Angiotensin Converting Enzyme 2 Expression in Human Type II Pneumocytes is Influenced by Renin-Angiotensin System Blockade

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Abstract

Angiotensin converting enzyme 2 (ACE2) acts as the receptor for the type 2 coronavirus that causes severe acute respiratory syndrome (SARS-CoV2), the etiological agent of coronavirus-19 disease (COVID-19). There has been a growing concern that renin-angiotensin (RAS) blockade increases the expression of ACE2 and then elevate patient susceptibility to SARS-CoV-2. Here, we report that age, smoking and RAS blockade influence on the percentage of human ACE2-expressing type II pneumocytes. These findings may explain the increased susceptibility to COVID-19 with age and in subjects with treated cardiovascular-related pathologies. Enhanced ACE2-expressing type II pneumocytes would facilitate SARS-CoV2 infection, thus increasing patient susceptibility to acute respiratory distress syndrome development because of an unbalance between the pressor and depressor axis of the RAS due to ACE2 sequestration by the virus.

Introduction

The renin-angiotensin system (RAS) is composed of two axis with opposing functions. The depressor and protective axis, represented by angiotensin (Ang) (1-7) and its Mas receptor counterbalancing the classic pressor axis represented by Ang II and the angiotensin type 1 receptor (AT1R). Overexpression of the pressor axis Ang II/AT1R promotes cardiovascular, renal, pulmonary and brain organ damage due to its oxidative stress, proinflammatory, fibrotic and hypertrophic effects among others¹. Angiotensin converting enzyme 2 (ACE2) is a key element of the protective axis of the RAS. ACE2 catalyzes Ang II degradation into Ang-(1-7), a component of the RAS that exerts hypotensive, anti-oxidant, anti-fibrotic, anti-inflammatory and protective effects opposite to those displayed by Ang II^{2,3}. ACE2 is widely expressed in the human body with strong expression in the gastrointestinal tract, heart and kidney^{4,5}. In addition to its enzymatic function counterbalancing the opposing arms of the RAS, ACE2 acts as a receptor for the type 2 coronavirus that causes severe acute respiratory syndrome (SARS-CoV2), the etiological agent of coronavirus-19 disease (COVID-19)^{6,7}. Through single-cell ACE2 RNA sequencing, organs identified at risk are lung, heart, esophagus, kidney, bladder, and ileum, and located specific cell types (i.e., type II alveolar cells, myocardial cells, proximal tubule cells of the kidney, ileum and esophagus epithelial cells, and bladder urothelial cells), which are vulnerable to SARS-CoV2 infection⁶.

Type II alveolar cells (pneumocytes) are key cells to maintain lung homeostasis⁸. Type II pneumocytes are cuboidal secreting cells responsible for secretory functions of the peripheral lung, thus reducing the surface tension of the alveoli and preventing them from collapsing, and constitute the progenitor cells of the epithelium⁸. ACE2 is mainly expressed in type II pneumocytes⁹⁻¹² and protects against acute lung injury in several animal models of acute respiratory distress syndrome (ARDS)¹³. The pressor arm of the RAS is implicated in the pathogenesis of acute lung injury, thus, the protective role of ACE2 results from Ang II downregulation and Ang-(1-7) upregulation¹³⁻¹⁶.

Antihypertensive drugs targeting the pressor axis of the RAS such as ACE inhibitors (ACEIs) or type 1 Ang II receptor blockers (ARBs) are extensively used worldwide to treat many cardiovascular disorders. Evidence in animals show that ACEIs and ARBs increase ACE2 expression^{17–20}. Regarding human tissues, hypertension and RAS blockers are not associated with ACE2 expression in the kidney²¹ but ACE2 expression is slightly reduced in human upper (nasal) respiratory tract of patients taking ACEI compared to matched controls¹⁰. In contrast, human intestine luminal ACE2 expression is increased by ACEI²². Thus, evidence in animals gave rise to the concern that antihypertensive treatment with ACEIs or ARBs may be harmful because hypertensive subjects under those treatments would be more susceptible to be infected by the virus. In contrast, other authors postulate that these antihypertensive treatments would be beneficial because Ang II levels decrease, thus decreasing the harmful effect of overexpression of the pressor arm of the RAS, while the levels of the protective component of the RAS, Ang-(1-7), increase. Although clinical studies have shown that these antihypertensive treatment did not increase the percentage of deaths or those hospitalized with COVID-19^{23–26}, up to date there is no experimental evidence showing whether ACEIs or ARBs induce changes in ACE2 in human lung. In this study we investigate Ang II and Ang-(1-7) levels in lung parenchymal and ACE2 protein expression in type II pneumocytes from hypertensive subjects treated with ACEI/ARB compared to normotensive subjects.

Results

Baseline Characteristics of the Total Study Population. We analyzed lung samples obtained from white subjects, normotensive (n=42) and hypertensive subjects treated with ACEI/ARB (n= 41). The mean age of normotensive subjects was 51 ± 17 and of ACEI/ARB-treated subjects was 63 ± 9 years old. The male-to-female ratio was 42/41. Characteristics of the population investigated are presented under table 1.

Lung Ang II levels are Influenced by Age, Sex, Smoking and ACEI/ARB Treatment. In order to investigate ACE2 activity as the ratio between Ang-(1-7) and Ang II, we measured Ang II and Ang-(1-7) levels by radioimmunoassay. Figure 1 shows Ang II levels in pg/g tissue in lung parenchyma of normotensive and hypertensive subjects treated with ACEI/ARB. We found that age, sex, smoking and ACEI/ARB treatment influence on Ang II levels ($p < 0.05$). Within the normotensive population, subjects under 60 years old, smokers or never smokers, showed no difference in Ang II levels irrespective of their sex. Conversely, Ang II levels were higher in smoker and former smoker men older than 60 years old compared to smoker and former smoker women of the same age. In addition, Ang II levels were higher in older smokers men compared to younger smoker and never smoker women. Unfortunately, our study lacks samples from never smoker subjects older than 60 years old to make a comparison between older never smoker and smokers. Regarding Ang-(1-7) levels, we observed a trend ($p = 0.08$) to reduce Ang-(1-7) levels in normotensive older and smoker subjects, female and male compared to younger smoker subjects, that is, age and smoking showed a tendency to induce a decrease in Ang-(1-7) levels in normotensive subjects (Figure 2). Altogether, our results showed that normotensive older smoker or former smoker men

displayed higher Ang II levels compared to women, while Ang-(1-7) levels were not changed by sex at the same age. In contrast, younger smoker women showed slightly higher Ang II levels compared to men.

Age and smoking also influence Ang II levels in those subjects under RAS blockade ($p= 0.001$). We found that Ang II levels were reduced in those hypertensive subjects treated with ACEI/ARB, never smoker or smoker, under 60 years old compared to subjects older than 60 years old, independently of their sex (Figure 1). RAS blockade treatment showed a tendency to increase Ang-(1-7) levels in subjects under 60 years old of any sex, smoker or never smoker compared to older subjects ($p= 0.08$). Instead, antihypertensive treatment did not change Ang-(1-7) levels in subjects over 60 years old (Figure 2).

Because ACE2 catalyzes the conversion of Ang II into Ang-(1-7), we then evaluated Ang-(1-7)/Ang II ratio as an indirect measurement of ACE2 activity. We observed no difference in Ang-(1-7)/Ang II ratio between the investigated groups, suggesting no change in ACE2 activity (Figure 3).

ACE2 is Influenced by Age, Smoking and ACEI/ARB Treatment. Because type II pneumocytes are key cells to maintain lung homeostasis⁸ and that ACE2 is mainly expressed in these cells⁹⁻¹², we measured the percentage of ACE2-expressing type II pneumocytes by immunohistochemistry in lung parenchymal from normotensive and hypertensive subjects under ACEI/ARB treatment. If we consider only normotensive and hypertensive treated subjects, we found no difference in the percentage of type II pneumocytes that expresses ACE2 (Figure 4A), being ACE2 present in $61.8 \pm 7.5 \%$ of type II pneumocytes from normotensive subjects and in $62.4 \pm 8.6 \%$ of type II pneumocytes from ACEI/ARB-treated subjects. We also did not find significant differences if we only compare never smokers, former smokers or smokers (Figure 4B) or male and female (Figure 4C). However, we observed that subjects over 60 years old exhibited lower percentage of ACE2-expressing type II pneumocytes ($p= 0.012$) (Figure 4D). When we performed statistical analysis comparing all the variables at the same time, that is, smoking, sex, age and antihypertensive treatment, we found a significant interaction between ACEI/ARB treatment and smoking on the percentage of ACE2-expressing type II pneumocytes ($p= 0.026$). This means that the percentage of ACE2-expressing type II pneumocytes depends on whether the subject is a smoker, former smoker or never smoker, under or not RAS blockade treatment. Regarding smokers subjects, those under RAS blocking treatment exhibited higher percentage of ACE2-expressing type II pneumocytes than normotensive ones (Figure 5). Within the group of hypertensive subjects under ACEI/ARB treatment, the percentage of ACE2-expressing type II pneumocytes was higher in smokers and former smokers compared to never smokers (Figure 5). Altogether, these data suggest that smoking and RAS blocking treatment enhances the percentage of ACE2-expressing type II pneumocytes.

ACE2 Protein Levels are Influenced by Age and Smoking. We evaluated ACE2 immunostaining intensity, indicative of ACE2 protein levels. We found a significant association between ACE2 immunostaining

intensity and smoking on subjects equal or older than 60 years old ($p = 0.05$), that is, older smokers and former smokers exhibited higher ACE2 protein levels compared to never smokers (Figure 6B).

We next evaluated whether RAS blockade and age are associated with ACE2 protein immunostaining intensity. Although the p value of that association was between 0.05 and 0.1, this fact shows a tendency to older hypertensive subjects treated with ACEI/ARB to display higher ACE2 protein expression levels compared to normotensive subjects of the same age or younger hypertensive subjects under RAS blockade (Figure 6C). Thus, age and RAS blockade trends to enhance ACE2 protein expression levels.

Discussion

We report novel findings in this translational research study: 1) age, sex, smoking and RAS blockade influence lung Ang II levels, 2) age, smoking and ACEI/ARB treatment influence the percentage of human ACE2-expressing type II pneumocytes, 3) age and smoking enhance ACE2 protein expression levels, and 4) RAS blockade shows a tendency to enhance ACE2 protein levels in older subjects. Figure 7 resumes the most relevant conclusions of our study. The effect of RAS blockade on human lung Ang II levels and ACE2 has never been previously reported to our knowledge.

We found that Ang II levels were higher in smoker men older than 60 years old compared to smoker and former smoker women of the same age or younger subjects, but they were reduced under ACEI/ARB treatment. Conversely, a trend to have their Ang-(1-7) levels reduced were observed in normotensive older and smoker subjects. Altogether these data suggest that smoking and aging induces RAS disbalance. In accord, chronic cigarette smoking and aging are associated with an upregulation of the pressor arm and downregulation of the compensatory ACE2/Ang-(1-7)/MasR axis of the RAS²⁷⁻²⁹. In a chronic cigarette smoke-induced pulmonary arterial hypertension rat model, lung Ang II levels and ACE expression were augmented while ACE2 expression was diminished^{30,31}. The ARB losartan reduced lung Ang II levels, restored ACE2 expression and ameliorated the deleterious effects induced by cigarette smoking in this model^{30,31} suggesting an AT1R-dependent mechanism. Accordingly, here we report that Ang II levels were augmented in lung from older smoker men but they were reduced in subjects under RAS blockade. Furthermore, we found that the percentage of ACE2-expressing type II pneumocytes were lower in older subjects compared to younger, reinforcing the concept of a RAS disbalance with aging^{28,29}. Accordingly, aging caused a reduction in ACE2 expression in various organs of mice, being the lowest in the lungs³².

We observed that smoking and RAS blockade influence on the percentage of human lung ACE2-expressing type II pneumocytes. The percentage of ACE2-expressing type II pneumocytes was similar between never smokers and smokers in normotensive subjects, but increased in smokers under RAS blockade. Furthermore, we also found higher ACE2 protein expression levels in type II pneumocytes from older smokers and former smokers compared to never smokers. Controversies exist regarding ACE2 expression and smoking. Some reports showed that current smokers and never smokers have closely similar bronchial epithelial cell mRNA levels of ACE2³³, no change in ACE2 protein levels in both bronchial and alveolar epithelial cells³⁴ and in nasal ciliary cells¹⁰. In contrast, others have reported higher ACE2

expression in smokers compared to never-smokers³⁵⁻³⁷. ACE2 RNA expression in the human lung at single-cell resolution showed that ACE2 expression was higher in the small airway epithelia of smokers than never smokers and that male smokers exhibited higher ACE2 RNA levels than female smokers or never smokers of either sex³⁸. In contrast, Smith et al. showed that lung ACE2 levels do not vary by age or sex, but smokers exhibit upregulated ACE2³⁷. Purkayastha and colleagues, through modeling the direct effects of cigarette smoke on SARS-CoV-2 infection, showed that acute cigarette smoke exposure increases the number of infected and apoptotic cells, prevents the normal airway basal stem cell repair response, suggesting that smoke increases COVID-19 severity³⁹. However, it has been shown that smoking was not associated with increased mortality in COVID-19 pneumonia^{40,41}, but cumulative smoking exposure, as measured by pack/years, was associated with a higher risk of hospitalization and mortality from COVID-19 in a dose-dependent manner⁴². In contrast, cigarette smoke-pretreatment potentially abrogates SARS-CoV-2 replication in an vitro model³⁴. An ongoing clinical trial focused on nicotine and smoking in COVID-19 subjects listed on ClinicalTrials.gov (NCT04429815) should address that concept.

There has been a growing concern that the use of ACEI and/or ARBs blockers by subjects with cardiovascular-related pathologies could increase the expression of ACE2 and then elevate patient susceptibility to SARS-CoV-2 infection. Recently an observational multicenter cohort study with patients from USA and Spain showed no clinically significant increased risk of hospital admission with COVID-19 associated with ACEI or ARB²⁶. In a small sample size of patients taking ACEI, upper respiratory tract ciliary ACE2 expression was slightly decreased compared to matched controls¹⁰. In contrast, we found that smoking and RAS blockade influence on the percentage of ACE2-expressing type II pneumocytes, being higher in smokers under ACEI/ARB treatment. Differences between our study and that from Lee et al.¹⁰ may be due to the area investigated (alveolar vs upper respiratory tract), the type of cell (type II pneumocytes vs ciliary cells) and the number of samples under study. Furthermore, those authors used upper respiratory tract and ciliary cells, an area that lacks type II pneumocytes⁴³. Pneumocytes together with alveolar macrophages are essential to maintain lung homeostasis⁷. We also found a trend for subjects older than 60 years old under ACEI/ARB treatment to exhibit higher ACE2 expression in type II pneumocytes compared to normotensive subjects of the same age or younger subjects under ACEI/ARB treatment. The increased ACE2 expression in type II pneumocytes would be protective due to Ang II downregulation. In fact, older ACEI/ARB-treated subjects exhibited lower Ang II levels (present results). But at the same time, the increased percentage of ACE2-expressing type II pneumocytes in older smokers under RAS blocking treatment would facilitate SARS-CoV2 infection, thus increasing patient susceptibility to uncontrolled ARDS development because of an unbalance between the pressor and depressor axis of the RAS due to ACE2 sequestration by the virus.

Our study is limited by a retrospective design, which is vulnerable to uncontrolled biases. We were unable to assess the impact of time since smoking cessation in the former smokers subjects population. We also do not consider the amount of cigarettes that each subject usually consumes in the smokers subjects population, the pathology by which the subject underwent lung surgery and the doses of ACEIs

or ARBs taken by each subject. Another limitation was the lack of lung samples from uncontrolled hypertensive subjects and from never smoker older subjects.

In conclusion, we found that older smoker men displayed the highest Ang II levels in their lungs while they were reduced under RAS blockade. Hypertensive smokers under ACEI/ARB treatment displayed higher percentage of ACE2-expressing type II pneumocytes, which may increase their susceptibility to be infected by SARS-CoV2. Thus, due to ACE2 sequestration by the virus there will be a disbalance in the RAS with the consequent ARDS development.

Methods

Human lung samples. The present is an observational retrospective study with insensitive information, thus informed consent was not required. The Ethics and Clinical Research Committee of Hospital Provincial del Tórax “Dr. A. Cetrángolo” and Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (PV-2020-00851131) have approved the present study.

Lung parenchymal samples from normotensive subjects (n=42) and hypertensive subjects treated with the ACEIs enalapril (n=22) or perindopril (n= 1), or the ARBs valsartan (n= 9), losartan (n= 7), telmisartan (n= 1) or candesartan (n= 1) (total ARB-treated subjects= 18) who underwent resection (segmentectomy or lobectomy) were obtained from the unified archives of the Pathology Service from Hospital Municipal de Vte. López “Prof. B. Houssay and Hospital Provincial del Tórax “Dr. A. Cetrángolo”. Those were resection samples obtained from subjects undergoing surgery for various reasons (cancer, interstitial lung diseases, bullas, bronchiectasis, infections and others). Tissues were only used if characterized as non-diseased.

Ang II and Ang-(1-7) levels. Ang II and Ang-(1-7) endogenous levels were determined in 84 samples (42 from normotensive subjects and 41 from ACE/ARB-treated hypertensive subjects). Angs levels were measured by radioimmunoassay as previously described^{44–46}. Briefly, samples were dewaxed, rehydrated and homogenized in acid ethanol (0.1 mol/L HCl/80% ethanol) containing 0.44 mmol/L o-phenanthroline, 1 mmol/L Na+para-chloromercuribenzoate, 0.12 mmol/L pepstatin A and 25 mmol/L EDTA. Homogenates were centrifuged at 20000 xg for 30 min at 4°C. Proteins in the supernatant were quantified. The supernatant was subsequently lyophilized and Angs extraction and recovery was performed as previously described^{45,46}. Each sample was corrected for each recovery. Angs levels were quantified by radioimmunoassay using Angs labelled in our laboratory as previously described⁴⁷. Radioimmunoassay for Ang-(1-7) has been previously validated⁴⁸. Intra-assay and inter-assay variability were $13.7 \pm 2.3\%$ and $12.4 \pm 3.1\%$, respectively. Results were expressed as pg/g tissue.

ACE2 Immunohistochemistry. Immunohistochemistry staining was performed on a Discovery Ultra VENTANA systems (Roche) automated Stainer. Fifty seven samples were analyzed (20 from normotensive subjects and 37 from ACE/ARB-treated hypertensive subjects). Samples were exposed to a rabbit polyclonal ACE2 antibody (Abcam cat. ab15348, lot GR3333640-5 dilution 1:500) and with a mouse monoclonal antibody TTF-1 (Cell Marque clone 8G7G3/1, lot 077115 dilution 1:500) to label type II pneumocyte marker. Antibody binding to ACE2 and type II pneumocyte was visualized by incubation with the ultraView Universal Alkaline Phosphatase Red Detection Kit (Roche cat. 0785269814001) and the ultraView Universal DAB Detection Kit (Roche cat. SAP 5269806001), respectively. Images were taken on a Olympus CX31 microscope. Two independent qualified pathologists specialized in human lung analyzed ACE2 staining in type II pneumocyte in a blinded fashion. Percentage of ACE2-expressing type II pneumocytes was measured in representative lung fields sufficient to count no fewer than 450 pneumonocytes. ACE2 intensity was classified from 1 to 3 (1+, very weak; 2+, moderate and 3+, strong).

Statistical Analysis. All analyses were conducted using the IBM SPSS Statistics 19 software. To analyze differences between the different groups investigated for each of the following variables: Ang II (pg/g tissue), Ang-(1-7) (pg/g tissue), Ang-(1-7)/Ang II, percentage of ACE2-expressing type II pneumocytes and ACE2 intensity, the age variable was coded (AgeCod) as equal to 1 if the patient was <60 years old or equal to 0 if the patient was ≥ 60 years old. Kolmogorov-Smirnov test was applied to verify whether the variables followed a normal distribution. Because the variables Ang II (pg/g tissue), Ang-(1-7) (pg/g tissue) and Ang-(1-7)/Ang II did not follow a normal distribution, they were transformed into neperian logarithm (NL). Under this transformation, they followed a normal distribution. The homogeneity between groups was verified in each case using the Levene test.

To detect differences between groups that conform the variables AgeCod, sex, ACEI/ARB-treated hypertensive (HT) and smoking, the general linear model for each of the dependent variables was applied, that is, for NL (Ang II (pg/g tissue) , NL (Ang-(1-7) (pg/g tissue), NL (Ang-(1-7)/Ang II), percentage of ACE2-expressing type II pneumocytes and ACE2 intensity. The following results were obtained:

- For NL Ang II (pg/g tissue) an interaction between AgeCod and smoking ($p = 0.001$) and AgeCod and sex ($p = 0.013$) was verified. This means that each factor has an effect on the variable response but the size of that effect depends on the level of the other factor. For example, if we take AgeCod and smoking, the presence of interaction indicates that AgeCod and smoking have an effect on the variable Ang II (pg/g tissue) but the size of the effect of age depends on whether the person is a smoker, former smoker or never smoker. In the case of HT, the existence of interaction between AgeCod and smoking was also detected ($p = 0.001$).
- For NL (Ang-(1-7) (pg/g tissue) the existence of interaction between AgeCod and smoking ($p = 0.086$) and AgeCod and hypertensive treated subjects ($p = 0.08$) was verified. Although the p value in both cases is greater than 0.05 but less than 0.1, this result shows a trend that these factors have an

effect on the variable. In the case of HT, no significant differences were found between the groups for the same response variable.

- For NL (Ang-(1-7)/Ang II no significant differences were found between the groups for the same response variable.
- There is a significant statistically difference between subjects under 60 years old ($n = 27$) compared to subjects equal or over 60 years old ($n = 30$) ($p = 0.012$)
- We verified the existence of interaction between HT and smoking ($p = 0.026$) on the percentage of ACE2-expressing type II pneumocytes ($n = 57$, which were 8 normotensive never smokers, 9 normotensive smokers, 3 normotensive former smokers, 9 never smokers HT, 17 smokers HT and 11 former smokers HT).

Finally, through the stratified Fisher's Exact Test we found an association between the variables ACE2 intensity, smoking and subjects equal or older than 60 years old of $p = 0.05$) and between ACE2 intensity, age and ACEI/ARB hypertensive treated of $p = 0.09$. Although this last p value is greater than 0.05 but less than 0.1, this result shows a trend that these factors are associated.

Data Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. It is also included in the SI file.

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Declarations

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Author contributions

M.M.G. conceived and coordinated the study. M.G.S. and G.R.C. designed and performed the experiments. N.L.F., L.I. and G.R.G. evaluated human samples and performed immunohistochemistry, microscopy

imaging and ACE2 quantification. G.C.T. and R.F.S collected information regarding each clinic history. M.N. conducted statistical analyses. M.M.G. wrote the manuscript with contributions by J.A. The first coauthors, M.G.S., N.F. and G.R.C. contributed equally and have the right to list their name first in their CV. All authors reviewed and agreed with the content of this manuscript.

Competing interests

The authors declare no competing interest.

Table

Table 1. Baseline Characteristics of the Investigated Population

	Normotensive subjects	Hipertensive subjects treated with ACEI/ARB
Number of subjects, n (%)	42 (100)	41 (100)
Age (year), $\bar{x} \pm SD$	51 $\pm$ 17	63 $\pm$ 9
< 60 years old, n (%)	28 (67)	12 (29)
$\geq$ 60 years old, n (%)	14 (33)	29 (71)
Treated with ACEI, n (%)	0 (0)	23 (55)
Treated with ARB, n (%)	0 (0)	18 (44)
Female, n (%)	19 (45)	22 (54)
Male, n (%)	23 (55)	19 (46)
Never smoker, n (%)	16 (38)	9 (22)
Smoker, n (%)	18 (43)	17 (41)
Former smoker, n (%)	8 (19)	15 (37)
Chronic obstructive pulmonary disease, n (%)	1 (2)	9 (22)
Type 2 diabetes, n (%)	1 (2)	6 (15)
Cardiovascular disease, n (%)	0 (0)	5 (12)
Chronic kidney disease, n (%)	0 (0)	0 (0)
Lung cancer, n (%)	6 (14)	12 (29)

Figures

Figure 1

Ang II levels are Influenced by Age, Sex and Smoking. Ang II levels expressed as pg/g tissue in lung parenchyma of normotensive or ACEI/ARB-treated hypertensive women (pink) or men (blue), smokers (S), never smokers (NS) or former smokers (FS) under 60 years old (<60) or equal to or over 60 years old (≥60). Because the number of samples of normotensive females never smokers < 60 years old and of males former smokers <60 years was very low, they were obviated to be represented in the graph but they were included in the statistical analysis. Statistical analysis (general linear model) showed a significant interaction between age and smoking ($p = 0.001$) and age and sex ($p = 0.013$) on Ang II levels. The bottom and top of the box plots represent the 25th and 75th percentiles, respectively. The bands within the box show the median value, the dot is the media and the whiskers extending from both ends of the boxes are minimum and maximum values.

Figure 2

Ang-(1-7) Levels Showed a Trend to Be Influenced by Age and Smoking. Ang-(1-7) levels expressed as pg/g tissue in lung parenchyma of normotensive or ACEI/ARB-treated hypertensive women (pink) or men (blue), smokers (S), never smokers (NS) or former smokers (FS) under 60 years old (<60) or equal to or over 60 years old (≥ 60). Because of the low n in normotensive female never smokers ≥ 60 years old and in males former smokers <60 years old they were obviated to be represented in the graph but they were included in the statistical analysis. Statistical analysis (general linear model) showed an interaction between age and smoking ($p = 0.086$) and age and ACEI/ARB treatment ($p = 0.08$) on Ang-(1-7) levels. Although the p value in both cases is greater than 0.05 but less than 0.1, this result shows a trend that these factors have an effect on the variable. The bottom and top of the box plots represent the 25th and 75th percentiles, respectively. The bands within the box show the median value, the dot is the media and the whiskers extending from both ends of the boxes are minimum and maximum values.

Figure 3

Ang-(1-7)/Ang II Ratio did not Change in ACEI/ARB-treated Hypertensive Compared to Normotensive Subjects. Results are expressed as the ratio between Ang-(1-7) (pg/g tissue) and Ang II (pg/g tissue) levels in lung parenchyma of normotensive (n= 42) and ACEI/ARB-treated hypertensive subjects (n= 41).

Figure 4

ACE2-Expressing type II Pneumocytes are Influenced by Age. Percentage of ACE2-expressing type II pneumocytes of lung parenchyma of normotensive and ACEI/ARB-treated hypertensive subjects (A), never smokers, former smokers or smokers (B), male or female (C) or from subjects under 60 years old (<60 y) or equal or over 60 years old (≥ 60 y). Statistical analysis (general linear model) showed a significant difference by age ($p= 0.012$). The bottom and top of the box plots represent the 25th and 75th percentiles, respectively. The bands within the box show the median value, the dot is the media and the whiskers extending from both ends of the boxes are minimum and maximum values. Black points outside the box are outliers.

Figure 5

ACE2-Expressing type II Pneumocytes are Influenced by Smoking and ACEI/ARB Treatment. Percentage of ACE2-expressing type II pneumocytes of lung parenchyma of normotensive and ACEI/ARB-treated hypertensive never smokers, former smokers or smokers. Statistical analysis (general linear model) showed a significant interaction between ACEI/ARB treatment and smoking ($p = 0.026$) on the percentage of ACE2-expressing type II pneumocytes. The bottom and top of the box plots represent the 25th and 75th percentiles, respectively. The bands within the box show the median value, the dot is the media and the whiskers extending from both ends of the boxes are minimum and maximum values. Black points represent outliers.

Figure 6

ACE2 Protein Expression is Greater in Older Smokers. Percentage of subjects with ACE2 immunostaining intensity (+1 or +2) in type II pneumocytes of lung parenchyma from never smokers and smokers + former smokers subjects under 60 years old (<60 y) or equal or over 60 years old (≥ 60 y) (B) or from normotensive and ACEI/ARB-treated hypertensive subjects under 60 years old (<60 y) or equal or over 60 years old (≥ 60 y) (C). Representative images (40x) of surgically resected lung tissue stained for ACE2 protein (red) and type II pneumocytes (brown) counterstained with hematoxylin (blue) (A). ACE2 intensity was classified from +1 to +3. Positive ACE2 staining in type II pneumocytes and in alveolar macrophages is indicated by blue and green arrows, respectively. ACE2 intensity corresponding to the score +3 was not presented because of the low number of samples with that classification, but they were included when statistical analysis was performed. Percentage of subjects with ACE2 intensity of +1 or +2 in each group is displayed inside the box. The stratified Fisher's Exact Test shows an association between ACE2 intensity, smoking and subjects equal or older than 60 years old ($p = 0.05$) and between ACE2 intensity, age and ACEI/ARB hypertensive treated ($p = 0.09$). Although this last p value is greater than 0.05 but less than 0.1, this result shows a trend that these factors are associated

Figure 7

Scheme that resumes the conclusions raised in the present work