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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. In addition to its enzymatic function counterbalancing the pressor arm of the renin-angiotensin system (RAS), angiotensin-converting enzyme (ACE) 2 acts as the receptor for SARS-CoV-2. Viral fusion and cellular entry require ACE2 and the transmembrane protease serine 2 (TMPRSS2). Some evidence in tissue animals showed that RAS blockade by either ACE inhibitors (ACEIs) or type 1 angiotensin receptor blockers (ARBs) influence ACE2, though evidence in human lungs is lacking. Our aim was to evaluate ACE2 and TMPRSS2 in type II pneumocytes, the key cells that maintain lung homeostasis, present in lung parenchymal of ACEI/ARB-treated subjects compared to untreated control subjects.

Methods and Results. ACE2 and TMPRSS2 protein expression was evaluated by immunohistochemistry. The percentage of ACE2-expressing type II pneumocytes were not different between male and female. We found a significant interaction between ACEI/ARB treatment and smoking on ACE2-expressing type II pneumocytes ($P = 0.026$). Subjects with a positive history of smoking and ACEI/ARB treatment contained higher number of ACE2-expressing type II pneumocytes. Furthermore, ACE2 protein content correlated positively with smoking habits and age ($P = 0.05$). On the other hand, the percentage of TMPRSS2-expressing type II pneumocytes were higher in males than females ($P = 0.026$) and in subjects under 60 years of age ($P = 0.04$) and not significantly influenced by ACEI/ARB treatment ($p=0.06$). There was a positive association of TMPRSS2 protein content with age ($P = 0.001$) and smoking ($P = 0.039$) in ACEI/ARB-treated subjects with high TMPRSS2 protein levels most evident in ACEI/ARB-treated older adults and smokers.

Conclusions. We conclude that ACEI/ARB treatment influences human lung ACE2 and TMPRSS2 but this effect depends on the age and smoking habits of the subject.

Keywords: angiotensin-converting enzyme 2; TMPRSS2; angiotensin; hypertension; type II pneumocytes; lung

Translational Perspective:

We found that subjects with a positive history of smoking and ACEI/ARB treatment contained higher number of ACE2-expressing type II pneumocytes. Furthermore, ACE2 protein content correlated positively with smoking habits and age. On the other hand, there was a positive association of TMPRSS2 protein content with age and smoking in ACEI/ARB-treated subjects. This finding may help explain the increased susceptibility to COVID-19 in smokers subjects 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 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^{4,7-9} and protects against acute lung injury in several animal models of acute respiratory distress syndrome.^{10,11} 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.^{10,11}

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.¹² Regarding human tissues, RAS blockers have not been associated with ACE2 expression in human kidney,¹³ 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 intestine¹⁴ and cardiomyocytes of subjects treated with ACEIs showed a significantly higher ACE2 expression.¹⁵ 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^{16–19} and randomized clinical trials investigating the impact of discontinuation of RAS blockers on patients hospitalized with COVID-19 showed inconsistent results.^{19,20} However, to date, there is no experimental evidence on RAS blockade and ACE2 expression in human lungs. In this study, we investigated for the first time 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. Methods

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

2.1. Human Lung Samples

This study was an observational retrospective study of de-identified material, thus informed consent was not required. 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 and conformed to National Institutes of Health guidelines. 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 unified archives of 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. 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.

2.2. Ang II and Ang-(1–7) levels

Angiotensin levels were measured by radioimmunoassay as previously described.^{21,22} 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 angiotensins extraction and recovery was performed as previously described.²² Each sample was corrected for each recovery. Angiotensin levels were quantified by radioimmunoassay using angiotensins labelled in our laboratory as previously described.²³

Radioimmunoassay for Ang-(1–7) has been previously validated.²³ 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.

2.3. 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 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. 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. Percentage of ACE2- or TMPRSS2-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). 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.

2.4. Statistical Analysis

All analyses were conducted using the IBM SPSS Statistics 19 software. 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). 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. The stratified Fisher's exact test was used to investigate the association between variables. $P < 0.05$ was considered statistically significant. A $P \geq 0.05$ and ≤ 0.1 indicated a trend, suggesting that the sample size must be increased to reach significance.

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. 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 (Figure 1). However, we found a significant interaction between age and smoking ($P = 0.001$) and age and sex ($P = 0.013$) 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 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. Figure S1 showed Ang II levels in the lung parenchyma of the investigated population.

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 ($P = 0.018$; Figure 2). No differences in Ang-(1–7) levels were found when data were stratified by ACEI/ARB treatment, sex or smoking habits (Figure 2). However, an interaction between age and RAS-blocking treatment ($P = 0.042$) 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 also detected an interaction between age and smoking on Ang-(1–7) levels ($P = 0.068$), which, though not statistically significant, indicated that there was a trend of both factors influencing Ang-(1–7) levels. Figure S2 showed Ang-(1–7) levels in the lung parenchyma of the investigated population.

We observed no difference in ratios of Ang-(1–7) to Ang II between the investigated groups (Figure S3).

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 homeostasis⁶ 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 (Figure 3A). There were no significant differences in ACE2-expressing type II pneumocytes between never smokers, former smokers, and smokers (Figure 3B) or male and female subjects (Figure 3C). 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 , $P = 0.012$; Figure 3D). 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 ($P = 0.026$). 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. For example, subjects who were smokers and undergoing RAS-blocking treatment exhibited higher percentages of ACE2-expressing type II pneumocytes than untreated subjects. Within the group of ACEI/ARB-treated subjects, ACE2-expressing type II pneumocytes were higher in smokers compared to never smokers (Figure 3). Altogether, these data suggest that smoking and RAS-blocking treatment enhances levels of ACE2-expressing type II pneumocytes.

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 (Figure 4A).

We next evaluated whether RAS blockade and age were associated with ACE2 protein levels. Though not significant, there was a trend in older ACEI/ARB-treated subjects to have highest ACE2 protein content ($P = 0.09$; Figure 4B).

3.5. TMPRSS2-Expressing Type II Pneumocytes are Influenced by Age and Sex

TMPRSS2-expressing type II pneumocytes were significantly lower in females ($P = 0.026$) and older subjects ($P = 0.04$), and though not significant, there was a trend towards increased TMPRSS2-expressing type II pneumocyte levels in ACEI/ARB-treated subjects compared to untreated controls ($P = 0.06$; Figure 5). There was no difference in TMPRSS2-expressing type II pneumocytes between never smokers, smokers, and former smokers (Figure 5).

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 (Figure 6A). Similarly, there was an association of TMPRSS2 protein levels with age ($P = 0.001$; Figure 6B) and smoking ($P = 0.039$; Figure 6C) 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 regarding 1) the influence of age, sex, and smoking on lung Ang II levels; 2) the influence of age on Ang-(1–7) levels; 3) the influence of age, smoking, and ACEI/ARB treatment on human ACE2-expressing type II pneumocytes; 4) the enhancement of ACE2 protein content due to age and smoking; 5) the influence of age and sex on human TMPRSS2-expressing type II pneumocytes; and 6) the enhancement of TMPRSS2 protein content by RAS blockade in older and smoking subjects. Figure 7 illustrates the most relevant conclusions of our study. To our knowledge, human lung angiotensin levels and the effect of RAS blockade on ACE2 and TMPRSS2 have not been previously reported.

Our study demonstrated that the influence of age on Ang II depends on sex and smoking. Chronic 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.^{24–26} 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.^{27,28} 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.^{25,26} 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.^{29,30}

We observed that smoking and RAS blockade influence human ACE2-expressing type II pneumocytes. Although the number of ACE2-expressing type II pneumocytes were similar between untreated never smokers and smokers, they were enhanced in smokers under RAS

blockade. Furthermore, we found that older smokers and former smokers comprised the largest percentage of subjects exhibiting higher ACE2 protein content. Some reports have shown that current smokers and never smokers have similar levels of bronchial epithelial cell mRNA ACE2³¹ and ACE2 protein in both bronchial and alveolar epithelial cells³² as well as nasal ciliary cells.¹⁰ In contrast, others have reported higher ACE2 expression in smokers compared to never smokers.^{18,33,34} 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 sex.³⁵ In contrast, Smith et al. showed that lung ACE2 levels do not vary by age or sex, though smokers exhibit upregulated ACE2.¹⁸

Regarding RAS blockade and ACE2 expression, 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. In contrast, we found that smokers under RAS blockade exhibited enhanced ACE2-expressing type II pneumocytes. The differences in findings may be due to Lee et al.¹⁰ studying the ciliary cells of the upper respiratory tract while our study investigated the type II pneumocytes of the alveoli; the upper respiratory tract is an area that lacks type II pneumocytes.³⁶ Pneumocytes together with alveolar macrophages are essential to the maintenance of lung homeostasis.⁷ We also found a trend of subjects 60 years of age and older under ACEI/ARB treatment exhibiting higher ACE2 expression in type II pneumocytes compared to younger subjects. The increased ACE2 expression in type II pneumocytes would be a protective mechanism due to Ang II downregulation.

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

275 decreasing age-related trend in TMPRSS2 expression in human lungs.³⁷ In contrast, increased
276 TMPRSS2 expression with aging has been reported in both mice and humans.³⁸ The difference
277 between these findings and ours may be related to the age range and the cell type investigated (type
278 I versus type II pneumocytes). We also observed that TMPRSS2-expressing type II pneumocytes
279 were higher in men than women. In contrast, other studies have identified no statistically significant
280 differences in TMPRSS2 expression in lung tissues when stratifying for gender.³⁷ Again, these
281 differences may be due to the type of sample under investigation.

282 Regarding TMPRSS2 protein content in the type II pneumocytes of ACEI/ARB-treated subjects, we
283 found that subjects 60 years of age and older and subjects who were smokers made up the largest
284 percentage of people with higher protein content; thus, RAS blockade together with aging and
285 smoking influence TMPRSS2 protein content. In contrast, it has been shown that RAS inhibition
286 did not affect mRNA abundance of ACE2 or TMPRSS2 in male C57BL/6J mice administered
287 ACEI or ARB.³⁹ Differences with our results may be due to differences in species (rat versus
288 human) and the sample investigated (whole lung versus type II pneumocytes). In addition, the
289 mouse model did not include exposure to smoke. Evidence shows that smoking increases
290 TMPRSS2^{40,41}, which is in accordance with our results.

291 Our study was limited by a retrospective design, which is vulnerable to uncontrolled biases. We
292 were unable to assess the impact of time since smoking cessation in the former smokers subject
293 population or the usual quantity of cigarette consumption in the smokers subject population.

294 Another limitation was the lack of lung samples from uncontrolled hypertensive subjects and from
295 never smoker older subjects. We also did not consider the dosage of ACEIs or ARBs prescribed or
296 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.

In conclusion, we found that ACE2 and TMPRSS2 in type II pneumocytes are influenced by smoking, age and ACEI/ARB treatment. Theoretically, higher ACE2 protein expression may facilitate SARS-CoV2 entry but on the other hand, it would have a protective effect on alveolar function. This finding may help explain the increased susceptibility to COVID-19 in smokers subjects with treated cardiovascular-related pathologies, though this is only a hypothesis. Lung samples from people who died from COVID-19 would be necessary to test this hypothesis.

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7. Conflict of Interest

None declared.

8. References

1. Santos RAS, Sampaio WO, Alzamora AC, Motta-Santos D, Alenina N, Bader M, Campagnole-Santos MJ. The ACE2/Angiotensin-(1-7)/MAS Axis of the Renin-Angiotensin System: Focus on Angiotensin-(1-7). *Physiol Rev* 2018;98:505–553.

DOI:10.1152/physrev.00023.2016

2. Forrester SJ, Booz GW, Sigmund CD, Coffman TM, Kawai T, Rizzo V, Scalia R, Eguchi S. Angiotensin II Signal Transduction: An Update on Mechanisms of Physiology and Pathophysiology. *Physiol Rev* 2018;98:1627–1738. DOI:10.1152/physrev.00038.2017
3. Gheblawi M, Wang K, Viveiros A, Nguyen Q, Zhong JC, Turner AJ, Raizada MK, Grant MB, Oudit GY. Angiotensin-Converting Enzyme 2: SARS-CoV-2 Receptor and Regulator of the Renin-Angiotensin System: Celebrating the 20th Anniversary of the Discovery of ACE2. *Circ. Res.* Lippincott Williams and Wilkins; 2020. p. 1456–1474.
4. Zou X, Chen K, Zou J, Han P, Hao J, Han Z. Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. *Front Med* Higher Education Press; 2020;14:185–192. DOI:10.1007/s11684-020-0754-0
5. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* Cell Press; 2020;181:271-280.e8. DOI:10.1016/j.cell.2020.02.052
6. Bissonnette EY, Lauzon-Joset JF, Debley JS, Ziegler SF. Cross-Talk Between Alveolar Macrophages and Lung Epithelial Cells is Essential to Maintain Lung Homeostasis. *Front. Immunol.* Frontiers Media S.A.; 2020.
7. Zhao Y, Zhao Z, Wang Y, Zhou Y, Ma Y, Zuo W. Single-Cell RNA Expression Profiling of ACE2, the Receptor of SARS-CoV-2. *Am. J. Respir. Crit. Care Med.* American Thoracic Society; 2020. p. 756–759.

8. Lee IT, Nakayama T, Wu CT, Goltsev Y, Jiang S, Gall PA, Liao CK, Shih LC, Schürch CM, McIlwain DR, Chu P, Borchard NA, Zarabanda D, Dholakia SS, Yang A, Kim D, Chen H, Kanie T, Lin C Der, Tsai MH, Phillips KM, Kim R, Overdevest JB, Tyler MA, Yan CH, Lin CF, Lin YT, Bau DT, Tsay GJ, Patel ZM, et al. ACE2 localizes to the respiratory cilia and is not increased by ACE inhibitors or ARBs. *Nat Commun Nature Research*; 2020;11. DOI:10.1038/s41467-020-19145-6
9. Ziegler CGK, Allon SJ, Nyquist SK, Mbano IM, Miao VN, Tzouanas CN, Cao Y, Yousif AS, Bals J, Hauser BM, Feldman J, Muus C, Wadsworth MH, Kazer SW, Hughes TK, Doran B, Gatter GJ, Vukovic M, Taliaferro F, Mead BE, Guo Z, Wang JP, Gras D, Plaisant M, Ansari M, Angelidis I, Adler H, Sucre JMS, Taylor CJ, Lin B, et al. SARS-CoV-2 Receptor ACE2 Is an Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is Detected in Specific Cell Subsets across Tissues. *Cell* Cell Press; 2020;181:1016-1035.e19. DOI:10.1016/j.cell.2020.04.035
10. Kuba K, Imai Y, Penninger JM. Angiotensin-converting enzyme 2 in lung diseases. *Curr. Opin. Pharmacol. Curr Opin Pharmacol*; 2006. p. 271–276.
11. Ferrario CM, Ahmad S, Groban L. Twenty years of progress in angiotensin converting enzyme 2 and its link to SARS-CoV-2 disease. *Clin. Sci. Portland Press Ltd*; 2020. p. 2645–2664.
12. Ferrario CM, Ahmad S, Groban L. Mechanisms by which angiotensin-receptor blockers increase ACE2 levels. *Nat. Rev. Cardiol. Nature Research*; 2020. p. 378.
13. Jiang X, Eales JM, Scannali D, Nazgiewicz A, Prestes P, Maier M, Denniff M, Xu X, Saluja S, Cano-Gamez E, Wystrychowski W, Szulinska M, Antczak A, Byars S, Skrypnik

- D, Glyda M, Król R, Zywiec J, Zukowska-Szczechowska E, Burrell LM, Woolf AS, Greenstein A, Bogdanski P, Keavney B, Morris AP, Heagerty A, Williams B, Harrap SB, Trynka G, Samani NJ, et al. Hypertension and renin-angiotensin system blockers are not associated with expression of angiotensin-converting enzyme 2 (ACE2) in the kidney. *Eur Heart J* Oxford University Press (OUP); 2020; DOI:10.1093/eurheartj/ehaa794
14. Vuille-Dit-Bille RN, Camargo SM, Emmenegger L, Sasse T, Kummer E, Jando J, Hamie QM, Meier CF, Hunziker S, Forras-Kaufmann Z, Kuyumcu S, Fox M, Schwizer W, Fried M, Lindenmeyer M, Götze O, Verrey F. Human intestine luminal ACE2 and amino acid transporter expression increased by ACE-inhibitors. *Amino Acids* Springer-Verlag Wien; 2015;47:693–705. DOI:10.1007/s00726-014-1889-6
15. Nicin L, Abplanalp WT, Mellentin H, Kattih B, Tombor L, John D, Schmitto JD, Heineke J, Emrich F, Arsalan M, Holubec T, Walther T, Zeiher AM, Dimmeler S. Cell type-specific expression of the putative SARS-CoV-2 receptor ACE2 in human hearts. *Eur. Heart J.* Oxford University Press; 2020. p. 1804–1806.
16. Pirola CJ, Sookoian S. Estimation of Renin-Angiotensin-Aldosterone-System (RAAS)-Inhibitor effect on COVID-19 outcome: A Meta-analysis. *J Infect* W.B. Saunders Ltd; 2020;81:276–281. DOI:10.1016/j.jinf.2020.05.052
17. Morales DR, Conover MM, You SC, Pratt N, Kostka K, Duarte-Salles T, Fernández-Bertolín S, Aragón M, DuVall SL, Lynch K, Falconer T, Bochove K van, Sung C, Matheny ME, Lambert CG, Nyberg F, Alshammari TM, Williams AE, Park RW, Weaver J, Sena AG, Schuemie MJ, Rijnbeek PR, Williams RD, Lane JCE, Prats-Urbe A, Zhang L, Areia C, Krumholz HM, Prieto-Alhambra D, et al. Renin–angiotensin system blockers

and susceptibility to COVID-19: an international, open science, cohort analysis. *Lancet Digit Heal* Lancet Digit Health; 2020; DOI:10.1016/S2589-7500(20)30289-2

18. Smith JC, Sausville EL, Girish V, Yuan M Lou, Vasudevan A, John KM, Sheltzer JM. Cigarette Smoke Exposure and Inflammatory Signaling Increase the Expression of the SARS-CoV-2 Receptor ACE2 in the Respiratory Tract. *Dev. Cell*. Cell Press; 2020. p. 514-529.e3.
19. Singh S, Offringa-Hup AK, Logtenberg SJJ, Linden PD Van der, Janssen WMT, Klein H, Waanders F, Simsek S, Jager CPC de, Smits P, Feltz M van der, Jan Beumer G, Widrich C, Nap M, Pinto-Sietsma S-J. Discontinuation of Antihypertensive Medications on the Outcome of Hospitalized Patients With Severe Acute Respiratory Syndrome-Coronavirus 2. *Hypertension* Lippincott Williams & Wilkins Hagerstown, MD; 2021;78:165–173. DOI:10.1161/HYPERTENSIONAHA.121.17328
20. Lopes RD, Macedo AVS, Barros E Silva PGM De, Moll-Bernardes RJ, Santos TM Dos, Mazza L, Feldman A, D'Andréa Saba Arruda G, Albuquerque DC De, Camiletti AS, Sousa AS De, Paula TC De, Giusti KGD, Domiciano RAM, Noya-Rabelo MM, Hamilton AM, Loures VA, Dionísio RM, Furquim TAB, Luca FA De, Santos Sousa ÍB Dos, Bandeira BS, Zukowski CN, Oliveira RGG De, Ribeiro NB, Moraes JL De, Petriz JLF, Pimentel AM, Miranda JS, Jesus Abufaiad BE De, et al. Effect of Discontinuing vs Continuing Angiotensin-Converting Enzyme Inhibitors and Angiotensin II Receptor Blockers on Days Alive and out of the Hospital in Patients Admitted with COVID-19: A Randomized Clinical Trial. *JAMA - J Am Med Assoc* American Medical Association; 2021;325:254–264. DOI:10.1001/jama.2020.25864

- 408 21. Gironacci MM, Brosnihan KB, Ferrario CM, Gorzalczany S, Verrilli MAL, Pascual M,
409 Taira C, Peña C. Increased hypothalamic angiotensin-(1-7) levels in rats with aortic
410 coarctation-induced hypertension. *Peptides* Peptides; 2007;28:1580–1585.
411 DOI:10.1016/j.peptides.2007.06.016
- 412 22. Brosnihan KB, Chappell MC. Measurement of angiotensin peptides: HPLC-RIA. *Methods*
413 *in Molecular Biology* Humana Press Inc.; 2017. p. 81–99.
- 414 23. Lopez Verrilli MA, Pirola CJ, Pascual MM, Dominici FP, Turyn D, Gironacci MM.
415 Angiotensin-(1-7) through AT2 receptors mediates tyrosine hydroxylase degradation via
416 the ubiquitin-proteasome pathway. *J Neurochem* J Neurochem; 2009;109:326–335.
417 DOI:10.1111/j.1471-4159.2009.05912.x
- 418 24. Oakes JM, Fuchs RM, Gardner JD, Lazartigues E, Yue X. Nicotine and the renin-
419 angiotensin system. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* American
420 Physiological Society; 2018. p. R895–R906.
- 421 25. Yoon HE, Kim EN, Kim MY, Lim JH, Jang IA, Ban TH, Shin SJ, Park CW, Chang YS,
422 Choi BS. Age-Associated Changes in the Vascular Renin-Angiotensin System in Mice.
423 *Oxid Med Cell Longev* Hindawi Limited; 2016;2016. DOI:10.1155/2016/6731093
- 424 26. Conti S, Cassis P, Benigni A. Aging and the renin-angiotensin system. *Hypertension*
425 Lippincott Williams & WilkinsHagerstown, MD; 2012;60:878–883.
426 DOI:10.1161/HYPERTENSIONAHA.110.155895
- 427 27. Han SX, He GM, Wang T, Chen L, Ning YY, Luo F, An J, Yang T, Dong JJ, Liao Z lin,
428 Xu D, Wen FQ. Losartan attenuates chronic cigarette smoke exposure-induced pulmonary
429 arterial hypertension in rats: Possible involvement of angiotensin-converting enzyme-2.

430 *Toxicol Appl Pharmacol* Toxicol Appl Pharmacol; 2010;245:100–107.
431 DOI:10.1016/j.taap.2010.02.009

432 28. Yuan YM, Luo L, Guo Z, Yang M, Ye RS, Luo C. Activation of renin-angiotensin-
433 aldosterone system (RAAS) in the lung of smoking-induced pulmonary arterial
434 hypertension (PAH) rats. *JRAAS - J Renin-Angiotensin-Aldosterone Syst* SAGE
435 Publications Ltd; 2015;16:249–253. DOI:10.1177/1470320315576256

436 29. Bártová E, Legartová S, Krejčí J, Arcidiacono OA. Cell differentiation and aging
437 accompanied by depletion of the ACE2 protein. *Aging (Albany NY)* Impact Journals LLC;
438 2020;12:22495–22508. DOI:10.18632/aging.202221

439 30. Xudong X, Junzhu C, Xingxiang W, Furong Z, Yanrong L. Age- and gender-related
440 difference of ACE2 expression in rat lung. *Life Sci* Elsevier Inc.; 2006;78:2166–2171.
441 DOI:10.1016/j.lfs.2005.09.038

442 31. Voinsky I, Gurwitz D. Smoking and COVID-19: Similar bronchial ACE2 and TMPRSS2
443 expression and higher TMPRSS4 expression in current versus never smokers. *Drug Dev*
444 *Res* Wiley-Liss Inc.; 2020; DOI:10.1002/ddr.21729

445 32. Tomchaney M, Contoli M, Mayo J, Baraldo S, Shuaizhi L, CR C, Bull D, Lick S, Malo J,
446 Knoper S, Kim S, Tram J, Rojas-Quintero J, Kraft M, Ledford J, Tesfaigzi Y, Martinez F,
447 Thorne C, Kheradmand F, Campos S, Papi A, Polverino F. Paradoxical effects of cigarette
448 smoke and COPD on SARS-CoV2 infection and disease. *bioRxiv* bioRxiv; 2020;Preprint
449 posted online December 07, 2020. DOI:10.1101/2020.12.07.413252

450 33. Cai G, Bossé Y, Xiao F, Kheradmand F, Amos CI. Tobacco smoking increases the lung
451 gene expression of ACE2, the Receptor of SARS-CoV-2. *Am. J. Respir. Crit. Care Med.*

American Thoracic Society; 2020. p. 1557–1559.

34. Brake SJ, Barnsley K, Lu W, McAlinden KD, Eapen MS, Sohal SS. Smoking Upregulates Angiotensin-Converting Enzyme-2 Receptor: A Potential Adhesion Site for Novel Coronavirus SARS-CoV-2 (Covid-19). *J Clin Med* MDPI AG; 2020;9:841. DOI:10.3390/jcm9030841

35. Zhang H, Rostami MR, Leopold PL, Mezey JG, O’Beirne SL, Strulovici-Barel Y, Crystal RG. Expression of the SARS-CoV-2 ACE2 Receptor in the Human Airway Epithelium. *Am J Respir Crit Care Med* American Thoracic Society; 2020;202:219–229. DOI:10.1164/rccm.202003-0541OC

36. Uraih LC, Maronpot RR. Normal histology of the nasal cavity and application of special techniques. *Environ Health Perspect* Environmental Health Perspectives; 1990;85:187–208. DOI:10.1289/ehp.85-1568325

37. Piva F, Sabanovic B, Cecati M, Giulietti M. Expression and co-expression analyses of TMPRSS2, a key element in COVID-19. *Eur J Clin Microbiol Infect Dis* Springer Science and Business Media Deutschland GmbH; 2021;40:451–455. DOI:10.1007/s10096-020-04089-y

38. Schuler BA, Habermann AC, Plosa EJ, Taylor CJ, Jetter C, Negretti NM, Kapp ME, Benjamin JT, Gulleman P, Nichols DS, Braunstein LZ, Hackett A, Koval M, Guttentag SH, Blackwell TS, Webber SA, Banovich NE, Kropski JA, Sucre JMS. Age-determined expression of priming protease TMPRSS2 and localization of SARS-CoV-2 in lung epithelium. *J Clin Invest* American Society for Clinical Investigation; 2021;131. DOI:10.1172/JCI140766

39. Wu C, Ye D, Mullick A, Li Z, Danser AHJ, Daugherty A, Lu H. Effects of Renin-Angiotensin Inhibition on ACE2 and TMPRSS2 Expression: Insights into COVID-19. *bioRxiv Prepr Serv Biol* Cold Spring Harbor Laboratory Preprints; 2020; DOI:10.1101/2020.06.08.137331
40. Pinto BGG, Oliveira AER, Singh Y, Jimenez L, Gonçalves ANA, Ogawa RLT, Creighton R, Peron JPS, Nakaya HI. ACE2 expression is increased in the lungs of patients with comorbidities associated with severe COVID-19. *J Infect Dis* Oxford University Press; 2020;222:556–563. DOI:10.1093/infdis/jiaa332
41. Saheb Sharif-Askari N, Saheb Sharif-Askari F, Alabed M, Temsah MH, Heialy S Al, Hamid Q, Halwani R. Airways Expression of SARS-CoV-2 Receptor, ACE2, and TMPRSS2 Is Lower in Children Than Adults and Increases with Smoking and COPD. *Mol Ther - Methods Clin Dev* Cell Press; 2020;18:1–6. DOI:10.1016/j.omtm.2020.05.013

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 ± 17	63 ± 9
< 60 years old, n	17	11
≥ 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
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

507 Figure Legends

508

509 **Figure 1. Ang II and Ang-(1-7) levels in human lung parenchyma.** Ang II levels expressed as

510 pg/g tissue in lung parenchyma of (A) untreated (n = 38) and ACEI/ARB-treated subjects (n =

511 38); (B) subjects less than 60 years of age (< 60 y) (n = 37) and subjects 60 years of age and

512 older (≥ 60 y) (n = 39); (C) female (n = 34) and male (n = 42); and (D) subjects never smokers (n

513 = 26), smokers (n = 30) and former smokers (n = 20). Ang-(1-7) levels expressed as pg/g tissue

514 in lung parenchyma of (E) untreated and ACEI/ARB-treated subjects; (F) subjects less than 60

515 years of age (< 60 y) and subjects 60 years of age and older (≥ 60 y); (G) female and male; and

(H) subjects never smokers, smokers and former smokers. The number of samples were the same as those for Ang II. 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. Statistical analysis (General Linear Model) showed a significant difference by age in Ang-(1–7) levels.

Figure 2. ACE2-Expressing Type II Pneumocytes are Influenced by Age. 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); and (D) subjects less than 60 years of age (n = 28) and subjects 60 years of age and older (n = 32). The General Linear Model was used for statistical analysis, which 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 band and black triangle symbol within the box show the median and mean values, respectively. The whiskers extending from both ends of the boxes indicate minimum and maximum values. Points outside the box are outliers.

537

538 **Figure 3. ACE2-Expressing Type II Pneumocytes are Influenced by Smoking and ACEI/ARB**

539 **Treatment.** Percentage of ACE2-expressing type II pneumocytes in the lung parenchyma of

540 untreated and ACEI/ARB-treated never smokers (n = 10 untreated and 9 ACEI/ARB-treated),

541 smokers (n = 9 untreated and 17 ACEI/ARB-treated) and former smokers (n = 3 untreated and 12

542 ACEI/ARB-treated). Statistical analysis (General Linear Model) showed a significant interaction

543 between ACEI/ARB treatment and smoking ($P = 0.026$) on percentage of ACE2-expressing type II

544 pneumocytes. The bottom and top of the box plots represent the 25th and 75th percentiles,

545 respectively. The bands within the box show the median value, the black triangle indicates the

546 mean, and the whiskers extending from both ends of the boxes correspond to minimum and

547 maximum values.

548

A

B

550

551 **Figure 4. ACE2 Protein Content is Greater in Older Smokers.** Percentage of subjects with

552 ACE2 immunostaining intensity of +1 to +3 in type II pneumocytes of lung parenchyma of **(A)**

553 never smokers, smokers, and former smokers stratified by age (less than 60 years of age and 60

554 years of age and older) and **(B)** untreated and ACEI/ARB-treated subjects stratified by age (less

555 than 60 years of age and 60 years of age and older). Representative images of surgically resected

556 lung tissue stained for ACE2 protein are presented in Figure S4 in the Data Supplement. The

557 stratified Fisher's exact test showed an association between ACE2 intensity, smoking, and subjects

558 60 years of age and older ($P = 0.05$). Association between ACE2 intensity, age, and ACEI/ARB

559 treatment showed a $P = 0.09$. Although this P value is greater than 0.05, it is less than 0.1, which

560 shows a trend that these factors are associated.

561

Figure 5. 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.

572 The General Linear Model was used for statistical analysis, which showed a significant difference
573 by sex ($P = 0.026$) and age ($P = 0.040$).

574

575

576

A

B

C

Figure 6. 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 and 60 years of age and older; (B) untreated and ACEI/ARB-treated subjects under 60 years of age and 60 years of age and older; and (C) untreated and ACEI/ARB-treated 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 are presented in Figure S5 in the Data Supplement. Tmprss2 immunostaining intensity of +3 was not detected in 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.

Figure 7. Scheme highlighting the conclusions drawn from the present work

Figures

Figure 1

Ang II and Ang-(1-7) 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). Ang-(1–7) levels expressed as pg/g tissue in lung parenchyma of (E) untreated and ACEI/ARB-treated subjects; (F) subjects less than 60 years of age (< 60 y) and subjects 60 years of age and older (≥ 60 y); (G) female and male; and 26 (H) subjects never smokers, smokers and former smokers. The number of samples were the same as those for Ang II. 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. Statistical analysis (General Linear Model) showed a significant difference by age in Ang-(1–7) levels.

Figure 2

ACE2-Expressing Type II Pneumocytes are 524 Influenced by Age. 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); and (D) subjects less than 60 years of age (n = 28) and subjects 60 years of age and older (n = 32). The General Linear Model was used for statistical analysis, which 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 band and black triangle symbol within the box show the median and mean values, respectively. The whiskers extending from both ends of the boxes indicate minimum and maximum values. Points outside the box are outliers.

Figure 3

ACE2-Expressing Type II Pneumocytes are Influenced by Smoking and ACEI/ARB Treatment. Percentage of ACE2-expressing type II pneumocytes 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). Statistical analysis (General Linear Model) showed a significant interaction between ACEI/ARB treatment and smoking (P = 0.026) on 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 black triangle indicates the mean, and the whiskers extending from both ends of the boxes correspond to minimum and maximum values.

Figure 4

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, smokers, and former smokers stratified by age (less than 60 years of age and 60 years of age and older) and (B) untreated and ACEI/ARB-treated subjects stratified by age (less than 60 years of age and 60 years of age and older). Representative images of surgically resected lung tissue stained for ACE2 protein are presented in Figure S4 in the Data Supplement. The stratified Fisher's exact test showed an association between ACE2 intensity, smoking, and subjects 60 years of age and older ($P = 0.05$). Association between ACE2 intensity, age, and ACEI/ARB treatment showed a $P = 0.09$. Although this P value is greater than 0.05, it is less than 0.1, which shows a trend that these factors are associated.

Figure 5

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, which showed a significant difference by sex ($P = 0.026$) and age ($P = 0.040$).

Figure 6

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 and 60 years of age and older; (B) untreated and ACEI/ARB treated subjects under 60 years of age and 60 years of age and older; and (C) untreated and ACEI/ARB-treated 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 are presented in Figure S5 in the Data Supplement. TMPRSS2 immunostaining intensity of +3 was not detected in 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.

Figure 7

Scheme highlighting the conclusions drawn from the present work