Smoking Helps Open Gateway to Coronavirus Infection, Study Shows

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- Cigarette use may elevate enzymes that allow virus to get in
- Study by St. Paul's Hospital published in medical journal

Photographer: Hollie Adams/Bloomberg

Smoking may raise the risk of Covid-19 by elevating enzymes that allow the coronavirus to gain access into lung cells, according to a new study.

Smokers and people with chronic obstructive pulmonary disease may have elevated levels of an enzyme called ACE-2, which helps the virus enter cells in their lungs, where it replicates, a study published in the European Respiratory Journal Thursday showed.

Obesity, diabetes, high blood pressure and other chronic conditions have emerged as factors that make people vulnerable to Covid-19, the disease caused by the coronavirus that's sweeping the world in a pandemic.

The research, led by Janice Leung, a respirologist at St. Paul's Hospital in Vancouver, is an observational study based on data emerging from China and was peer reviewed. In China, where the mortality rate is higher for men than women, about half of males smoke, according to the World Health Organization. That compares with some 2% for females.

"There has never been a better time to quit smoking to protect yourself from Covid-19, " Leung said.

Read more: Stay-at-Home Smoking Helps Tobacco Sales During Virus Lockdowns

Samples were taken from the lungs of 21 patients with COPD and 21 people not suffering from COPD. Higher levels of ACE-2 were found both in COPD patients and current smokers.

The researchers also cross-referenced their findings with two existing study groups that include more test subjects, and came to the same conclusion.

Read more: European Smokers, Vapers Still Get Their Fix During Lockdowns

Michael R. Bloomberg, founder and majority owner of Bloomberg News parent Bloomberg LP, has campaigned and given money in support of a nationwide ban on flavored e-cigarettes and tobacco.

Coronavirus May ‘Reactivate’ in Cured Patients, Korean CDC Says

As of Wednesday, South Korea had 10,384 virus cases, with 6,776 released from hospital, according to data compiled by Johns Hopkins University and Bloomberg News.

Epidemiologists around the world are in a race to find out more about the virus that causes Covid-19. The pathogen’s rapid global spread has recently seen the focus shift to patients who contract the virus but display few or atypical symptoms. Korea has been at the forefront of tracking these cases, which are causing particular concern in China, where the epidemic is showing signs of coming under control.

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Early View

Research letter

ACE-2 Expression in the Small Airway Epithelia of Smokers and COPD Patients: Implications for COVID-19

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This manuscript has recently been accepted for publication in the *European Respiratory Journal*. It is published here in its accepted form prior to copyediting and typesetting by our production team. After these production processes are complete and the authors have approved the resulting proofs, the article will move to the latest issue of the ERJ online.

ACE-2 Expression in the Small Airway Epithelia of Smokers and COPD Patients:

Implications for COVID-19

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The World Health Organization (WHO) has declared coronavirus disease 2019 (COVID-19) as a pandemic [1]. COVID-19 is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). COVID-19 displays symptoms ranging from mild to severe (pneumonia) that can lead to death in some individuals [2-4]. As of March 24, 2020, there have been 422,566 cases of COVID-19 worldwide and 18,887 deaths [5]. SARS-CoV-2 uses the angiotensin converting enzyme II (ACE-2) as the cellular entry receptor[6]. While the virus can infect individuals of any age, to date, most of the severe cases have been described in those over the age of 55 years and with significant co-morbidities such as chronic obstructive pulmonary disease (COPD) [7]. Here, we determined whether patients with COPD have increased expression of ACE-2 in bronchial epithelial cells in lower respiratory tract.

Patients undergoing bronchoscopy at St. Paul's Hospital (SPH), Vancouver, Canada for clinical purposes were enrolled. The protocol was approved by the University of British Columbia/Providence Health Care Ethics Board (UBC/PHC REB H15-02166). All patients were required to be 19 years of age or older, who underwent spirometry according to international guidelines[8]. Patients with COPD were defined as those having a clinical diagnosis of COPD made by a board-certified respiratory physician and either a forced expiratory volume in 1 second (FEV1)/forced vital capacity (FVC) <70% or clear evidence of emphysema on computed tomographic (CT) imaging on visual inspection. Cytologic brushings were obtained in subsegmental airways (6^{th}-8^{th} generation) of the lung that were unaffected by the patient's underlying clinical indication for bronchoscopy.

Total RNA was extracted from cytologic brushings using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Transcriptomic sequencing was performed on the NovaSeq 6000 (Illumina, San Diego, CA) at a sequencing depth of 55 million reads. Raw sequencing reads were quality controlled with FastQC[9] and aligned to the GENCODE (version 31) GRCh37 genome reference using STAR (Spliced Transcripts Alignment to a Reference) [10]. After alignment, the data were quantified using RSEM (RNA-Seq by Expectation Maximization) to obtain the read counts. Limma voom[11] was applied to normalize the counts to log2 counts per million reads (CPM), which was used in the downstream analysis.

Two cohorts were used for validation; the details of which are provided in a previous publication [12]. First, we used 16 datasets obtained from bronchial brushings of 10^{th}-12^{th} generation bronchi collected at a single center; transcriptome measurement was performed using the U133 Plus 2.0 microarray (denoted as the Cornell Dataset)[13]. Second, we used dataset GSE37147 consisting of bronchial brushings from the 6^{th}-8^{th} generation airways with gene

expression profiles generated from the GeneChip Human Gene 1.0 ST microarray[14]. This dataset was denoted as British Columbia Cancer Agency (BCCA) cohort.

We also determined protein expression of ACE-2 in resected lung tissue specimens. These samples were obtained from 10 current smokers with COPD (FEV1/FVC 61±7%), 9 non-smoker controls (FEV1/FVC 85±2%), and 8 healthy current smokers (FEV1/FVC 78±6%). Human lung tissue samples were obtained with informed consent from patients undergoing thoracic surgery as part of the James Hogg Lung Registry (UBC/PHC REB Protocol H00-50110). Formalin-fixed paraffin-embedded human lung tissues were stained with antibody against ACE-2 (Ab15348; Abcam) using the Bond Polymer Refine Red Detection kit on a Leica Bond Autostainer as previously described[15]. Airway epithelial-specific ACE-2 protein intensity was quantified using the Aperio imaging system with normalization to the length of the basement membrane (Leica Biosystem; Concord, Ontario).

For the primary study population, log2 CPM of ACE-2 was the principal outcome of interest. Robust linear models were used to determine whether 1) ACE-2 was differentially expressed in patients with COPD and in smokers after adjustment for age and sex and 2) ACE-2 expression was significantly correlated with lung function. All analyses were performed in R (version 3.5.0). In the immunohistochemistry dataset, Kruskal-Wallis with Dunn's Multiple Comparisons tests was used. Continuous data are expressed as mean±SD, unless otherwise indicated.

The average age of the SPH cohort was 64.8±12.0 years; 55% were females and 24% were current smokers. Compared to control subjects (N=21), those with COPD (N=21) had lower FEV1% (72.0±15.6 vs 85.9±17.9% predicted; p=0.011) and FEV1/FVC (64.1±7.9 vs 76.3±5.9%; 2.621×10^{-6}). Most (79%) underwent bronchoscopy for investigation of lung nodules, followed by chronic cough (7%) and lymphadenopathy (7%). ACE-2 expression in the epithelial cells was significantly increased in COPD versus non-COPD subjects (COPD=2.52±0.66 versus non-COPD=1.70±0.51; $p=7.62\times10^{-4}$; **Figure 1A**). There was a significant inverse relationship between ACE-2 gene expression and FEV1% of predicted (r=-0.24; p=0.035; **Figure 1B)**. Interestingly, smoking status was also significantly related to ACE-2 gene expression levels in airways of these participants with current smokers having a significantly higher gene expression than never smokers (current smokers=2.77±0.91 versus never smokers=1.78±0.39; p=0.024). Former smokers had gene expression levels in-between that of never and current smokers (former smokers=2.00±1.23; **Figure 1C**). Conditional on the smoking status, the association between ACE-2 expression and COPD was still significant (Adjusted Mean±SE of non-COPD: 0.90±0.65 versus COPD: 1.75±0.82, p=0.016).

Next, we validated the above findings in: 1) the Cornell Cohort (N= 211) and 2) the BCCA cohort (N=238). The average age of the Cornell Cohort was 43.6±10.5 years with 33.2% of the cohort being females. There were 32.2% who were never smokers and 67.8% who were current smokers at the time of the bronchoscopy. The average age of the BCCA cohort was 64.5±5.9 years with 43.3% of the cohort being females. All were heavy smokers with at least 30 pack-years of smoking. Of these, 41.6% were current smokers at the time of the bronchoscopy and the remaining were former smokers.

In both the Cornell and BCCA cohorts, current smokers had increased ACE-2 gene expression levels in the airways compared with never smokers (in the Cornell cohort; current smokers=4.34±0.45 versus never smokers=4.15±0.36; $p=1.92\times10^{-3}$) and with former smokers (in the BCCA cohort; current smokers=6.05±0.53 versus former smokers=5.57±0.37; $p<2\times10^{-16}$). In the BCCA cohort, pre-bronchodilator FEV1 was measured and it was significantly related to ACE-2 gene expression level (r=-0.10; p=0.037).

Representative images of epithelial-specific ACE-2 protein expression in non-smokers, healthy smokers and smokers with COPD are shown in Figure 1D. ACE-2 expression in the human small airway epithelium was significantly increased in COPD compared to non-smokers but not in healthy smokers (Figure 1D). ACE-2 protein staining was largely restricted to the airway epithelium of COPD and cells in the submucosal compartment.

There is a worldwide outbreak of COVID-19 coronavirus. Although most patients infected and diagnosed with CVOID-19 disease have mild symptoms, approximately 20% of individuals have demonstrated severe or critically severe disease including symptoms and signs of pneumonia, respiratory failure, septic shock and multi-organ failure. The estimated case-fatality rate is 1-2% [2, 3]. Importantly, nearly all deaths have occurred in those with significant underlying chronic diseases including COPD and cardiovascular diseases [4]. The reason for this observation is largely unknown.

One possibility is differential expression of ACE-2, which is the main receptor used by SARS-CoV-2 to gain entry into the host mucosa and cause active infection. Here, we investigated gene expression levels of ACE-2 in the airways of individuals with and without COPD and found that COPD and current smokers had significantly increased expression of ACE-2. Importantly, gene expression levels of ACE-2 were inversely related to individual's FEV1, suggesting a dose-dependent response. These findings were observed in 3 different cohorts, indicating their generalizability and robustness.

ACE-2 is a type I transmembrane metallocarboxypeptidase with homology to angiotensin converting enzyme (ACE). In contrast to ACE, which converts angiotensin I to the active vasoconstrictor, angiotensin II, ACE-2 breaks down angiotensin II to its metabolites including angiotensin-(1–9) and angiotensin-(1–7), which are potent vasodilators, and thus may be a negative regulator of the renin-angiotensin system[16]. ACE-2 is expressed in a variety of different tissues including both the upper and lower respiratory tract, myocardium and the gastrointestinal mucosa [17]. Although its role in human health and disease has not been fully elucidated, it appears to have an important regulatory role in blood pressure and cardiac function. The physiologic role of ACE-2 in the airways is largely unknown. However, in mice, ACE-2 has been shown to protect animals from severe lung injury related to aspiration and sepsis [18].

To our knowledge, our study is the first to demonstrate increased ACE-2 expression in airways of current (but not former) smokers and those with COPD. These results are also consistent with previous observations in small animals wherein smoke exposure has been shown to upregulate both the expression and activity of ACE-2 in the airways [19, 20]. While the up-regulation of ACE-2 may be useful in protecting the host against acute lung injury, chronically, this may predispose individuals to increased risk of coronavirus infections, which uses this receptor to gain entrance into epithelial cells. This may in part explain the increased risk of viral respiratory tract infection in active smokers and virus-related exacerbations in those with COPD.

There were limitations to the study. First, the study was cross-sectional and as such, we could not determine whether interventions such as inhaled corticosteroids or bronchodilators (for those with COPD) could modulate ACE-2 gene expression in the airways. Second, as receptor expression is one of many host factors that govern infection risk among individuals, the precise attributable risk (for coronavirus infections) imposed by cigarette smoking and COPD is uncertain. Third, although the airway epithelia is the major source of entry for COVID-19, the virus can gain host entry through other ports including gastrointestinal mucosa, which was not evaluated in this study. Fourth, we did not have access to upper airway tissues, which may also become infected with SARS-CoV-2.

In summary, active cigarette smoking and COPD up-regulate ACE-2 expression in lower airways, which in part may explain the increased risk of severe COVID-19 in these populations. These findings highlight the importance of smoking cessation for these individuals and increased surveillance of these risk subgroups for prevention and rapid diagnosis of this potentially deadly disease.

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Figure 1A: A violin plot of ACE-2 expression in small airways of COPD and non-COPD subjects in the St. Paul's Hospital Cohort.

The red box indicates the median and the interquartile range. The P-value was obtained from the robust linear model

Abbreviations: ACE-2, angiotensin converting enzyme II; COPD, chronic obstructive pulmonary disease; CPM, counts per million reads

Figure 1B: A scatter plot of ACE-2 expression in small airways according to FEV1 % Predicted in the St. Paul's Hospital Cohort.

Abbreviations: ACE-2, angiotensin converting enzyme II; COPD, chronic obstructive pulmonary disease; CPM, counts per million reads

ACE-2 gene expression in airway epithelia is inversely related to FEV1% predicted (p=0.0348)

Figure 1C. A violin plot of ACE2 expression in small airways of never, former and current smokers in the St. Paul's Hospital Cohort.

The red box indicates the median and the interquartile range. The P-value was obtained from the robust linear model.

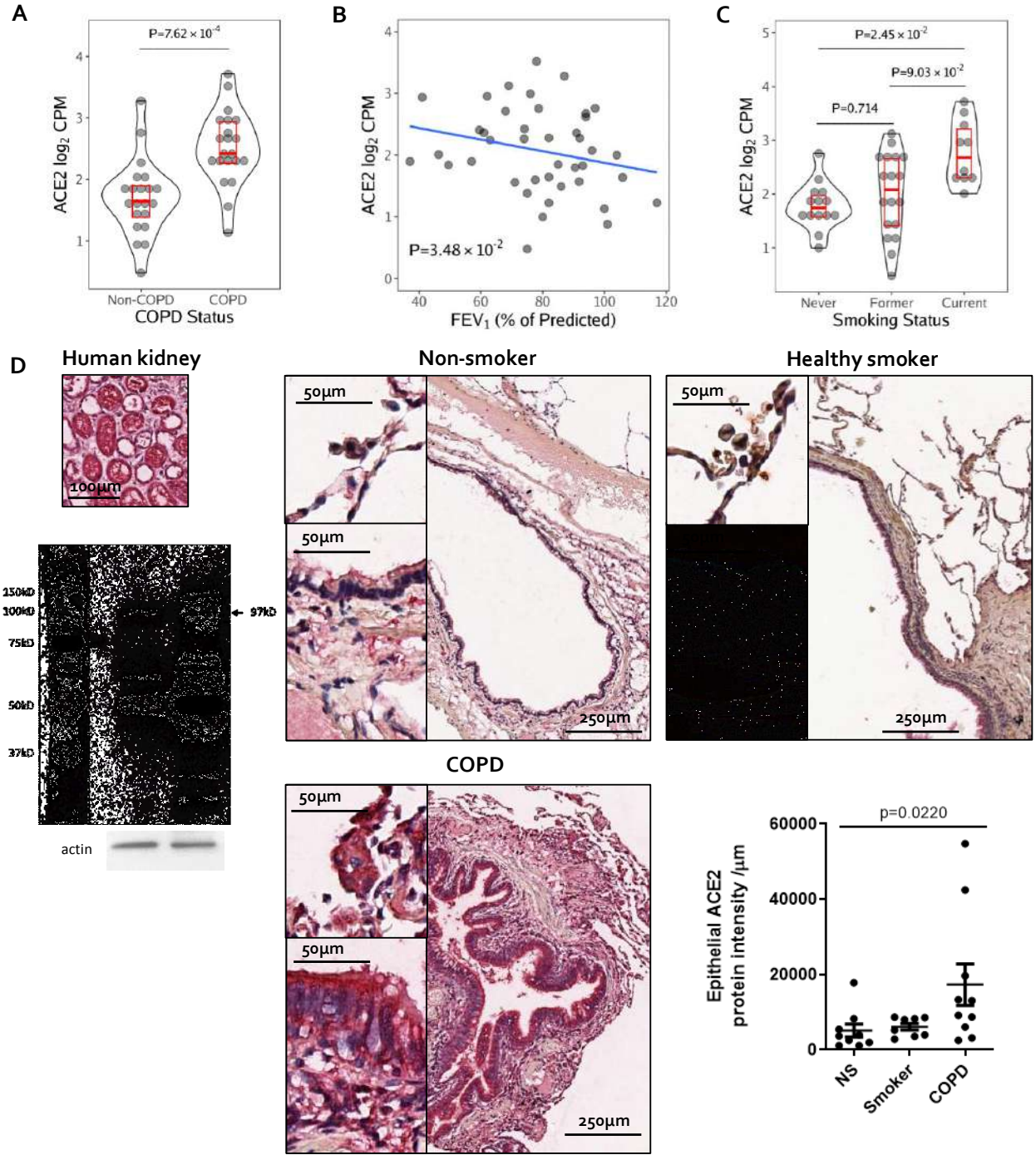
Abbreviations: ACE-2, angiotensin converting enzyme II; CPM, counts per million reads

Figure 1D. Protein staining of ACE2 in airways of Individuals with and without COPD

Human kidney slide was the positive control for ACE-2. The specificity of the antibody against ACE-2 was determined using an immunoblot assay with HEK2 cell lysates as a positive control. The expected molecular weight of ACE-2 is 90-100 kDa.

In airways, most of the protein expression was noted in the epithelial layer, most pronounced in those with COPD.

Abbreviations: ACE-2, angiotensin converting enzyme II; NHBE, normal human bronchial epithelial cells; NS, non-smoker


A
P=7.62 × 10-4
ACE2 log2 CPM
Non-COPD
COPD
COPD Status
B
P=3.48 × 10-2
ACE2 log2 CPM
FEV1 (% of Predicted)
C
P=2.45 × 10-2
P=9.03 × 10-2
P=0.714
ACE2 log2 CPM
Never
Former
Current
Smoking Status
D
Human kidney
100μm
150kD
100kD
75kD
50kD
37kD
97kD
actin
Non-smoker
Healthy smoker
COPD
50μm
250μm
p=0.0220
Epithelial ACE2 protein intensity /μm
NS
Smoker
COPD

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Editorial

Smoking Upregulates Angiotensin-Converting Enzyme-2 Receptor: A Potential Adhesion Site for Novel Coronavirus SARS-CoV-2 (Covid-19)

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Abstract: The epicenter of the original outbreak in China has high male smoking rates of around 50%, and early reported death rates have an emphasis on older males, therefore the likelihood of smokers being overrepresented in fatalities is high. In Iran, China, Italy, and South Korea, female smoking rates are much lower than males. Fewer females have contracted the virus. If this analysis is correct, then Indonesia would be expected to begin experiencing high rates of Covid-19 because its male smoking rate is over 60% (Tobacco Atlas). Smokers are vulnerable to respiratory viruses. Smoking can upregulate angiotensin-converting enzyme-2 (ACE2) receptor, the known receptor for both the severe acute respiratory syndrome (SARS)-coronavirus (SARS-CoV) and the human respiratory coronavirus NL638. This could also be true for new electronic smoking devices such as electronic cigarettes and "heat-not-burn" IQOS devices. ACE2 could be a novel adhesion molecule for SARS-CoV-2 causing Covid-19 and a potential therapeutic target for the prevention of fatal microbial infections, and therefore it should be fast tracked and prioritized for research and investigation. Data on smoking status should be collected on all identified cases of Covid-19.

Keywords: ACE2 receptor; SARS-CoV-2; Covid-19; Smoking; COPD; Electronic cigarettes; Vaping; Heat-Not-Burn; IQOS

Little attention has been given to the role of smoking in either the transmission of the novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, actual virus) or mortality rate of Covid-19 (name of the disease caused). Smokers contract more respiratory ailments, including colds (commonly rhinoviruses, but also coronaviruses) than non-smokers. Smokers also show double the influenza rate and increased rates of bacterial pneumonia and tuberculosis [1–5]. The damage caused to the lungs by smoking makes patients more susceptible to pulmonary infections, both bacterial and viral [6]. Smokers are 34% more likely than non-smokers to contract the flu [6]. Han and colleagues conclude that literature evidence showed that smoking was consistently associated with a higher risk of hospital admissions after influenza infection [7]. Smoking is the primary etiological factor behind chronic obstructive pulmonary disease (COPD) in the developed world, but environmental pollution and degrading air quality are also responsible in developing countries. It is now the fourth leading cause of death in the world [8]. Vaccination against influenza is strongly recommended for patients with COPD, as the frequency and progression of exacerbations are strongly linked to respiratory viruses

in 30% of cases [1]. Rubin et al. found that COPD patients who were prone to viral infections had higher exacerbation rates, more inflammation, and loss of lung function compared to those with existing exacerbating disease conditions [9]. Symptomatology and mortality in influenza-infected smokers were also enhanced [9]. According to the WHO, comorbidities are associated with a high percentage of Covid-19 related deaths [10,11]. In conjunction with the complications arising from comorbidities in patients who smoke [12], we put forth the question of whether smoking, smoking-induced health conditions, and comorbidities, in combination, is culminating in a high risk demographic for both contraction of the virus and the severe presentation of Covid-19.

China has a high male smoking rate at around 50% in rural areas and is estimated to be about 44.8% overall [13]. Most of the deaths identified from the epicenter of the Covid-19 outbreak were in men from older age groups and those with underlying conditions such as chronic respiratory disease, cancer, hypertension, diabetes, or cardiovascular disease. The initial age distribution of Covid-19 cases was skewed towards older age groups with a median age of 45 years (IQR 33–56) for patients who were alive or who had an unknown outcome at the time of reporting. The median age of patients who had died at the time of reporting was 70 years (IQR 65–81) as reported by Sun and colleagues [14]. This data was also supported by an early epidemiological study of 99 Covid-19 cases from Wuhan, China [14].

Fatality rates are given as the percentage of the defined group with confirmed Covid-19 that died, and therefore will not add up to 100%. The Table 1 was adapted from Coronavirus Disease (Covid-19) Research and Statistics [15].

Table 1. Risk factor-based fatality rates of Covid-19 from early data in China.

Age group	Fatality rates
0–9 years	0%
10–19 years	0.2%
20–29 years	0.2%
30–39 years	0.2%
40 - 49 years	0.4%
50–59 years	1.3%
60–69 years	3.6%
70–79 years	8%
80 years and above	14.8%
Underlying health conditions	
Cardiovascular disease	10.5%
Diabetes	7.3%
Chronic respiratory disease	6.3%
Hypertension	6%
Cancer	5.6%
No underlying health conditions	0.9%

The term “coronaviruses” arose from their crown-like appearance when imaged, the Latin for crown being corona. The distinguishing crown-like feature of coronaviruses is attributed to the presence of large type 1 transmembrane spike (S) glycoproteins. This heavily glycosylated cell surface protein contains two distinct functional domains (S1 and S2) which are thought to mediate host cell entry by the virus. The S1 domain contains the angiotensin-converting enzyme-2 (ACE2) receptor-binding domain and is responsible for first stage host cell entry [16]. The S2 domain facilitates fusion between cell and virus membrane, required for cellular infiltration [17]. S proteins are enzymatically modified, exposing the fusion site for cellular adhesion. This is achieved through cleavage by cellular proteases, mediated by protein convertase called “furin” [17,18]. Furin is expressed significantly in the lungs, and respiratory viruses also utilize this system to convert their surface proteins [17]. Although the S protein cleavage site is less observed in coronavirus with similar genomic sequence [17], it is essential to note that more pathogenic influenza viruses share similar cleavage sites [19].

The ACE2 receptor provides a human cell-binding site for the S protein for the SARS-coronavirus (SARS-CoV) [20–22] (a virus that was first identified in 2003 in a southern province of China [23–25]), the coronavirus NL63 [20,26], and now SARS-CoV-2 [27]. Recent studies have found that the modified S protein of SARS-CoV-2 has a significantly higher affinity for ACE2 and is 10- to 20-fold more likely to bind to ACE2 in human cells than the S protein of the previous SARS-CoV [28,29]. This increase in affinity may enable easier person-to-person spread of the virus and thus contribute to a higher estimated R0 for SARS-CoV-2 than the previous SARS virus. The ACE2 protein is expressed on the surface of lung type-2 pneumocytes [30]. It could thus act as a novel adhesion molecule for Covid-19 and be a potential therapeutic target for the prevention of fatal microbial infections in the community.

An early suggestion is that ACE2 is upregulated on the airway epithelium of smokers. Guoshuai Cai recently reported higher ACE2 gene expression in smoker samples compared to never-smokers. Zhao et al. observed that ACE2 is expressed explicitly in type-2 pneumocytes, in which genes regulating viral reproduction and transmission are highly expressed [31]. This indicates that smokers may be more susceptible to infection by SARS-CoV-2, and possibly Covid-19. We recently identified enhanced ACE2 expression in resected lung tissue from patients with COPD and healthy lung function smokers, albeit comparably less in the latter, while entirely absent in heathy non-smoking individuals (Figure 1). ACE2 expression was quite evident in the type-2 pneumocytes, alveolar macrophages, and the apical end of the small airway epithelium. COPD patients showed significantly higher levels of ACE2, suggesting that COPD further exaggerates ACE2 and potential SARS-CoV-2 adhesion site. ACE2 expression could also be true for patients with another chronic lung disease such as idiopathic pulmonary fibrosis [32]. The attachment of the virus to cell surface ACE2 protects them from immune surveillance mechanisms, leaving them tagged to the host for relatively longer periods, thus making them an efficient carrier and vulnerable host for future infections and spread. The eventual engulfment of ACE2 further provides the virus access to the host cells system, thus providing a flourishing environment, not just to sustain and proliferate but also to mutate and modify host evasion mechanisms. Previous observations using in vivo knockout mice models suggest that SARS-CoV-2 adhesion on ACE2 could also downmodulate the expression of ACE2 itself. This, in turn, increases the production and activation of other related ACE enzymes. This differential modulation and the drastic reduction in ACE2 results in severe acute respiratory failure [33,34].

Wang et al. also noted an ACE2 connection to smoking and Covid-19 [35]. The increases seen in smokers further raises the question of whether this is also true for people engaged in waterpipe smoking [36] and those switching over to the more recent alternatives such as electronic cigarettes and "heat-not-burn" IQOS devices. It is essential to recognize that these devices are not "safer", they are still a tobacco product that produces vapor or smoke and similarly could cause infectious lung damage as we see with traditional cigarettes [37–39].

Further research on these products and their influence on the virulence of coronaviruses is urgently needed. Following the outbreak in New York City, Mayor Bill de Blasio announced that "If you are a smoker or a vaper that does make you more vulnerable," urging that now is the perfect time to quit [40]. Smokers, as a vulnerable group, must be supported to quit and should be advised to avoid areas where they may be liable to be exposed to Covid-19, especially smokers with pre-existing respiratory health concerns. Smokers should be prioritized for vaccination when a vaccine is developed, particularly if it is found they are a key transmission source.

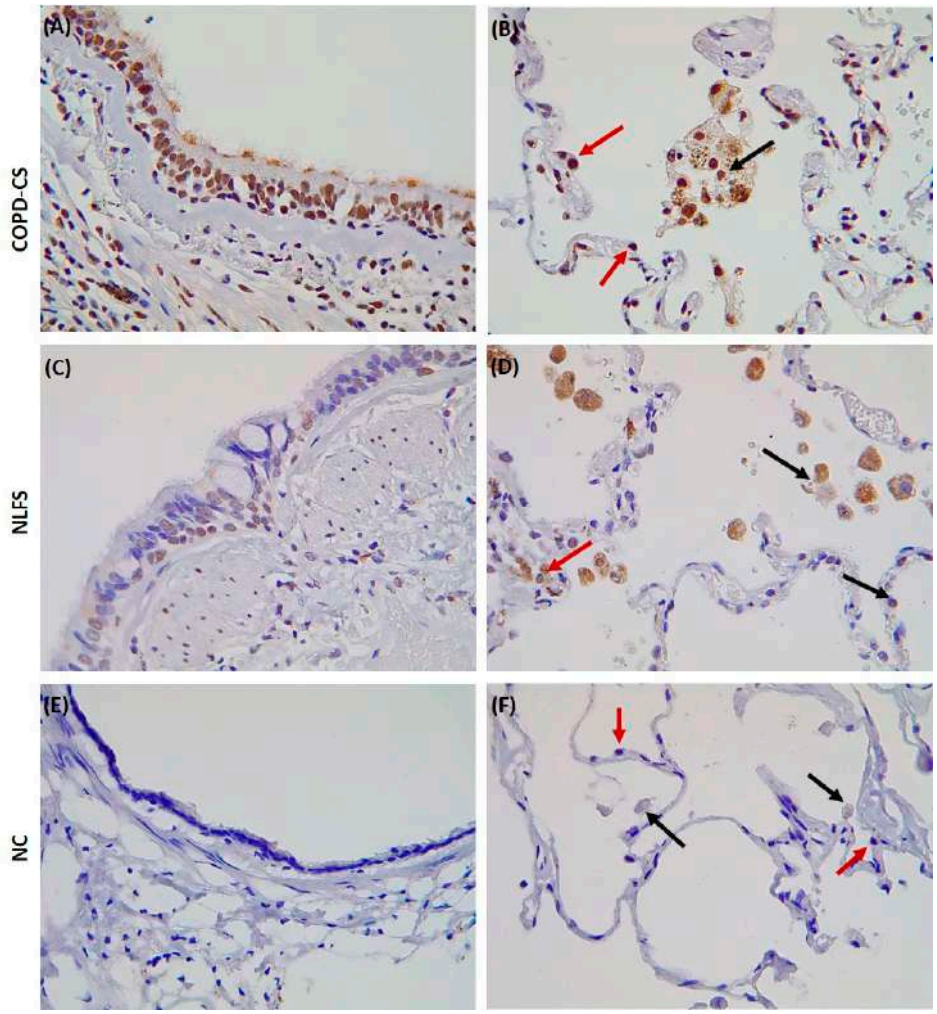

Figure 1. Surgically resected lung tissue stained for the angiotensin-converting enzyme-2 (ACE2) receptor. Current smoker with chronic obstructive pulmonary disease (COPD-CS), (**A**) showing positive staining in the small airway epithelium but also apical including cilia (**B**) red arrows indicating positive staining in type-2 pneumocytes and black arrows showing alveolar macrophages positive for the ACE2 receptor. Normal lung function smoker (NLFS), (**C**) and (**D**) showing similar pattern for COPD-CS although a little less staining is observed. Normal controls (NC), (**E**) and (**F**) no staining observed in any of the areas. **This is the first immunohistochemical human lung evidence for ACE2 receptor expression in smokers and patients with COPD.**

Research on smoking and potential exacerbations of Covid-19 transmission and mortality should include waterpipes, electronic smoking devices, and "heat-not-burn" devices, such as IQOS devices. Further compounding this link between smoking and Covid-19 vulnerability are the comorbidities that have been identified as a significant increased risk factor for severe and fatal Covid-19. The link between smoking and comorbidities, such as diabetes and cardiovascular disease, have long been established [12]. As a research community, we must ask the questions:

(1) Are COPD and other smoking-related illnesses associated with fatal Covid-19 cases?
(2) Are smokers more likely to contract and transmit SARS-CoV-2 than non-smokers?
(3) Are demographics with high smoking rates more vulnerable to Covid-19 outbreaks?

WHO and all countries should ensure that the smoking status of patients identified with Covid-19, including deaths, is recorded and incorporated in data sets, so the smoker's relationship to Covid-19 can be determined.

Status data collection could be simple in four categories,

1. active smoker,
2. passive smoker (those living in households with smokers or working in smoky environments),
3. former smoker (12 months or longer abstinence),
4. non-smoker.

Governments should act to reduce smoking rates in all countries in accordance with the WHO Framework Convention on Tobacco Control (FCTC), and initiate a stimulus package for health, as they have done for business, at the time of this outbreak/pandemic including all communicable pulmonary diseases and Covid-19, as it is possible that smoking exacerbates contraction, transmission, and mortality. It appears that smoking has the potential to upregulate the ACE2 receptor, making smokers and COPD patients more vulnerable to Covid-19. The new electronic smoking devices also do not seem to be safer options. ACE2 thus could be a potential therapeutic target for SARS-CoV-2 and should be prioritized for further research.

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VIEWPOINT

Is nicotine exposure linked to cardiopulmonary vulnerability to COVID-19 in the general population?

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Keywords
addiction; infectious Disease; pandemic; public Health; tobacco

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The recent emergence of COVID-19 has resulted in a worldwide crisis, with large populations locked down and transportation links severed. While approximately 80% of infected individuals have minimal symptoms, around 15–20% need to be hospitalized, greatly stressing global healthcare systems. As of March 10, the death rate appears to be about 3.4%, although this number is highly stratified among different populations. Here, we focus on those individuals who have been exposed to nicotine prior to their exposure to the virus. We predict that these individuals are 'primed' to be at higher risk because nicotine can directly impact the putative receptor for the virus (ACE2) and lead to deleterious signaling in lung epithelial cells.

Our theory for how nicotine consumption represents a special risk factor in coronavirus disease 2019 (COVID-19) was spurred by an earlier outbreak. In 2002, reports emerged from the Guangdong Province in southern China of a new fatal atypical pneumonia termed severe acute respiratory syndrome (SARS). Its rapid expansion throughout Southeast Asia prompted scientific efforts that identified a phylogenetically distinct coronavirus (SARS-CoV) through genomic sequencing [1]. To date, there is no treatment for SARS, and scientists are now grappling for potential vaccines and therapeutics that can target SARS-CoV—or its host cell components involved in viral replication—as both short- and long-term therapeutic strategies.

In order to gain access to cells, CoV binds host cell receptors via their viral envelope and are internalized into the cell through what appear to be traditional clathrin-mediated processes [2,3]. The *ACE2* gene encodes angiotensin-converting enzyme-2 (ACE2), which has been found to be the target receptor for both SARS-CoV and the human respiratory coronavirus NL63. Studies indicate that ACE2 is now likely to be the host receptor for the coronavirus 2019-nCoV/SARS-CoV-2 (COVID-19) [4,5]. ACE2 is a relatively newly described type I transmembrane metallo-carboxypeptidase with overall homology to more classical ACE enzymes that regulate vascular tone and hormone secretion within the renin–angiotensin system (RAS) [6]. ACE2 appears to play both protective and pathogenic roles within RAS pathways, and its direct mechanisms of function in cells remain less understood [7,8]. ACE2 is a critical mediator of RAS signaling throughout the body but particularly in the heart, lung, kidney, and gastrointestinal tract [9], which are known sites for SARS-CoV infection. Findings now suggest that common ACE inhibitors used in the treatment of disease such as diabetes can upregulate ACE2

Abbreviations
ACE2, angiotensin-converting enzyme-2; COVID-19, Coronavirus disease 2019; e-cig, electronic cigarette; RAS, renin–angiotensin system; SARS, severe acute respiratory syndrome; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

expression and that ACE2 can also be increased by chronic use of drugs such as thiazolidinediones and ibuprofen [10]. Thus, it is not a coincidence that many of the same symptoms that account for COVID-19-related illnesses and fatalities parallel those that emerge from RAS dysfunction in humans and animal models, including congestive heart failure, acute and chronic lung diseases, and cardiorenal metabolic syndrome [11-13].

In the absence of long-term immunization or effective therapies for COVID-19, public health management must rely on rapid responses for the identification, treatment, and management of the infection and extra care for vulnerable (high-risk) populations. Emergent evidence supports the involvement of smoking as a key predisposing factor for COVID-19-related illness severity and mortality based on a recent study of 1,590 patients from 575 hospitals in 31 province/autonomous regions/provincial municipalities across China [14]. Age- and sex-matched comparisons indicate that mortality and symptom severity are higher in smokers and former smokers. These findings may begin to shed light on mechanisms that account for responses of infected individuals such as the old vs. young and males vs. females in China and now elsewhere. In a recent report based on 1099 patients with COVID-19 from 552 hospitals in 30 provinces in China, 58% of the patients were men, indicating that there might be a sex predisposition to COVID-19, with men more prone to being affected. However, it is more likely that this sex predisposition reflects the higher smoking rate in men than in women in China (288 million men and 12.6 million women were smokers in 2018) [15].

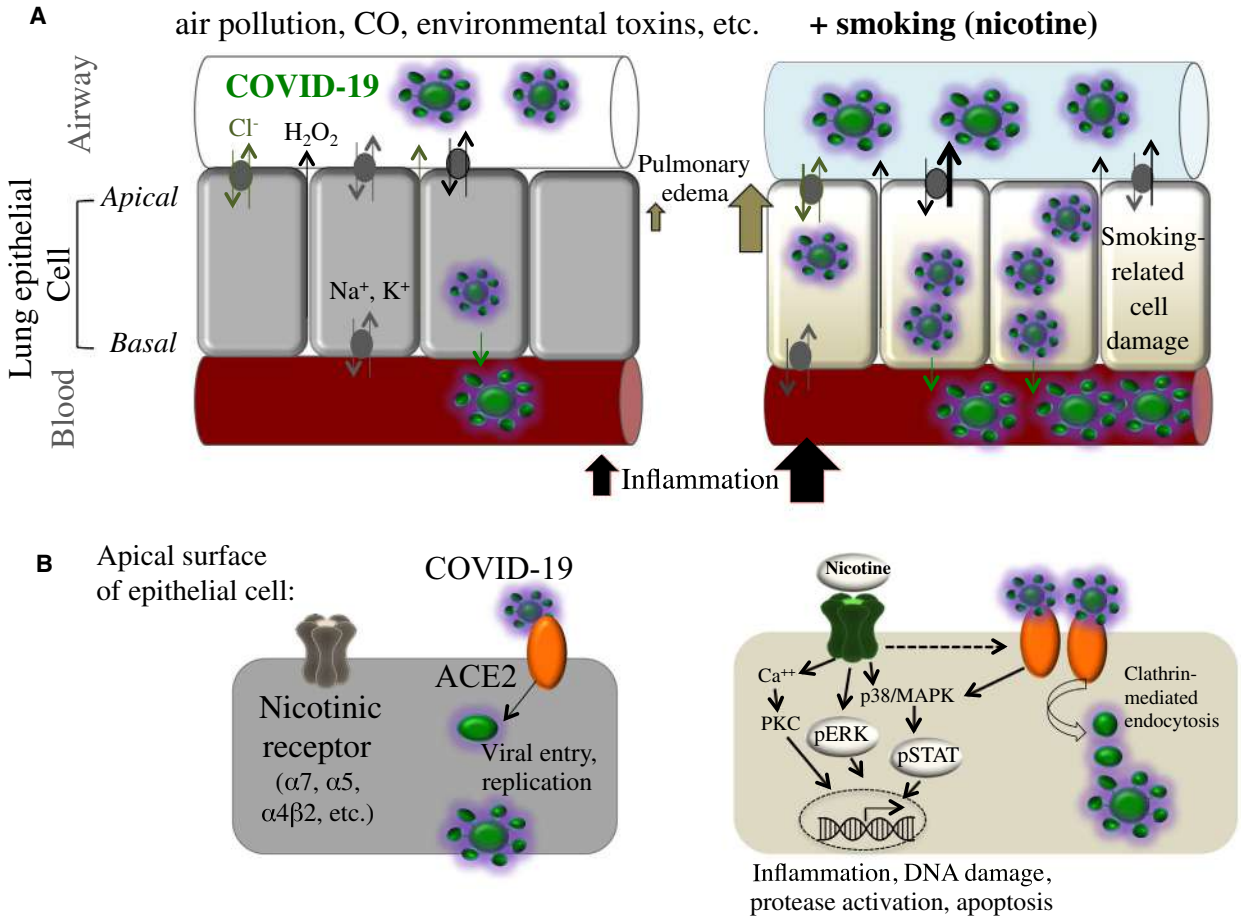

Fig. 1. A schematic model for how nicotine exposure augments risk of COVID-19 entry into the human host lung. (A) Pulmonary and immune responses to COVID-19 infection in epithelial cells of smokers (right) and nonsmokers (left). (B) Cellular mechanisms of nicotinic receptor activity that promotes COVID-19 entry and proliferation in epithelial cells through co-expression of ACE2. Nicotine activation of nicotinic receptors can lead to enhanced protease activation, cell death (apoptosis), and inflammatory signaling through mechanisms that converge on ACE2 regulation and signaling.

Smoking has long been known to be a key causative agent of cardiovascular and pulmonary illnesses through its direct actions on various types of nicotinic receptors expressed in cardiac tissue, lungs, and blood vessels [16-18]. Smoking is also significantly associated with high mortality rates in infections of various respiratory viruses including those that underlie annual (seasonal) influenza [19,20]. Interaction between nicotine exposure, nicotinic receptor signaling, and modulation of the RAS has been recognized, yet remains understudied. In this case, however, smoking appears to participate in a *direct cellular process* that effects COVID-19 infection and possible outcome, in a mechanism involving the ability of the nicotinic receptor to regulate ACE2 protein expression in cells [21-23]. Smoking is also known to cause lung damage through the activation of inflammatory cytokines and programmed cell death in the pulmonary tissue and direct actions on circulating immune cells such as T cell [24]. Of particular interest, lung AT2 cells exposed to nicotine show altered expression of the ACE2 protein that may underlie enhanced exposure of the putative receptor to COVID-19 spike protein, and recent analysis of a large dataset from RNA-seq and DNA microarray supports the finding that smoking is associated with increased ACE2 expression in the lung [25]. Prolonged nicotine exposure systemically—through various kinds of smoking habits—may thus provide a cellular mechanism for viral susceptibility and illness severity during the course of the infection in the lungs as well as other organ systems (Figure 1).

Tobacco formulations are not just nicotine and often contain a varied mixture of >5000 chemicals, with potential carcinogenic, cardiovascular, and respiratory properties [26,27]. In addition to nicotine, cigarettes contain toxins such as carbon monoxide and polycyclic aromatic hydrocarbons, which also perturb the function of the cardiovascular, pulmonary, and

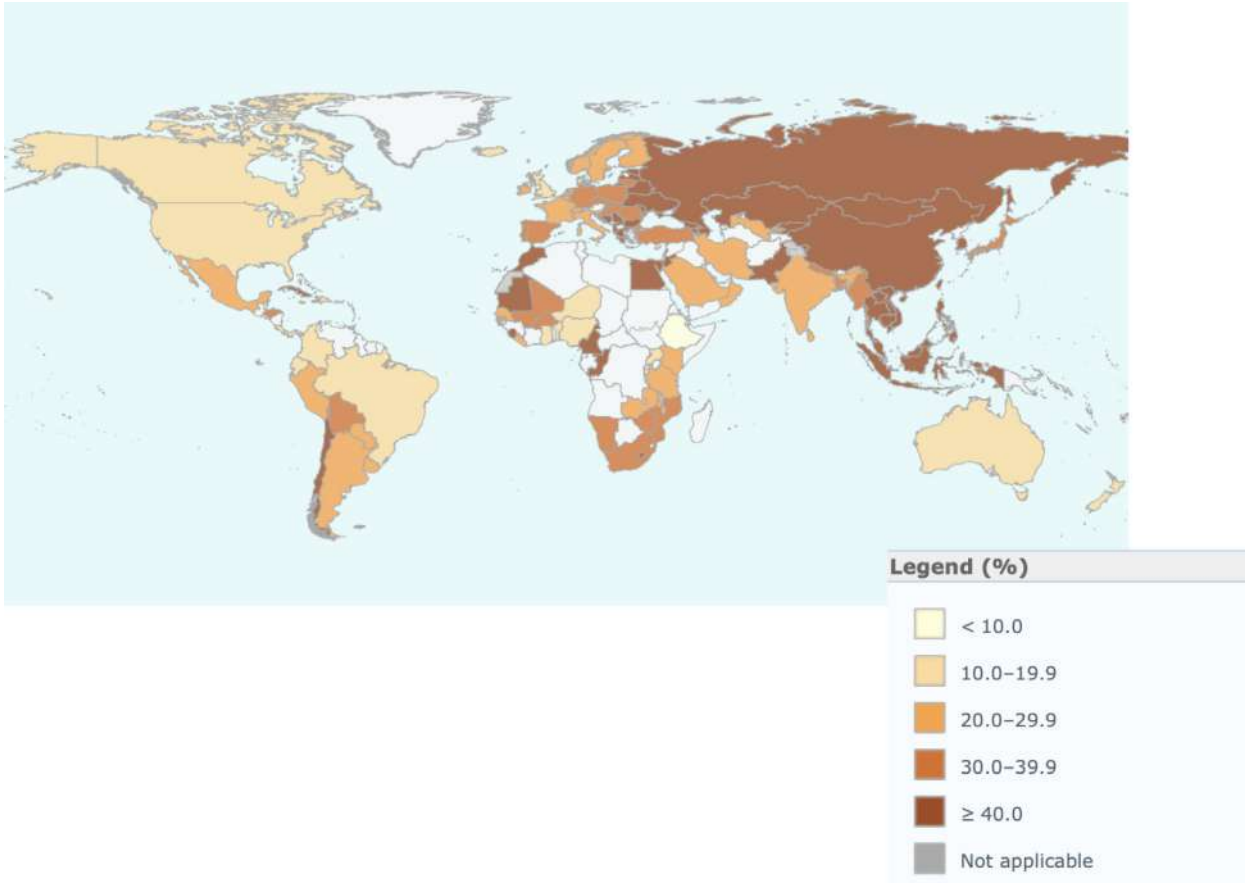

Fig. 2. World Health Organization data on global smoking prevalence (percentage of smokers of overall population) segmented by country. Note how China, South Korea, and Italy represent local 'hot spots' for smoking of nicotine cigarettes. These nations were also early epicenters for COVID-19. If our hypothesis is correct, nicotine consumption heterogeneities in Latin America and Africa predict future health challenges for at-risk populations as the epidemic proceeds.

immune systems, and at this point, such toxins may also contribute to COVID-19 disease outcome. Chronic nicotine use, systemically through cigarette and other tobacco products or indirectly possibly through second-hand smoke, now presents an important factor in COVID-19 vulnerability in various populations. Alarming increases in nicotine consumption and mortalities throughout Asia (Figure 2) and rises in the distribution of electronic cigarette (e-cig) formulations worldwide point to augmenting vulnerability to respiratory infecting viruses such as COVID-19 [28]. With more data rapidly emerging in various countries and locations, it will be important to critically consider evidence between virus-related infection and illness as well as resilience in relation to nicotine consumption. These findings aim to prioritize public health efforts of identifying populations at risk and aid in venues for critical intervention.

Summary

- Cigarette smoking or nicotine inhalation in volunteers correlates with an acute increase in systolic and diastolic blood pressure as well as increased plasma ACE activity.
- In many lung cells (including bronchial epithelial cells, alveolar macrophages, pulmonary endothelial cells, and interstitial fibroblasts), nicotinic receptors are co-expressed with most components of the RAS.
- ACE2-mediated activation of the JAK/STAT pathway drives epigenetic changes that underlie lung damage through inflammation and protease activation. These pathways are also activated by nicotinic receptors.
- Studies on the effects of e-cigs on RAS are inconclusive, and thus, it is not yet clear how the use of these devices will impact infection and prognosis in COVID-19 cases.
- Interaction sites between ACE2 and SARS-CoV have been identified at the atomic level and may provide a promising target site for antibodies or small molecules.

Conflict of interest

The authors declare no conflict of interest.

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Novel insights in the pathogenesis of renal interstitial damage during ACE inhibition

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CHAPTER 5

Tissue distribution of ACE2 protein, the functional receptor for SARS Coronavirus

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Abstract

Severe acute respiratory syndrome (SARS) is an acute infectious disease that spreads mainly via the respiratory route. A distinct coronavirus (SARS-CoV) has been identified as the etiological agent of SARS. Recently, a metallopeptidase named angiotensin converting enzyme 2 (ACE2) has been identified as the functional receptor for SARS-CoV. Although ACE2 mRNA is known to be present in virtually all organs, its protein expression is largely unknown. Since identifying the possible route of infection has major implications for understanding the pathogenesis and future treatment strategies for SARS, we investigated the localization of ACE2 protein in various human organs (oral and nasal mucosa, nasopharynx, lung, stomach, small intestine, colon, skin, lymph nodes, thymus, bone marrow, spleen, liver, kidney and brain).

The most remarkable finding is the surface expression of ACE2 protein on lung alveolar epithelial cells and enterocytes of the small intestine. Furthermore, ACE2 is present in arterial and venous endothelial cells and arterial smooth muscle cells in all studied organs.

In conclusion, ACE2 is abundantly present in epithelia of the lung and small intestine in humans, which might provide possible routes of entry for the SARS-CoV. This epithelial expression, together with the presence of ACE2 in vascular endothelium, also provides a first step in understanding the pathogenesis of the main SARS disease manifestations.

Introduction

Severe acute respiratory syndrome (SARS) is an acute infectious disease that spreads mainly via the respiratory route. Recently, a distinct coronavirus (SARS-CoV) has been identified as the etiological agent of SARS[1-4]. The spike proteins of this RNA virus associate with cellular receptors of sensitive cells, to mediate infection of their target cells after which it starts replicating in the cytoplasm. The main targets of SARS-CoV are the lungs, immune organs and systemic small vessels, resulting in systemic vasculitis, decreased immune function and respiratory distress caused by extensive pulmonary consolidation and diffuse alveolar damage with hyaline membrane formation[5], which causes death in 10% of infected individuals[6].

Recently, Li et al identified a metallopeptidase named angiotensin converting enzyme 2 (ACE2), isolated from SARS-CoV – permissive Vero-E6 cells, that effectively binds to the S1 domain of the SARS-CoV protein. ACE2 transfected 293T cells formed multinucleated syncytia with cells expressing S proteins. The virus was shown to replicate effectively in ACE2-transfected, but not in mock-transfected 293T cells. ACE2 antibodies, but not ACE1 antibodies, blocked the viral replication on Vero E6 cells[7]. These data indicated convincingly that ACE2 is a functional receptor for SARS-CoV.

Although real-time PCR revealed that ACE2 messenger RNA expression is present in 72 human tissues[8], ACE2 protein expression has thus far been identified only in heart, kidney and testis[9-12]. Since identifying the possible route of infection has major implications for understanding the pathogenesis and future treatment options for SARS, we investigated the immunolocalization of ACE2 protein in various human organs.

Methods

Human tissue specimen

Human tissues from 15 different organs were obtained from patients undergoing biopsy procedures for diagnostic purposes or surgery for various reasons, predominantly cancer. Additional tissue was obtained from unused donor organs (because of technical reasons; often in case unilateral transplantation with lack of an adequate acceptor for the other lung). Extensive specification of the diagnosis is given for the lung and small intestine only (see below). Brain tissue was obtained from autopsies. Tissues were chosen to represent organ systems were the SARS virus has been detected in human[13] and in experimentally infected macaques[3]. Routine morphology was evaluated by hematoxylin and eosin by a qualified pathologist. Tissues were only used if characterized as non-diseased. Tissues were investigated from 93 different subjects: lung (cancer n=4, unused donorlung n=5, alpha 1 antitrypsin deficiency n=1); skin (n=6); oral mucosa (n=4); nasal mucosa (n=5); nasopharynx (n=6), gastric cardia and corpus (n=9); different parts of the small intestine: duodenum (cancer n=2, ulcus n=2), jejunum (chronic inflammation n=1, atresia n=1, cancer n=1, resection ileostoma n=1) and ileum (resection ileostoma n=1, chronic inflammation n=1, metastatic cancer n=3, primary cancer n=1, M.Hirschsprung n=1,

angiodysplasia n=1); colon (n=5); spleen (n=4); thymus (n=4); lymph nodes (n=6); bone marrow (n=5); liver (n=6); kidney (n=4); and brain (n=3). All procedures and use of (anonymized) tissue were performed according to recent national guidelines.
The lung type II alveolar epithelial cell line A 549 and fibrotic lung tissue from patients (n=4) with usual interstitial pneumonia were used to confirm the findings on type II pneumocytes.

Immunohistochemistry and ACE2 localization

Tissues were deparaffinized, rehydrated and subjected to heat induced antigen retrieval by overnight incubation in 0.1 M Tris/HCl buffer pH 9 at 80°C. Endogenous peroxidase was blocked with 0.075% H_2O_2 in phosphate-buffered saline (PBS, pH 7.4) for 30 minutes. Cytospin prepreations from A549 cells were fixed in PBS buffered paraformaldehyde (2%) at 4ºC for 10 minutes. Subsequently, they were dried and stained for ACE2. A polyclonal rabbit anti-ACE2 antiserum (Millenium Pharmaceuticals, Inc, Cambridge, MA)[10] diluted in PBS and supplemented with 1% bovine serum albumin was used in a concentration of 1:1000 for 1 hr at room temperature. Antibody binding was detected using sequential incubations with peroxidase-labeled goat anti-rabbit and peroxidase-labeled rabbit anti-goat antibodies (GARPO/RAGPO Dako, Glostrup, Denmark). Human AB serum (1%) was added to the secondary antibodies. Peroxidase activity was developed by using 3,3-diaminobenzidine tetrachloride (DAB) for 10 minutes. Counterstaining was performed using Mayer's hematoxylin. Three types of control tests were performed to determine the specificity of the antibody. First, control sections were incubated with anti-ACE2 antibody solutions, which were pre-incubated with the synthetic peptide to which the antibody was raised (peptide sequence: NTNITEENVQNMNNAGDKW aa51-69, Pepscan Systems BV, Lelystad, The Netherlands). Second, sections were incubated with unrelated rabbit polyclonal antibodies (anti-alpha 1 Inhibitor 3 or anti-nitrotyrosine) and third, sections were incubated with PBS in the absence of the primary antibodies. These control sections did not reveal any staining (Figure 1F and 2F). A qualified pathologist analyzed the staining for structures positive for ACE2.

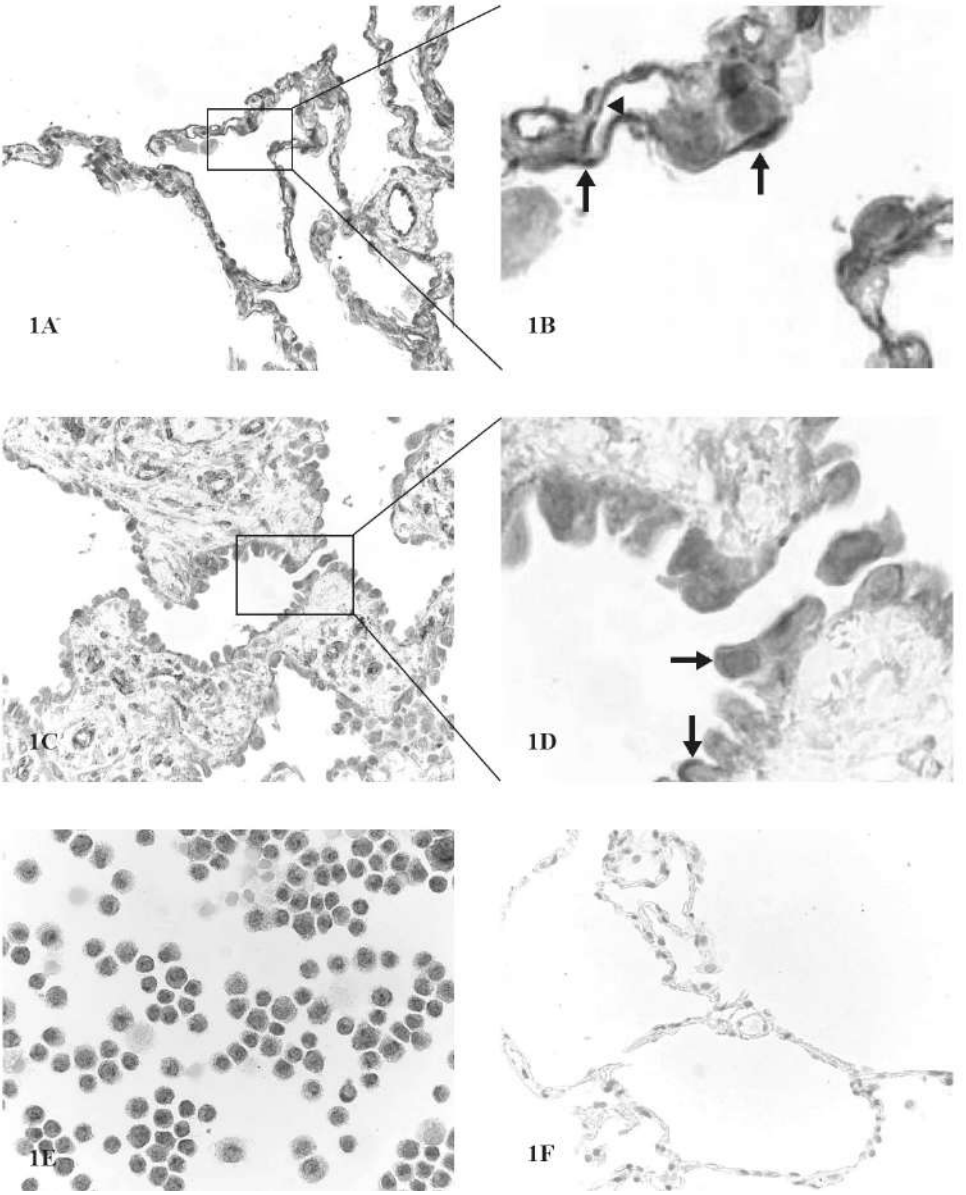

Figure 1. *Normal lung tissue on overview* ***(A)*** *and larger magnification* ***(B)****. Positive staining for ACE2 is clearly present on alveolar epithelial cells (arrow) and capillairy endothelium (arrow-head). Fibrotic lung tissue* ***(C)*** *and a larger magnification* ***(D)****. Positive staining for ACE2 is clearly present on type II cells (arrow). Cultured lung type II alveolar epithelial cells (A549) stain strongly positive for ACE2* ***(E)****. Control section stained with anti-ACE2 in the presence of the synthetic ACE2 peptide shows negative staining in lung tissue* ***(F)****.*

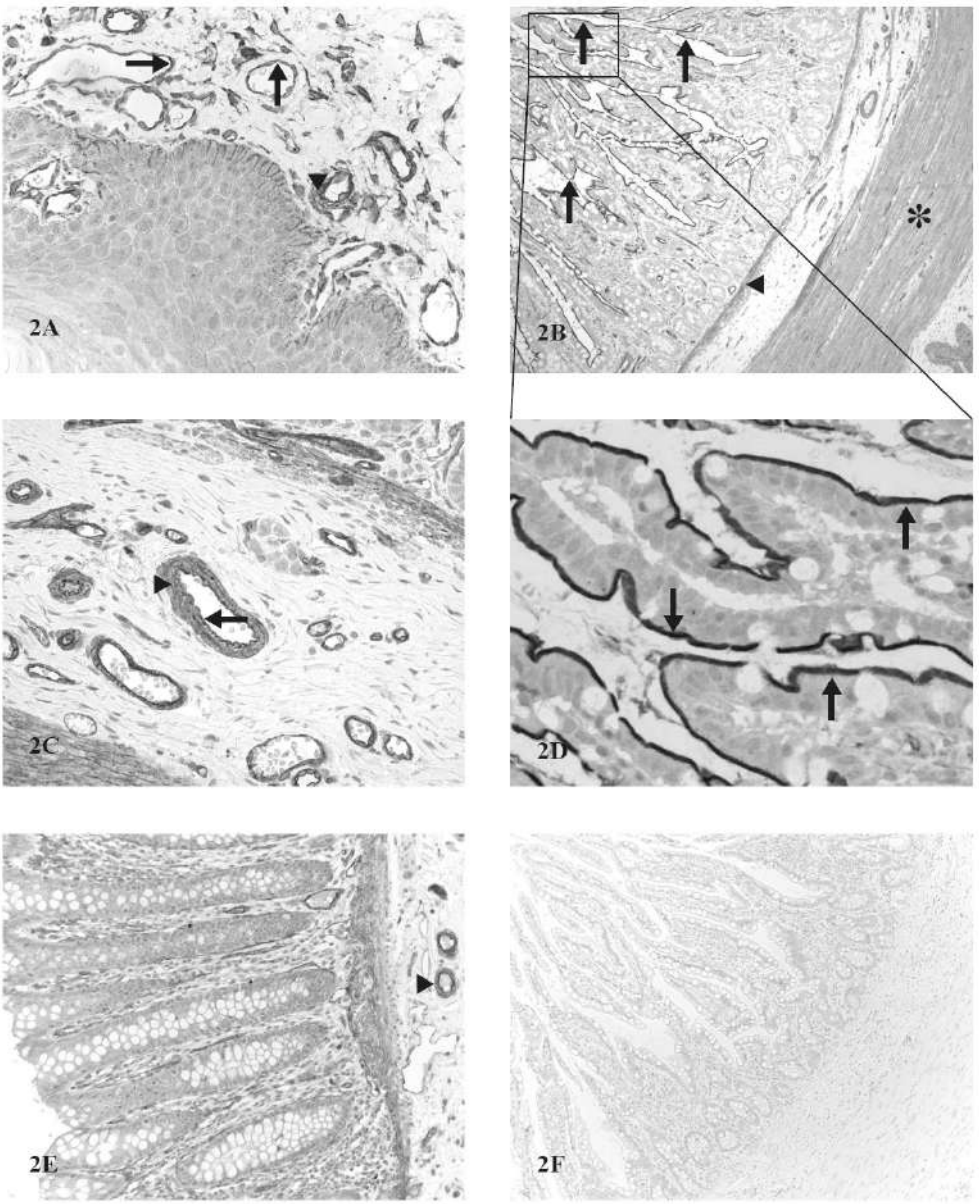

Figure 2. *Oral mucosa on overview* ***(A)****. Strong staining is observed in vascular endothelium (arrow) and vascular smooth muscle cells (arrow-head). Granular ACE2 staining is present in the basal layer of the epithelium. In the small intestine (ileum)* ***(B)*** *strong staining can be seen in the villous brush border (arrow), the muscularis mucosae (arrow-head) and the muscularis propria (star). In a larger magnification of the submucosa* ***(C)****, strong staining is present in vascular endothelium (arrow) and vascular smooth muscle cells (arrow-head). In a larger magnification of the villi* ***(D)****, abundant staining is seen on the brush border of the enterocytes (arrow). In the colon* ***(E)****, ACE2 staining is present in endothelium and vascular smooth muscle cells from the blood vessels (arrow-head) and in the muscular layers. Control section stained with anti-ACE2 in the presence of the synthetic ACE2 peptide shows no staining in the small intestine (ileum)* ***(F)****.*

Results

The mean age of patients (n=93) was 52 ± 22 year and the male-to-female ratio was 50/43. The ACE2 staining pattern was consistent in the same type of tissues regardless of the pathological condition of the organ and disease status of the patient.

The first remarkable finding was that ACE2 is present in endothelial cells from small and large arteries and veins in all studied tissues. Moreover, arterial smooth muscle cells were consistently positive for ACE2. Positive staining for ACE2 was also noted in myofibroblasts and the membrane of fat cells in various organs. Furthermore, ACE2 was found at specific sites in each organ as described below.

Marked ACE2 immunostaining was found in type I and type II alveolar epithelial cells in normal lungs (Figure 1A and B). This finding was confirmed by ACE2 expression in the lung type II alveolar epithelial cell-line A549 (Figure 1E) and by lungs with fibrotic changes which revealed abundant staining of type II epithelial cells (Figure 1C and D). Cytoplasm of bronchial epithelial cells showed also weak positive ACE2 staining.

In nasal and oral mucosa and the nasopharynx, we found ACE2 expression in the basal layer of the non-keratinizing squamous epithelium (Figure 2A).

Beside ACE2 localization in the smooth muscle cells and endothelium of the vessels from stomach, small intestine, and colon we found ACE2 in smooth muscle cells of the muscularis mucosae and the muscularis propria (Figure 2B, C, E). Remarkably, ACE2 was abundantly present in the enterocytes of all parts of the small intestine including duodenum, jejunum and ileum, but not in enterocytes of the colon. The staining in enterocytes was confined to the brush border (Figure 2B and D).

In the skin, ACE2 is present in the basal cell layer of the epidermis extending to the basal cell layer of hair follicles (Figure 3A, C and D). Smooth muscle cells surrounding the sebaceous glands were also positive for ACE2. Weak cytoplasmic staining was observed in sebaceous gland cells. Strong granular staining pattern for ACE2 was seen in cells of the eccrine glands (Figure 3B).

Consistent with findings in other organs, the brain only revealed endothelial and smooth muscle cell staining (Figure 4A). Despite the clear endothelial staining of many small vessels, the endothelial lining of the sinusoids in the liver was negative for ACE2. Surface staining in bile ducts was occasionally observed. Kupffer cells and hepatocytes were negative (Figure 4B).

In the spleen, thymus, lymph nodes, and bone marrow, cells of the immune system such as B and T lymphocytes, and macrophages were consistently negative for ACE2 (Figure 4C). In some lymph nodes, we noted positive staining in sinus endothelial cells in a granular staining pattern.

In the kidney, weak glomerular visceral ACE2 staining was observed, whereas the parietal epithelial cells were moderately positive. Despite the clear endothelial staining of vessels, the mesangium and glomerular endothelium were negative for ACE2. Abundant staining was seen in the brush border of the proximal tubular cells, whereas the cytoplasm of these cells was weakly

positive. Epithelial cells from the distal tubules and collecting ducts showed weak cytoplasmic staining (Figure 4D).

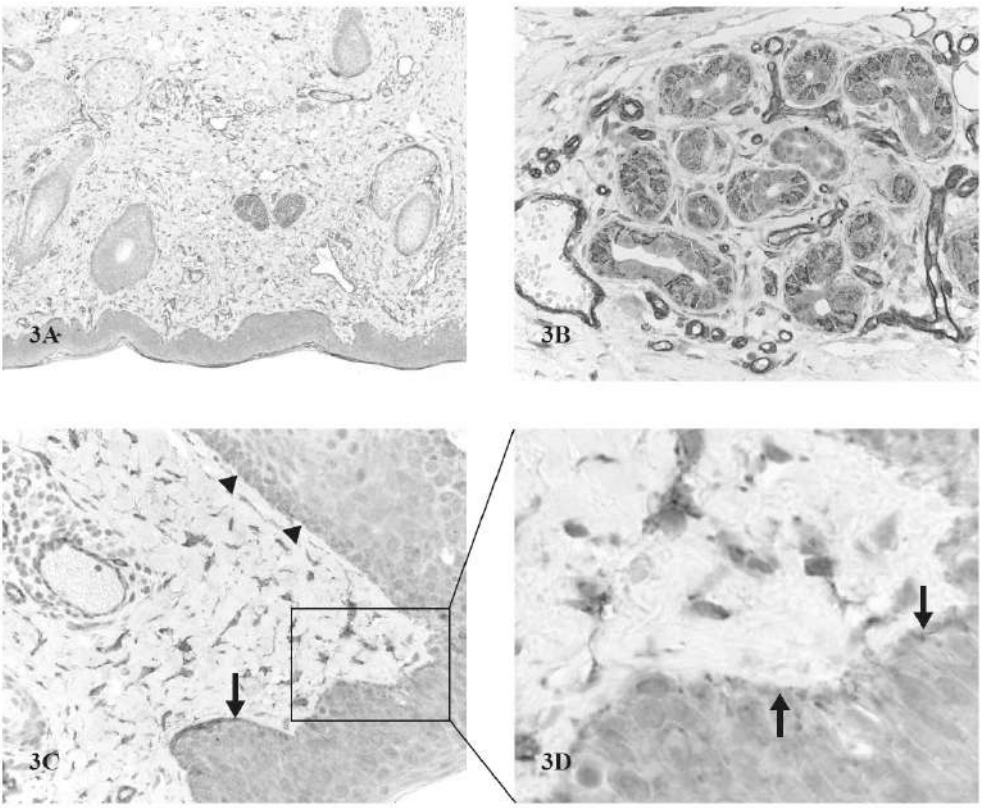

Figure 3. *Skin tissue* ***(A)*** *with larger magnification* ***(C and D).*** *Staining is abundantly present in blood vessels/ capillaries and in the basal layer of epidermis of the skin (arrow) and hair follicles (arrow-head). Eccrine glands are also positive for ACE2* ***(B)****.*

Discussion

In the present paper we report the immunolocalization of Angiotensin-converting enzyme 2 (ACE2), the functional receptor for SARS-CoV, in human tissues. The most remarkable finding is the surface expression of ACE2 protein on lung alveolar epithelial cells and enterocytes of the small intestine, i.e. cells in contact with the external environment. Furthermore, ACE2 is present in arterial and venous endothelial cells and arterial smooth muscle cells in all studied organs. These data are consistent with previous findings that low levels of ACE2 mRNA are found in many tissues and that ACE2 mRNA is highly expressed in renal, cardiovascular and gastrointestinal tissues[8,10,12].

The physiological role of ACE2 in most tissues has not been elucidated, although ACE2 is thought

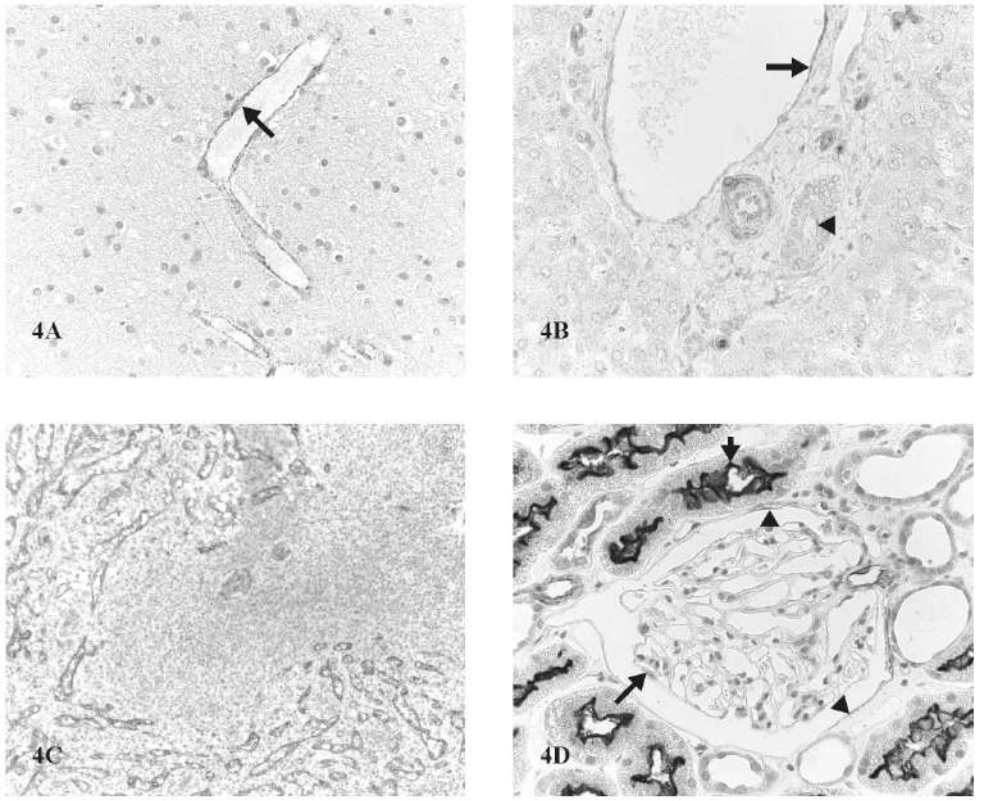

Figure 4. *In the brain* ***(A)****, ACE2 is expressed only in endothelium (arrow) and smooth muscle cells of the vessels. In the liver* ***(B)*** *Kupffer cells, hepatocytes and the endothelium of sinusoids were negative. Surface staining in bile ducts was occasionally observed (arrow-head). Vascular endothelium (arrow) and smooth muscle cells were positive. In the spleen* ***(C)*** *ACE2 was not expressed in cells of the immune system. Vascular- and red pulp sinus endothelium was positive. In the kidney* ***(D)*** *ACE2 is present in visceral (arrow) and parietal (arrow-head) epithelium, in the brush border (short arrow) and cytoplasm of proximal tubular cells and in cytoplasm of distal tubules and collecting ducts.*

to be an essential regulator of cardiac function and blood pressure control[9], possibly by acting as a natural counterpart of ACE1[14]. ACE2 has recently been identified as the functional receptor for SARS-CoV[7]. Li et al showed that ACE2 can be immunoprecipitated by the S1 domain of the SARS-CoV virus and that ACE2 can promote viral replication. The demonstration of ACE2 expression in human organs can potentially identify the possible routes of infection for SARS-CoV, and possible routes of spread and replication throughout the body.

SARS is mainly a lower respiratory tract disease, causing pulmonary lesions and respiratory distress[5]. Furthermore, SARS-CoV is spread via the respiratory tract. Recent studies in autopsy series using viral isolation, culture techniques and *in-situ* hybridization showed that SARS-CoV is present in pneumocytes[13]. Transmission electron microscopy revealed presence of coronavirus-like particles and viral inclusion bodies in pneumocytes. We found that type I and type II

pneumocytes are markedly positive for ACE2 and that bronchial epithelial cells only show weak staining. The type II alveolar epithelial cell line A549 confirmed the presence of ACE2 protein in type II pneumocytes. This data, combined with the fact that ACE2 is the functional receptor for SARS-CoV, indicates that alveolar pneumocytes in the lung are a possible site of entrance for SARS-CoV. Furthermore, this expression pattern provides a possible explanation for the pathologic lung manifestations and its rapid progression. Initial viral entrance may cause cytopathological changes at the epithelial alveolo-capillary interface, initially resulting in induction of type II alveolar cells as a first attempt to repair. In case of SARS, the abundant expression of ACE2 in type II alveolar cells may cause a base for rapid viral expansion and a vicious circle of local alveolar wall destruction, resulting in rapidly progressive severe diffuse alveolar damage.

Upper respiratory tract symptoms occur in the minority of SARS patients and SARS-CoV RNA can be detected in nasopharyngeal aspirates[15]. However, tissues of the upper respiratory tract, like oral and nasal mucosa and nasopharynx did not show ACE2 expression on the surface of epithelial suggesting that these tissues are not the primary site of entrance for SARS-CoV. The upper respiratory tract symptoms cannot be explained by our findings, but patients with SARS might be susceptible for secondary infections[16]. Moreover, SARS-CoV RNA detected in nasopharyngeal aspirates might be derived from infected lower respiratory tract.

Extrapulmonary manifestations of SARS-CoV infection like gastrointestinal symptoms have been reported and include watery diarrhoea[15,17,18,18,19]. Using *in-situ* hybridization, To et al. found SARS-CoV in the surface of small intestine enterocytes[13]. Active viral replication in the enterocytes of the small intestine has been reported by Leung et al[19] and SARS-CoV RNA can be detected in stool of patients[15,17,19]. We showed that ACE2 protein is abundantly expressed in the brush border of enterocytes of all parts of the small intestine, including duodenum, jejenum and ileum. Surprisingly, other organs of the digestive tract as stomach and colon did not show this brush border staining. The presence of ACE2 as a functional receptor for SARS-CoV and the presence of SARS-CoV in enterocytes of the small intestine, combined with the fact that virus is present in stool of patients is consistent with the possibility of oral-faecal transmission.

In addition to pulmonary and gastrointestinal problems, SARS-CoV infection also causes massive necrosis of the spleen and lymph nodes. Furthermore, most patients develop lymphopenia[20] which, in analogy with respiratory syncytial virus disease, measles and sepsis has been ascribed to increased apoptosis of lymphocytes[21]. The consistent absence of ACE2 in immune cells in all haemato-lymphoid organs suggests that direct viral infection is unlikely to be the cause of these manifestations and that the pathological changes seen in these organs are probably related to the systemic effects of the abnormal immune reactions towards the virus.

Other SARS-CoV related manifestations include systemic vasculitis, apoptosis and swelling of endothelial cells and inflammation in various organs like heart, kidney, liver and adrenal glands[5]. The abundant expression of ACE2 on endothelia and smooth muscle cells in virtually all organs

suggests that the SARS-CoV, once present in the circulation, can spread easily through the body. The absence, however, of SARS-CoV in these organs as shown by in situ hybridization studies[13] is at variance with this assumption. The vascular abnormalities and inflammatory changes in various organs might therefore be related to systemic toxic effects of the immune reactions elicited by SARS-CoV infection.

It is remarkable that despite the presence of ACE2 in endothelia of all organs and SARS-CoV in blood plasma of infected individuals, so few organs become virus positive. This may imply that, in analogy with HIV infection, where the current general model of viral entry requires not only binding of the viral envelope to a cell surface receptor (CD4), but also to a chemokine co-receptor [CXCR4 or CCR5(BBA)][22], SARS-CoV also needs the presence of a co-receptor for cellular entry. Future studies have to elucidate whether SARS-CoV binding to a co-receptor in addition to ACE2 might be involved in the specific infection of lung and small intestine .

In conclusion, ACE2 is abundantly present in epithelia of the lung and small intestine in humans, which might provide possible routes of entry for the SARS-CoV. This epithelial expression, together with the presence in vascular endothelium, also provides a first step in understanding the pathogenesis of the main SARS disease manifestation, in particular in the lung. Whether the abundant expression in the vascular systems may also serve as a route of spread and replication, should be further investigated in functional studies applying blockade of the ACE2 protein.

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Tobacco-use disparity in gene expression of ACE2, the receptor of 2019-nCov

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Abstract

In current severe global emergency situation of 2019-nCov outbreak, it is imperative to identify vulnerable and susceptible groups for effective protection and care. Recently, studies found that 2019-nCov and SARS-nCov share the same receptor, ACE2. In this study, we analyzed four large-scale datasets of normal lung tissue to investigate the disparities related to race, age, gender and smoking status in ACE2 gene expression. No significant disparities in ACE2 gene expression were found between racial groups (Asian vs Caucasian), age groups (>60 vs <60) or gender groups (male vs female). However, we observed significantly higher ACE2 gene expression in smoker samples compared to non-smoker samples. This indicates the smokers may be more susceptible to 2019-nCov and thus smoking history should be considered in identifying susceptible population and standardizing treatment regimen.

Key words

Wuhan 2019-nCov, ACE2, expression, susceptibility, race, age, gender, smoking

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Introduction

In the past two decades, pathogenic coronaviruses (CoVs) have cause pandemic infections, including the server acute respiratory syndrome (SARS)-CoV outbreak in 2003, the Middle East respiratory syndrome (MERS) outbreak in 2012 and the current novel Wuhan 2019-nCov outbreak. We have learned from SARS-Cov and MERS-Cov that human populations showed disparities in susceptibility to these viruses. For example, epidemiology studies found that males had higher incidence and mortality rates than females.[1,2] We believe that the susceptibility to the novel 2019-nCov is also different among population groups. In current severe global emergency situation of 2019-nCov outbreak, it is imperative to identify vulnerable and susceptible groups for effective protection and care.

Recently, Xu et.al. computationally modelled protein interactions and identified a putative cell entry receptor of 2019-nCov, angiotensin-converting enzyme 2 (ACE2), which is also a receptor for SARS-nCov.[3] Zhou et.al. further confirmed this virus receptor in the HELA cell line.[4] Interestingly, Zhao et al. found ACE2 is specifically expressed in a subset of type II alveolar cells (AT2), in which genes regulating viral reproduction and transmission are highly expressed.[5] They also found that an Asian male has much higher ACE2-expressing cell ratio than other seven white and African American donors, which may indicate the higher susceptibility of Asian. However, the sample size was too small to draw conclusion on this racial disparity. Here, we analyzed four large-scale datasets of normal lung tissue to investigate the disparities related to race, age, gender and smoking status in ACE2 gene expression.

Methods

Two RNA-seq datasets and two DNA microarray datasets from lung cancer patients were analyzed in this study, including a Caucasian RNA-seq dataset from TCGA (https://www.cancer.gov/tcga), an Asian RNA-seq dataset from Gene Expression Omnibus (GEO) with the accession number GSE40419[6], an Asian microarray dataset from GEO with the accession number GSE19804[7] and a Caucasian microarray dataset from GEO with the accession number GSE10072[8]. Both RNA-seq datasets were generated with the Illumina

HiSeq platform and both microarray datasets were generated with the Affymetrix GeneChip Human Genome U133 Array. The details and processing of data were described in our previous study[9]. All datasets contain samples from tumor and normal pairs and we only use the normal samples in this study. In total, 54 samples in the TCGA dataset, 77 samples in the GSE40419 dataset, 60 samples in the GSE19804 dataset and 33 samples in the GSE10072 dataset were analyzed. We studied the Reads per kilobase per million mapped reads (RPKM) values for RNA-seq data and Robust Multi-Array Average (RMA)[10] values for microarray data. All data were log2 transformed to improve normality. The data means across samples in each dataset from the same platform were highly correlated (Pearson correlation coefficient r=0.9 for microarray datasets and r=0.97 for RNA-seq datasets, Fig. S1), indicating no significant system variation in datasets from the same platform.

Simple linear regressions were used to test the association of ACE2 gene expression with each single variable of age, gender, race and smoking status. Also, multiple linear regression was used to test the association of ACE2 expression with multiple factors (age, gender, race, smoking status and data platform). All data management, statistical analyses and visualizations were accomplished using R 3.6.1.

Results

Racial Disparity

Inconsistent with the study of Zhao et al.[5], we observed no significant difference in ACE2 expression in Caucasian lung tissue samples compared to Asian lung tissue samples in the RNAseq datasets (*p*-value=0.45, Fig 1A). In the microarray datasets, a higher ACE2 expression was observed in Caucasian samples compared to Asian samples (*p*-value=0.03, Fig 1A). Given that the GSE19804 RNA-seq study focused on female non-smokers while the TCGA dataset includes samples from both males and females and both smokers and non-smokers, we believe that the observed disparity may be due to other factors other than race, such as smoking, gender and unknown factors. Therefore, we performed multiple linear

regression on multiple independent variables (age, gender, race, smoking status and platform) and found no significant difference between racial groups (*p*-value=0.36, Fig. 1E).

Tobacco-related disparity

We found a significant higher ACE2 gene expression in smoker samples compared to non-smoker samples in the TCGA (*p*-value=0.05) and GSE40419 datasets (*p*-value=0.008, Fig. 1B). Smokers in GSE10072 showed a higher mean of ACE2 gene expression than non-smokers. The difference is not significant (*p*-value=0.18), which may be due to its small sample size (n=33) with insufficient power to detect the difference. The GSE19804 data with only smoker samples available was not included into the analysis. Adjusted by other factors (age, gender, race and platforms) in multivariate analysis, smoking still shows a significant disparity in ACE2 gene expression (*p*-value=0.008, Fig. 1E).

Age and Gender

We didn't observe a disparity between age groups (>60 vs <60) or gender groups (male vs female) in ACE2 gene expression in each available study (Fig. 1C, D). Consistently, multivariate analysis didn't reject the null hypothesis that there is no difference between groups of age or gender after other variables (age/gender, race, smoking status and platforms) were adjusted (*p*-value=0.90 for age, *p*-value=0.35 for gender, Fig. 1E). We also consistently found no difference between male and female healthy lung tissue samples from GTEx[12] (Fig. S2).

Discussion

In this study, we investigated the disparities related to race, age, gender and smoking status in ACE2 gene expression and found significantly higher ACE2 gene expression in lung tissue of smokers compared to that of non-smokers. This may explain the reason why more males (56% of 425 cases) were found in a recent epidemiology report of 2019-nCov early transmission by China CDC[11]. We didn't observe significant disparities in ACE2 gene expression between racial groups (Asian vs Caucasian), age groups (>60 vs <60) or gender groups (male vs female).

This study has several limitations. First, the data analysed in this study were from the normal lung tissue of patients with lung adenocarcinoma, which may be different with the lung tissue of healthy people. Although we observed no difference between male and female healthy samples from GTEx, further validation studies are required for other factors. Second, our analysis was based on the average expression from bulk tissue. This may lead to a power loss in detecting the expression from particular cell types such as the AT2 cells in which ACE2 are specifically highly expressed.

Whether ACE2 is the only or major receptor of 2019-nCov is unknown. The reason(s) for the tobacco-related disparity in ACE2 expression is unknown. Despites current limited knowledge, this study indicates the smokers may be more susceptible to 2019-nCov and thus smoking history should be considered in identifying susceptible population and standardizing treatment regimen. Wuhan, stay strong.

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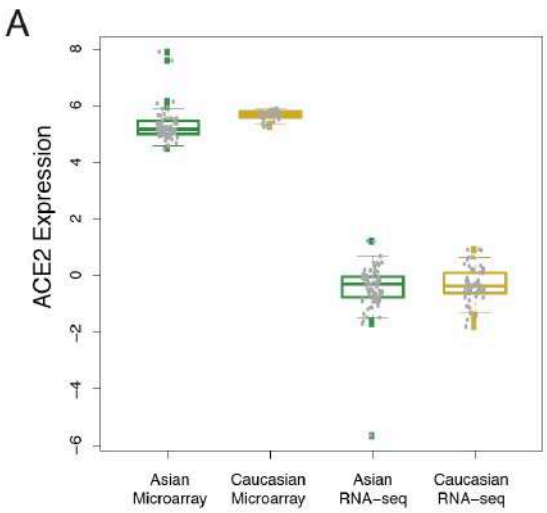
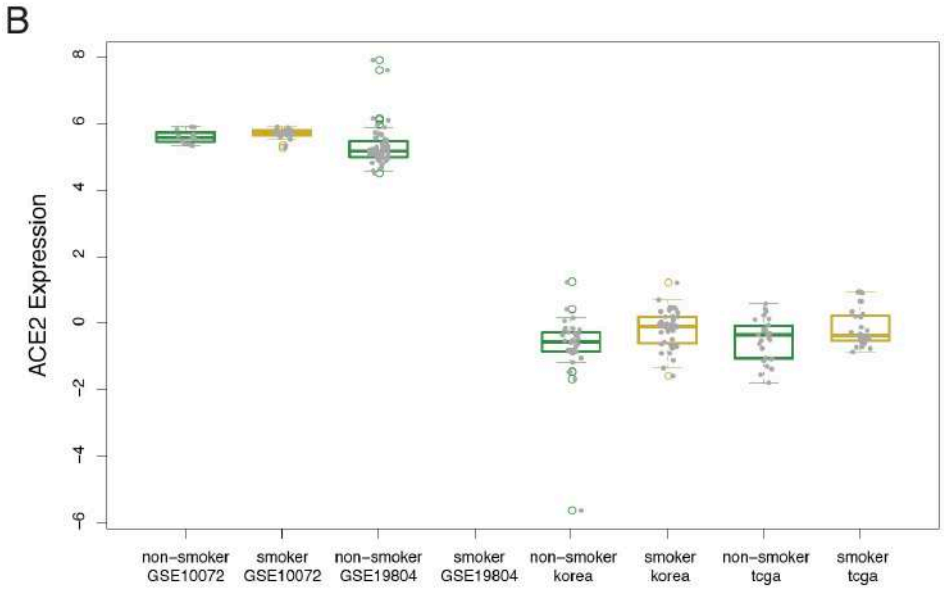
Figure



E

Variable	Beta	P-value
Platform: RNA-seq	-5.940	<2e-16
Race: Caucasian	0.088	0.359
Age: >60	-0.012	0.900
Gender: Male	0.105	0.352
Smoking: Smoker	0.313	**0.008**



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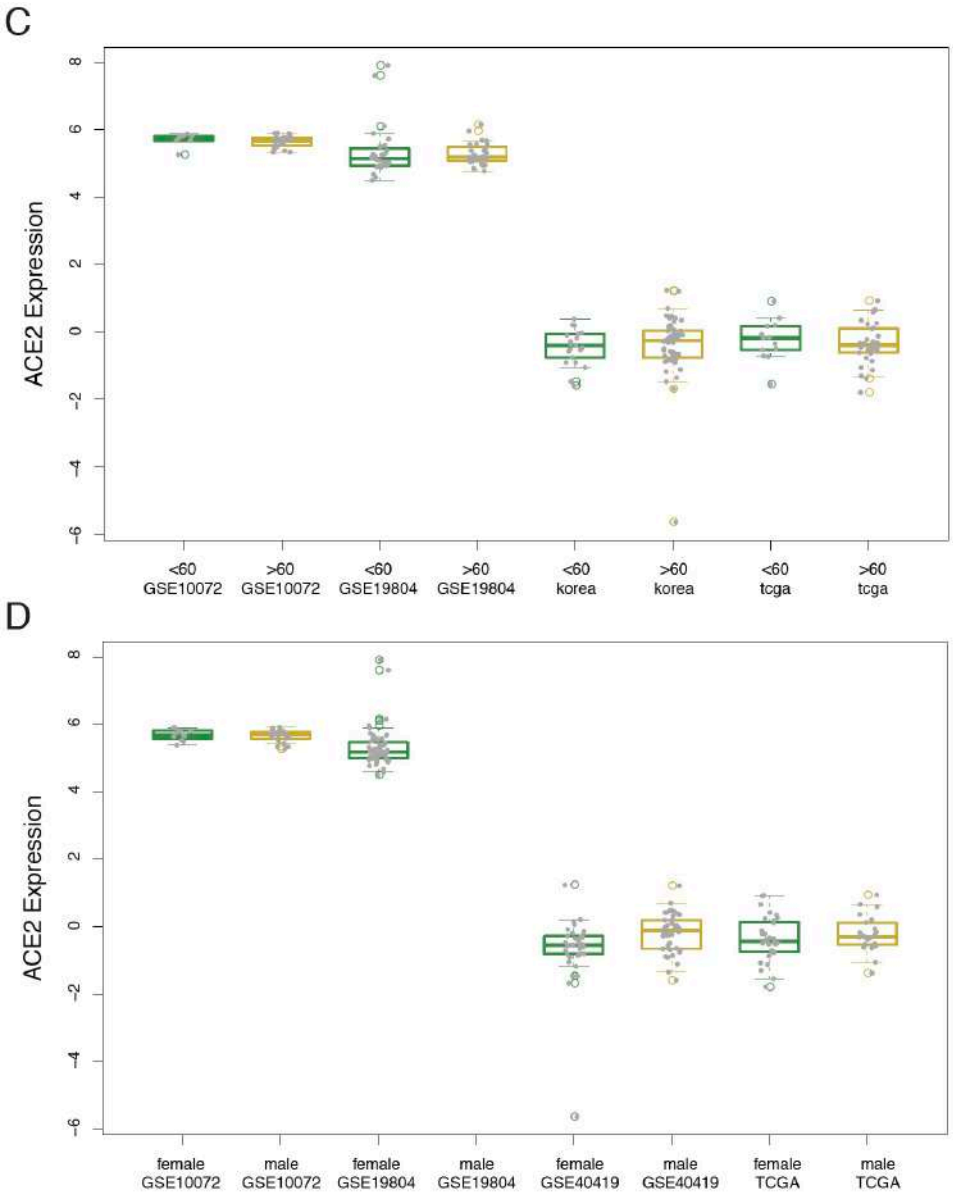

Figure 1. ACE2 gene expression profiling in groups.
A-D shows groups in race (Caucasian vs Asian), smoking (smoker vs non-smoker), age (>60 vs <60) and gender (male vs female). E shows the result from multivariate analysis with all factors including age, gender, race, smoking and platforms.

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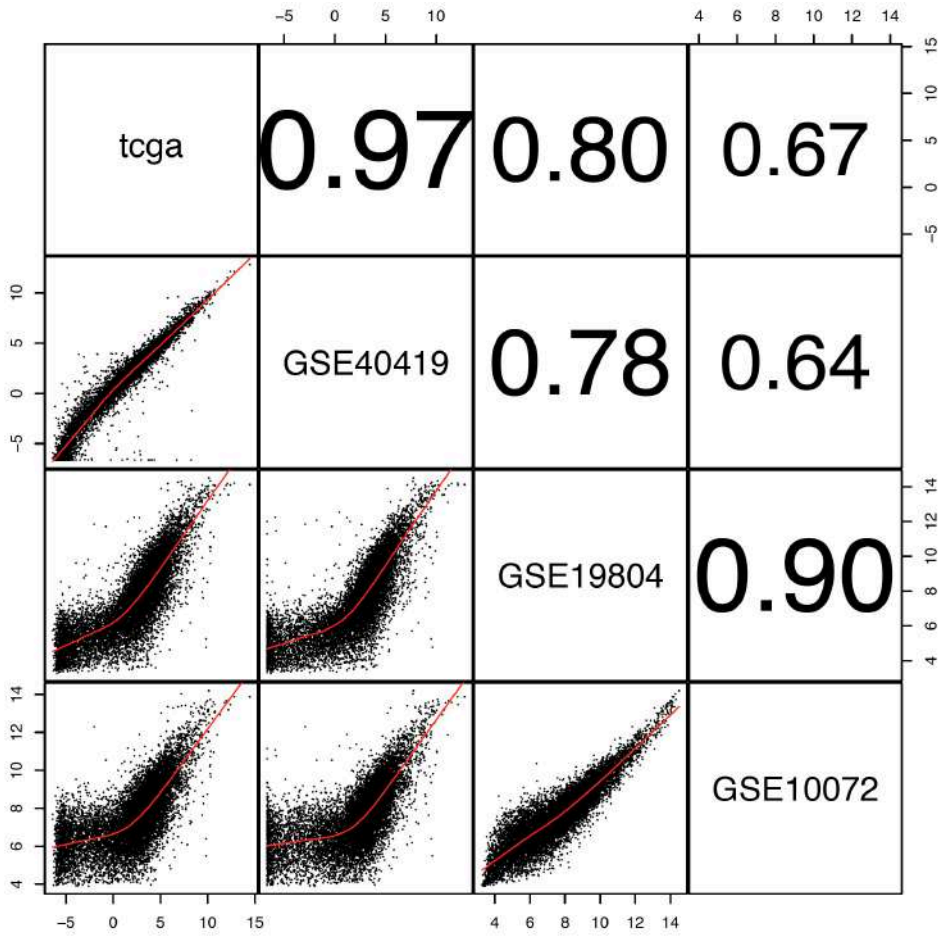

Figure S1. Correlation of four datasets.
Lower panel shows pairwise scatter plots of data mean across samples in each dataset.

Upper panel shows their corresponding Pearson correlation coefficients.

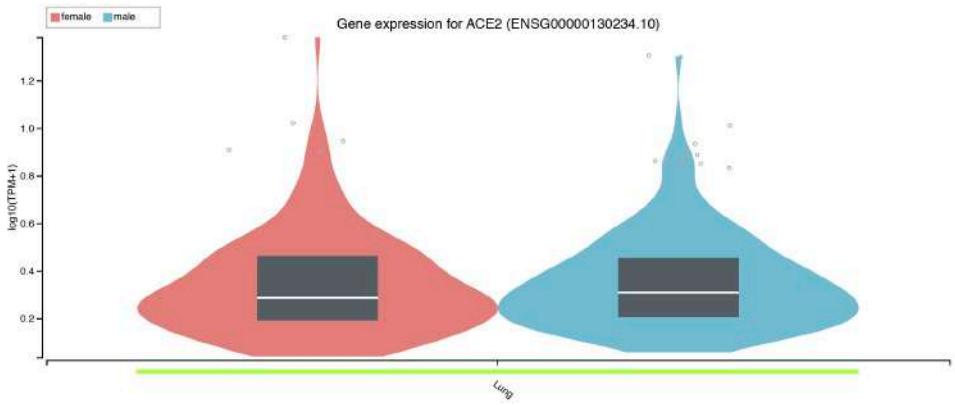

Figure S2. ACE2 gene expression in GTEx female and male lung tissues.
y-axix shows the log10 scaled RNA-seq Transcript Per Million (TPM) values.

COVID-19, propelled by smoking, could destroy entire nations

ersnet.org/covid-19-blog/covid-19--propelled-by-smoking--could-destroy-entire-nations

Blog Author(s): Kathryn Barnsley [1] and Sukhwinder Singh Sohal [2] / 30 March, 2020

Smoking is a risk factor for many diseases. COVID-19 is in a whole new class of its own (1). Many countries are just starting to come to terms with the fact that smokers are 14 times more likely to die from COVID-19 (2, 3), but few have issued warnings to quit smoking (4) and to our knowledge, no country has ramped up its tobacco control prevention measures. In the face of this pandemic we know of no country that has widely distributed free nicotine replacement therapy or cessation support drugs, yet many countries have implemented “economic support packages”, and less developed nations face the threat of disaster (4, 5).

However, in its seventh update on COVID-19, the European Centre for Disease Prevention and Control (ECDC) recently suggested that possible preventable determinants of severe COVID-19 such as smoking and medications should be identified, as they may contribute to an increase in the number of severe cases and thus impact hospital capacity (6).

It is ironic that the World Health Organization (WHO) Framework Convention on Tobacco Control (FCTC) Secretariat is part of the same WHO currently leading action on COVID-19, yet we have not yet heard any of the WHO COVID-19 leadership team urge countries to adopt the FCTC to ramp up action on tobacco control and to urge smokers to quit. The WHO coronavirus website mentions smoking, but not as one of the things making it more likely for people to become severely ill (7, 8), yet it refers to pre-existing conditions (9). Two of the countries which failed to ratify the FCTC, the USA and Indonesia, are recording high death rates (10). Furthermore, the FCTC has a strategy for 2020 that has apparently not been mentioned as a priority by the WHO coronavirus leadership team (11).

There is an abundance of evidence regarding the vulnerability of smokers and those with COPD to contract influenza, tuberculosis and other lung conditions (12-16). References in WHO and country COVID-19 literature for members of the public and the media refer elliptically to “pre-existing conditions”, without expanding on this adequately, nor clarifying the causal link to smoking, including cardiovascular disease, cancer, chronic obstructive pulmonary disease (COPD) and diabetes. Hopefully, this will be updated soon.

We found, as some others recently noted, that angiotensin-converting enzyme-2 (ACE2) receptor increases in the lungs of smokers and patients with COPD, a potential therapeutic target for COVID-19 and the importance of smoking cessation. We reported that ACE2 is upregulated in the lungs of smokers and patients with COPD (17). In this

early report, we found that ACE2 receptor is upregulated in small airway epithelium including brush borders, type-2 pneumocytes and alveolar macrophages (17). The expression was more in patients with COPD compared to normal lung function smokers, and none or little in never smokers, which shows that smoking upregulates ACE2 expression and having COPD further exaggerates it, hence more susceptibility for COVID-19 in this population.

The histopathology suggested that there are multiple binding sites, and not just the small airway epithelium. This is the first early immunohistochemical evidence of ACE2 receptor in the tissue from smokers and patients with COPD (17). This has been recently confirmed by JM Leung and colleagues in a very elegant study with a larger cohort, reporting ACE2 gene and protein expression increases in the airway epithelium obtained from cytologic brushings of sixth to eighth generation airways in individuals with and without COPD (18). The authors also reported that there was a significant inverse relationship between ACE2 gene expression and FEV1% of predicted, indicating implications for lung function decline in this situation (18).

Guoshuai Cai reported higher ACE2 gene expression in smokers compared to never-smokers (19). Zhao et al. observed that ACE2 is expressed explicitly in type-2 pneumocytes, in which genes regulating viral reproduction and transmission are highly expressed, similar to what we found in the tissue from smokers and patients with COPD (20). Wang et al. also noted an ACE2 connection to smoking and Covid-19 (21). ACE2 expression could also be true for patients with other chronic lung diseases such as idiopathic pulmonary fibrosis (22).

The increases seen in smokers further raises the question of whether this is also true for people engaged in waterpipe smoking and those switching to electronic cigarettes and “heat-not-burn” IQOS devices (17, 23-25). It is essential to recognise that these devices are not “safer”, they are still a tobacco product that produces vapor or smoke and similarly could cause infectious lung damage as we see with traditional cigarettes (26, 27). Use of waterpipes and e-cigarettes are a risk for transmission of COVID-19, as the user exhales vapour droplets, which would carry SARS-Cov-2 (24).

The attachment of the virus to cell surface ACE2 protects them from immune surveillance mechanisms, leaving them tagged to the host for relatively longer periods, thus making them an efficient carrier and vulnerable host for future infections and spread. The eventual engulfment of ACE2 further provides the virus access to the host cells system, thus providing a flourishing environment, not just to sustain and proliferate but also to mutate and modify host evasion mechanisms. Taken together these studies grant preliminary *but* very importantly suggest that smokers and patients with COPD are at increased risk of serious COVID-19 infection and highlight the importance of smoking cessation in reducing the risk.

COVID-19 is a dress rehearsal for the next pandemic, and the next, and the one after that - the new normal. Wang *et al* comment on emerging zoonotic viruses (EZV): "Now is not a time for blame. Rather, there are lessons the global health community can and

should learn and act on so that we can better respond to the next EZV event, which is almost certain to happen again. These lessons are definitely not unique to China (28)."

Veterinary and other scientific experts are in no doubt that zoonoses will continue to materialise, and dishearteningly, there are few paths to predict when or where (29, 30). However, if countries allocate additional resources to zoonoses pandemic prediction research, and implement recommendations for action, there might be progress (31). The old aphorism, "when you are up to your ears in alligators, remember your first objective was to drain the swamp", applies here. Smoking is an additional invisible immediate threat, which is a major contributor driving the more severe cases of COVID-19 which in turn will almost certainly bankrupt entire countries, decimate health systems, collapse hospitals, disarticulate social cohesion, undermine political systems and kill thousands – perhaps millions – of citizens.

We strongly recommend that the WHO and countries act to advance their efforts to reduce smoking, vaping and waterpipe use; that they re-examine the provisions of the FCTC; and develop comprehensive strategies to implement all aspects of the FCTC. During a pandemic it is difficult to focus on anything other than the immediate threat. The "primacy of rescue" has overwhelmed preventive action (32, 33). Additional research into the relationship of smoking to infection, transmission and progression of COVID-19 is required. As ACE2 could be a novel adhesion molecule for COVID-19 and potential therapeutic target for prevention of fatal microbial infections it should be fast-tracked and prioritised for further research.

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Chronic nicotine inhalation increases susceptibility to cardiovascular and pulmonary diseases through inhibition of local compensatory mechanisms Gardner, Jason D. Lazartigues, Eric D. Yue, Xinping Louisiana State Univ Hsc New Orleans, New Orleans, LA, United States

Nicotine is the addictive component of tobacco-derived products. Through binding to a family of nicotinic acetylcholine receptors (nAChRs), nicotine influences a diverse range of cellular mechanisms involved in homeostasis and disease. Although cigarette smoking is a major risk factor for cardiovascular and pulmonary diseases (CVPD) including hypertension, vascular dysfunction and fibrosis, the direct effects and the molecular mechanisms of nicotine in the pathogenesis of these diseases have not been elucidated. Our preliminary data show that cigarette smoke or direct nicotine inhalation disrupts the homeostasis of the renin-angiotensin system (RAS). The role of the RAS in the regulation of blood pressure and the development of CVPD through neurovascular and cardiopulmonary mechanisms has been firmly established. Angiotensin (Ang)-II, by means of its type 1 receptor (AT1R), promotes increased sympathetic activity, salt and water reabsorption, vasoconstriction, aldosterone and vasopressin release and inflammation, contributing to tissue fibrosis, endothelium dysfunction and hypertension. Angiotensin Converting Enzyme type 2 (ACE2) cleaves Ang-II into the vasodilator peptide Ang-(1-7), hence a pivotal player in the ACE2/Ang-(1-7)/Mas receptor compensatory axis of the RAS. AT2R, another receptor for Ang-II, opposes the deleterious effects of AT1R activation, and ACE2-formed Ang-(1-7) was recently shown to activate not only the Mas receptor but also AT2R. Our pilot data suggest that cigarette smoke or nicotine inhalation inhibits the expression of ACE2/AT2R in multiple organs including the brain, heart and lungs, thus disrupting the balance within the RAS. Accordingly, the central hypothesis of this application is: Chronic nicotine inhalation disrupts RAS homeostasis through inhibition of local compensatory mechanisms, leading to increased susceptibility to cardiovascular and pulmonary diseases. Taking advantage of the combined expertise of our multidisciplinary team, we will use state- of-the-art molecular, cellular and pharmacological tools combined with novel transgenic and knockout murine models (ACE2 overexpression and knockout) to assess the direct effects of inhaled nicotine on cardiovascular, autonomic and pulmonary functions. We will address the following Specific Aims: 1) Chronic nicotine inhalation impairs local compensatory activity within the RAS; 2) Chronic nicotine inhalation increases susceptibility?to CVPD; 3) Chronic

nicotine inhalation adversely affects the treatment for CVPD. Findings from this study will advance our understanding of the pathogenic mechanisms linked to inhaled nicotine, and set the basis for future development of improved treatment to preserve RAS compensatory activity in CVPD.

Public Health Relevance

Cardiovascular and associated pulmonary diseases (CVPD) are the number one cause of death in industrialized countries, and smoking is a major risk factor for CVPD. The present study examines how nicotine, the addictive component of cigarette smoke, alters the homeostasis of the renin-angiotensin system and predisposes nicotine users to CVPD. Findings from this study will help us better inform the public of the danger associated with nicotine use, and may also lead to improved treatment for nicotine-associated CVPD.