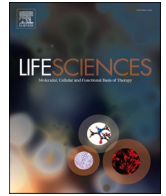




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Renin-angiotensin system blockade on angiotensin-converting enzyme 2 and TMPRSS2 in human type II pneumocytes

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ABSTRACT

Aims: Angiotensin-converting enzyme (ACE) 2 is the receptor for severe acute respiratory syndrome coronavirus 2 which causes coronavirus disease 2019 (COVID-19). Viral cellular entry requires ACE2 and transmembrane protease serine 2 (TMPRSS2). ACE inhibitors (ACEIs) or angiotensin (Ang) receptor blockers (ARBs) influence ACE2 in animals, though evidence in human lungs is lacking. We investigated ACE2 and TMPRSS2 in type II pneumocytes, the key cells that maintain lung homeostasis, in lung parenchymal of ACEI/ARB-treated subjects compared to untreated control subjects.

Main methods: Ang II and Ang-(1-7) levels and ACE2 and TMPRSS2 protein expression were measured by radioimmunoassay and immunohistochemistry, respectively.

Key findings: We found that the ratio Ang-(1-7)/Ang II, a surrogate marker of ACE2 activity, as well as the amount of ACE2-expressing type II pneumocytes were not different between ACEI/ARB-treated and untreated subjects. ACE2 protein content correlated positively with smoking habit and age. The percentage of TMPRSS2-expressing type II pneumocytes was higher in males than females and in subjects under 60 years of age but it was not different between ACEI/ARB-treated and untreated subjects. However, there was a positive association of TMPRSS2 protein content with age and smoking in ACEI/ARB-treated subjects, with high TMPRSS2 protein levels most evident in ACEI/ARB-treated older adults and smokers.

Significance: ACEI/ARB treatment influences human lung TMPRSS2 but not ACE2 protein content and this effect is dependent on age and smoking habit. This finding may help explain the increased susceptibility to COVID-19 seen in smokers and older patients with treated cardiovascular-related pathologies.

1. Introduction

The renin-angiotensin system (RAS) is composed of two axes with opposing functions. The depressor and protective axis, represented by angiotensin-(1-7) [Ang-(1-7)] and its Mas receptor, counterbalances the classic pressor axis, represented by angiotensin II (Ang II) and the

angiotensin type 1 receptor (AT1R) [1]. Overexpression of the Ang II/AT1R pressor axis promotes cardiovascular, renal, pulmonary, and brain organ damage due to its oxidative stress and proinflammatory, fibrotic, and hypertrophic effects, among others [2]. 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) [1]. ACE2 is widely

Abbreviations: ACE2, angiotensin-converting enzyme 2; ACEIs, angiotensin-converting enzyme inhibitors; Ang, angiotensin; ARBs, type 1 angiotensin receptor blockers; COVID-19, coronavirus disease 2019; RAS, renin-angiotensin system; SARS-CoV-2, type 2 coronavirus that causes severe acute respiratory syndrome; TMPRSS2, transmembrane protease serine 2.

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expressed in the human body with strong expression in the gastrointestinal tract, heart, and kidney [3]. In addition to its enzymatic function counterbalancing the pressor arm of the RAS, ACE2 acts as a receptor for the type 2 coronavirus that causes severe acute respiratory syndrome (SARS-CoV-2), the etiological agent of coronavirus-19 disease (COVID-19) [4,5]. The spike protein on SARS-CoV-2 binds ACE2 and is primed by the transmembrane protease serine 2 (TMPRSS2), allowing viral fusion and cellular entry [5].

Through single-cell ACE2 RNA sequencing, specific cell types that are vulnerable to SARS-CoV-2 infection have been identified, including type II alveolar cells, myocardial cells, proximal tubule cells of the kidney, ileum and esophagus epithelial cells, and bladder urothelial cells [4]. Type II alveolar cells, or pneumocytes, are key cells that maintain lung homeostasis [6]. Type II pneumocytes are cuboidal cells responsible for the secretory functions of the lung, reducing the surface tension of the alveoli, and preventing them from collapsing [6]. ACE2 is primarily expressed in type II pneumocytes [4,7,8] and protects against acute lung injury in several animal models of acute respiratory distress syndrome [9,10]. 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) upregulation [9,10].

Antihypertensive drugs targeting the pressor axis of the RAS such as ACE inhibitors (ACEIs) and type 1 Ang II receptor blockers (ARBs) are extensively used worldwide to treat many cardiovascular disorders. Evidence in rat kidney and heart showed that ACEIs and ARBs increase ACE2 gene transcripts [11]. Regarding human tissues, RAS blockers have not been associated with ACE2 expression in human kidney [12], though ACE2 expression has been shown to be slightly reduced in the upper (nasal) respiratory tract of patients taking ACEIs compared to matched controls [8]. In contrast, human intestine [13] and cardiomyocytes of subjects treated with ACEIs showed a significantly higher ACE2 expression [14]. Because of evidence in animals, it was suggested that RAS blockers may increase patient vulnerability to SARS-CoV-2 infection. However, several clinical studies argue against this hypothesis [15-18] and randomized clinical trials investigating the impact of discontinuation of RAS blockers on patients hospitalized with COVID-19 showed inconsistent results [18,19]. However, to date, there is no experimental evidence on RAS blockade and ACE2 expression in human lungs. In this study, we investigated ACE2 and TMPRSS2 protein expression in type II pneumocytes from lung parenchyma of subjects treated with ACEI/ARB compared to untreated control subjects. Those samples were from resected lung tissue. We also analyzed Ang II and Ang-(1-7) levels in subjects' lung parenchyma to employ the ratio of Ang-(1-7) to Ang II as a surrogate marker of ACE2 activity.

2. Material and methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

2.1. Ethics statement

This study was an observational retrospective study of de-identified material, thus informed consent was not required. The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Approval for the study was obtained from the Ethics and Clinical Research Committee of Hospital Provincial del Tórax and Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires.

2.2. Human lung samples

Lung parenchymal samples from untreated control subjects ($n = 22$) and subjects treated with ACEI ($n = 21$) or ARB ($n = 17$; total ACEI/ARB-treated subjects = 38) who underwent resection (segmentectomy or lobectomy) were obtained from the Pathology Service from Hospital

Municipal de Vte. López and Hospital Provincial del Tórax. The resection samples obtained were from subjects who underwent surgery for various reasons (e.g., suspected cancer, interstitial lung diseases, bullae, bronchiectasis, infections). No subject was under chemotherapy. Tissues were only used if they were characterized as non-diseased. Two cohorts were selected, comprising lungs from untreated and ACEI/ARB-treated subjects. As lung samples were the tissue under investigation, smoking was considered during data analysis. Characteristics of the study population are presented in Table 1.

The same sample was analyzed for Ang II and Ang-(1-7) levels and ACE2 and TMPRSS2 protein content; that is, 22 tissue samples from subjects and 38 from ACEI/ARB-treated subjects. Experimenters were blind to group assignment and outcome assessment, for all experiments. Because angiotensin level measurement by radioimmunoassay is a quantitative method, the dispersion in the data would be greater compared to a semiquantitative method such as immunohistochemistry. For this reason, we decided to increase the untreated control group sample size to 38 for angiotensin level analysis.

2.3. Ang II and Ang-(1-7) levels

Angiotensin levels were measured by radioimmunoassay. 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 20,000 $\times g$ for 30 min at 4 °C. Proteins in the supernatant were quantified. The supernatant was subsequently lyophilized and angiotensins extraction and recovery was performed as previously described [20]. Each sample was corrected for each recovery. Angiotensin levels were quantified by radioimmunoassay using angiotensins labelled in our laboratory as previously described [21]. Radioimmunoassay for Ang-(1-7) has been previously validated [21]. Intra-assay and inter-assay variability were $13.7 \pm 2.3\%$ and $12.4 \pm 3.1\%$, respectively. To validate Angs measurement in dewaxed tissue, we performed the same experimental condition with no-waxed tissue, that is, tissue immersed in formol as soon it was taken off the patient, and we obtained similar results to those when tissue was dewaxed.

2.4. ACE2 and TMPRSS2 immunohistochemistry

Immunohistochemistry staining was performed on a Discovery Ultra VENTANA systems (Roche) automated Stainer. Samples were exposed to a rabbit polyclonal anti-ACE2 antibody (Abcam cat. ab15348, lot

Table 1
Baseline characteristics of the investigated population.

	Untreated subjects	ACEI/ARB-treated subjects
Number of subjects, n	22	38
Age (year) $\bar{X} \pm SD$	48 \pm 17	63 \pm 9
<60 years old, n	17	11
\geq 60 years old, n	5	27
Treated with ACEI, n	0	21
Treated with ARB, n	0	17
Female, n	9	18
Male, n	13	20
Never smoker, n	10	9
Smoker, n	9	15
Former smoker, n	3	14
Chronic obstructive pulmonary disease, n	2	9
Type 2 diabetes, n	0	6
Cardiovascular disease, n	0	5
Chronic kidney disease, n	0	0
Lung cancer, n	1	12

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin type 1 receptor blocker.

GR3333640-5 dilution 1:500) or to a rabbit monoclonal anti-TMPRSS2 antibody (Abcam cat. ab92323, lot GR3344246-11, dilution 1:1500) together with a mouse monoclonal antibody TTF-1 (Cell Marque clone 8G7G3/1, lot 077115 dilution 1:500) to label type II pneumocytes. TTF-1 is a nuclear homeodomain transcription factor that in the lungs is expressed only in type II pneumocytes and Clara cells [22]. Antibody binding to ACE2 or TMPRSS2 and type II pneumocytes 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. ACE2 and TMPRSS2 quantification was performed according to Allred score [23]. Percentage of ACE2- or TMPRSS2-expressing type II pneumocytes was measured in representative lung fields sufficient to count no fewer than 450 pneumocytes. ACE2 intensity was classified from 1 to 3 (1+, very weak; 2+, moderate and 3+, strong). Two independent qualified pathologists specialized in human lung analyzed ACE2 or TMPRSS2 staining in type II pneumocyte in a blinded fashion to the patient's condition following principles for reproducible tissue scores.

2.5. Statistics

The data were analyzed by a statistician (M.N., chair of Mathematics and Biostatistics at the Faculty of Pharmacy and Biochemistry, University of Buenos Aires). All analyses were conducted using the IBM SPSS Statistics 19 software. The Kolmogorov–Smirnov test was applied to verify normal distribution of the variables. Data from ACE2 and TMPRSS2 followed normal distribution. Data from Ang II and Ang-(1–7) were transformed to logarithm to reach normal distribution. Homogeneity between groups was verified by the Levene test. To investigate differences, the General Linear Model was applied, which is an analysis of variance for multiple dependent variables by one or more covariates or factor variables, which divide the population into groups. Using this model, it is possible to test null hypotheses regarding the effects of factor

variables on the means of various clusters of a joint distribution of dependent variables. In addition, this model can be used to investigate the interactions between the factors as well as the individual effects of the factors. Potential confounders were sex, age, smoking and antihypertensive treatment and because of these potential confounders, the General Linear Model was applied. The stratified Fisher's exact test was used to investigate the association between variables. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline characteristics of the total study population

We analyzed lung samples obtained from subjects, untreated ($n = 22$) and treated with ACEI/ARB ($n = 38$) (Table 1). Mean subject systolic and diastolic pressures were 120 ± 5 and 67 ± 2 mmHg in the untreated group and 124 ± 5 and 73 ± 4 mmHg in the ACEI/ARB-treated group, respectively. The mean age of untreated subjects was 48 ± 17 years old and the mean age of ACEI/ARB-treated subjects was 63 ± 9 years old ($P < 0.05$). The difference in age was due because hypertension is more prevalent with age [24,25]. However, 11 of 22 samples from untreated subjects were from people over 57 years old. The male-to-female ratio was 13:9 in the untreated group and 20:18 in the ACEI/ARB-treated group.

3.2. Lung Ang II levels are influenced by age, sex, smoking, and ACEI/ARB treatment

Lung parenchymal Ang II levels were not changed when data were stratified by ACEI/ARB-treatment, sex, age or smoking habits (Fig. 1A–D). However, we found a significant interaction between age and smoking ($P < 0.001$) and age and sex ($P = 0.01$) on lung Ang II levels; that is, Ang II levels were influenced by the subject's age, but the effect of age on Ang II levels depended on whether the subject was a

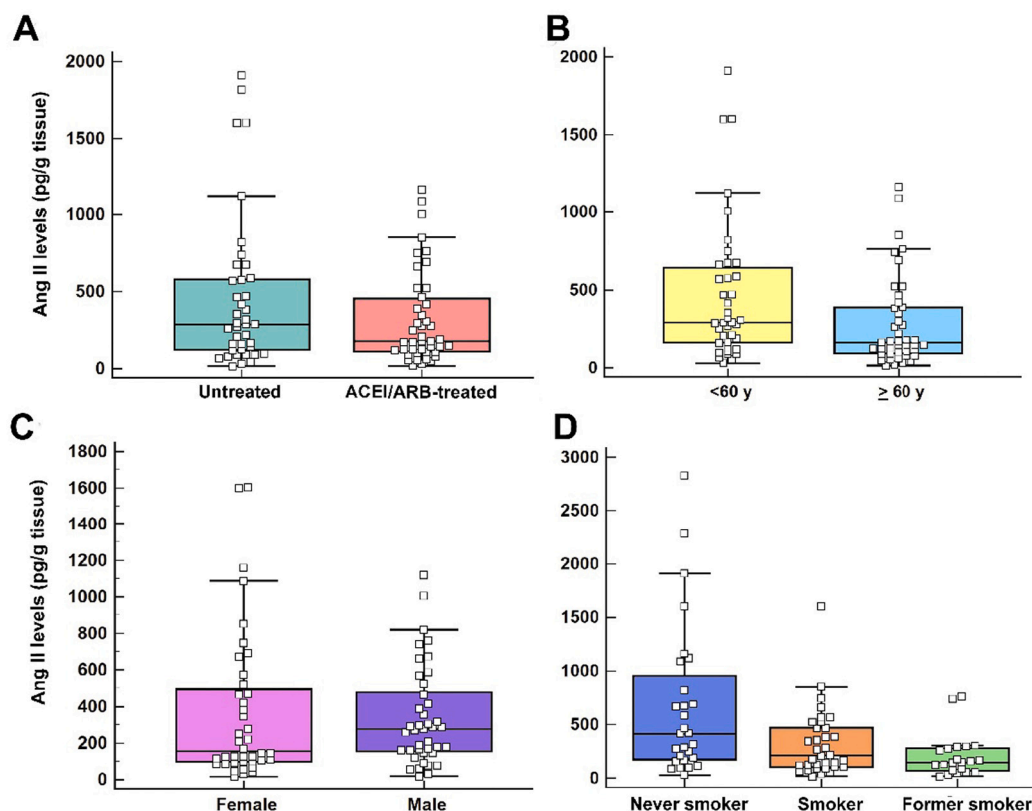


Fig. 1. Ang II levels in human lung parenchyma. Ang II levels expressed as pg/g tissue in lung parenchyma of (A) untreated ($n = 38$) and ACEI/ARB-treated subjects ($n = 38$); (B) subjects less than 60 years of age (<60 y) ($n = 37$) and subjects 60 years of age and older (≥ 60 y) ($n = 39$); (C) female ($n = 34$) and male ($n = 42$); and (D) subjects never smokers ($n = 26$), smokers ($n = 30$) and former smokers ($n = 20$). The bottom and top of the box plots represent the 25th and 75th percentiles, respectively. The bands within the box show the median value and the whiskers extending from both ends of the boxes are minimum and maximum values. The General Linear Model was applied for statistical analysis.

never smoker, former smoker, or smoker and whether the subject was female or male. An interaction between age and smoking on Ang II levels was also observed in subjects under RAS blockade ($P < 0.001$); lung Ang II levels in ACEI/ARB-treated subjects were influenced by the subject's age, though also depended on whether the subject was a never smoker, former smoker, or smoker.

Regarding Ang-(1–7) levels, we observed a significant difference by age, with subjects 60 years of age and older presenting with lower lung Ang-(1–7) levels compared to subjects younger than 60 years of age ($P = 0.02$). No differences in Ang-(1–7) levels were found when data were stratified by ACEI/ARB treatment, sex or smoking habits (Fig. 2A–D). However, an interaction between age and RAS-blocking treatment ($P = 0.04$) on lung Ang-(1–7) levels was detected. Thus, the effect of age on Ang-(1–7) levels in parenchymal lung depended on whether the subject was untreated or under RAS blockade treatment.

We observed no difference in ratios of Ang-(1–7) to Ang II by ACEI/ARB treatment, sex, age, and smoking habits (Fig. 3).

3.3. ACE2-expressing type II pneumocytes are influenced by age, smoking, and ACEI/ARB treatment

Because type II pneumocytes are critical cells in lung homeostasis [6] and ACE2 is primarily expressed in these cells [7,8], we measured the percentage of ACE2-expressing type II pneumocytes in the lung parenchyma of untreated and ACEI/ARB-treated subjects. There was no difference in the percentage of ACE2-expressing type II pneumocytes between untreated and ACEI/ARB-treated subjects, with ACE2 present in $61.8\% \pm 7.5\%$ of type II pneumocytes of untreated subjects and in $62.4\% \pm 8.6\%$ of type II pneumocytes of ACEI/ARB-treated subjects

(Fig. 4A). There were no significant differences in ACE2-expressing type II pneumocytes between never smokers, former smokers, and smokers (Fig. 4B) or male and female subjects (Fig. 4C). However, we observed that subjects 60 years of age and older exhibited lower percentages of ACE2-expressing type II pneumocytes (60.5 ± 8.1 vs 64.0 ± 8.0 , $P = 0.01$; Fig. 4D). Since the statistical analysis employed compared all variables (i.e., smoking, sex, age, and antihypertensive treatment) simultaneously, we found a significant interaction between ACEI/ARB treatment and smoking on the percentage of ACE2-expressing type II pneumocytes ($P = 0.03$). This means that the percentage of ACE2-expressing type II pneumocytes depended on whether the subject was a smoker, former smoker, or never smoker and whether the subject was under RAS-blockade treatment. Within the group of untreated or ACEI/ARB-treated subjects, ACE2-expressing type II pneumocytes were slightly higher in smokers compared to never smokers (Fig. 4E). Untreated former smokers exhibited higher amount of ACE2-expressing type II pneumocytes than untreated never smokers or former smokers under ACE/ARB treatment.

3.4. ACE2 protein content is influenced by age and smoking

We evaluated ACE2 immunostaining intensity, which indicates ACE2 protein levels. We found a significant association between ACE2 immunostaining intensity and smoking in subjects who were 60 years of age and older ($P = 0.05$), with the largest percentage of subjects exhibiting higher ACE2 protein levels being older smokers and former smokers (Fig. 5A).

We next evaluated whether RAS blockade and age were associated with ACE2 protein levels. We found no significant association between

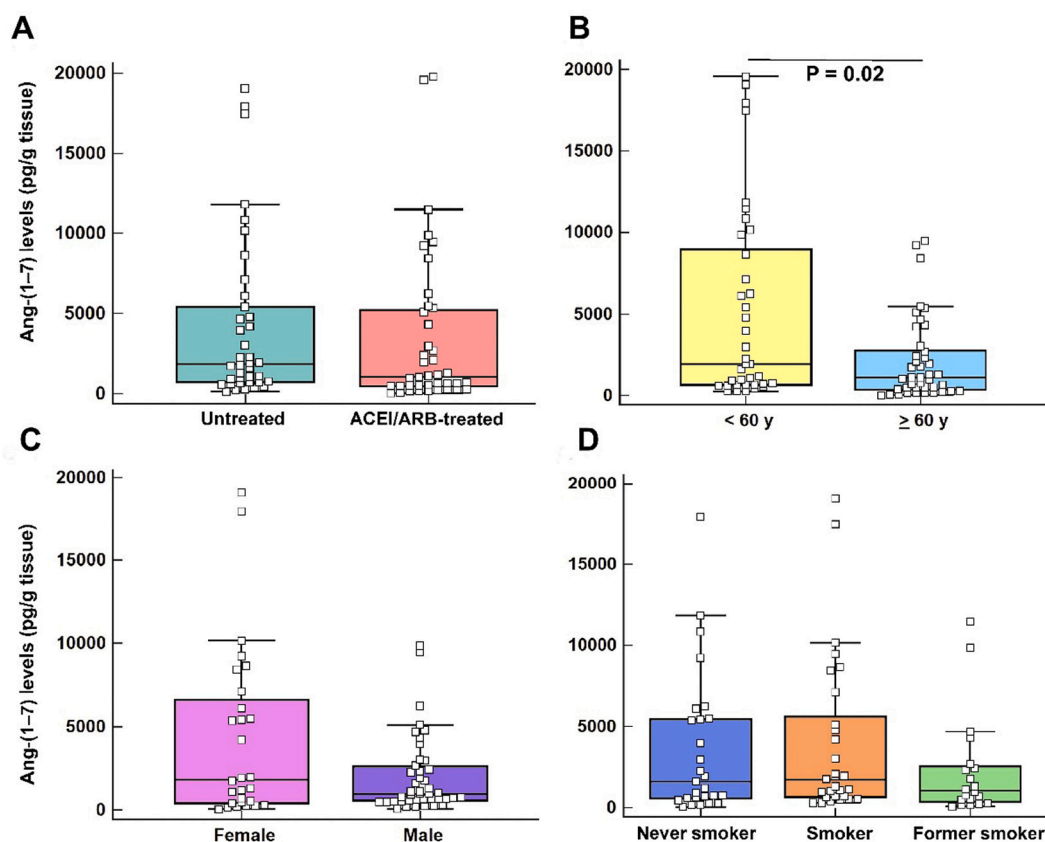


Fig. 2. Ang-(1–7) levels in human lung parenchyma. Ang-(1–7) levels expressed as pg/g tissue in lung parenchyma of (A) untreated ($n = 38$) and ACEI/ARB-treated subjects ($n = 38$); (B) subjects less than 60 years of age (<60 y) ($n = 37$) and subjects 60 years of age and older (≥ 60 y) ($n = 39$); (C) female ($n = 34$) and male ($n = 42$); and (D) subjects never smokers ($n = 26$), smokers ($n = 30$) and former smokers ($n = 20$). The bottom and top of the box plots represent the 25th and 75th percentiles, respectively. The bands within the box show the median value and the whiskers extending from both ends of the boxes are minimum and maximum values. The General Linear Model was applied for statistical analysis.

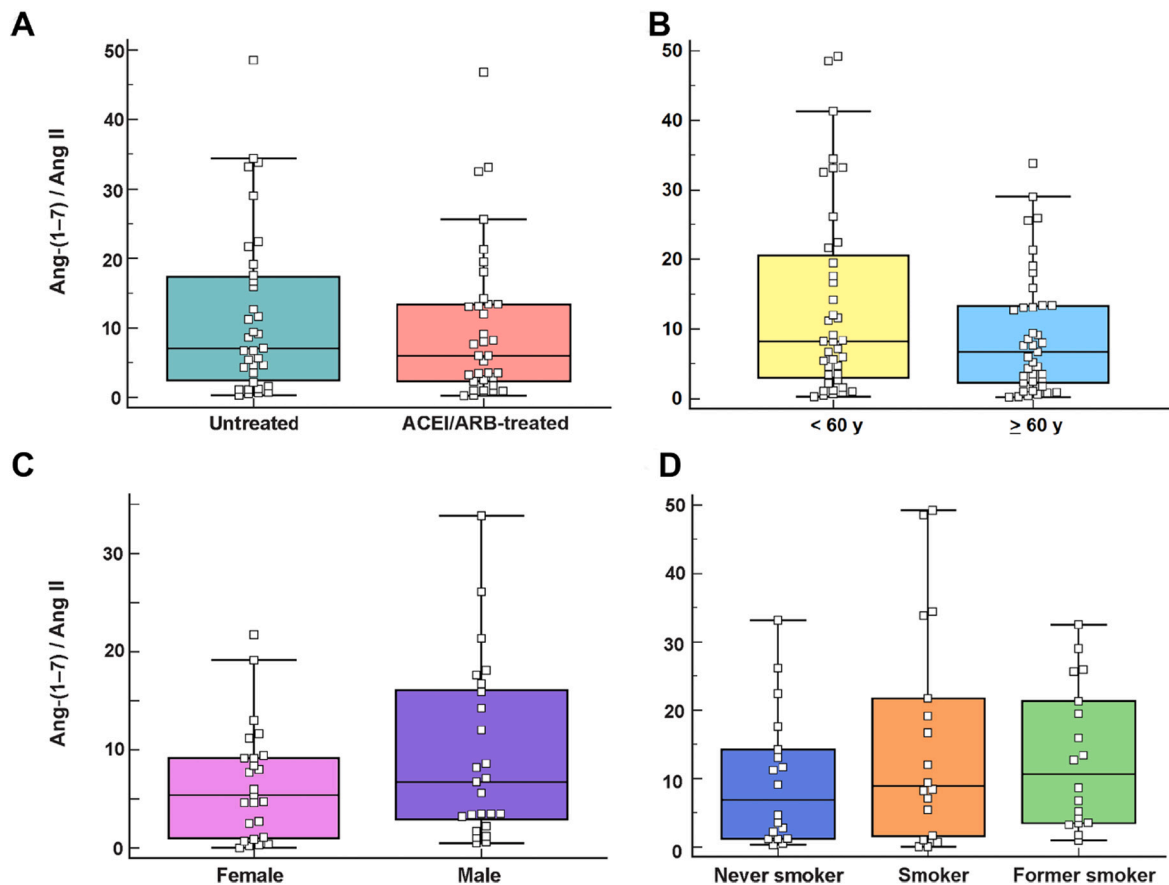


Fig. 3. The conversion of Ang II to Ang-(1-7) was not different between ACEI/ARB-treated and untreated subjects. Ratio between Ang-(1-7) and Ang II levels in lung parenchyma of (A) untreated ($n = 38$) and ACEI/ARB-treated subjects ($n = 38$); (B) subjects less than 60 years of age (<60 y) ($n = 37$) and subjects 60 years of age and older (≥ 60 y) ($n = 39$); (C) female ($n = 34$) and male ($n = 42$); and (D) subjects never smokers ($n = 26$), smokers ($n = 30$) and former smokers ($n = 20$). The bottom and top of the box plots represent the 25th and 75th percentiles, respectively. The band within the box shows the median. The whiskers extending from both ends of the boxes indicate minimum and maximum values. Points outside the box are outliers. The General Linear Model was used for statistical analysis.

age and ACEI/ARB treatment on ACE2 protein content ($P = 0.09$; Fig. 5B).

3.5. *TMPRSS2*-expressing type II pneumocytes are influenced by age and sex

TMPRSS2-expressing type II pneumocytes were significantly lower in females ($P = 0.03$) and older subjects ($P = 0.04$), and though not significant, there was a trend toward increased *TMPRSS2*-expressing type II pneumocyte levels in ACEI/ARB-treated subjects compared to untreated controls ($P = 0.06$; Fig. 6). There was no difference in *TMPRSS2*-expressing type II pneumocytes between never smokers, smokers, and former smokers (Fig. 6).

3.6. *TMPRSS2* protein content is influenced by age, smoking, and RAS blockade

We then evaluated the association between *TMPRSS2* protein content and age, sex, smoking, and ACEI/ARB treatment. We found a significant association of *TMPRSS2* protein content with age and smoking ($P < 0.001$); that is, smokers and subjects 60 years of age and older exhibited higher *TMPRSS2* protein levels (Fig. 7A). Similarly, there was an association of *TMPRSS2* protein levels with age ($P < 0.001$; Fig. 7B) and smoking ($P = 0.04$; Fig. 7C) in ACEI/ARB-treated subjects; the largest proportion of ACEI/ARB-treated subjects exhibiting high *TMPRSS2* protein levels were subjects who were older and smokers.

4. Discussion

We report novel findings in this translational research study: 1) age influences ACE2, *TMPRSS2* and Ang-(1-7) levels, that is, lung Ang-(1-7) and the number of ACE2- and *TMPRSS2*-expressing type II pneumocytes are lower in older subjects; 2) age and smoking habit influence ACE2 and *TMPRSS2* protein content, that is, older smokers exhibits the highest ACE2 and *TMPRSS2* protein content; 3) smoking habits together with RAS blockade marginally influence ACE2, that is, smokers under ACEI/ARB treatment display a slightly enhanced number of ACE2-expressing type II pneumocytes compared to never smokers; and 4) RAS blockade influences *TMPRSS2* protein content, that is, *TMPRSS2* protein content was the highest in older and smokers under ACEI/ARB treatment. Fig. 8 illustrates the most relevant conclusions of our study. To our knowledge, human lung angiotensin levels and the effect of RAS blockade on human lung ACE2 and *TMPRSS2* have not been previously reported.

The nasal cavity is the first site of SARS-CoV-2 infection [26]. Nasal ciliated cells, which express ACE2 at the highest levels [27] are primary targets for SARS-CoV-2 replication. Due to virus infection, innate immunity response is activated. SARS-CoV-2 avoids the innate immune responses associated with type I and type III interferons [27]. Thus, COVID-19 severity and duration result from an early virus evasion of innate immune recognition and lack of timely T cell responses [28]. This maladapted induction of the immune response to the infection leads to the “cytokine storm” [28]. Due to the escape of the virus from the innate immune response, SARS-CoV-2 migrates from the infected epithelial cells of the upper airway to those of the lungs [29]. In the lung, type II

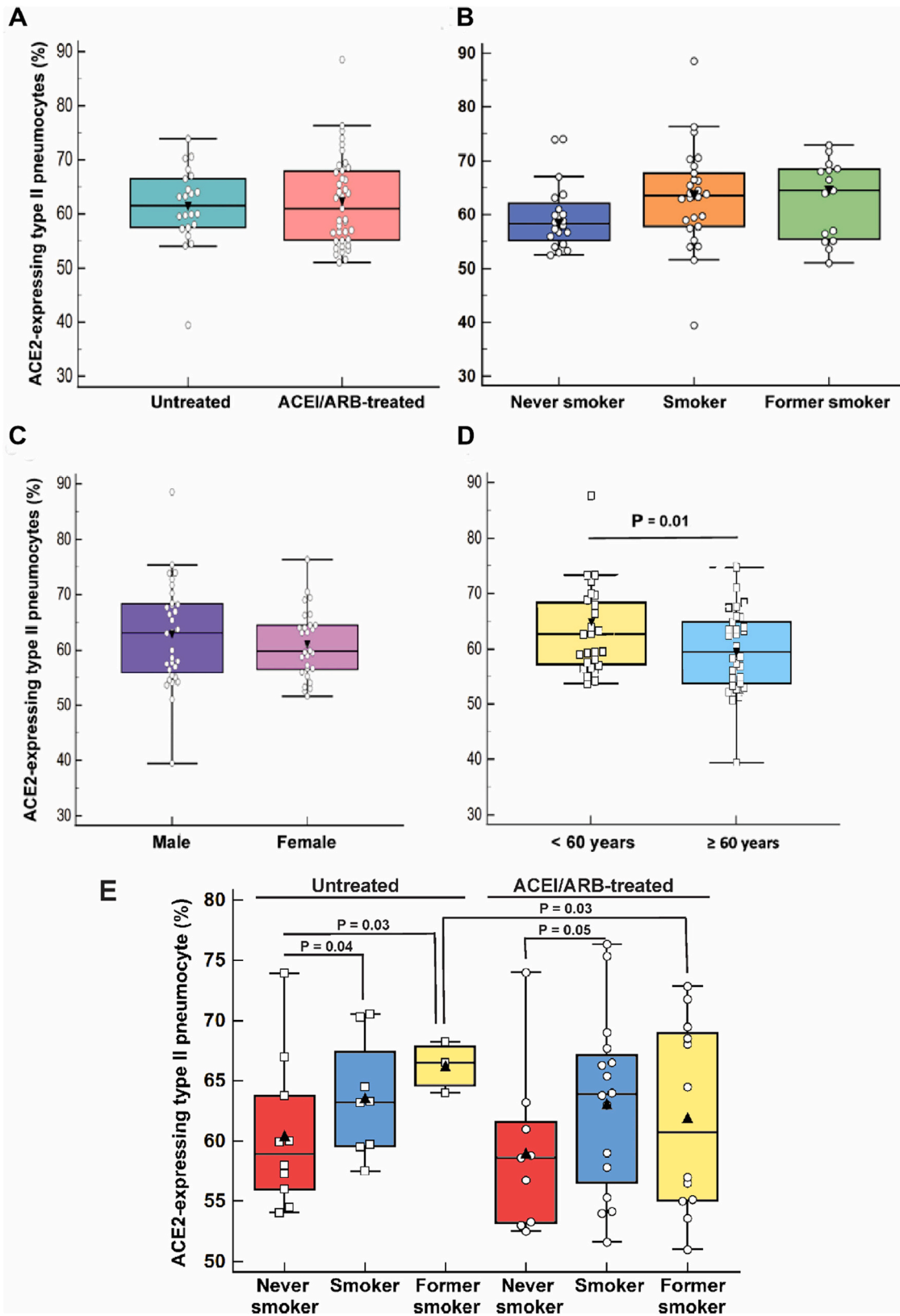


Fig. 4. ACE2-expressing type II pneumocytes are influenced by age, smoking and ACEI/ARB treatment. Percentage of ACE2-expressing type II pneumocytes in the lung parenchyma of (A) untreated ($n = 22$) and ACEI/ARB-treated subjects ($n = 38$); (B) never smokers ($n = 19$), smokers ($n = 24$) and former smokers ($n = 17$), and (C) males ($n = 33$) and females ($n = 27$); (D) subjects less than 60 years of age ($n = 28$) and subjects 60 years of age and older ($n = 32$); and (E) in the lung parenchyma of untreated and ACEI/ARB-treated never smokers ($n = 10$ untreated and 9 ACEI/ARB-treated), smokers ($n = 9$ untreated and 17 ACEI/ARB-treated) and former smokers ($n = 3$ untreated and 12 ACEI/ARB-treated). The bottom and top of the box plots represent the 25th and 75th percentiles, respectively. The band within the box shows the median. The whiskers extending from both ends of the boxes indicate minimum and maximum values. Points outside the box are outliers. The General Linear Model was used for statistical analysis.

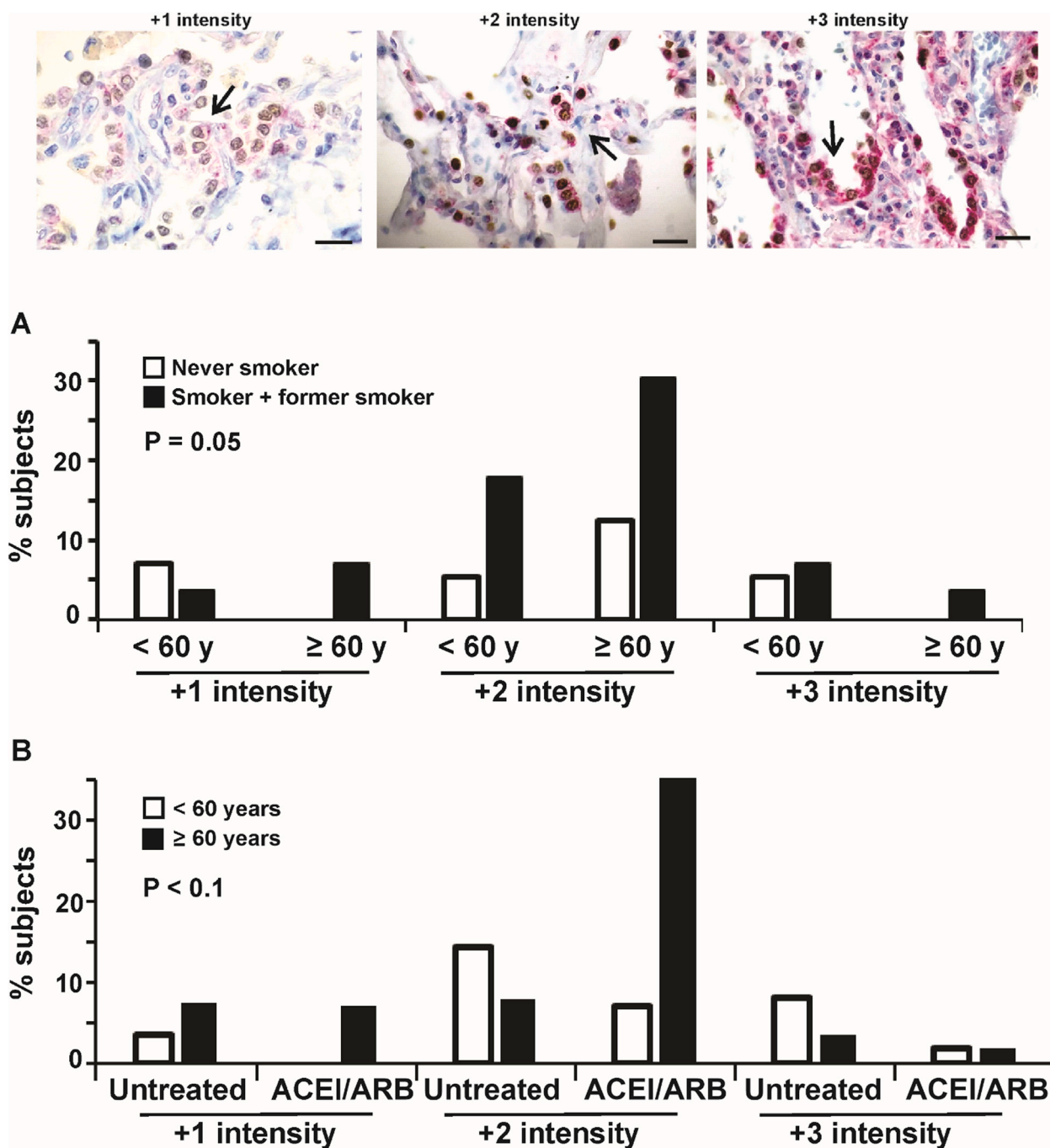


Fig. 5. ACE2 protein content is greater in older smokers. Percentage of subjects with ACE2 immunostaining intensity of +1 to +3 in type II pneumocytes of lung parenchyma of (A) never smokers and smokers plus former smokers stratified by age less than 60 years of age (<60 y) and 60 years of age and older (≥60 y) and (B) untreated and ACEI/ARB-treated (ACEI/ARB) subjects stratified by age (less than 60 years of age (<60 y) and 60 years of age and older (≥60 y)). Representative images of surgically resected lung tissue stained for ACE2 protein (red, as indicated by black arrows) and type II pneumocytes (brown) counterstained with hematoxylin (blue) are presented (scale bar: 20 μm). The stratified Fisher's exact test was applied for statistical analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pneumocytes express high amount of ACE2 [4,7,30]. SARS-CoV-2 enters type II pneumocytes via ACE2 in the respiratory system leading to rapid virus replication as well as to a proinflammatory state with elevated levels of cytokines with the subsequent accumulation of pathogenic inflammatory neutrophils and macrophages in the lung, resulting in an excessive inflammatory and immune response leading to acute respiratory distress syndrome (ARDS), pulmonary edema, and apoptosis of epithelial cells [28]. Recently, it has been shown that SARS-CoV-2 not only replicates in multiciliated cells but also induces their

dedifferentiation leading to loss of mucociliary clearance function of these cells which facilitates viral spread to deeper regions of the bronchial tree until the virus reaches the alveoli and triggers pneumocyte damage [31]. Thus, not only evasion of the immune response by the virus allow infection in type II pneumocytes [28,29] but also SARS-CoV2-induced dedifferentiation in nasal ciliated cells [31] would allow the virus to infect the alveoli and induce ARDS.

Our study showed that human lung Ang II levels are influenced by age but the effect of age on Ang II depends on smoking habits. Chronic

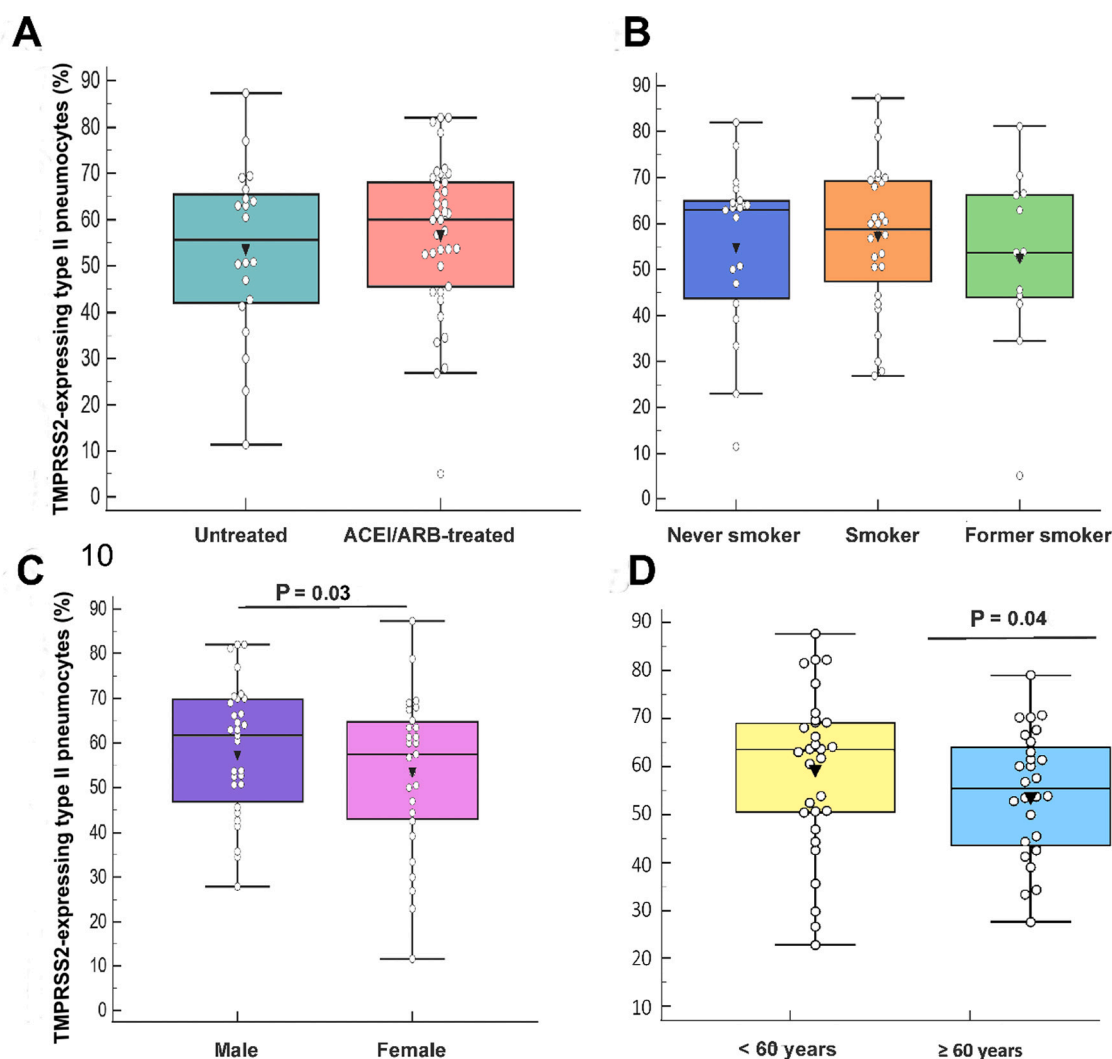


Fig. 6. TMPRSS2-expressing type II pneumocytes are influenced by age and sex. Percentage of TMPRSS2-expressing type II pneumocytes in the lung parenchyma of (A) untreated ($n = 22$) and ACEI/ARB-treated subjects ($n = 38$); (B) never smokers ($n = 19$), smokers ($n = 24$) and former smokers ($n = 17$), and (C) males ($n = 33$) and females ($n = 27$); and (D) subjects less than 60 years of age ($n = 28$) and subjects 60 years of age and older ($n = 32$). 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 black triangle indicates the mean, and the whiskers extending from both ends of the boxes correspond to minimum and maximum values. Points outside the box are outliers. The General Linear Model was used for statistical analysis.

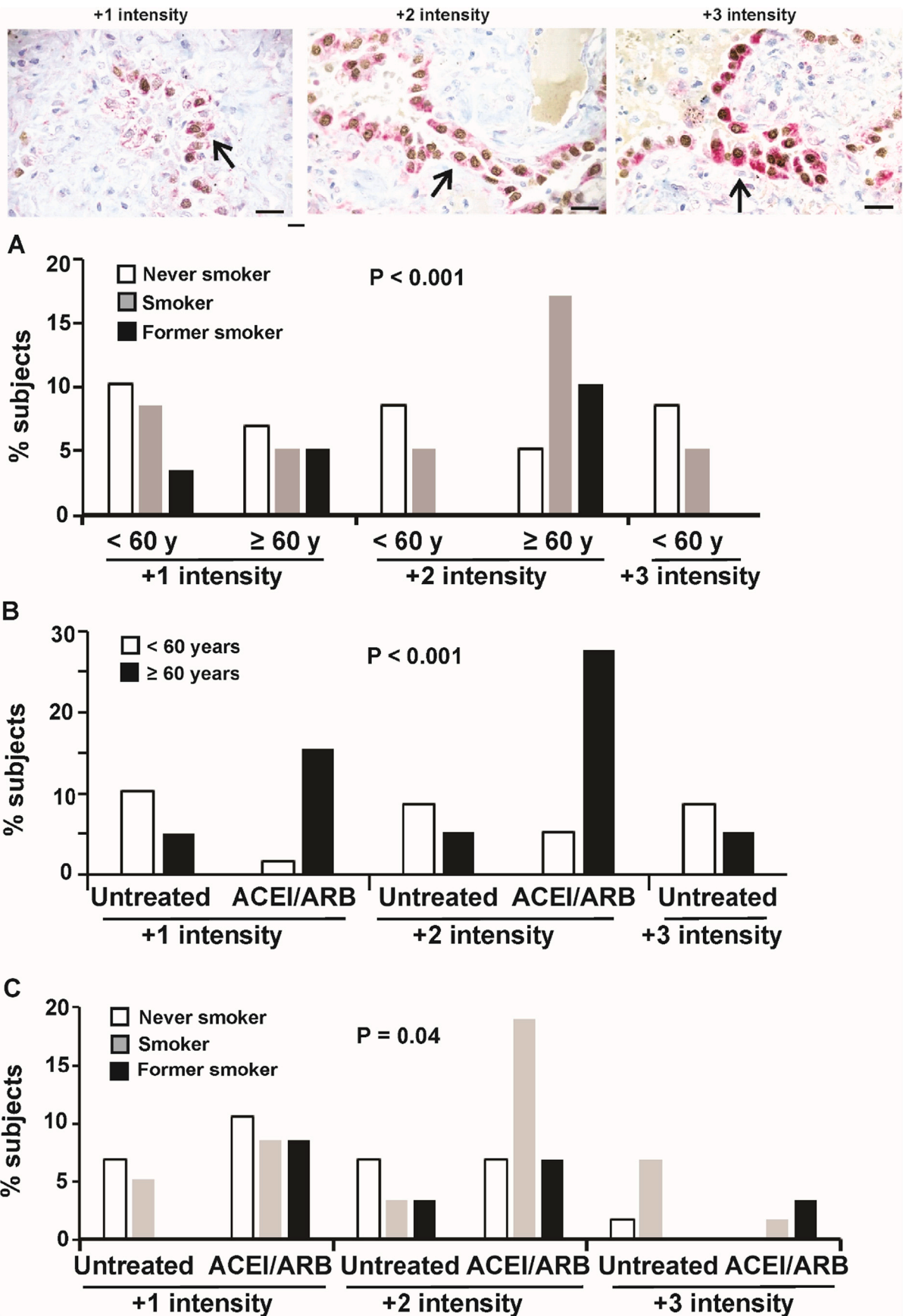
cigarette smoking and aging are associated with an upregulation of the pressor arm and downregulation of the compensatory ACE2/Ang-(1–7)/Mas receptor axis of the RAS [32–34]. This is consistent with the augmented lung Ang II levels and diminished ACE2 expression observed in a chronic cigarette smoke-induced pulmonary arterial hypertension rat model [35,36]. We also found that lung Ang-(1–7) levels and ACE2-expressing type II pneumocytes were lower in older subjects compared to younger subjects, reinforcing the concept of RAS imbalance with aging [33,34]. This finding is in line with the aging related decrease in ACE2 expression in various organs of mice, with the lowest levels occurring in the lungs [37,38].

Telomerase is a ribonucleoprotein complex that add a telomere repeat sequence to the 3' end of telomeres on chromosomal ends, which are protective structures for chromosome stabilization. Decreased telomerase is associated with type II pneumocytes apoptosis [39]. Selective telomerase deficiency in type II alveolar epithelial cell diminished their proliferation and induced cellular senescence [40] and alveolar stem cell failure [41]. Older patients usually present telomerase deficiency in type 2 pneumocytes [42] and this fact may influence in part the lower percentage of ACE2-expressing type II pneumocytes in older people

observed in our study.

We found that older smokers and former smokers comprised the largest percentage of subjects exhibiting higher ACE2 protein content. In agreement, higher ACE2 expression in smokers compared to never smokers have been reported [17,43–45]. In contrast, other reports have shown that current smokers and never smokers have similar levels of bronchial epithelial cell mRNA ACE2 [46] and ACE2 protein in both bronchial and alveolar epithelial cells [47] as well as nasal ciliary cells [9]. The difference may be due to the area of the respiratory tract investigated.

Regarding RAS blockade and ACE2 expression we found that smokers under RAS blockade exhibited a slightly enhanced amount of ACE2-expressing type II pneumocytes compared to never smokers. Supporting our results, recently it has been shown that ARBs increased SARS-CoV-2 replication in Vero E6 cells that correlated with the ARBs-induced up-regulation of ACE2 expression [45]. In contrast, in a study by Lee et al. [8] using a small sample size of patients taking ACEIs, upper respiratory tract ciliary ACE2 expression was slightly decreased compared to matched controls. The differences in findings may be due to Lee et al. [8] studying the ciliary cells of the upper respiratory tract



(caption on next page)

Fig. 7. TMPRSS2 protein content is higher in older smokers, older subjects, and smokers under RAS blockade. Percentage of subjects with TMPRSS2 immunostaining intensity of +1, +2, and +3 in type II pneumocytes of lung parenchyma of (A) never smokers, smokers, and former smokers under 60 years of age (< 60 y) and 60 years of age and older (≥ 60 y); (B) untreated and ACEI/ARB-treated (ACEI/ARB) subjects under 60 years of age (<60 y) and 60 years of age and older (≥ 60 y); and (C) untreated and ACEI/ARB-treated (ACEI/ARB) never smokers, smokers, and former smokers. TMPRSS2 intensity was classified from +1 to +3. Representative images of surgically resected lung tissue stained for TMPRSS2 protein (red, as indicated by black arrows) and type II pneumocytes (brown) counterstained with hematoxylin (blue) are presented (scale bar: 20 μ m). TMPRSS2 immunostaining intensity of +3 was not detected in never smokers, smokers, or former smokers 60 years of age and older or in untreated or ACEI/ARB-treated subjects. The stratified Fisher's exact test was applied to verify the associations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

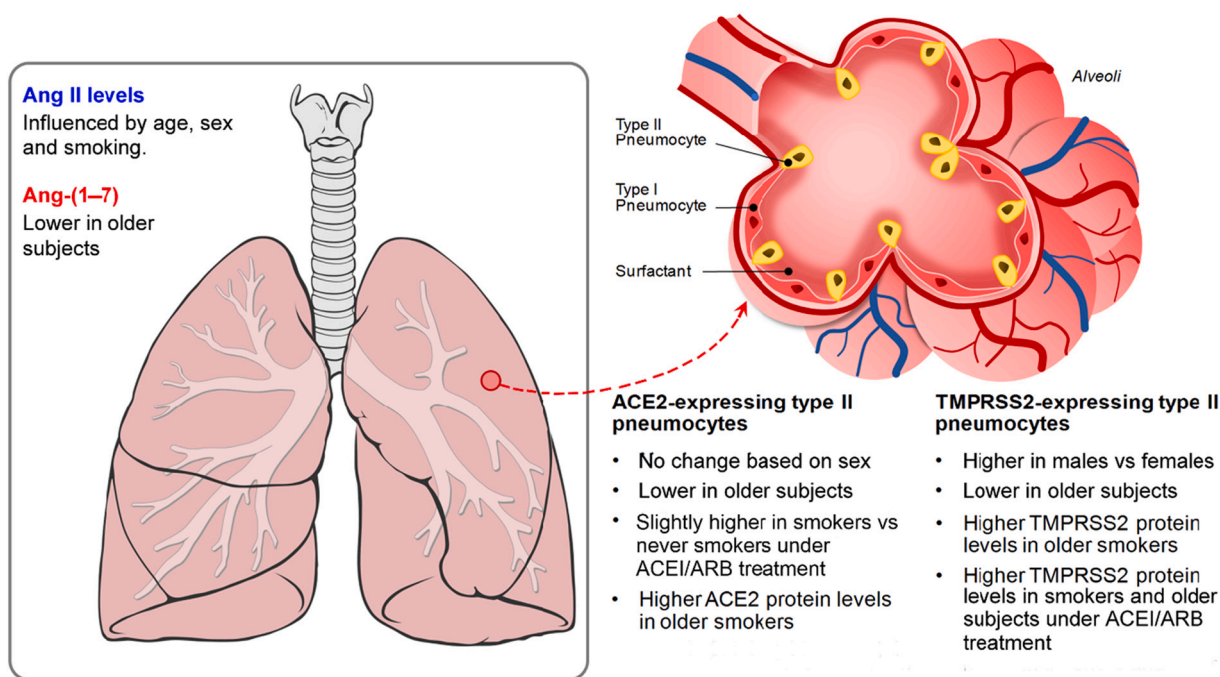


Fig. 8. Scheme highlighting the conclusions drawn from the present work.

while our study investigated the type II pneumocytes of the alveoli; the upper respiratory tract is an area that lacks type II pneumocytes [48]. Pneumocytes together with alveolar macrophages are essential to the maintenance of lung homeostasis [6].

TMPRSS2 is a key protein in SARS-CoV-2 entry [5]. We observed that TMPRSS2-expressing type II pneumocytes decreased with age, which is in agreement with other recent data showing a weak decreasing age-related trend in TMPRSS2 expression in human lungs [49]. In contrast, increased TMPRSS2 expression with aging has been reported in both mice and humans [50]. The difference between these findings and ours may be related to the age range and the cell type investigated (type I versus type II pneumocytes). We also observed that TMPRSS2-expressing type II pneumocytes were higher in men than women. In contrast, other studies have identified no statistically significant differences in TMPRSS2 expression in lung tissues when stratifying for sex [49]. Again, these differences may be due to the type of sample under investigation.

Regarding TMPRSS2 protein content in the type II pneumocytes of ACEI/ARB-treated subjects, we found that subjects 60 years of age and older and subjects who were smokers made up the largest percentage of people with higher protein content; thus, RAS blockade together with aging and smoking influence TMPRSS2 protein content. In contrast, it has been shown that RAS inhibition did not affect mRNA abundance of ACE2 or TMPRSS2 in male C57BL/6 J mice administered ACEI or ARB [51]. Differences with our results may be due to differences in species (mouse versus human) and the sample investigated (whole lung versus type II pneumocytes). In addition, the mouse model did not include exposure to smoke. Evidence shows that smoking increases TMPRSS2 [52,53], which is in accordance with our results.

Our study was 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 subject population or the usual quantity of cigarette consumption in the smokers subject population. Another limitation was the lack of lung samples from uncontrolled hypertensive subjects and from never smoker older subjects. Because the majority of pulmonary specimens from patients treated with antihypertensive drugs included in the study had some degree of inflammation, such as a history of smoking, chronic obstructive pulmonary disease, diabetes or lung cancer which are known to induce inflammatory responses, alterations in ACE2 may also be influenced by the inflammatory arena. We also did not consider the dosage of ACEIs or ARBs prescribed, the type of ACEI or ARB, and dosing schedules for each of the tested agents or the pathology causing the subjects to undergo lung surgery. Thus, though we employed healthy tissue we could not disregard the impact of suspected lung cancer in those changes observed in the present study. Several studies have shown an association between cancer progression and ACE2, although conflicting results have been reported. ACE2 influences tumors progression and metastasis via inhibiting tumor cell proliferation, invasion, migration, and angiogenesis [54]. ACE2 upregulation was associated with favorable survival in cancer suggesting a potential protective role for ACE2 in cancer progression [55–57]. In addition, ACE2 expression and cancer prognosis was correlated with immune cell infiltration [58]. ACE2 was not highly expressed in lung cancer cells [57,59]. RNA-sequencing showed that ACE2 and TMPRSS2 RNA were expressed at higher levels in human colorectal tumor and normal tissue samples than in human tumor or normal tissue samples of lung, esophagus, stomach, and liver [60]. In contrast, through computational studies it has been reported that ACE2 gene is up-regulated in

lung cancer which may lead to lung carcinoma progression [61], suggesting that the high expression of ACE2 may lead to higher susceptibility of lung cancer patients toward COVID-19. Thus, ACE2 may display both positive and negative roles in cancer progression which seem to depend on the cancer type [54].

5. Conclusions

In conclusion, we found that ACE2 and TMPRSS2 in type II pneumocytes are somehow influenced by smoking, age, and ACEI/ARB treatment. This finding may *help improve our understanding of* the increased susceptibility to COVID-19 in older and smokers subjects with treated cardiovascular-related pathologies. Lung samples from people who died from COVID-19 would be necessary to confirm our results. Unfortunately we do not have access to those samples.

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CRediT authorship contribution statement

Mauro G. Silva: Data curation, Methodology, Investigation, Validation. **Nora L. Falcoff:** Data curation, Methodology, Investigation, Visualization. **Gerardo R. Corradi:** Data curation, Methodology, Investigation. **José Alfie:** Writing – review & editing, Data curation. **Rolando F. Seguel:** Resources. **Gabriela C. Tabaj:** Resources. **Laura I. Iglesias:** Methodology. **Myriam Nuñez:** Formal analysis. **Gabriela R. Guman:** Data curation, Methodology, Investigation. **Mariela M. Gironacci:** Conceptualization, Methodology, Supervision, Project administration, Visualization, Funding acquisition, Writing – original draft.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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