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Biomarkers of Response to Nintedanib in IPF

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Abstract

IPF is an idiopathic, chronic and progressive interstitial pneumonia, in which there are currently none serum biomarkers with diagnostic and prognostic implications. However, they could become valuable tools to facilitate diagnosis, monitor disease progression and assess treatment efficacy. The pathogenesis of IPF involves repeated pulmonary damage on alveolar epithelial cells. Several studies have shown that in the bronchoalveolar lavage (BAL) of patients with IPF, eosinophilia greater than 10% is observed. Additionally, some studies have reported that nitric oxide (NO) plays a crucial role in the angiogenesis process in fibrotic lungs and upregulates the expression of Vascular Endothelial Growth Factor (VEGF). The aim of our study is to evaluate the role of blood eosinophil counts (BECs) and FeNO (fractional exhaled NO) as potential biomarkers for IPF. A total of 38 patients with IPF (mean age 75.5 ± 6.7 years) followed at the University of Ferrara-Italy was enrolled. We monitored BECs and FeNO at baseline (T0, time of diagnosis and initiation of antifibrotic therapy) and after 12 months (T12) of treatment. Patients with IPF undergoing therapy with Nintedanib exhibit, after one year of therapy, a higher BECs (0.54 ± 0.13 x10³/mcrl) compared to patients treated with Pirfenidone (0.13 ± 0.10 x10³/mcrl) or those not receiving antifibrotic treatment (0.02 ± 0.01 x10³/mcrl). Moreover, after one year of treatment, the FeNO concentration was also higher in patients on Nintedanib (29.42 ± 2.99 ppb) compared to those on Pirfenidone therapy (20.8 ± 20.5 ppb). BECs and FeNO are potential biomarkers of response to Nintedanib.

Keywords: IPF; eosinophils; FeNO; Nintedanib; BECs; NO; DLCO; BAL; VEGF; Pirfenidone

Abbreviations: IPF = Idiopathic Pulmonary Fibrosis; BECs= Blood eosinophil counts; FeNO = Fractional exhaled nitric oxide; BAL= Bronchoalveolar lavage; AEC= Alveolar epithelial cell; VEFG-R=Vascular Endothelial Growth Factor Receptor; NO= Nitric oxide; eNOS= endothelial nitric oxide synthase; cNOS= constitutive nitric oxide synthase; iNOS= inducible nitric oxide synthase; UIP= Usual Interstitial Pneumonia; TGF- β: transforming growth factor beta

Introduction

Idiopathic Pulmonary Fibrosis:

Idiopathic Pulmonary Fibrosis (IPF) is a chronic and progressive interstitial pneumonia with unknown etiology. It affects mainly adult males and it's characterized by functional progression and chronic cough. Diagnosis is made if clinical parameters are compatible and if other causes are excluded. Without treatment, the mean life expectancy is between 4,5 years [1,2]. Unfortunately, the prognosis is unpredictable [3,4]. IPF is characterized by

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a UIP (*Usual Interstitial Pneumonia*) pattern both regarding radiology and histopathology [5]. IPF incidence in Europe and North America vary between 2,8 -18 cases per 100.000/ year [1-6]; in Italy, incidence is approximately around 9,3 cases per 100.000 and prevalence around 31,6 per 100.000 [7,8]. In patients with IPF, there are higher levels of apoptosis (due to aging, oxidative stress, telomere shortening) of Type 2 alveolar epithelial cells (AEC2 cells), which should repair the damaged type I alveolar epithelial cells (AEC1). The result of repeated damaging stimuli is a miscommunication between epithelial cells and fibroblasts, leading to the deposition of fibroblastic foci (aggregates of mesenchymal cells within a myxoid matrix) and interstitial remodelling [9,10]. Clinically, IPF manifests with dyspnoea (initially on exercise-related and then resting) and non-productive cough; some patients remain stable for years, while others progress rapidly [5,11,12]. The 2018 ATS guidelines made a strong recommendation on the importance of a multidisciplinary approach in diagnosing IPF [5]. If a radiologic diagnosis of UIP definite or probable pattern is made with High-resolution chest CT (HRCT) in a compatible clinical context, it is already possible to diagnose IPF without the need for a lung biopsy [13]. The introduction of antifibrotic drugs (Pirfenidone and Nintedanib) has led to a significant reduction in mortality, although their prescribing is limited to strict functional criteria and they often cause gastrointestinal effects (nausea, vomiting, dyspepsia, diarrhoea), that lead to the discontinuation of therapy [14].

Th2-inflammation in IPF:

Eosinophils are bone marrow-derived granulocytes and are found in low numbers in the peripheral blood of healthy subjects. In type 2 inflammatory diseases, eosinopoiesis in the bone marrow is increased, resulting in a rise in the number of mature eosinophils released in the circulation.

Eosinophils can have different phenotypes and activation patterns in humans. While resident eosinophils (rEOS) are present in both healthy and chronically inflamed lungs with an immunomodulatory role, inflammation-activated eosinophils (iEOS), whose activation is driven by IL-5, were more present in both blood (BECs) and lungs of asthmatic patients, and have a clear pathogenic role [15]. From the blood, eosinophils can migrate in multiple tissues and organs under both physiological and pathological conditions. Eosinophils exert their various functions through the synthesis and release of a variety of granule proteins and pro-inflammatory mediators (granular proteins, Charcot-Leyden crystals); autoantibodies anti-eosinophilic granular proteins have a correlation with disease severity in patients with eosinophilic airway disease [16-18].

In IPF pathogenesis, the damage to airway epithelial cells induces the release of cytokines (IL-33, IL-25 and Thymic Stromal Lymphopoietin-TSLP), which activate the Th2 inflammatory cascade with the release of profibrotic factors [transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), matrix metallopeptidase 7 (MMP7)] that drive fibroblasts to differentiate into myofibroblasts, responsible for the deposition of extracellular matrix [19-21]. Fibroblasts further differentiate into myofibroblasts also with support of other profibrotic mediators [interleukin 4, interleukin 13 (IL-4, IL-13)] and mitogenesis mediators (vascular endothelial growth factor (VEGF)] [22-26]. In 1995, K Fujimoto et al published on Chest journal an article about BAL eosinophilia in patients with IPF; they measured the concentration of eosinophilic cationic protein (ECP) in the bronchoalveolar lavage fluid (BALF), finding that patients with IPF who had elevated eosinophilia in the BAL (more than 20%) exhibited a progressive phenotype and more frequent acute exacerbation events, the authors suggested that the cytotoxic effects of eosinophils may contribute to lung damage and the development of fibrosis [27].

FeNO:

Nitric oxide (NO) is a gaseous molecule that acts as a key mediator in numerous physiological processes (such as smooth muscle dilation, protection against bronchoconstrictive stimuli and ciliary motility) [28]. The presence of NO in exhaled air (FeNO) was first demonstrated by chemiluminescence analysis and mass spectrometry by Gustafsson in 1991 [29,30]. Over the past two decades, FeNO has particularly emerged as one of the most important biomarkers in the management of bronchial asthma, and the procedure for its measurement was standardized for the first time in 2005 by the ATS and the ERS [31]. In asthmatic patients, FeNO indicates the degree of eosinophilic inflammation in the airways and is an indicator of response to inhaled corticosteroid therapy (ICS) and biologics drugs [32-34]. In the lung, NO is produced by various types of cells (epithelial cells, mast cells, vascular endothelial cells). It is synthesized through the enzyme nitric oxide synthase (NOS). Three different isoforms have been cloned so far: endothelial (eNOS) and neuronal (nNOS), together referred to as constitutive forms (cNOS), and the inducible form (iNOS). The increase in NO levels in the exhaled air of asthmatic patients is attributed to the "over-expression" of the enzyme iNOS [35-37].

The concentrations of NO in exhaled air, expressed in parts per billion (ppb), are flow-dependent. For this reason, ERS and ATS recommend performing at least three reproducible measurements (with a variability of about 5%). The subject must inspire to total lung capacity with purified air to avoid contaminating the sample with potentially high levels of environmental NO. The subject then performs a 10-second exhalation at a pressure of 5-20 cmH2O, which ensures the closure of the soft palate, minimizing the risk of contamination with NO from the paranasal sinuses.

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An exhalation is considered adequate if a stable eNO concentration is reached at a flow of 50 ml/s. When measuring NO concentrations in exhaled air from the lower airways, it is important to exclude the contribution of nitric oxide produced in the nasal cavities, as the concentrations of nasal NO are much higher than those found in bronchial exhaled air. To minimize nasal "contamination," the exhalation is performed at a constant flow against an expiratory resistance. Nitric oxide is quantified in "parts per billion" (ppb), with values under 12 ppb considered normal in young non-asthmatic individuals.

Values up to about 25 ppb in adults and around 20 ppb in children are still considered normal, while values between 25 and 50 ppb in adults and between 20 and 35 ppb in children are regarded as moderately elevated [38,39]. Values above 50 ppb in adults and above 35 ppb in children are considered indicative of significant inflammation [32].

FeNO and IPF:

The role of NO in the pathogenesis of interstitial lung diseases (ILDs) is not fully understood [40-41]. NO is considered a key mediator in the processes of healing and repair of epithelial damage, as most of the cells involved in these mechanisms (fibroblasts, epithelial cells) can produce NO [42,43]. The first study related to increased production of NO and its derivatives in the lungs of IPF patients was published in 1997, when Saleh and colleagues reported significant overexpression of iNOS in inflammatory cells and alveolar epithelium, associated with a marked production of nitrotyrosine compared to healthy subjects [44]. Some studies have indicated that NO plays a crucial role in the aberrant angiogenesis process in fibrotic lungs. Regarding this aspect, Iyer and colleagues demonstrated that NO is the key molecule in Vascular Endothelial Growth Factor (VEGF) upregulated expression. These findings were also confirmed by proteomic studies and are particularly interesting since Nintedanib is a non-specific competitive antagonist of (VEGF), Platelet-Derived Growth Factor (PDGF), and Fibroblast Growth Factor (FGF) receptors. Despite these studies, the role of NO in pulmonary fibrogenesis remains, to date, controversial [45].

Materials and Methods

Study population:

A total of 38 IPF patients (34 males, 29 former smokers; mean age 75.5 ± 6.7 years) followed from March 2023 to October 2024 at the Respiratory Department of the Saint Anna Hospital of Ferrara University were enrolled in the study (Table 1).

The diagnosis of IPF was made according to the ATS/ERS/JRS/ALAT guidelines, in a multidisciplinary setting. Clinical, functional, and immunological data were collected from all patients. Laboratory parameters included complete blood count with leukocyte differential, lipid profile, coagulation profile, lymphocyte typing on peripheral venous blood, serum protein electrophoresis and iron status. The enrolled patients underwent functional monitoring of forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC), total lung capacity (TLC), diffusing capacity of the lung for carbon monoxide (DLCO, absolute values expressed in milliliters and as percentage of predicted) every 6 months, according to the study protocol. The study also recorded the main comorbidities across the entire population under study, shown in Table 2.

Table 1: Demographic characteristics of study population.

	IPF (38)
Age years (mean ± SD)	75,5 ± 6,7
Male (%)	34 (89%)
Smokers former/current	29/0

IPF= idiopathic pulmonary fibrosis

Table 2: Main comorbidities in the study population.

	IPF
Hypertension	21 (55%)
Hypercholesterolemia	10 (26%)
Ischemic heart disease	8 (21%)
ATS	4 (11%)
Hepatic steatosis	1 (3%)
Mild OSAS	1 (3%)
Diabetes mellitus type II	7 (18%)
Atrial Fibrillation	8 (21%)
Visceral obesity	2 (5%)
Bronchial Asthma	1 (3%)
GER	3 (8%)

IPF= idiopathic pulmonary fibrosis; ATS= atherosclerosis; OSAS= obstructive sleep apnoea syndrome; GER= gastroesophageal reflux disease.

The study was approved by the ethics committee (protocol lung-biomarkers, CE: 303/2023/Oss/AOUFE) of the University of Ferrara. All patients were Caucasian and signed informed consent for participation in the study, which was approved by the above-mentioned ethics committee. Data were entered into a specific database along with survival data. Patients enrolled in the study were invited to undergo the procedure for the detection of nitric oxide in exhaled air (FeNO) using the Medisoft device from Sensor-Medics, exactly 12 months after starting antifibrotic therapy.

They were specifically encouraged to perform a maximal exhalation, and once the residual volume was reached, they were instructed to take a maximal inspiration, placing the

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mouthpiece of the device in their mouths until the residual volume was reached. Following this, a normal controlled exhalation monitored by the device allowed the assessment of the FeNO. During their exhalation, a tachometer appeared on the monitor, with a needle indicating the intensity at which the patient needed to exhale in order to maintain a stable exhalation flow (50 ml/sec), allowing for as precise a measurement as possible.

Currently, FeNO is not used in the diagnosis or follow-up of IPF. However, the study examined the FeNO progression in two patient groups: patients with IPF treated with Pirfenidone and patients with IPF treated with Nintedanib, exploring whether its utilization could become a useful biomarker both regarding survival and response-to-therapy approaches. In this study, the blood eosinophil counts (BECs) was considered for the patients with IPF at baseline (T0 = time of diagnosis and initiation of antifibrotic therapy) and after 12 months (T12) of antifibrotic treatment. The aim was to assess the changes in BECs during antifibrotic therapy in IPF patients and to evaluate potential differences between the Pirfenidone and Nintedanib group, to understand whether BECs could serve as a marker of therapy response and whether it correlates with functional progression. The patients with IPF were categorized based on their antifibrotic therapy (Pirfenidone or Nintedanib), and their responses to therapy were evaluated at baseline (T0) and after 6 and 12 months of antifibrotic treatment (T6 and T12) in terms of functional progression and side effects. Furthermore, the patients were subdivided according to their radiological patterns (*UIP definite* or *probable UIP*) to assess whether there was a difference in disease progression between these two patterns. This approach allowed for a comprehensive evaluation of the role of BECs in response to antifibrotic treatments, the impact of treatment on functional status and any potential radiological differences that could influence the progression of IPF.

Statistical analysis:

The results were expressed as means ± standard deviation. Since the data were not normally distributed, the one-way Kruskal-Wallis's analysis of variance and the Dunn test were used for multiple comparisons. The Mann-Whitney test was used for pairwise comparisons of variables. Receiver Operating Characteristic (ROC) curve areas were also calculated, and the Youden index was used to obtain cut-off values with the best sensitivity and specificity. The Spearman test was used to search for correlations between variables. A P-value lower than 0.05 was considered statistically significant. Statistical analysis and data graphing were performed using GraphPad Prism 9.0 software.

Results

The results obtained from the study are as follows: 18 patients on Pirfenidone therapy (IPF-Pirf), 13 patients on Nintedanib therapy (IPF-Nint), 7 patients not on antifibrotic therapy due to significant comorbidities (IPF).

In the IPF-Pirf group (18 patients), we observed: one patient died due to disease progression; the measurement of FeNO after 12 months of therapy (T12) was 20.8 ± 20.5 ppb; 4 patients (22%) experienced gastrointestinal side effects (diarrhea, nausea, anorexia, weight loss), but these were not severe enough to discontinue therapy; 3 patients (17%) had an acute exacerbation of the disease after 12 months of Pirfenidone treatment; 12 patients (66%) had a definite UIP pattern on HRCT chest scan at baseline (T0); 6 patients (33%) had a probable UIP pattern on HRCT chest scan at baseline (T0); of these, 2 had concomitant apical pulmonary emphysema. Blood eosinophil counts (BECs) at baseline (T0) was 0.16 ± 0.15 x10³/mcrl; BECs after 12 months of therapy (T12) was 0.13 ± 0.10 x10³/mcrl.

The functional worsening (FVC and DLCO as a percentage of predicted values) at T0, T6 and T12, in the three IPF groups (Pirfenidone/Nintedanib/no therapy) is shown in Table 3, Figure 1, Figure 2.

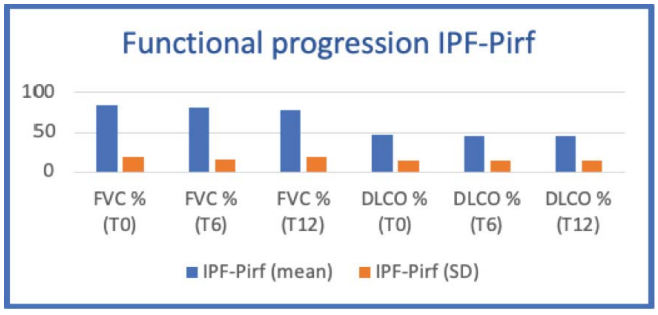

Figure 1: The progression of respiratory function at baseline (T0 = time of diagnosis and initiation of antifibrotic therapy), after 6 months of treatment (T6), after 12 months of treatment (T12) in IPF-Pirf. IPF-Pirf= patients with IPF on Pirfenidone treatment; FVC= Forced Vital Capacity; DLCO= Diffusion Capacity for Carbon Monoxide; IPF= idiopathic pulmonary fibrosis.

Table 3: Functional worsening in the study population at baseline (T0 = time of diagnosis and starting antifibrotic therapy), after 6 months of treatment (T6), after 12 months of treatment (T12). IPF-Pirf= patients with IPF on Pirfenidone treatment; IPF-Nint= patients with IPF on Nintedanib treatment; IPF= patients with IPF without antifibrotic treatment; FVC= Forced Vital Capacity; DLCO= Diffusion Capacity for Carbon Monoxide.

	IPF- Pirf	IPF-Nint	IPF
FVC % del pred T0	83,4 ± 18,49	79 ± 15,6	58 ± 7,93
FVC % del pred T6	80,33 ± 15,61	81 ± 16,3	//
FVC % del pred T12	77,9 ± 19,47	80,17 ± 14,8	65,5 ± 10,6
DLCO % del pred T0	46,92 ± 14,5	57,1 ± 18,03	56 ± 8,10
DLCO % del pred T6	45,76 ± 14,47	57 ± 14,48	//
DLCO % del pred T12	45,76 ± 14,47	58,3 ± 24,51	44 ± 6,37

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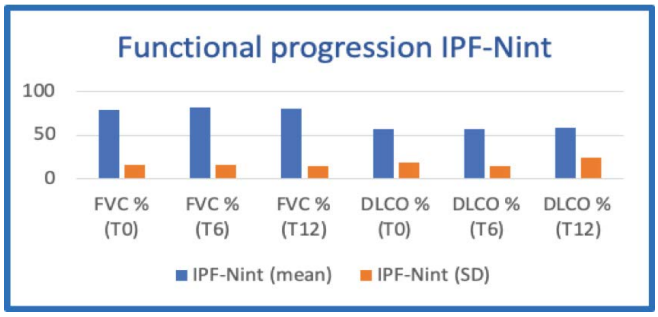

Figure 2: The progression of respiratory function at baseline (T0 = time of diagnosis and initiation of antifibrotic therapy), after 6 months of treatment (T6), after 12 months of treatment (T12) in IPF-Nint. IPF-Nint= patients with IPF on Nintedanib treatment; FVC= Forced Vital Capacity; DLCO= Diffusion Capacity for Carbon Monoxide; IPF= idiopathic pulmonary fibrosis.

From these Figures and Tables, it is evident that patients with IPF on Pirfenidone and Nintedanib treatment, enrolled in our study population, maintained functional stability over the 12-month treatment period, which is consistent with the data in the literature.

In the IPF-Nint group (13 patients), we observed: 2 patients died due to disease progression (after approximately 1 year of therapy; these patients were not candidates for Pirfenidone therapy due to functional criteria). The measurement of FeNO after 12 months of therapy (T12) was 29.42 ± 2.99 ppb; 8 patients (62%) experienced gastrointestinal side effects (diarrhea, weight loss, renal failure), but these were not severe enough to discontinue therapy. No patients experienced an acute exacerbation of the disease after starting Nintedanib therapy; 11 patients (85%) had a definite UIP pattern on HRCT chest scan at baseline (T0); 2 patients (15%) had a probable UIP pattern on HRCT chest scan at baseline (T0); BECs at baseline (T0) was 0.19 ± 0.03 x10³/mcrl; BECs after 12 months (T12) of Nintedanib treatment was 0.54 ± 0.13 x10³/mcrl.

In the IPF group not on antifibrotic therapy (7 patients), we observed: 2 patients died due to disease progression approximately one year after diagnosis; one patient had an acute exacerbation of the disease approximately one year after diagnosis; 6 patients (85%) had a definite UIP pattern on HRCT chest scan at baseline (T0); one patient (14%) had a probable UIP pattern on HRCT chest scan at baseline (T0). BECs at baseline (T0) was 0.08 ± 0.07 x10³/mcrl; BECs after 12 months without antifibrotic therapy (T12) was 0.02 ± 0.01 x10³/mcrl.

Our results show that analyzing the complete blood count in IPF patients at baseline (T0) and after 12 months (T12) of antifibrotic treatment, we found higher BECs in patients on Nintedanib treatment compared to those on Pirfenidone or not on treatment. Furthermore, after one year of antifibrotic treatment, we observed a higher concentration of FeNO in patients treated with Nintedanib compared to the Pirfenidone group (Table 4).

Table 4: Blood eosinophil counts (BECs) in patients with IPF at baseline (T0) and after 12 months of antifibrotic therapy.

	BECs x10^3/mcrl (T0)	BECs x10^3/mcrl (T12)	FeNO ppb (T12)
IPF	0,08 ± 0,07	0,02 ± 0,01	//
IPF-Pirf	0,16 ± 0,15	0,13 ± 0,10	20,8 ± 20,5
IPF-Nint	0,19 ± 0,03	0,54 ± 0,13	29,4 ± 2,99

IPF-Pirf= patients with IPF on Pirfenidone treatment; IPF-Nint= patients with IPF on Nintedanib treatment; IPF= patients with IPF without antifibrotic treatment; exhaled nitric oxide (FeNO) concentration after 12 months of antifibrotic therapy (T12); mcrl= microliter; ppb= parts per billions

Moreover, the increase in BECs after one year of treatment with Nintedanib (IPF-Nint T12) was statistically significant (P value < 0,0001) compared to eosinophilia at the time of diagnosis (IPF-Nint T0). The difference in BECs after one year of Nintedanib treatment was also statistically significant compared to the IPF-Pirf group at T12 and to the IPF patients not receiving treatment at T12 (P value < 0,0001) (Figure 3).

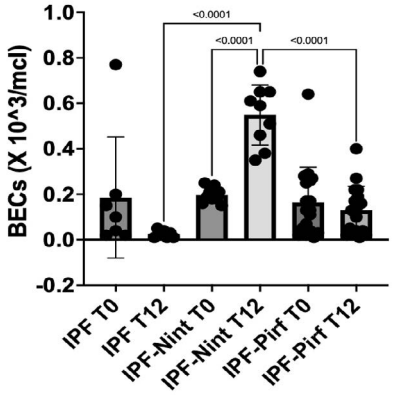

Figure 3: The graphical representation of BECs in patients with IPF at baseline (T0) and after 12 months of antifibrotic therapy (T12). BECs in IPF-Nint T12 was statistically significant (P value < 0,0001) compared to IPF-Nint T0. BECs in IPF-Nint T12 was statistically significant compared to the IPF-Pirf T12 and to the IPF T12 (P value < 0,0001). BECs= blood eosinophil counts; IPF= patients with IPF without antifibrotic treatment; IPF-Nint= patients with IPF on Nintedanib treatment; IPF-Pirf= patients with IPF on Pirfenidone treatment; mcrl= microliter.

We also measured FeNO after 12 months of antifibrotic therapy (T12) and we observed a statistically significant difference (P value = 0.0106) in concentration between the IPF-Pirf and IPF-Nint groups, with a higher FeNO concentration in patients treated with Nintedanib (Figure 4). The FeNO data in the IPF group one year after diagnosis was not available due to the insufficient number of patients.

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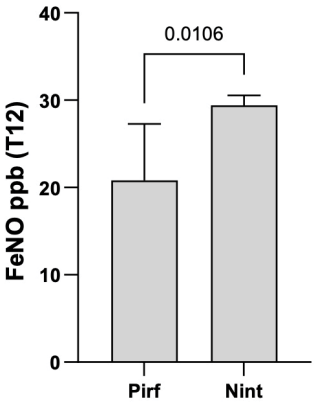

Figure 4: Graphical representation of exhaled nitric oxide (FeNO) concentration after 12 months of antifibrotic therapy (T12) in patients with IPF on Pirfenidone treatment (Pirf) and on Nintedanib therapy (Nint). Statistically significant difference (P value = 0.0106) between the Pirf and Nint group, with a higher FeNO concentration in Nint (patients with IPF treated with Nintedanib). ppb=parts per billion.

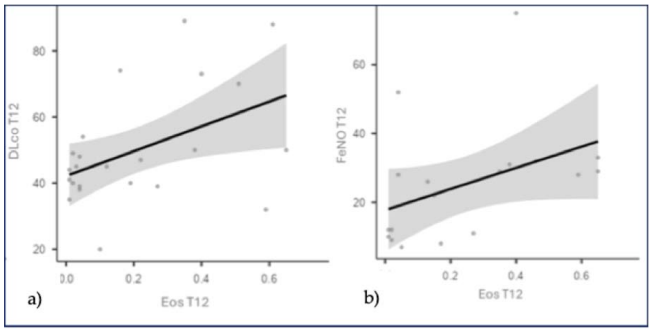

Figure 5: In the IPF-Nint group, statistically significant correlations between: a) EosT12 and DLCO T12: r=0.430, P value=0.0406; b) EosT12 and FeNO T12: r=0.575, P value=0.0126. IPF-Nint= patients with IPF on Nintedanib treatment; DLCO= Diffusion Capacity for Carbon Monoxide percentage; T12= after 12 months of Nintedanib treatment; FeNO= fraction of exhaled nitric oxide; Eos= blood eosinophil counts; IPF= idiopathic pulmonary fibrosis.

Considering the IPF-Nint group, we found statistically significant correlations between: BECs (T12) and DLCO as a percentage (T12): r=0.430, P value=0.0406; BECs (T12) and FeNO (T12): r=0.575, P value=0.0126 (Figure 5). These statistical correlations suggest that as BECs increases after 12 months of treatment, there is also an increase in FeNO, as well as an improvement in DLCO function, supporting our hypothesis that eosinophils, when confined to the bloodstream, do not act at the site of extracellular matrix remodeling, leading to radiological and functional stability (probably due to the endothelial action mechanism of Nintedanib). Therefore, we could hypothesize that blood eosinophilia and FeNO could be considered potential biomarkers of response to antifibrotic treatment with Nintedanib.

Discussion

It is well known in the literature that pulmonary fibrosis is the result of persistent alveolitis with abnormal deposition of extracellular matrix. Alveolitis consists of infiltration by inflammatory cells, including eosinophils, which release cytokines and stimulate the proliferation, migration, and activation of mesenchymal cells, increasing extracellular matrix synthesis. The results of our study show that patients with idiopathic pulmonary fibrosis, either on Pirfenidone therapy or untreated, have a very low BECs (both at diagnosis and after one year of antifibrotic treatment). This low BECs in IPF is likely a consequence of alveolar tissue eosinophilia, which is the site where the fibrogenic process involving eosinophils occurs. The interesting results of our study, for the first time in literature, show that BECs increases during antifibrotic therapy with Nintedanib. This finding may be interpreted as an effect of the antifibrotic drug on the endothelial side. We could hypothesize that Nintedanib induces a shift of eosinophils from the alveolar tissue to the vascular compartment, leading to functional stability of the disease. Furthermore, in the group of IPF patients on Nintedanib treatment, we observed a higher FeNO concentration compared to the Pirfenidone-group, after one year of antifibrotic treatment. We know that FeNO reflects the degree of inflammation in the proximal airways rather than in the alveolar space; however, it is well established that oxidative stress plays a role in the fibrogenic process in IPF, as well as the pro-angiogenic aspects of NO in the fibrotic lung. NO increases the expression of VEGF, inducing neoangiogenesis in the fibrogenic process. Moreover, the VEGF receptor is a target of Nintedanib. It appears that eNOS is protective against vascular remodeling, whereas iNOS, overexpressed by inflammatory cells such as alveolar macrophages, contributes to the development of interstitial fibrosis. Therefore, FeNO could be considered a potential marker of response to Nintedanib antifibrotic therapy in IPF. Additionally, since 2017, attention has been drawn to CaNO (alveolar nitric oxide concentration), measured by multiple exhalations of FeNO (at least three exhalations at a flow rate of 50 ml/sec) in the context of pulmonary interstitial diseases, as a marker reflecting the degree of alveolar inflammation [45].

In our study, however, we observed a statistically significant correlation after one year of treatment with Nintedanib between FeNO levels and the percentage of DLCO. Our hypothesis is that the increase in FeNO is secondary to the activity of iNOS, which is stimulated by alveolar macrophages activated by the initial damage on epithelial alveolar cells. Nintedanib, by inhibiting VEGF-R, prevents the process of neoangiogenesis and thus the deposition of extracellular matrix. Therefore, this elevated FeNO is concentrated in the upper airways, without

leading to endoalveolar inflammation, which is why it is associated with an improvement in DLCO in our patients. Furthermore, an increase in BECs after 12 months of therapy and an improvement in DLCO, supporting our hypothesis that eosinophils, when confined to the bloodstream, do not act at the site of extracellular matrix remodeling, resulting in radiological and functional stability (likely due to the endothelial action mechanism of Nintedanib). We could hypothesize that blood eosinophilia and FeNO might be considered potential biomarkers of response to antifibrotic therapy with Nintedanib (Figure 6).

In our study we also observed functional stability after one year of antifibrotic treatment in both the Pirfenidone and Nintedanib group. In the IPF-Pirf group, there was a lower incidence of gastrointestinal side effects but more episodes of acute exacerbation of the disease, while in the IPF-Nint group, no exacerbations of IPF were observed. Among patients with definite and probable UIP radiological patterns, we found the same degree of radiological and functional progression after one year of antifibrotic treatment, confirming what is known in the literature that the probable UIP pattern warrants early antifibrotic therapy, as the definite UIP pattern.

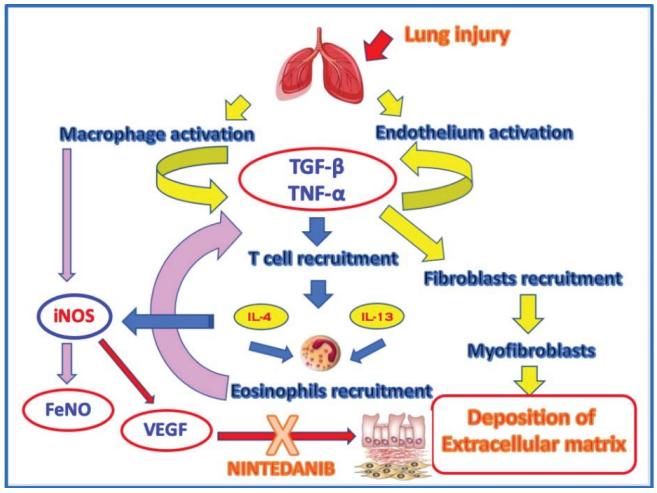

Figure 6: Possible mechanism of action of eosinophils and FeNO in idiopathic pulmonary fibrosis. TGFβ= transforming growth factor beta; TNFα= tumor necrosis factor alpha; T cell= T lymphocytes; IL-4= interleukin 4; IL-13= interleukin 13; iNOS= inducible nitric oxide synthase; FeNO= fraction of exhaled nitric oxide; VEGF= Vascular Endothelial Growth Factor.

Conclusions

The results of our research project on biomarkers in IPF are particularly interesting, especially the role of eosinophils and FeNO as potential biomarkers of response to antifibrotic therapy (Nintedanib), on which there are currently no data in the literature. This needs to be further demonstrated in a larger population of patients. Monitoring FeNO and blood eosinophilia in patients treated with Nintedanib could be a valuable tool to determine whether the patient is a responder to therapy or if alternative treatment options (such as experimental protocols) should be considered.

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Author Contributions: L.V., M.R., were responsible for the day-to-day running of and completion of the study along with the final written manuscript. M.d'A. also contributed significantly to the final written manuscript. The study design, protocol, and intellectual property of the study were conceived by L.V., M.R., I.G., M.d'A., A.C.,A.P. Specialist statistical support was received by M.d'A. M.R., I.G., contributed significantly to the physical recruitment and completion of the study. L.V., A.P., were responsible for the writing, review and editing. All authors significantly contributed to the interpretation of data, drafting and critically revising the manuscript for intellectual content, and when agreeing to a final version.

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