

Acknowledgment: The authors thank Janel L. Martinez, M.S., Valerie Clarkson Keever, and Bree Mabley, as well as the staff of the Pulmonary Physiology Laboratory and the Center for Post COVID Care at National Jewish Health, Denver, Colorado, for their contribution to this study.

Esther de Boer, M.D., Ph.D.
Irina Petrache, M.D.*

National Jewish Health
Denver, Colorado
and

University of Colorado Anschutz Medical Campus
Aurora, Colorado

Nir M. Goldstein, M.D.
J. Tod Olin, M.D.

National Jewish Health
Denver, Colorado

Rebecca C. Keith, M.D.
National Jewish Health
Denver, Colorado
and

University of Colorado Anschutz Medical Campus
Aurora, Colorado

Brian Modena, M.D.
National Jewish Health
Denver, Colorado

Michael P. Mohning, M.D.
Zulma X. Yunt, M.D.

National Jewish Health
Denver, Colorado
and

University of Colorado Anschutz Medical Campus
Aurora, Colorado

Inigo San-Millán, D.Sc.†
University of Colorado Anschutz Medical Campus
Aurora, Colorado
and

University of Colorado
Colorado Springs, Colorado

Jeffrey J. Swigris, D.O., M.S.‡
National Jewish Health
Denver, Colorado
and

University of Colorado Anschutz Medical Campus
Aurora, Colorado

ORCID IDs: 0000-0003-4024-6366 (E.d.B.); 0000-0003-1094-2600 (I.P.).

*Corresponding author (e-mail: petrachei@njhealth.org).

†These authors contributed equally to this work.

References

1. Del Rio C, Collins LF, Malani P. Long-term health consequences of COVID-19. *JAMA* 2020;324:1723–1724.
2. Baratto C, Caravita S, Faini A, Perego GB, Senni M, Badano LP, et al. Impact of COVID-19 on exercise pathophysiology: a combined cardiopulmonary and echocardiographic exercise study. *J Appl Physiol (1985)* 2021;130:1470–1478.
3. San-Millán I, Brooks GA. Assessment of metabolic flexibility by means of measuring blood lactate, fat, and carbohydrate oxidation responses to exercise in professional endurance athletes and less-fit individuals. *Sports Med* 2018;48:467–479.
4. Wasserman DH, Halseth AE. An overview of muscle glucose uptake during exercise: sites of regulation. *Adv Exp Med Biol* 1998;441: 1–16.
5. American Thoracic Society; American College of Chest Physicians. ATS/ACCP Statement on cardiopulmonary exercise testing. *Am J Respir Crit Care Med* 2003;167:211–277.
6. Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 1983;55:628–634.
7. Parikh S, Goldstein A, Koenig MK, Scaglia F, Enns GM, Saneto R, et al. Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet Med* 2015; 17:689–701.
8. Puthuchery ZA, Astin R, Mcphail MJW, Saeed S, Pasha Y, Bear DE, et al. Metabolic phenotype of skeletal muscle in early critical illness. *Thorax* 2018;73:926–935.
9. Dos Santos C, Hussain SN, Mathur S, Picard M, Herridge M, Correa J, et al.; MEND ICU Group; RECOVER Program Investigators; Canadian Critical Care Translational Biology Group. Mechanisms of chronic muscle wasting and dysfunction after an intensive care unit stay: a pilot study. *Am J Respir Crit Care Med* 2016;194:821–830.
10. Pleguezuelos E, Del Carmen A, Llorensi G, Carcole J, Casarramona P, Moreno E, et al. Severe loss of mechanical efficiency in COVID-19 patients. *J Cachexia Sarcopenia Muscle* 2021;12: 1056–1063.
11. Rinaldo RF, Mondoni M, Parazzini EM, Baccelli A, Pitari F, Brambilla E, et al. Severity does not impact on exercise capacity in COVID-19 survivors. *Respir Med* 2021;187:106577.

Copyright © 2022 by the American Thoracic Society



Combustible and Electronic Cigarette Exposures Increase ACE2 Activity and SARS-CoV-2 Spike Binding

To the Editor:

The outbreak of coronavirus disease (COVID-19) has extensively impacted global health. The spike protein on the surface of the causative pathogen severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) binds to the angiotensin-converting enzyme 2 (ACE2) receptor, a metalloprotease, which is expressed in both mACE2 (membrane-anchored ACE2) and sACE2 (soluble ACE2) forms in the lung. Although mACE2 is responsible for viral

‡This article is open access and distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives License 4.0. For commercial usage and reprints, please e-mail Diane Gern (dgern@thoracic.org).

Supported by NIH/Food and Drug Administration grants HL120100 and HL153698, NIH/NHLBI grant HL135642, NIH grants R01CA237652 and 1DP5OD021385, an American Cancer Society Research Scholar Grant (J.M.S.), and a New York Community Trust grant (J.M.S.). Research reported in this publication was supported in part by the NIH and the Family Smoking Prevention and Tobacco Control Act. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the Food and Drug Administration.

Originally Published in Press as DOI: 10.1164/rccm.202106-1377LE on November 8, 2021

entry, recent observations also suggest that sACE2 is involved, by its interaction with the spike protein, followed by receptor-mediated endocytosis of the viral particles (1). Tobacco use has been speculated as a risk factor for contracting SARS-CoV-2 infection and subsequent disease severity (2, 3), and electronic cigarettes (e-cigarettes) have been shown to induce harmful proteomic and immune changes in the lungs of vapers (4). In addition, the effect of vaping, and the role of nicotine, in the regulation of ACE2 expression has been demonstrated in animal models and cell culture systems (5–8). We therefore tested the hypothesis that combustible tobacco (e.g., cigarettes) and noncombustible e-cigarettes could affect ACE2 activity and subsequent SARS-CoV-2 infection. Some of our data have been reported previously at an international conference (9) and deposited in a preprint repository (<https://doi.org/10.1101/2021.06.04.447156>).

We first evaluated sACE2 activity in concentrated bronchoalveolar lavage fluid (BALF) samples from nonsmokers (age, 28.5 ± 9.5 yr; body mass index [BMI], 28.8 ± 7.2 kg/m²; FVC% predicted, 98.0 ± 25.0 ; FEV₁% predicted, 101.6 ± 14.5), smokers (age, 34.4 ± 8.2 yr; BMI, 27.5 ± 7.8 kg/m²; FVC% predicted, 104.2 ± 11.2 ; FEV₁% predicted, 97.9 ± 16.1), and vapers (age, 29.3 ± 9.1 yr; BMI, 29.6 ± 8.0 kg/m²; FVC% predicted, 106.2 ± 11.2 ; FEV₁% predicted, 104.5 ± 7.7). Details of the subject recruitment criteria and the study design have been published (4, 10, 11). We measured cleavage of an ACE2-specific fluorogenic peptide (SFQ-3819-PI; vivitide) by using a microplate reader to evaluate ACE2 activity as described (4). sACE2 activity was significantly increased in BALF from both smokers and vapers compared with age-matched nonsmokers (Figure 1A), indicating heightened vulnerability toward SARS-CoV-2 infection in both groups.

To evaluate the impact of tobacco smoke on ACE2 activity, we cultured primary human bronchial epithelial cells (HBECs) at the air–liquid interface until they were well differentiated into ciliated and goblet cells (12) and then exposed them to 14 puffs of smoke per day from Kentucky research cigarettes (1R6F) for 1 day (acute) and 4 days (chronic) as previously reported (13). Acute smoke exposure significantly increased both mACE2 and sACE2 activity (Figure 1B). After chronic smoke exposure, both mACE2 and sACE2 activity remained significantly elevated in smoke-exposed cultures compared with air-exposed controls (Figure 1B). Consistent with these observations, ACE2 protein was significantly increased in chronically smoke-exposed cultures compared with air-exposed controls (Figure 1C), indicating that more receptors were potentially available for SARS-CoV-2 binding. As an orthogonal approach, we developed a pseudovirus by using recombinant vesicular stomatitis virus Δ G (a gift from Pengfei Wang, Columbia University) that contains the SARS-CoV-2 spike protein (145032; Addgene) in its membrane and expressed DsRed for the identification of infected cells. We exposed HBECs to acute and chronic tobacco smoke, which was followed by infection with 3×10^4 U/ml pseudovirus, and measured DsRed expression by using a microplate reader. Cigarette smoke exposure significantly increased DsRed fluorescence (increased viral infection; Figure 1D). We then incubated chronically air- and smoke-exposed HBECs with fluorescently labeled recombinant SARS-CoV-2 S1 protein. Consistent with the observed increase in ACE2 activity, smoke exposure caused a significant (20%, $n = 9$, $P = 0.024$) increase in S1 binding. Taken together, these data indicate that cigarette smoke exposure upregulates ACE2 activity and increases SARS-CoV-2 binding.

Next, to compare the potential impact of smoking and vaping on SARS-CoV-2 pseudovirus infection in cells from both the lower and

upper airways, we explored the impact of cigarette smoke condensate (CSC) and e-liquid exposure on pseudovirus infection on ACE2 gene-expressing primary tracheobronchial cells (PCS-300-010; American Type Culture Collection) and small airway epithelial cells (PCS-301-010; American Type Culture Collection). We have previously demonstrated that these cell types express ACE2 (14). To ensure that these different cell types were studied at the same time, they were cultured to confluence on plastic instead of on semipermeable membranes; treated with a range of different doses of CSC (Murty Pharmaceuticals) or JUUL Virginia tobacco-flavored e-liquid (59 mg/ml nicotine) for 24 hours, which corresponded to a final nicotine concentration of 21.6–176 μ M for CSC and 364–3,641 μ M for JUUL e-liquid; and infected them with pseudovirus ($2.5\text{--}5 \times 10^5$ U/ml). After all exposures, cellular viability was confirmed by positive culture characteristics under light microscopy. We then used fluorescent microscopy and flow cytometry to visualize and quantify this infection, respectively. Acute exposure to both CSC and JUUL e-liquid significantly increased DsRed fluorescence, indicating increased pseudovirus infection compared with control cells (Figures 2A–2D). These data correlate with our BALF data (Figure 1A) and indicate that both cigarettes and e-cigarettes increase the potential for SARS-CoV-2 infection.

Several meta-analyses have been performed to look at tobacco use and COVID-19 disease severity, which have reported contradictory findings. A “nicotinic hypothesis” was proposed, which suggested that nicotine exerted a protective role against SARS-CoV-2 infection (15). Conversely, other reports confirmed a strong association between smoking and COVID-19 symptoms (2, 3). Increased ACE2 expression in smokers has been proposed as a risk factor for COVID-19 (16). Moreover, tobacco smoking is a risk factor for several other respiratory viruses, including influenza (17). Our current study provides further *in vivo* evidence that sACE2 is upregulated in smokers’ and vapers’ BALF. However, our study has some limitations. First, our study was cross-sectional and of a relatively low sample size, and we were unable to probe our data sets to understand the impact of sex, race, and other factors. A second limitation of our study is that we only measured sACE2, and not mACE2, *in vivo*. However, sACE2 facilitates SARS-CoV-2 infection through interactions with the renin–angiotensin system, and elevated sACE2 in smokers and vapers is expected to contribute toward SARS-CoV-2 infection (1). The increase in sACE2 most likely reflects an overall increase in cellular ACE2 expression. Indeed, ACE2 mRNA expression is elevated in smokers’ pulmonary cells relative to those derived from nonsmokers, suggesting that both mACE2 and sACE2 may be increased (14). Moreover, elevated sACE2 was positively associated with COVID-19 severity (18), demonstrating the utility of sACE2 as a marker of COVID-19 risk. Our *in vitro* CSC exposure represented the nicotine concentrations typically reached in smokers’ lungs (19). JUUL e-liquids contain 5% nicotine (~ 300 mM). However, nicotine concentrations in the human lung after JUUL e-liquid inhalation are not known. Therefore, we added $\sim 0.1\text{--}1\%$ of the JUUL e-liquid to cells on the basis of the assumption that 0.1–1% of e-liquid is deposited in the lung after vaping, owing to nicotine dilution kinetics. However, additional studies will be required to define JUUL nicotine concentrations in the lung and to understand whether different e-liquids affect ACE2 expression and SARS-CoV-2 infection in a similar fashion.

Nicotine, which is present in both combustible cigarettes and e-cigarettes, increases cellular Ca²⁺ concentrations (4). Elevated Ca²⁺

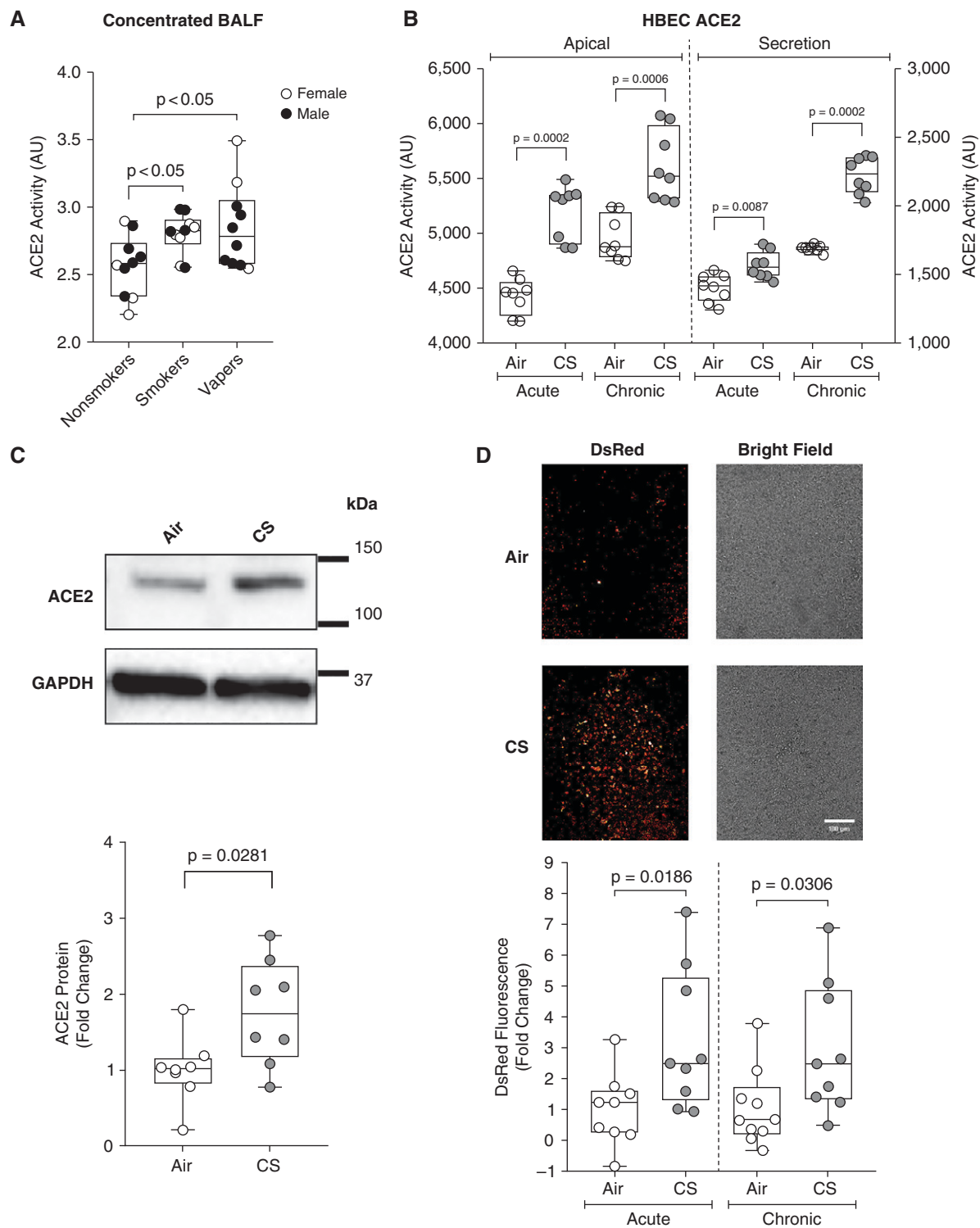


Figure 1. Cigarette smoke (CS) increases ACE2 (angiotensin-converting enzyme 2) activity and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. BAL fluids from nonsmokers, smokers, and vapers ($n = 10$ each) were concentrated, and ACE2 activity was measured by using 50- μM specific fluorogenic substrates. (A) Cleaved substrate-derived fluorescence shown as arbitrary units following 1 hour of reaction. HBECs were cultured at the air-liquid interface and exposed to smoke from one Kentucky research cigarette (1R6F) per day for 1 day (acute) or 4 days (chronic). The apical surface was washed with PBS to collect the mucosal secretion and 50- μM fluorogenic substrate was added on the apical surface to evaluate apical ACE2 activity. (B) ACE2 activity on the apical side of acutely and chronically smoke-exposed cultures and the corresponding apical washes. Activities are presented as accumulated fluorescence in arbitrary units of the cleaved products following 10 minutes of reaction. Whole-cell lysates were collected from chronically CS-exposed cultures, and ACE2 and GAPDH

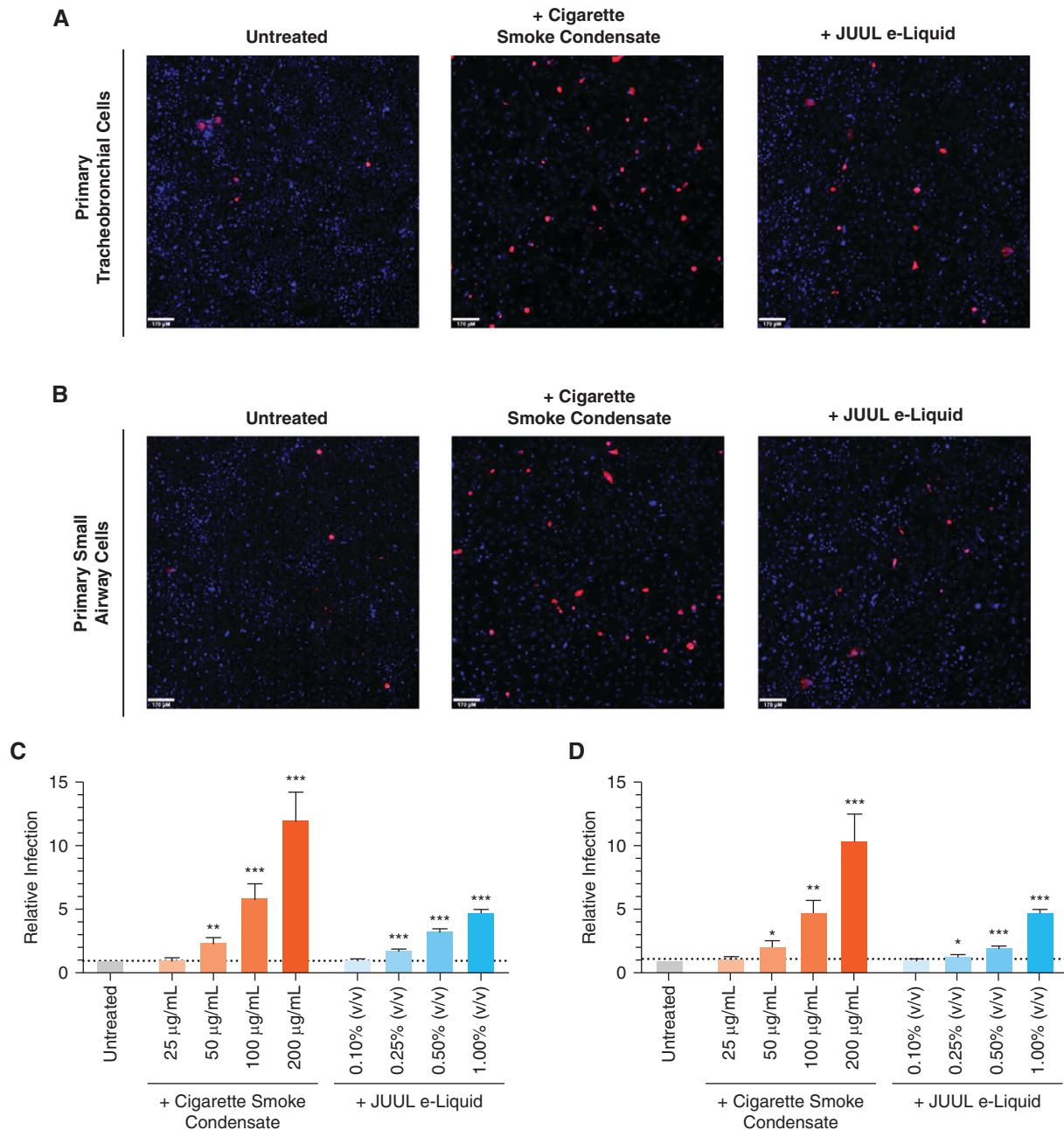


Figure 2. Cigarette smoke condensate (CSC) exposure increases severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pseudovirus infection. (A) Primary tracheobronchial cells and (B) small airway cells were pretreated with 200 μg/ml CSC (middle) and 1.00% JUUL e-liquid (right) for 24 hours and challenged with SARS-CoV-2 pseudovirus. After incubation for 48 hours, live cells were imaged on a spinning-disc confocal microscopy system (UltraVIEW Vox; PerkinElmer). Cell nuclei were stained with Hoechst dye, and infected cells are shown in red. Flow cytometry quantification of relative SARS-CoV-2 pseudovirus infection reported as the fold change in (C) primary tracheobronchial cells and (D) small airway cells pretreated with different doses of CSC and JUUL e-liquid; $n = 11-12$ separate experiments. Bars represent the mean \pm SEM. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$, unpaired t test. Scale bars, 170 μm.

Figure 1. (Continued). protein expression were evaluated by using immunoblotting. (C) Representative and GAPDH-normalized ACE2 expression in air- and smoke-exposed cultures shown as the fold change. (D) Acutely or chronically smoke-exposed cultures were apically exposed to a Spike pseudovirus suspension, and the resulting DsRed marker fluorescence was recorded after 3 days. Representative confocal microscopic images (SP8; Leica Microsystems) with the corresponding bright fields are shown above the DsRed fluorescence fold changes. Scale bar, 100 μm. Data are presented as the median and lower and upper quartiles; $n = 8-9$ per group from 3-4 different donors. P values are provided on the graph. AU = arbitrary unit; BALF = BAL fluid; CS = cigarette smoke; HBEC = human bronchial epithelial cell.

signaling is well recognized to play a vital role in facilitating viral entry, gene and protein processing, and subsequent viral release (20). Our studies demonstrate that tobacco and e-liquid exposures increase both ACE2 and SARS-CoV-2 spike protein binding. Thus, we hypothesize that nicotine may be responsible for the increased spike protein binding and subsequent pseudovirus infection. However, the underlying mechanisms, and the purported link to nicotine, will require additional study. We further speculate that acute but persistent increases in ACE2 and spike protein binding place tobacco users at risk of SARS-CoV-2 infection. Overall, these observations indicate that tobacco product use elevates ACE2 activity and increases the potential for SARS-CoV-2 infection through enhanced spike protein binding. Importantly, our results strongly urge consideration of vaping as a risk factor for COVID-19. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

Arunava Ghosh, Ph.D.*
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

Vishruth Girish, B.S.*
Cold Spring Harbor Laboratory
Cold Spring Harbor New York

Monet Lou Yuan
Cold Spring Harbor Laboratory
Cold Spring Harbor New York
and
Johns Hopkins University
Baltimore, Maryland

Raymond D. Coakley, M.D.
Joe A. Wrennall, Ph.D.
Neil E. Alexis, Ph.D.
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

Erin L. Sausville, B.S.
Anand Vasudevan, Ph.D.
Cold Spring Harbor Laboratory
Cold Spring Harbor New York

Alexander R. Chait, B.S.
Cold Spring Harbor Laboratory
Cold Spring Harbor New York
and
University of Rochester
Rochester, New York

Jason M. Sheltzer, Ph.D.
Cold Spring Harbor Laboratory
Cold Spring Harbor New York

Robert Tarran, Ph.D.†
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

ORCID ID: 0000-0002-8598-2642 (R.T.).

*Co-first authors.

†Corresponding author (e-mail: robert_tarran@med.unc.edu).

References

- Yeung ML, Teng JLL, Jia L, Zhang C, Huang C, Cai JP, *et al*. Soluble ACE2-mediated cell entry of SARS-CoV-2 via interaction with proteins related to the renin-angiotensin system. *Cell* 2021;184:2212–2228, e12.
- Farsalinos K, Barbouni A, Poulas K, Polosa R, Caponnetto P, Niaura R. Current smoking, former smoking, and adverse outcome among hospitalized COVID-19 patients: a systematic review and meta-analysis. *Ther Adv Chronic Dis* 2020;11:2040622320935765.
- Karanasos A, Aznaouridis K, Latsios G, Synetos A, Plitaria S, Tousoulis D, *et al*. Impact of smoking status on disease severity and mortality of hospitalized patients with COVID-19 infection: a systematic review and meta-analysis. *Nicotine Tob Res* 2020;22:1657–1659.
- Ghosh A, Coakley RD, Ghio AJ, Muhlebach MS, Esther CR Jr, Alexis NE, *et al*. Chronic e-cigarette use increases neutrophil elastase and matrix metalloproteinase levels in the lung. *Am J Respir Crit Care Med* 2019;200:1392–1401.
- Wang Q, Sundar IK, Li D, Lucas JH, Muthumalage T, McDonough SR, *et al*. E-cigarette-induced pulmonary inflammation and dysregulated repair are mediated by nAChR $\alpha 7$ receptor: role of nAChR $\alpha 7$ in SARS-CoV-2 COVID-19 ACE2 receptor regulation. *Respir Res* 2020;21:154.
- Naidu V, Zeki AA, Sharma P. Sex differences in the induction of angiotensin converting enzyme 2 (ACE-2) in mouse lungs after e-cigarette vapor exposure and its relevance to COVID-19. *J Invest Med* 2021;69:954–961.
- Masso-Silva JA, Moshensky A, Shin J, Olay J, Nilaad S, Advani I, *et al*. Chronic E-cigarette aerosol inhalation alters the immune state of the lungs and increases ACE2 expression, raising concern for altered response and susceptibility to SARS-CoV-2. *Front Physiol* 2021;12:649604.
- McAlinden KD, Lu W, Ferdowsi PV, Myers S, Markos J, Larby J, *et al*. Electronic cigarette aerosol is cytotoxic and increases ACE2 expression on human airway epithelial cells: implications for SARS-CoV-2 (COVID-19). *J Clin Med* 2021;10:1028.
- Ghosh A, Coakley RD, Wrennall JA, Sheltzer JM, Alexis NE, Tarran R. Tobacco smoke exposure increases ACE2 activity. Presented at the 27th Society for Research on Nicotine and Tobacco Annual Meeting. February 24–27, 2021, Virtual. Rapid Response Abstracts, Rapid Session 4: Late Breaking: Pre-Clinical, POD18-1:15.
- Ghosh A, Ahmad S, Coakley RD, Sassano MF, Alexis NE, Tarran R. Lipid-laden macrophages are not unique to patients with e-cigarette or vaping product use-associated lung injury. *Am J Respir Crit Care Med* 2021;203:1030–1033.
- Ghosh A, Coakley RC, Mascenik T, Rowell TR, Davis ES, Rogers K, *et al*. Chronic e-cigarette exposure alters the human bronchial epithelial proteome. *Am J Respir Crit Care Med* 2018;198:67–76.
- Fulcher ML, Gabriel S, Burns KA, Yankaskas JR, Randell SH. Well-differentiated human airway epithelial cell cultures. *Methods Mol Med* 2005;107:183–206.
- Ghosh A, Abdelwahab SH, Reeber SL, Reidel B, Marklew AJ, Garrison AJ, *et al*. Little cigars are more toxic than cigarettes and uniquely change the airway gene and protein expression. *Sci Rep* 2017;7:46239.
- Smith JC, Sausville EL, Girish V, Yuan ML, Vasudevan A, John KM, *et al*. Cigarette smoke exposure and inflammatory signaling increase the expression of the SARS-CoV-2 receptor ACE2 in the respiratory tract. *Dev Cell* 2020;53:514–529, e3.
- Changeux JP, Amoura Z, Rey FA, Miyara M. A nicotinic hypothesis for COVID-19 with preventive and therapeutic implications. *C R Biol* 2020;343:33–39.
- Li G, He X, Zhang L, Ran Q, Wang J, Xiong A, *et al*. Assessing ACE2 expression patterns in lung tissues in the pathogenesis of COVID-19. *J Autoimmun* 2020;112:102463.
- Jiang C, Chen Q, Xie M. Smoking increases the risk of infectious diseases: a narrative review. *Tob Induc Dis* 2020;18:60.
- Swärd P, Edsfieldt A, Reepalu A, Jehpsson L, Rosengren BE, Karlsson MK. Age and sex differences in soluble ACE2 may give insights for COVID-19. *Crit Care* 2020;24:221.
- Clunes LA, Bridges A, Alexis N, Tarran R. In vivo versus in vitro airway surface liquid nicotine levels following cigarette smoke exposure. *J Anal Toxicol* 2008;32:201–207.
- Zhou Y, Frey TK, Yang JJ. Viral calciomics: interplays between Ca²⁺ and virus. *Cell Calcium* 2009;46:1–17.

Copyright © 2022 by the American Thoracic Society