refers to the presence of a hazy and diffuse homogeneous increase in lung density and, when located akin dense fibrotic areas, may represent mild/initial fibrosis[146]. However, ground glass attenuation may also be associated with the presence of inflammatory cells in the alveolar or interstitial space (i.e., alveolitis)[147], which is often more evident in cases with more aggressive disease as we showed in our previous studies.
Currently, longitudinal HRCT is used predominantly in clinical practice to identify complications of IPF, such as lung cancer or indirect signs of pulmonary hypertension. To the best of our knowledge, this is the first study that explores the role of change over time in CT scores and its correlation with functional decline in IPF patients on antifibrotic therapy. Our findings, we believe, are of particular interest as they may potentially help in early detection of disease progression by identifying subtle abnormalities that are not captured by a lung function test.

In conclusion during my years of PhD work I, along with my peers, have been able to study important aspects of the inflammatory reaction in COPD with novel results which added to previous knowledge in the areas of innate and adaptive inflammation and clarified to some degree the role of macrophages and eosinophils. We have also

starting to unravel the possible role of the peripheral blood lymphocytes in the outcome of smokers specially their possible role in the development of cancer.
Our idea that an inflammatory reaction possibly could be an important part of IPF has produced interesting results showing the possible implication of an adaptive immune response in the mechanisms of this disease. Combining clinical, morphometric pathology and radiology we have been able to possibly define the inflammatory component of the disease by the use of the HRCT. I believe that the important part of the work is that has provided the basis for future research in these smoking-induced diseases.

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