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Impact of angiotensin II type 1 and G-protein-coupled Mas receptor expression on the pulmonary performance of patients with idiopathic pulmonary fibrosis

Débora Raupp [a], Renata Streck Fernandes [a], Krist Helen Antunes [b], Fabíola Adélia Perin [c], Katya Rigatto [a,*]

[a] Laboratório de Fisiologia Translacional, Curso de Pós-Graduaçao em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, Brazil
[b] Laboratório de Imunologia Clínica e Experimental da Pontifícia, Universidade Católica do Rio Grande do Sul, Brazil
[c] Complexo Hospitalar da Irmandade Santa Casa de Misericórdia de Porto Alegre, Brazil

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a severe interstitial disease with a mean survival of about 2.5–5 years after diagnosis. Its pathophysiology is still a major challenge for science. It is known that angiotensin II (Ang-II) binds AT1 receptor (AT1R) and its overactivation induces fibrosis, inflammation and oxidative stress. In contrast, activation of the Mas receptor (Mas-R) by angiotensin 1–7 opposes the harmful effects induced by Ang-II. Thus, our innovative objective was to analyze, in patients' lung with IPF, the balance between AT1R and Mas-R expression and their possible association with pulmonary spirometric parameters: forced expiratory volume in the first second (FEV_1%) and forced vital capacity (FVC%). One cubic centimeter of lung tissue was obtained from IPF patients ($n = 6$) and from patients without IPF ($n = 6$) who underwent bronchial carcinoma resection. Receptor expression was quantified using western blot. AT1R expression was significantly higher (34 %) in patients with IPF ($P = 0.006$), whereas Mas-R was significantly less expressed (54 %) in these patients' lungs ($P = 0.046$). There was also a positive correlation between Mas-R expression and FEV_1% ($r = 0.62$, $P = 0.03$) and FVC % ($r = 0.58$, $P = 0.05$). Conversely, AT1R expression was negatively correlated with FEV_1% ($r = 0.80$, $P = 0.002$) and FVC% ($r = 0.74$, $P = 0.006$). In conclusion, our results demonstrated an increased expression of AT1R and reduced expression of Mas-R in the lung of patients with IPF. The dominance of AT1R expression is associated with reduced lung function, highlighting the role of the renin–angiotensin system peptides in the pathophysiology of IPF.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is defined as chronic fibrosing interstitial pneumonia of unknown etiology with a poor prognosis. The mean survival is about 2.5–5 years after diagnosis [1,2]. IPF is more common in men, current or former smokers, and it usually occurs between the sixth and seventh decades of life [1,3]. The known pathophysiology of IPF indicates that the disease originates from repetitive injuries in the pulmonary epithelium overlapped with accelerated aging of alveolar cells, which triggers failed repair mechanisms [4]. When activated in an uncontrolled manner, pulmonary epithelium produces mediators of fibroblast migration, proliferation and differentiation into active myofibroblasts [5]. Once in the injured areas, these myofibroblasts secrete exaggerated amounts of extracellular matrix (ECM) components and become resistant to apoptosis [5,6]. This results in ECM and fibrosis remodeling [7,8].

Due to the progressive loss of lung function caused by IPF, an individual's quality of life decreases as the disease progresses [9]. In

Abbreviations: IPF, idiopathic pulmonary fibrosis; RAS, renin-angiotensin system; ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; Ang-I, angiotensin I; Ang-II, angiotensin II; Ang-(1–7), angiotensin 1–7; AT1R, angiotensin receptor type 1; Mas-R, Mas receptor; BMI, body mass index; ECM, Extracellular matrix; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; JNK, protein kinase c-Jun N-terminal; TGFβ 1, transforming growth factor beta 1.

* Corresponding author at: Rua Sarmento Leite, 245, Porto Alegre, RS, 90050-170, Brazil.
E-mail address: kvr@ufcspa.edu.br (K. Rigatto).

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addition, due to the large number of associated comorbidities and the need to apply complex resources to manage them, IPF generates a substantial burden on health services [10,11]. Currently, the two drugs (pirfenidone and nintedanib) used to treat the disease are expensive and promote only a small reduction in lung function decline [12,13]. However, no therapy can modify the common progress of IPF, except for transplantation [1]. Therefore, understanding the pathophysiology of IPF is a major challenge for science and becomes extremely important in the search for new therapeutic targets not only for IPF but also for the new COVID-19 that causes fibrosis [14,15].

In this context, one of the important systems that play a key role in the homeostasis of different organs, such as heart, blood vessels, and especially lungs, is the renin–angiotensin system (RAS). Classically, it is known that the conversion of angiotensin I (Ang-I) to angiotensin II (Ang-II) occurs by angiotensin converting enzyme (ACE) located also in the lungs [16]. Ang-II is a potent vasoconstrictor that acts mainly via angiotensin type 1 receptor (AT1R), forming the ACE-Ang-II-AT1R axis [17]. When excessively activated, AT1R may induce deleterious effects such as fibrosis, apoptosis, angiogenesis, hypertrophy, oxidative stress and cell proliferation in several cell types [18–21].

On the other hand, angiotensin-converting enzyme 2 (ACE2) plays a role in cleaves Ang-II into angiotensin 1–7 [Ang-(1–7)], which binds to the Mas receptor (Mas-R), to form the ACE2–Ang-(1–7)–Mas-R axis. Acting as an antagonist peptide to the effects of Ang-II, Ang-(1–7) has a vasodilating, antiproliferative, antifibrotic and anti-inflammatory action [22–26]. Thus, the axis represented by Ang-(1–7) possibly induces effects that are opposite to those generated by the ACE-Ang-II-AT1R axis [27,28].

Surprisingly, although Ang-II has a well-described fibrotic effect [29–31], being activated in the lung [16], and Ang-(1–7) has actions that antagonize Ang-II effects [26,28], no study has been found in the literature demonstrating the expression of their receptors directly into lung tissue of patients with IPF. A possible cause for this lack of studies is that using Ang-II blockers has not demonstrated effective clinical results in disease improvement or survival [32–34].

However, considering the therapeutic potential of Ang-(1–7), shown in many fibrotic tissues [35–37], it is reasonable to believe that the imbalance between the axis of the RAS may be involved in the progression of IPF. It is possibly more important to stimulate the components of the protective axis represented by Ang-(1–7)–Mas-R than to block the components of the Ang-II-AT1R axis, whose overstimulation causes fibrosis.

Despite scientific advances, fibrotic diseases remain an important public health problem and further research is essential to improve understanding of the mechanisms involved in the pathophysiology of these diseases, including the involvement of RAS peptides. Hence, our objective was to analyze the expression of AT1 and Mas receptors in the lungs of patients with IPF and verify whether there is an association with the pulmonary function parameters.

2. Materials and methods

2.1. IPF patients and controls

Lung tissue samples were obtained from six patients with IPF who underwent lung transplantation and six patients who underwent bronchial carcinoma resection (control group). The control group included individuals without a diagnosis of IPF, preferably with age and gender similar to the group with IPF but not necessarily respecting a perfect pairing. Patients diagnosed with heart failure were excluded. Considering the complexity of the disease and the number of medications these patients could be taking at the time of surgery, exclusion criteria were not strict, even allowing the inclusion of patients who were using drugs that interfere with the RAS in both groups.

The study was approved by the research ethics committees of *Irmandade Santa Casa de Misericórdia de Porto Alegre* and Universidade Federal de Ciências da Saúde de Porto Alegre (approval numbers 2.691.887 and 2.619.738, respectively). Informed consent was given by all patients.

2.2. Tissue collection

A 1-cm^3 portion of lung tissue from each patient was collected and frozen in liquid nitrogen and then stored at -80 °C. IPF was later diagnosed using anatomopathological testing. Sample collection from the control group was carried out in the safety margin of the removed lung carcinoma, allowing analysis of tissue with similar characteristics to the lungs of a healthy individual.

2.3. Pulmonary function test

Spirometry was performed by the health service in the preoperative period and data were collected from the medical records. The spirometric parameters evaluated for analysis were: forced expiratory volume in the first second (FEV_1%), forced vital capacity (FVC%) and FEV_1/FVC % ratio.

2.4. Protein extraction

Protein was extracted from samples by manual homogenization in 50 µL of lysis buffer containing protease inhibitor: 10 mM Tris·HCl, pH 7.5; 1 mM $MgCl_2$; 1 mM ethylenediaminetetraacetic acid (EDTA); 0.1 mM phenylmethylsulfonyl fluoride (PMSF); 5 mM 2-mercaptoethanol; 0.5 % 3-[(3-cholamidopropyl) dimethylammonio] -1-propanesulfonate (CHAPS) and 10 % glycerol. To homogenize, the samples were vortexed for 30 s (four times at 10-min intervals) and then centrifuged for 1 h at a speed of 13,000 rpm and a temperature of 4 °C. After centrifugation, only the supernatant was carefully collected and frozen at –12 °C for further analysis. Protein quantification of the samples was done through spectrophotometry.

2.5. Western blot analysis

Protein samples (20 µg) were separated by one-dimensional 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes using buffer containing 20 mM Tris·HCl, 150 mM glycine, 20 % (v/v) methanol and 0.02 % (w/v) SDS (pH 8.2) in a cooled Bio-Rad transfer unit. After that, the nonspecific protein sites were blocked by 1 h of incubation in a blocking solution composed of 5% (v/v) skimmed milk in 0.1 % phosphate-buffered saline (PBS, 1×). Afterwards the membrane was stained with a 1:500 concentration of rabbit polyclonal anti-human anti-angiotensin II type-1 receptor antibody/AGTR1 (AAR-011, Alomone®, Israel) and a 1:250 concentration of rabbit polyclonal anti-human anti-angiotensin-(1–7) Mas receptor antibody (AAR-013, Alomone®, Israel) or mouse anti-human β-actin monoclonal antibody (A2228, Sigma Aldrich®, Germany), followed by secondary staining with a 1:1000 concentration of rabbit anti-mouse IgG (H + L)-HRP antibody (ThermoFisher Scientific®, MA, USA).

Washing steps were carried out with PBS (1x) and 0.05 % Tween-20. The western blots were visualized using enhanced chemiluminescence (GE Healthcare Life Sciences), band intensity was determined by densitometry analysis and ImageJ software was used for band quantification. The results were normalized using mouse anti-human β-actin monoclonal antibody (A2228, Sigma Aldrich®, Germany) at a concentration of 1:1000.

2.6. Statistical analysis

Data were submitted to the Shapiro-Wilk normality test to identify differences between tissue concentration of the peptides and Student's *t*-test was used to compare the results. Pearson's correlation coefficient

Table 1
Sample characterization and Pulmonary Function Test.

Variables	Control (n = 6)	Fibrosis (n = 6)	P
Age (years)	55.7 ± 14.1	54 ± 12.7	0.83
Gender (male)	3	5	
Weight (kg)	79.8 ± 20.57	72.5 ± 8.94	0.67
Height (m)	1.69 ± 0.12	1.72 ± 0.13	0.34
BMI (kg/m^2)	27.54 ± 3.86	24.7 ± 2.93	0.6
Previous smoking	2	3	0.6
FEV_1%	81.9 ± 14.5	46.5 ± 18.43	0.0041*
FVC%	86.3 ± 13	43.83 ± 16.9	0.0006**
FEV_1/FVC%	75.18 ± 6.36	70 ± 35.7	0.13

BMI = body mass index; kg = kilogram; m = meter; FEV_1=forced expiratory volume in the first second; FVC = forced vital capacity; %=Percentage. The data are presented as mean ± SD.

was used to detect associations. Quantitative variables were expressed as means and standard deviations. A P value of ≤0.05 was considered to be statistically significant. All analyses were performed using SPSS version 25 software.

3. Results

Controls and patients with IPF had similar age, weight, height and body mass index (BMI), indicating a homogeneous sample. The majority of patients with IPF were men (83 %) and only half (50 %) were smokers or former smokers. As expected, lung function in IPF patients was worse than in the control group, with a statistically significant difference for spirometric parameters FEV_1% and FVC% (Table 1). AT1R expression was significantly higher (34 %) in the tissue of patients with IPF ($P <$ 0.006), whereas Mas-R expression was significantly lower (54 %) in the same patients ($P < 0.046$) (Fig. 1).

Our data have also demonstrated a positive correlation between Mas-R expression and the spirometric parameters FEV_1% ($r = 0.62$, $P = 0.03$) and FVC% ($r = 0.58$, $P = 0.05$) (Fig. 2). When AT1R expression was compared to FEV_1% and FVC%, a negative correlation was found (FEV_1%: $r = 0.8$, $P = 0.002$; FVC%: $r = 0.74$, $P = 0.006$; Fig. 3). There was no correlation between receptor expression and FEV_1/FVC%.

4. Discussion

In the current study, we reported increased expression of AT1R and reduced expression of Mas-R in lung tissue of patients with IPF. In addition, we found negative and positive correlations between the spirometric parameters and AT1R Mas-R expression, respectively. Such evidence strengthens our hypothesis that the fibrotic process could be due to an imbalance between the RAS components in the lung in favor of the Ang-II axis. In addition, these results suggest that this imbalance could also be associated with the degree of pulmonary impairment.

Couluris et al. [38] have demonstrated the effect of losartan, an AT1R antagonist, on the progression of IPF in humans: lung function was stable in 12 of the 17 treated patients. This finding supports our hypothesis that AT1R activation might participate in the pathophysiology process of IPF. On the other hand, results from our laboratory demonstrated that the plasma concentration of Ang-II was similar between IPF patients and controls but the alamandine plasma concentration, which is part of the ACE2 anti-fibrotic axis, was 356 % lower in these patients [39]. This reasoning is consistent with the increased Ang-II-mediated AT1R stimulation in IPF patients.

Although the ACE blockers have not effectively improved the prognosis of patients with IPF blocking the ACE–Ang-II–AT1R axis [32,34], studies in an animal model have shown that the inhibition of AT1R signaling, attenuated pulmonary fibrosis induced by bleomycin [40] and improved respiratory compliance. These results agree with those of Königshoff et al. [41], who demonstrated that Ang-II–AT1R stimulated cell migration and proliferation in fibroblasts, both of which contribute to fibrogenesis. Collectively, these findings demonstrated the critical role of the ACE–Ang-II–AT1R axis in the development of experimental pulmonary fibrosis [30].

Considering current knowledge of the RAS, which highlights the participation of peptides with opposing effects to the Ang-II [27,42], we believe that, stimulating the antagonist axis ACE2–Ang-(1–7)–Mas-R may be more important than blocking the components of the fibrosis-promoting axis. Probably, in the future, Ang-(1–7) might be a

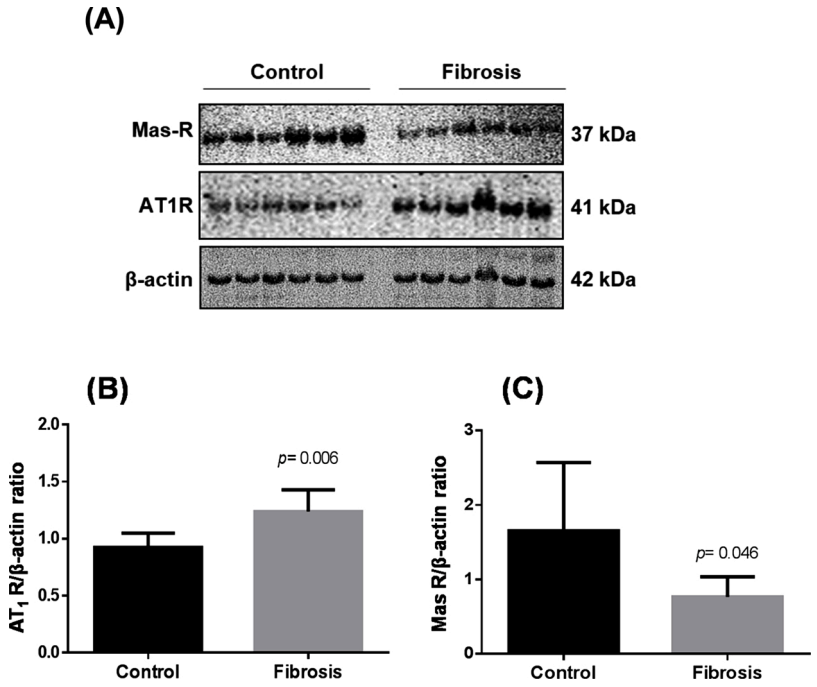

Fig. 1. Protein expression of AT1 and Mas receptors in lung tissue (n = 12). A: Expression of AT1 and Mas receptors in idiopathic pulmonary fibrosis lung tissue and control evaluated by western blot. B: Protein quantification of AT1 receptor in idiopathic pulmonary fibrosis and control lung tissue. C: Protein quantification of Mas receptor in idiopathic pulmonary fibrosis and control lung tissue.

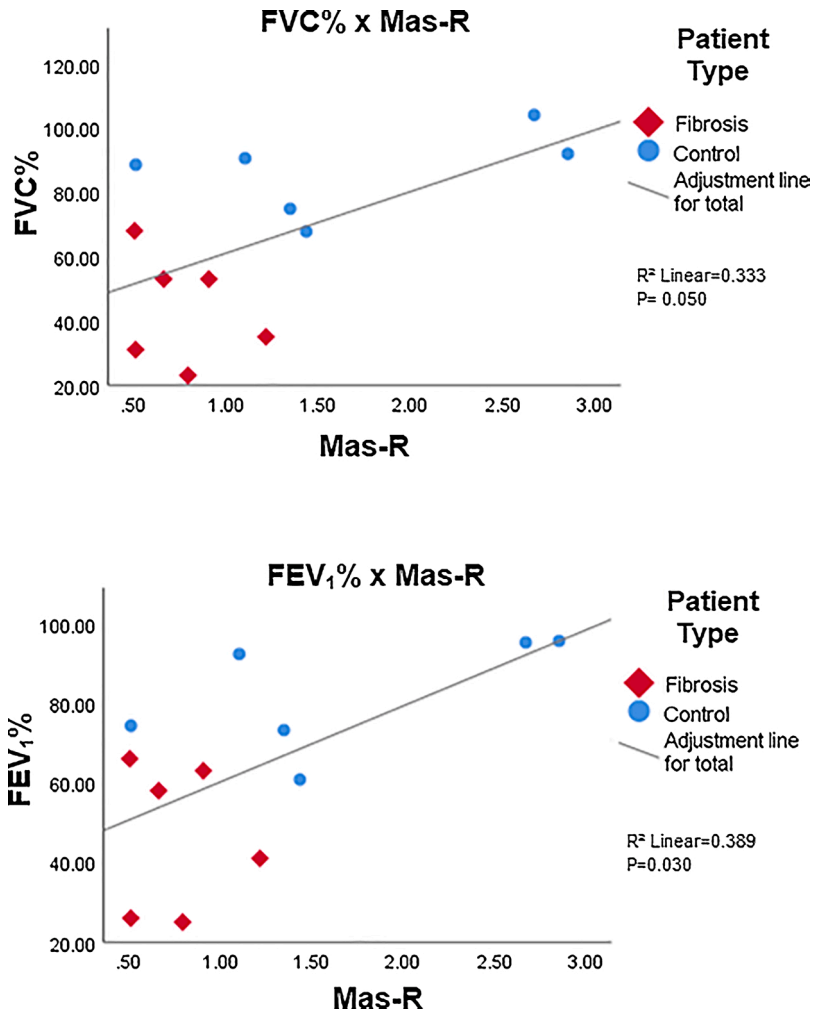

Fig. 2. Scatter plots of correlation analysis between spirometry values and Mas receptor quantification (n = 12). FEV_1=forced expiratory volume in the first second; FVC = forced vital capacity.

key component in the management of patients with IPF.

This rationale is supported by the literature, showing that in fibroblast culture of human lung and in mice with bleomycin-induced pulmonary fibrosis, Ang-(1–7) inhibit transforming growth factor-beta 1 (TGF-β1) activation, which is responsible for the transition of fibroblasts to myofibroblasts induced by Ang-II [43,44]. Moreover, Uhal et al. [45] observed that in experimental pulmonary fibrosis, Ang-(1–7) prevented activation of the protein kinase c-Jun N-terminal (JNK), which is responsible for triggering the apoptosis of alveolar epithelial cells. Collectively, these studies suggest that TGF-β and JNK protein activation contributes significantly to the anti-fibrogenic effects of the ACE2–Ang-(1–7)–Mas-R axis, reinforcing our hypothesis.

On the other hand, our study lacks results on Ang-II and Ang-(1–7) plasma and tissue concentration. A previous study [46] has demonstrated *in vitro* that the tissue concentration of RAS peptides is probably significantly higher than in plasma. This statement is confirmed by Dell'Italia et al. [47] who found that compared to the plasma concentration Ang-II levels can be >100-fold higher in the interstitial fluid of the heart. Moreover, although with modest affinity, Ang-(1–7) can also bind the AT1R [46]. In this sense, Ang-(1–7) could possibly reach sufficient concentrations in tissues to regulate AT1R activity in order to minimize harmful Ang-II effects. But, even in a local environment, given the higher affinity of Ang-II for AT1R compared with Ang-(1–7), the predominance of the Ang-(1–7)–AT1R axis will rely on even higher Ang-(1–7) concentrations.

Previously, Sipriani et al. [39] found a significant decrease in alamandine plasma concentration but not in Ang-I, Ang-II or Ang-(1–7), for IPF patients compared to the control group. Conversely, to justify the difference between our data and the results of Sipriani et al., as far as we know alamandine does not bind AT1R, AT2-R or Mas-R. Further studies should be conducted to understand these challenging findings.

Moreover, according to Joyner et al. [48], under certain conditions, such as pregnancy, the ratio between Ang-II and Ang-(1–7) does not change in the kidney. Pregnancy is a physiological period where there are significant blood volume and blood pressure fluctuations. These data demonstrate that for each tissue and pathophysiological condition, the balance among the RAS peptides can be quite specific.

In this sense, it is well documented that Ang-II production is increased in lungs of patients with IPF, whereas messenger RNA for ACE2 is reduced [49,50]. Li et al. [50] previously demonstrated that lung parenchyma controls Ang-II local generation (extravascular) through cleavage of angiotensinogen available in the tissue. These data and our findings demonstrate that, although there is no plasma difference between the RAS peptides, the imbalance between the expression of receptors and peptides in the lung tissue is singular and could play a key role in the pathophysiology of IPF.

Furthermore, our results also showed for the first time the significant functional impact of the imbalance of RAS components in the lungs. The patients' functional capacity was strongly associated with AT1R and Mas-R expression. The increase in AT1R is associated with worse lung function, as seen by the decrease in FVC% and FEV_1%, whereas the opposite association was found with Mas-R. The importance of these

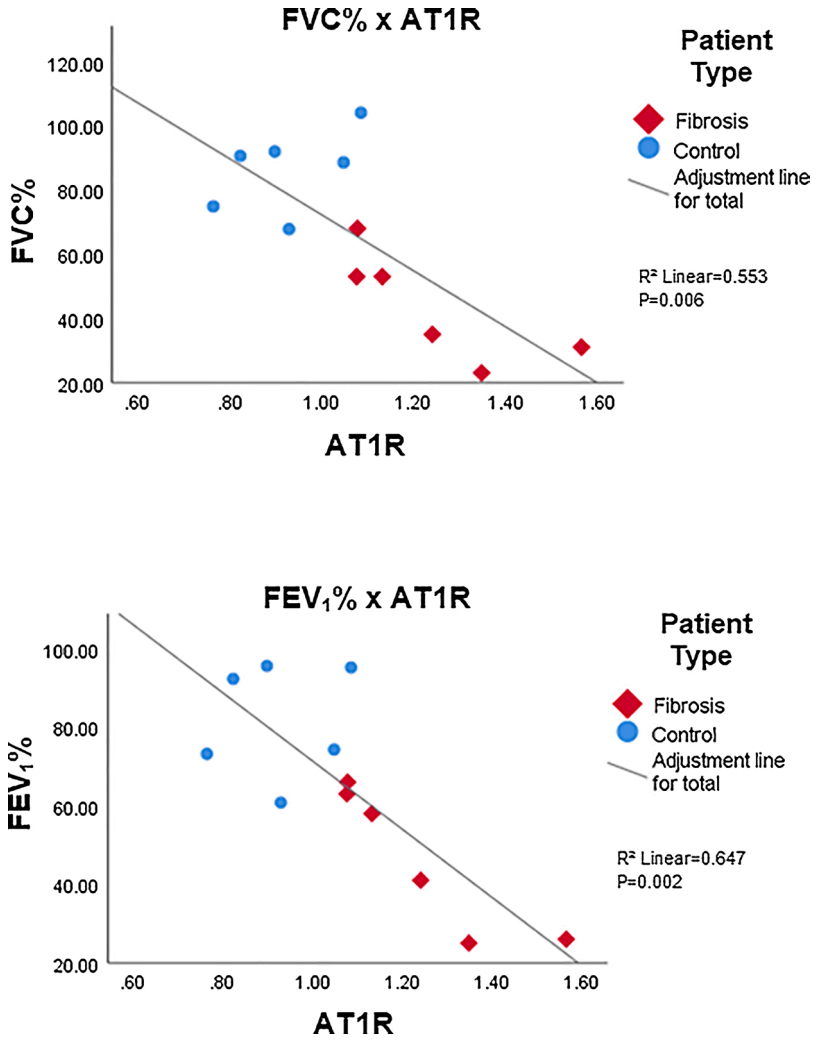

Fig. 3. Scatter plots of correlation analysis between spirometry values and AT1 receptor quantification (n = 12). FEV_1=forced expiratory volume in the first second; FVC = forced vital capacity.

results is reinforced by Bárczi et al. [9] who, on studying patients with IPF, found a positive correlation between quality of life and FVC ($r = 0.4$, $P < 0.05$), demonstrating that by improving functional parameters, the patients' well-being is also improved. Further studies should be conducted to confirm and amplify these important findings. The goal is to increase knowledge on the pathophysiology of the disease and improve the quality of life for those patients who have to live with the consequences of pulmonary fibrosis for the rest of their lives.

5. Conclusions

According to these data, it is doubtless that RAS involvement in IPF is far from clear. However, our results demonstrated increased expression of AT1R and reduced expression of Mas-R in the lung tissue of patients with IPF. We were able to show that the imbalance between receptors is associated with reduced lung function of the patients. Thus, these findings open new horizons for the role of RAS peptides in the pathophysiology of IPF, such that more studies need to be conducted to clarify the real implications of this system in disease development and progression.

Author contributions

K.R. conceived and designed the experimental protocol; D.R., R.S.F., K.H.A. and F.A.P. performed the experiments; D.R.; R.S.F., F.A.P. and K. R. data interpretation; D.R. and K.R. wrote the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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