ACE-2 expression in the small airway epithelia of smokers and COPD patients: implications for COVID-19

To the Editor:

The World Health Organization (WHO) has declared coronavirus disease 2019 (COVID-19) 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 18 April 2020, there have been 2 280 945 cases of COVID-19 worldwide and 156 354 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 >55 years of age and with significant comorbidities, such as COPD [7]. Here, we determined whether patients with COPD have increased expression of ACE-2 in bronchial epithelial cells in the lower respiratory tract.

Patients undergoing bronchoscopy at St Paul's Hospital (Vancouver, BC, 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 and undergoing 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 s (FEV_1)/forced vital capacity (FVC) <70% or clear evidence of emphysema on computed tomography imaging on visual inspection. Cytological brushings were obtained in subsegmental airways (6th–8th generation) of the lung that were unaffected by the patient's underlying clinical indication for bronchoscopy.

Total RNA was extracted from cytological brushings using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Transcriptomic sequencing was performed on the NovaSeq 6000 (Illumina, San Diego, CA, USA) 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 maximisation) to obtain the read counts. Limma voom [11] was applied to normalise the counts to log2 counts per million (CPM) reads, 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 10th–12th generation bronchi collected at a single centre; transcriptome measurement was performed using the U133 Plus 2.0 (ThermoFisher, Waltham, MA, USA) microarray (denoted as the Cornell cohort) [13]. Secondly, we used dataset GSE37147 consisting of bronchial brushings from the 6th–8th generation airways with gene expression profiles generated from the GeneChip Human Gene 1.0 ST microarray (ThermoFisher) [14]. This dataset was denoted as the British Columbia Cancer Agency (BCCA) cohort.

We also determined the protein expression of ACE-2 in resected lung tissue specimens. These samples were obtained from 10 current smokers with COPD (mean±SD FEV_1/FVC 61±7%), nine nonsmoker controls (FEV_1/FVC 85±2%) and eight healthy current smokers (FEV_1/FVC 78±6%). Human lung tissue samples were obtained with informed consent from patients undergoing thoracic surgery as part of the

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Smokers and those with COPD have increased airway expression of ACE-2, which is the entry receptor for the COVID-19 virus. This may explain the increased risk of severe COVID-19 in these subpopulations and highlight the importance of smoking cessation. https://bit.ly/3bC29es

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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, Cambridge, UK) using the Bond Polymer Refine Red Detection kit on a Leica Bond Autostainer (both Leica Biosystems, Concord, ON, Canada), as previously described [15]. Airway epithelial-specific ACE-2 protein intensity was quantified using the Aperio imaging system (Leica Biosystem) with normalisation to the length of the basement membrane.

For the primary study population, log2 CPM of ACE-2 was the principal outcome of interest. Robust linear models were used to determine: 1) whether ACE-2 was differentially expressed in patients with COPD and in smokers after adjustment for age and sex; and 2) whether ACE-2 expression was significantly correlated with lung function. All analyses were performed in R (version 3.5.0; https://cran.r-project.org). 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 St Paul's Hospital cohort was 64.8±12.0 years; 55% were females and 24% were current smokers. Compared with control subjects (n=21), those with COPD (n=21) had a lower FEV_1 % predicted (72.0±15.6 *versus* 85.9±17.9 % pred; p=0.011) and FEV_1/FVC (64.1±7.9 *versus* 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 FEV_1 % pred (r=−0.24; p=0.035; figure 1b). Interestingly, smoking status was also significantly related to ACE-2 gene expression levels in the 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 inbetween that of never- and current smokers (former smokers 2.00±1.23; figure 1c). Adjusted for 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; 33.2% of the cohort were female. 32.2% were never-smokers and 67.8% were current smokers at the time of bronchoscopy. The average age of the BCCA cohort was 64.5±5.9 years; 43.3% of the cohort were female. All were heavy smokers with ⩾30 pack-years of smoking. Of these, 41.6% were current smokers at the time of 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 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 FEV_1 was measured and 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 nonsmokers, 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 with nonsmokers, but not in healthy smokers (figure 1d). ACE-2 protein staining was largely restricted to the airway epithelium and cells in the submucosal compartment.

There is a worldwide outbreak of COVID-19. Although most patients infected and diagnosed with COVID-19 have mild symptoms, ∼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 is largely unknown.

One possibility is the 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. In this study, 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 an individual's FEV_1, suggesting a dose-dependent response. These findings were observed in three different cohorts, indicating their generalisability and robustness.

ACE-2 is a type I transmembrane metallocarboxypeptidase with homology to 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,

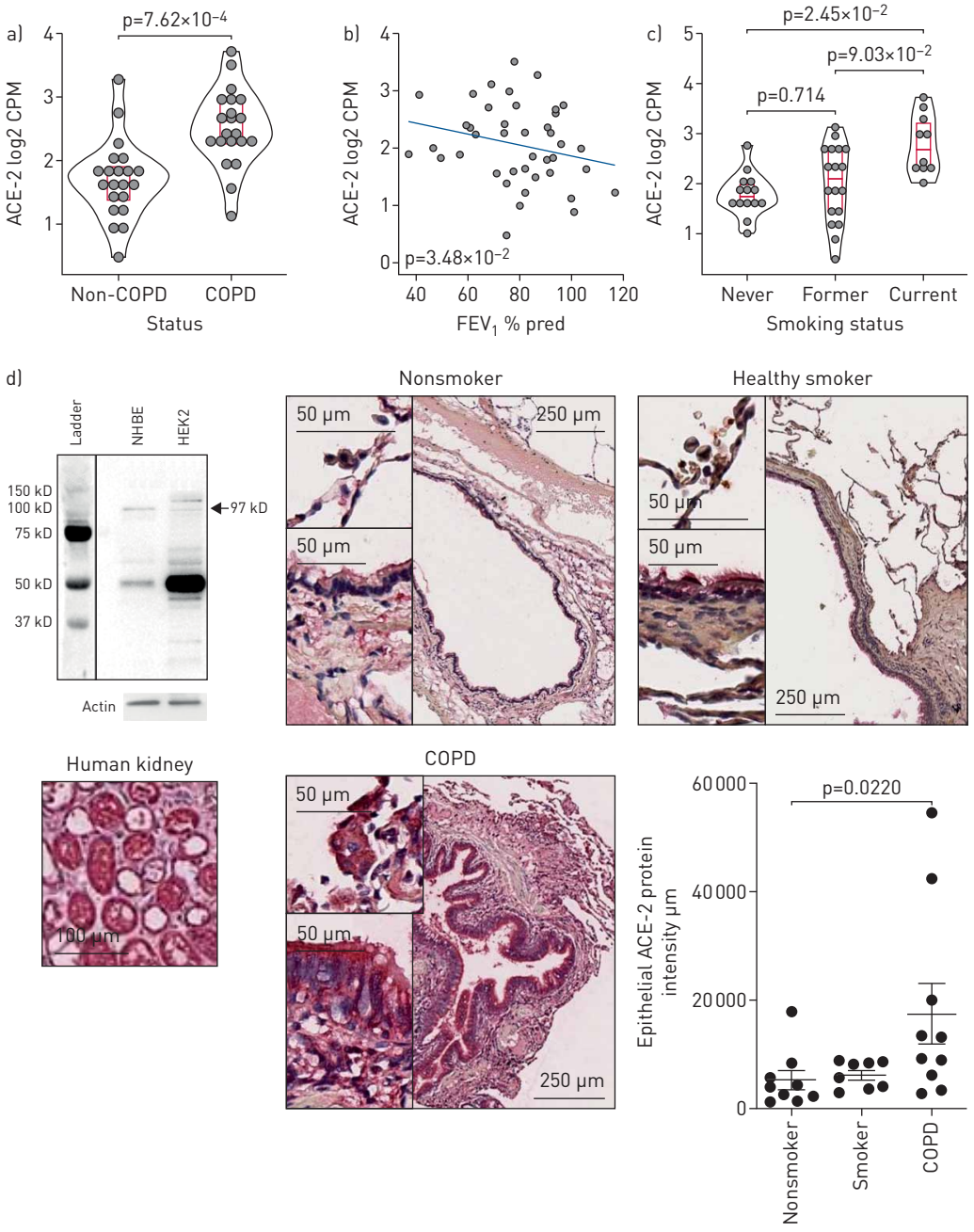

FIGURE 1 a) A violin plot of angiotensin-converting enzyme II (ACE-2) expression the in small airways of COPD and non-COPD subjects in the St Paul's Hospital (Vancouver, BC, Canada) cohort. The red box indicates the median and interquartile range. The p-value was obtained using a robust linear model. b) A scatterplot of ACE-2 expression in the small airways according to forced expiratory volume in 1 s (FEV_1) % predicted in the St Paul's Hospital cohort. ACE-2 gene expression in airway epithelia is inversely related to FEV_1 % pred (p=0.0348). c) A violin plot of ACE-2 expression in the 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 using a robust linear model. d) Protein staining of ACE-2 in the airways of individuals with and without COPD. A 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 the airways, most of the protein expression was noted in the epithelial layer, and most pronounced in those with COPD. CPM: counts per million; NHBE: normal human bronchial epithelial cells.

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 physiological

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 the 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 upregulation of ACE-2 may be useful in protecting the host against acute lung injury, chronically, this may predispose individuals to an increased risk of coronavirus infections, which use 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 are 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 upregulate ACE-2 expression in the 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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Data availability: Raw data available from the authors upon request. The data are also posted on GEO (Gene Expression Omnibus).

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