Dysregulation of endocytic machinery and ACE2 in small airways of smokers and COPD patients can augment their susceptibility to SARS-CoV-2 (COVID-19) infections

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Abstract

Lungs of smokers and chronic obstructive pulmonary disease (COPD) are severely compromised and are susceptible to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) attack. The dangerous combination of enhanced SARS-CoV-2 attachment receptor protein ACE2 along with an increase in endocytic vacuoles will enable viral attachment, entry, and replication. The objective of the study was to identify the presence of SARS-CoV-2 host attachment receptor angiotensin-converting enzyme-2 (ACE2) along with endocytic vacuoles, early endosome antigen-1 (EEA1), late endosome marker RAB7, cathepsin-L, and lysosomal associated membrane protein-1 (LAMP-1) as lysosome markers in the airways of smokers and COPD patients. The study design was cross-sectional and involved lung resections from 39 patients in total, which included 19 patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage I or GOLD stage II COPD, of which 9 were current smokers with COPD (COPD-CS) and 10 were ex-smokers with COPD (COPD-ES), 10 were normal lung function smokers, and 10 were never-smoking normal controls. Immunostaining for ACE2, EEA1, RAB7, and cathepsin-L was done. A comparative description for ACE2, EEA1, RAB7, and cathepsin-L expression pattern is provided for the patient groups. Furthermore, staining intensity for LAMP-1 lysosomes was measured as the ratio of the LAMP-1-stained areas per total area of epithelium or subepithelium, using Image ProPlus v7.0 software. LAMP-1 expression showed a positive correlation to patient smoking history while in COPD LAMP-1 negatively correlated to lung function. The active presence of ACE2 protein along with endocytic vacuoles such as early/late endosomes and lysosomes in the small airways of smokers and COPD patients provides evidence that these patient groups could be more susceptible to COVID-19.

ACE2; COVID-19; COPD; electronic cigarettes; SARS-CoV-2; smoking

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is attributed to COVID-19 infections causing high mortality and disruption to health systems worldwide (1). The SARS-CoV-2 has a strong affinity toward human angiotensin-converting enzyme 2 (ACE2) receptor expressed on host cells, through their densely glycosylated spike (S) protein, which is the initiation step for the virus to infect the human body (2). Susceptibility to infection from SARS-CoV-2 is multifold in patients with chronic lung disease and other comorbidities such as obesity and heart and kidney diseases, and the ACE2 receptor is known to play a pivotal role in these organs as well (3).
We previously and now other researchers have reported an increase in ACE2 expression in smokers and patients with chronic obstructive pulmonary disease (COPD) compared with nonsmokers (4, 5). Increased ACE2 expression facilitates attachment of the virus to susceptible host cells, consequently increasing their chances of infections. Several cell types are now known to express ACE2, including several epithelial cell types, smooth muscle, immune cells, and endothelial cells (6, 7).

SARS-CoV-2 are enveloped viruses and use to their advantage the host endocytosis processes. They are engulfed via endosomal routes, usually through clathrin-dependent or clathrin- and caveolae-independent entry pathways, for uptake and transport, before fusing with lysosomes (8). Endo-lysosomal fusion also activates lysosomal proteases such as cathepsins, which cleaves the viral surface S proteins, facilitating the fusion of the host and viral membranes, depositing the viral RNA into the host cells (9).

Considering the implications of the ACE2, endocytic pathways (lysosomes and endosomes) in SARS-CoV-2 infection, we evaluated the presence of these early and late endosomes EEA1 and RAB7, cathepsin-L and lysosomal marker, lysosomal associated membrane protein (LAMP-1) across a cross-sectional cohort consisting of normal nonsmoker controls and compared them with current and ex-smokers with normal associated membrane protein (LAMP-1) across a cross-sectional cohort consisting of normal nonsmoker controls, and rabbit serum (cat. no. X0903, Dako) at similar dilutions to primary antibodies were incorporated in every experiment to ensure the absence of false-positive staining. After the addition of horseradish peroxidase-tagged secondaries, the protein markers were visualized as brown color after the addition of substrate 3,3′-diaminobenzidine [Dako EnVision Detection System, Peroxidase/DAB +, Rabbit/Mouse (cat. no. K5007)]. All tissue was counterstained with hematoxylin (blue) for the nucleus.

### METHODS

#### Patient Demographics

We had surgically resected lung tissue from 39 patients from our biobank collected through Royal Hobart Hospital Tasmania after approvals from the Tasmanian Health and Medical Human Research Ethics Committee (ethics ID: EC00337). All subjects in the pathological cohorts had primary nonsmall cell lung cancer. Surgically resected tissue was taken well away from the primary tumor. Nineteen patients had Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage I or GOLD stage II COPD (forced expiratory ratio <70%) of which nine were current (COPD-CS), and ten were ex-smokers (COPD-ES) (>1 yr of smoking cessation). Ten were normal lung function smokers (NLFS), and 10 were never-smoking normal controls (NC). The normal controls were kindly provided by James Hogg Lung Registry, University of British Columbia with approval from the Providence Health Care Research Ethics Board (H00-50110).

| Groups       | NC     | NLFS   | COPD-CS | COPD-ES |
|--------------|--------|--------|---------|---------|
| Subjects     | 10     | 10     | 9       | 10      |
| Sex (F/M)    | 6/4    | 7/3    | 5/4     | 4/7     |
| Age (yr)     | 24 (19–63) | 70 (52–79) | 63 (59–78) | 68.5 (56–85) |
| Smoking, pack-years | 33.5 (0.3–60) | 28.5 (2–50) | 33 (18–36) |
| FEV1/FVC, %  | 76 (70–90) | 66 (59.9–70) | 64 (54.9–69) |
| FEF25–75%, L/s post-BD | 71 (47–116) | 35.5 (28–47) | 40.5 (20–55) |

Data expressed as medians and ranges. F, female; M, male; NC, normal control; NLFS, normal lung function smoker; COPD, chronic obstructive pulmonary disease; COPD-CS, COPD current smoker; COPD-ES, COPD ex-smoker; FEV1/FVC, forced expiration/forced vital capacity; FEF25–75% (L/s) post-BD, forced expiratory flow at 25–75% postbronchodilator.

### Immunohistochemical Staining and Analysis

As previously published (10), surgical resections were fixed in formalin within minutes of surgery. At processing, tissue blocks of small airways (<2.5-mm internal diameter) were separately embedded in paraffin for our analyses. In brief, tissue sections were sliced at 3.5-μm size and subjected to standard immunohistochemical staining techniques using rabbit anti-human polyclonal for ACE2 (cat. no. AB5348, Abcam 1:400), early endosomes with rabbit anti-human polyclonal early endosome antigen 1 (EEA1) (cat. no. AB2900, Abcam, 1:150), late endosome marker with rabbit anti-human polyclonal RAB7 (cat. no. PA52369, ThermoFisher, 1:100), rabbit anti-human polyclonal LAMP-1 (cat. no. AB24170 Abcam 1:200), and anti-human polyclonal cathepsin-L (cat. no. bs-1508R, Bioss Antibodies, 1:150). Staining intensity for all antibodies was determined using appropriate human tissue controls, and rabbit serum (cat. no. X0903, Dako) at similar dilutions to primary antibodies were incorporated in every experiment to ensure the absence of false-positive staining.

### Statistical Analysis

For all cross-sectional data, we tested their normal distributions using the D’Agostino-Pearson omnibus normality test. All the analysis is represented as medians and ranges. Nonparametric analyses of variance were performed using the Kruskal-Wallis test, which compared medians/ranges across all the groups of interest; correction for multiple comparisons were assessed using Dunn’s test. For correlations, we performed regression analyses using Spearman’s rank test. These statistical analyses were done using GraphPad.

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**ENDOCYTIC VACUOLES IN COPD: IMPLICATIONS FOR COVID-19**

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**RESULTS**

Detailed quantification of LAMP-1 in the epithelial and subepithelial (LP and SM) areas was done in the four groups. A significant increase in percent LAMP-1 staining in COPD-CS [median percentage 28, range 3.4–90; \( P < 0.001 \)], NLFS [median percentage 15.5, range 1.2–43; \( P < 0.001 \)] and COPD-ES [median percentage 4.5, range 0–18; \( P < 0.01 \)] when compared with NCs (median percentage 0 range 0–0.24) (Fig. 1). In comparison to COPD-CS, the ex-smokers had significantly lower expression of LAMP-1 (\( P < 0.05 \)). LAMP-1 percent change was observed in both LP and SM layer, albeit lower levels when compared with the epithelium; nonetheless, all pathological groups still showed a variable statistically significant increase over NCs (Fig. 1, ii and iii). Unlike epithelium LAMP-1 expression in the subepithelial areas showed no intragroup differences across the pathological cohorts. The increase in LAMP-1 expression positively correlates to the smoking history (pack-years) for both NLFS and COPD-CS [Spearman’s rho (rs) = −0.65, \( P < 0.01 \)] (Fig. 2, i and ii). Furthermore, percent LAMP-1 was observed to negatively correlate with the lung function parameters in COPD patients both current [Spearman’s rho (rs) = −0.4, \( P < 0.05 \)] and ex-smokers [Spearman’s rho (rs) = −0.6, \( P < 0.01 \)] (Fig. 2, i and ii).

For ACE2, EEA1, cathepsin-L, and RAB7, we provide here descriptive microscopic observations from a serial section from the same patient for each pathological cohort and healthy control in similar areas identified with LAMP-1 expression (Fig. 3). Our observations suggest an appreciable increase in of the SARS-CoV-2 receptor ACE2 along with

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**Figure 1.** Lysosome expression in small airway wall: significant increases in percent lysosome-associated membrane protein-1 (LAMP-1) expression was observed in the epithelium (i) and subepithelial lamina propria (ii) and smooth muscle (iii) areas in chronic obstructive pulmonary disease (COPD)-current smokers (CS) \([n = 9; \text{female (F)} 5/\text{male (M)} 4]\), normal lung function smokers (NLFS) \([n = 10; \text{F7/M3}],\) and COPD-ex-smokers (ES) \([n = 10; \text{F3/M7}]\) when compared with NC \([n = 10; \text{F6/M4}]\). No intra-group differences between pathological groups in subepithelial LAMP-1 expression were observed. Nonparametric analyses of variance were performed using the Kruskal-Wallis test, with correction of multiple comparisons assessed using Dunn’s test. \( P < 0.05 \) was considered statistically significant.

**Figure 2.** Regression analysis for small airway epithelial lysosome-associated membrane protein-1 (LAMP-1) vs. lung function (i) and obstruction (ii) in chronic obstructive pulmonary disease (COPD) [current (CS) and ex ES)] \([n = 19; \text{female (F)} 8/\text{male (M)} 11]\) group and with smoking history (pack-years) in current smokers (iii) (NLFS and COPD-CS) \([n = 19; \text{F12/M7}]\). Regression analyses were done using Spearman’s rank \( (r') \), with a \( P < 0.05 \) considered statistically significant. FEV1/FVC, forced expiration/forced vital capacity; FEF25–75, forced expiratory flow at 25–75%.
proteins involved in viral endocytic machinery including early and late endosomal marker EEA1, RAB7, and virus fusion modulator serine protease cathepsin-L expression in NLFS, COPD-CS, and COPD-ES, in both epithelium and subepithelium areas compared with NCs (Fig. 3). Similar increases in all were also observed in the parenchymal areas of the lung with prominent expression observed in all markers in both type-II pneumocytes and alveolar macrophages (Fig. 3, insets).

**DISCUSSION**

SAR-CoV-2, like other enveloped viruses, requires cellular ACE2 receptor binding for entry and membrane fusion to eject their RNA into the host system. These viruses are known to hijack the host endocytosis pathway entering via endosomes efficiently, proceed to lysosomes, and then fuse the viral and lysosomal membranes (11). An increase in endocytic protein would thus suggest dysregulated overactive

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**Figure 3.** Representative images of serial sections from surgical lung resections stained for angiotensin-converting enzyme-2 (ACE2) receptor, lysosomal associated membrane protein1 (LAMP-1), cathepsin-L, early endosomal marker 1 (EEA1), and late endosomal marker (RAB7). The observation was done using brightfield microscopy at a magnification of ×10 and enlarged insets at ×40 magnification for the small airway wall and parenchymal spaces. Normal nonsmoker (i–v) SA tissues observed to have a low expression for ACE-2, cathepsin-L, and endocytic markers when compared with NLFS (vi–xi), chronic obstructive pulmonary disease (COPD)-current smokers (CS) (xii–xv), and COPD-ex-smokers (ES) (xvi–xx). Compared with NLFS and COPS-CS, the expression levels of ACE-2 and endocytic makers were observed to be comparably lesser in ex-smokers across the 5 markers tested. A similar pattern was also observed in type 2 pneumocytes and macrophages across the patient population (insets). LAMP-1 expression in small airway epithelium appeared to be more diffused across the epithelium in COPD-CS (xii, inset) while in both normal lung function smokers (NLFS; vii inset) and COPD-ES (xvii, inset) the LAMP-1 was found to accumulate close to the nucleus. NC, normal control; LP lamina propria.
Cellular anomalies that could effectively facilitate viral entry and processing. This is the first study to our knowledge that characterizes the fault lines in the endocytic machinery in the small airways and parenchymal tissue areas of smokers and patients with COPD compared with healthy nonsmokers, with increases observed in early (EEA1)/late endosomes (RAB-7) and lysosomes (LAMP-1) and cathepsin-L. Interestingly, we noticed a concurrent rise in endosomal protein markers and ACE2 expression in similar areas of the airway tissue in smokers and COPD patients. A more recent finding establishes the role of ACE2 in inducing autophagy pathways and inflammation in acute lung injury (12, 13), and such association requires further investigation in the context of SARS-CoV-2 infections. Prominent expression of these endosomal markers and ACE2 was also observed in type-II pneumocytes and alveolar macrophages in all patient groups. Notably, in all these cell types, COPD ex-smokers had reduced expression of both ACE2 and the endocytic proteins compared with smokers with and without COPD.

Both lysosomes and endosomes are critical to cellular metabolism, breaking down biomolecules and cellular debris, and reprocessing nutrients for other cellular functions. The lysosomal and endosomal protease activities are central to their functionality (9). Cathepsins are one such group of endo-lysosomal proteases that include aspartyl, serine, and cysteine proteases with both endo- and exopeptidase activities (14, 15). The cathepsin enzymes work best within a low pH environment and facilitate viral S protein membrane fusion, which helps viral RNA entry into the host cytoplasm (8, 16). Among the several cathepsins, cathepsin-L is shown to play a crucial role in enhancing the SARS-CoV-2 infection through nonspecific degradation of the S1 domain on the virus S-protein, permitting conformational transition into the more stable S2 domain, which facilitates the virus fusion into the host cells (14, 17). Although cathepsin-L works best in the endo-lysosomal compartments, under pathological conditions, they along with TMPRSS2 and furin could activate the virus S-protein attached on cell surface ACE2, helping the virus’s quicker transition into the cell (18). Our observation of increased cathepsin-L expression along with lysosomes and early and late endosomal markers suggests that smoking does stimulate a highly conducive environment for SAR-CoV-2 entry and fusion in smokers and COPD patients (16).

The augmented presence of LAMP-1 lysosomes in the small airway epithelium in COPD patients was significantly associated with lower lung function and increase in small airway obstruction. An increased lysosome thus would mean that these individuals are more vulnerable to speedier deterioration when challenged with a viral infection such as the SAR-CoV-2. Furthermore, we observed that increase in the lysosomal protein expression had a direct link to the individual smoking rates. These factors further elaborate on the importance of smoking cessation, considering the deleterious situation these patients could face in severe infection.

The study was limited by the number of participants. Also, we have only quantified and presented LAMP-1+ lysosomes but further plan to quantify early endosomes and ACE2 expression in all patient groups and derive their relationships. Our study included a wider age range in normal subjects with median age lower than pathological subjects; however, we found no significant regression between LAMP-1 expression and age. The prominent presence of endocytic vacuoles and ACE2 expression in current smokers with NLFS and COPD provides evidence that these individuals may have a high susceptibility to SAR-CoV-2 infection. The epidemiological studies so far have demonstrated conflicting data that smokers may or may not be more susceptible to the COVID-19 virus, but the findings here further elaborate the need for future research in this area. The emergence of unsafe smoking alternatives like e-cigarettes and heat-not-burn (IQOS) device, could enhance user’s susceptibility to such infectious diseases (19–22). We believe lysosomotropic agents (23–25) and ACE2 inhibitors (26, 27) could potentially protect these patients from viral infections; however, smoking cessation remains the best option.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

M.S.E. and S.S.S. conceived and designed research; M.S.E., W.L., K.D.M., A.H., H.C.W., G.H., P.A.B.W., and S.S.S. analyzed data; M.S.E., W.L., T.L.H., and S.S.S. interpreted results of experiments; M.S.E. and S.S.S. prepared figures; M.S.E. drafted manuscript; M.S.E., W.L., T.L.H., G.K.S., H.C.W., G.H., P.A.B.W., C.C., and S.S.S. edited and revised manuscript; M.S.E., L.T.H., G.K.S., I.E.T., K.D.M., A.H., H.C.W., G.H., A.W., C.C., and S.S.S. approved final version of manuscript.

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