COVID-19 Related Genes in Sputum Cells in Asthma: Relationship to Demographic Features and Corticosteroids

Michael C. Peters M.D.[1], Satria Sajuthi, Ph.D.[2], Peter Deford, Ph.D.[2], Stephanie Christenson M.D.[1], Cydney L. Rios[2], Michael T. Montgomery[2], Prescott G. Woodruff, M.D.[1], David T. Mauger, Ph.D.[3], Serpil C. Erzurum, M.D.[4], Mats W. Johansson Ph.D.[5], Loren C. Denlinger M.D. Ph.D.[6], Nizar N. Jarjour M.D.[6], Mario Castro, M.D.[7], Annette T. Hastie Ph.D.[8], Wendy Moore M.D. [8], Victor E. Ortega M.D. Ph.D[8], Eugene R. Bleecker M.D.[9], Sally E. Wenzel M.D.[10], Elliot Israel, M.D.[11], Bruce D. Levy, M.D.[11], Max A. Seibold, Ph.D.[2,12,13] *, John V. Fahy, M.D. M.Sc.[1]*, and National Heart, Lung, and Blood Institute Severe Asthma Research Program-3 Investigators.

*These authors contributed equally.

Institutional Affiliations:

[1]Division of Pulmonary and Critical Care Medicine, Department of Medicine and the Cardiovascular Research Institute, University of California San Francisco, San Francisco, California.

[2] Center for Genes, Environment, and Health, National Jewish Health, Denver, Colorado

[3]Division of Statistics and Bioinformatics, Department of Public Health Sciences, Pennsylvania State University, Hershey, Pennsylvania.

[4]Department of Pathobiology, Cleveland Clinic, Cleveland, Ohio.

[5]Department of Biomolecular Chemistry, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin,

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

[6]Division of Allergy, Pulmonary and Critical Care Medicine, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin.

[7]Division of Allergy, Immunology and Pulmonary Medicine, University of Kansas, Kansas City, Kansas.

[8]Department of Internal Medicine, Pulmonary Section, School of Medicine, Wake Forest University, Medical Center Blvd, Winston-Salem, North Carolina.

[9]Division of Genetics, Genomics and Precision Medicine, University of Arizona Department of Medicine, Tucson, Arizona.

[10]Pulmonary, Allergy and Critical Care Medicine Division, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

[11]Division of Pulmonary and Critical Care Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts.

[12]Department of Pediatrics, National Jewish Health, Denver, CO, 80206 USA;

[13]Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado-AMC, Aurora, CO, 80045 USA

Address correspondence to: Michael C. Peters, M.D., Room M1292, UCSF, 505 Parnassus Avenue, San Francisco, California 94143, USA; Phone: 415.476.9940; Email: michael.peters@ucsf.edu.

Author contributions: M.C.P., and J.V.F. are the guarantors of the paper, taking responsibility for the integrity of the work as a whole from inception to published article.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

M.C.P., S.S., P.D. S.C, C.L.R., M.T.M., P.G.W., D.T.M., S.C.E., M.W.J., L.C.D., N.N.J., M.C., A.T.H., W.M., V.E.O., E.R.B., S.E.W., E.I., B.D.L., M.A.S., J.V.F. conceived and designed the study. M.C.P, S.S. P.D. M.A.S. and J.V.F did the primary analysis and made substantial contributions to the design of the study and interpretation of data. M.C.P, J.V.F. prepared the first draft of the manuscript and all authors revised the draft critically for important intellectual content. All authors gave final approval for the manuscript version to be published.

Funding/Support: This study was funded by the National Institutes of Health Grants: (PO1 HL107201, K23 HL138303, RO1 HL080414, RO1 HL135156, RO1 MD010443, RO1 HL 1288439, PO1 HL132821, PO1 HL107202, DOD W81WH-16-2-0018, P01 HL107202, R01 HL115118, P30 DK098722, U10 HL109146, U10 HL109164, U10 HL109172, U10 HL109086, U10 HL109250, U10 HL109168, U10HL109257, and U10 HL109152, by the UCSF Sandler Asthma Basic Research Center, and by grants from the Parker B Francis Foundation.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

ABSTRACT

Background: Coronavirus disease 2019 (COVID-19) is caused by SARS-coronavirus 2 (SARS-CoV-2). Angiotensin converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) mediate viral infection of host cells. We reasoned that differences in ACE2 or TMPRSS2 gene expression in sputum cells among asthma patients may identify subgroups at risk for COVID19 morbidity.

Methods: We analyzed gene expression for ACE2 and TMPRSS2, and for intercellular adhesion molecule 1 (ICAM-1)(rhinovirus receptor as a comparator), in sputum cells from 330 participants in the Severe Asthma Research Program-3 and 79 healthy controls.

Results: Gene expression of ACE2 was lower than TMPRSS2, and expression levels of both genes was similar in asthma and health. Among asthma patients, male gender, African Americans race, and history of diabetes mellitus, was associated with higher expression of ACE2 and TMPRSS2. Use of inhaled corticosteroids (ICS) was associated with lower expression of ACE2 and TMPRSS2, but treatment with triamcinolone acetonide (TA) did not decrease expression of either gene. These findings differed from those for ICAM-1, where gene expression was increased in asthma and less consistent differences were observed related to gender, race, and use of ICS.

Conclusion: Higher expression of ACE2 and TMPRSS2 in males, African Americans, and patients with diabetes mellitus provides rationale for monitoring these asthma subgroups for poor COVID19 outcomes. The lower expression of ACE2 and TMPRSS2

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

with ICS use warrants prospective study of ICS use as a predictor of decreased susceptibility to SARS-CoV-2 infection and decreased COVID19 morbidity.

Key words: Coronavirus; COVID-19; asthma; SARS-CoV-2; spike protein, S protein; angiotensin converting enzyme 2 receptor, ACE2; transmembrane protease serine 2, TMPRSS2; intercellular adhesion molecule 1, ICAM-1.

Word Count: 2914

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

INTRODUCTION

Coronavirus disease 2019, or COVID-19, caused by SARS-coronavirus 2 (SARS-CoV-2), may be more severe in patients with chronic lung disease, including patients with asthma (1). Among patients with asthma, it is possible that demographic or biological factors influence susceptibility to SARS-CoV-2 infection or severity of COVID19 disease. In this regard, the mechanism of SARS-CoV-2 infection is relevant. The spike (S) protein of SARS-CoV-2 binds the angiotensin converting enzyme 2 (ACE2) receptor to mediate virus attachment to host cell membranes, and virus cell entry also depends on S protein priming by host cell proteases, including transmembrane protease, serine 2 (TMPRSS2)(2,3). We reasoned that differences in ACE2 or TMPRSS2 gene expression in sputum cells among asthma patients may identify subgroups at risk for worse COVID19 outcomes. In particular, we hypothesized that co-morbidities such as diabetes mellitus or hypertension may affect ACE2 or TMPRSS2 gene expression in sputum cells because these diseases are reported to influence the severity of COVID19 or its outcome (1,4). In addition, we hypothesized that corticosteroids, a common treatment for asthma, may affect ACE2 or TMPRSS2 gene expression in sputum cells. To test these hypotheses, we studied participants in the Severe Asthma Research Program-3 (SARP), a longitudinal cohort study designed to uncover clinical and molecular phenotypes of asthma (5). Participants in SARP-3 have undergone detailed characterization and phenotyping, including assessment of treatment responses to bronchodilators and corticosteroids, and they have provided sputum cells, obtained by sputum induction, for gene profiling studies. To provide comparative data for the ACE2 and TMPRSS2 analyses, we also examined the expression of intercellular adhesion

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

molecule 1 (ICAM-1), the major intercellular protein that mediates binding of human rhinoviruses (HRVs) to the airway epithelium (6).

METHODS

Subjects

The Severe Asthma Research Program (SARP)-3 protocol is an ongoing, six visit, 3-year, longitudinal cohort study in which 60% of subjects have severe asthma as defined by the American Thoracic Society/European Respiratory Society (ATS/ERS) consensus (4,7). Sputum RNA that passed quality assurance measures was analyzed on 330 asthma participants, and 79 healthy control participants. Of the 330 asthma participants, 254 provided a sample at baseline, 121 provided a sample at 1 year, and 181 provided a sample at year three, for a total of 556 asthma samples (Supplemental Figure 1). Each healthy control subject provided one sample and included 22 subjects recruited by SARP-3 and 57 healthy subjects recruited by the Airway Clinical Research Center at UCSF.

UCSF Healthy Subjects: Fifty-seven healthy control subjects had been recruited to research studies in the UCSF Airway Clinical Research Center between 2005-2014. All studies included 1-2 baseline visits, which used standardized protocols for clinical characterization and collection, processing, and storage of blood and induced sputum. Healthy subjects had no history of pulmonary disease, no history of atopic disease or allergic rhinitis, and had normal airway responses to inhaled methacholine.

SARP-3 Healthy Subjects: Twenty-two healthy control subjects were recruited by SARP-3 centers between November 1, 2014 and February 1, 2015. Healthy subjects

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

had no history of pulmonary disease, no history of atopic disease or allergic rhinitis, and had normal airway responses to inhaled methacholine.

SARP-3 Asthma Subjects: The data reported here is from induced sputum cells collected at baseline (year 0) and at years 1 and 3. The visit structure of the SARP protocol is outlined in Supplementary Fig 1. The baseline visits (visits 1 and 2) included completion of medical history and asthma control questionnaires, spirometry, and biospecimen collection (induced sputum and blood). Baseline data collection included documentation of asthma medication use, including documentation of inhaled corticosteroid dosing consistent with ERS/ATS guidelines for no ICS, low/medium dose ICS, or high dose ICS (7). In addition, maximum bronchodilator reversibility tests (spirometry before and after 4–8 puffs of albuterol) were performed on baseline visits 2 and 3, and participants also underwent a systemic corticosteroid response test. The systemic corticosteroid response test involved an intramuscular injection of triamcinolone acetonide (40 mg) on baseline visit 2 and repeat characterization (including maximum bronchodilator reversibility tests, sputum induction, and blood draw) on visit 3 (2–4 weeks later)(4,8).

Sputum induction and processing

Subjects inhaled nebulized 3% saline through a mouthpiece for 12 minutes, as previously described and interrupted inhalation at 2-minute intervals to spit saliva into a saliva cup and induced sputum into a sputum cup(9). Saliva was discarded, and induced sputum was processed. A 10% solution of Sputolysin (EMD Millipore, Burlington, Mass) was added at a 1:1 gram per milliliter (sputum weight/Sputolysin) ratio

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

to the induced sputum, mixed with a serologic pipette, and placed in a 37°C shaking water bath for 15 minutes. Samples were removed at 5-, 10-, and 15-minute intervals for additional mixing with the pipette, and a portion of this sample was used to determine total and differential cell counts. The sample was then centrifuged in the cold (4°C) at 2000 rpm for 10 minutes. The cell pellet was then resuspended in 1 mL of Qiagen RNAprotect Saliva Reagent (Qiagen Hilden, Germany). Cell pellets were stored at −80°C and all RNA was shipped to the UCSF Sputum Core for RNA extraction.

RNA extraction

RNA was extracted from sputum cells with the RNeasy Qiagen kit (Qiagen), as previously described(10,11). RNA concentration and quality were measured with the Agilent 2100 bioanalyzer (Biogen, Weston, Mass), and samples with an RNA integrity number (RIN) less than 5 were considered degraded and excluded from analysis. Purified RNA was placed in aliquots and stored at − 80°C prior to RNA sequencing at National Jewish Health.

Whole Transcriptome RNA-seq

Library preparation and RNA sequencing was conducted at National Jewish Health, Denver CO. Briefly, KAPA mRNA HyperPrep (Roche) whole transcriptome libraries were constructed with 20 ng RNA input per sample, barcoded with Illumina Dual Index Adapters (IDT) and amplified for 16 cycles. Completed libraries were pooled together by concentration and sequenced using the Illumina NovaSeq® 6000 system.

Raw sequencing reads were trimmed using skewer(12) with parameters (end-quality=15, mean-quality=25, min=30). Trimmed reads were then aligned to the human

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

reference genome GRCh38 using HiSat2 with default parameters(13). Gene quantification was performed with htseq-count using GRCh38 ensemble v84 gene transcript model(14). Variance stabilization transformation implemented in DESeq2 was then carried out on the raw gene count matrix to create a variance stabilized gene expression matrix suitable for downstream analyses(15).

Statistical Methods

Analyses were performed using JMP 14 software package (SAS Institute, Cary, NC), Stata 15.1 (StataCorp College Station TX), and R statistical package (Vienna Austria). Two group comparisons between asthma participants and healthy participants were made using Student's t-test for continuous variables with roughly symmetric distributions, Wilcoxon's rank-sum test for continuous variables with skewed distributions, and Pearson's chi-square test for categorical variables. Pearson's correlation was used to assess the relationships between continuous variables. Using DESeq2 gene expression data were variance stabilizing transformation normalized. Multilevel mixed-effects linear regression models were utilized to evaluate the association between sputum gene expression and clinical and demographic variables(16,17). We selected covariates in the multivariate mixed effects models based upon two categories - covariates hypothesized as susceptibility factors for SARS-COV2 infection (age, body mass index, gender, race, diabetes, hypertension and use of inhaled corticosteroids) and covariates that represented outcomes of asthma control and severity (FEV1% predicted, Asthma Control Test scores [ACT], and asthma exacerbations). The covariates hypothesized as susceptibility factors were the primary variables of interest. Random and fixed effects were calculated by grouping the data at

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

the participant level with restricted maximum likelihood models. P-values <0.05 were considered statistically significant.

RESULTS

Subjects

The demographic and clinical features of the asthma patients and healthy controls are shown in Table 1.

Gene expression for SARS-Cov-2- and HRV-related genes in induced sputum cells from asthma patients and healthy controls

In induced sputum cells collected at the baseline visit, the expression levels of ACE2 were lower than the expression levels of TMPRSS2, and some sputum samples had undetectable ACE2 (Figure 1A). The expression of ACE2 and TMPRSS2 did not differ significantly in health and in asthma (Figure 1A,B). In contrast to the SARS-Co-V2-related genes, gene expression of ICAM1 was higher in asthma than in health (Figure 1C). The expression of ACE2 was strongly associated with the expression of TMPRSS2 in the healthy control subgroup (Figure 2A) and the asthma subgroup (Figure 2B), suggesting that these genes are expressed in similar cells(18).

Relationship between clinical and demographic variables and expression levels of SARS-Cov-2- and HRV-related genes in asthma patients

Here we analyzed gene expression data in the induced sputum samples collected at the baseline visit 2 and the follow up visits 4 (year 1) and 6 (year 3). The total number was 556 samples from 330 asthma subjects. ACE2 and TMPRSS2 expression levels

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

increased slightly with age, but were significantly higher in males than in females and in African Americans than in Caucasians (Figure 3, Tables S1,S2). In addition, ACE2 expression was higher in patients with diabetes mellitus than in those without (Figure 3, Tables S1,S2). These findings were qualitatively different from those for ICAM-1, where we found less consistent differences based on gender and race (Figure 3, Table S3). Although ICAM-1 expression differed by age and male gender, it did not differ in African Americans or in those with diabetes mellitus (Figure 3, Table S3). To exclude differences in sputum cell differentials as confounders of these findings, sputum cell differentials were added to the list of covariates in the multivariate mixed effects model. The addition of sputum cells did not change any findings (data not shown).

Expression of SARS-Cov-2- and HRV-related genes in asthma patients taking inhaled corticosteroids

Gene expression data in the induced sputum samples collected at the baseline visit 2 and the follow up visits 4 (year 1) and 6 (year 3) was analyzed, and three ICS subgroups were compared. The three subgroups were patients not taking ICS, patients taking low and medium ICS, and patients taking high doses of ICS. We considered the possibility of confounding by disease severity in these analyses and asthma severity factors such as FEV1, asthma control test scores, and asthma exacerbation history, were included as covariates in the analysis model for this reason. Using this analysis approach, ACE2 and TMPRSS2 expression levels were significantly lower in asthma patients taking ICS than in those not taking ICS, especially in those taking higher doses of ICS (Figure 4A, Table S1,S2). The ICS findings were qualitatively different from those for ICAM-1. Specifically, ICAM-1 levels were not significantly different among ICS

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

subgroups (Figure 4A, Table S3). These ICS findings prompted us to analyze SARS-Cov-2- and ICAM1 in sputum cells collected before and 2-4 weeks after intramuscular injection of triamcinolone acetonide (40 mg). We found no significant differences in expression levels of ACE2, TMPRSS2, or ICAM-1 before and after TA treatment (Figure 4B). As above for clinical and demographic features, the findings for ICS use remained similar and significant when sputum cells were included as covariates in the model.

Expression of SARS-Cov-2-related genes in asthma patients taking ACE inhibitor or Angiotensin Receptor Blockers (ARB)

Use of ACE inhibitors or Angiotensin receptor blockers data was available on 180 participants at Visit 6. To investigate if oral treatment with ACE inhibitor medications or ARBs influenced our findings we compared ACE2 and TMPRSS2 levels between subjects taking ARB (n=21) to participants not on ARB (n=159) and between participants taking ACE inhibitors (n=23) to participants not on ACE inhibitors (n=157). In this subgroup analysis we found no difference between ACE2 or TMPRSS2 sputum gene expression measures between subjects (Supplemental Figure S2).

DISCUSSION

ACE2 and TMPRSS2 expression mediate SARS-CoV-2 infection of host lung cells (3), and it is reasonable to infer that increases in their expression in lung cells will increase susceptibility to SARS-CoV-2 infection or lead to more severe COVID19 disease. Although gene expression for ACE2 and TMPRSS2 did not differ from health in asthma, we report that males, African Americans, and patients with diabetes mellitus have increased expression of ACE2 and TMPRSS2 in their sputum cells, and these patient

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

subgroups should therefore be monitored for poor COVID19 outcomes. In contrast, we report lower expression of ACE2 and TMPRSS2 in sputum cells from asthma patients taking ICS, and this finding warrants prospective research to determine if ICS use predicts decreased susceptibility to SARS-CoV-2 infection or decreased COVID19 morbidity.

Although recently identified as the SARS-CoV2 receptor, ACE2 has previously been studied for its role in angiotensin biology(2,3,19). Whereas angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II (Ang II), a decapeptide and potent vasoconstrictor, ACE2 catabolizes Ang II to Ang-(1-7) in the kidney and other tissues(19). Through these effects, ACE2 is thought to act as a natural brake on the adverse effects of ACE and Ang II in the pathophysiology of hypertension, renal disease, diabetes mellitus, and lung injury (19,20,20). The higher expression of ACE2 that we report in male patients is notable because of the high mortality of COVID19 in males (1,5). Perhaps relatedly, the ACE2 gene is on the X chromosome, and differences in sex chromosome dosage could affect ACE2 expression through X-inactivation or differences in parental imprinting. However, prior studies in rodents have found that ACE2 levels are relatively high in males because ACE2 levels are suppressed in females by female sex hormones(21).

We also report higher expression of ACE2 in patients with diabetes mellitus, and in African Americans. This finding is interesting because diabetes is a risk factor for severe morbidity or death from COVID19 (1). Hypertension is also a risk factor for COVID19 morbidity (1), but we did not find increases in ACE2 expression in sputum cells from asthma patients with and without hypertension. The higher expression of ACE2 in

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

African Americans is also noteworthy because COVID19 outcomes in African Americans, Africans, or other persons of African descent have not yet been reported in any detail. African Americans are known to have genetic risk factors for hypertension that relate to polymorphisms in renin-angiotensin cascade genes such as renin, angiotensinogen, type-1 angiotensin II receptor, and ACE(22–24). To our knowledge, little is known about polymorphisms that may affect ACE2.

TMPRSS2 is a transmembrane protease that modifies spike proteins in multiple viruses - including SARS-CoV, SARS-CoV2, MERS-CoV, and influenza A and B - to promote viral infection and spread (3). We found that TMPRSS2 gene expression in sputum cells was higher than ACE2 expression and that TMPRSS2 correlated strongly with ACE. We have previously found that strong correlations between genes in sputum cells indicate that the genes are co-expressed in same cell types (18). In addition, the changes in TMPRSS2 among asthma subgroups - including differences related to male gender, African American race, and Diabetes Mellitus - mirrored the changes for ACE2. The similar increases in both genes in the same patient subgroups provides a mechanism for a “double hit” susceptibility for SARS-CoV-2 infection and COVID19 morbidity in these patients.

ACE2 and TMPRSS2 expression was lower in patients taking inhaled corticosteroids (ICS) than in patients not taking ICS, a finding of high clinical relevance because of concern that the immunosuppressive effects of ICS could put asthma patients at risk during the COVID19 pandemic. The decrease in gene expression for ACE2 and TMPRSS2 provides some reassurance that ICS use will not increase the risk of infection with SARS-Cov2 or morbidity from COVID19, but this finding should be

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

explored in prospective studies. However, we did not find a reduction in ACE2 and TMPRSS2 expression in sputum cells collected before and 2-4 weeks after treatment with intramuscular triamcinolone acetonide (TA) injection. The lack of agreement for the effects of inhaled versus systemic corticosteroids and SARS-CoV2 genes in the SARP-3 cohort may be explained by multiple factors, including the possibility that the timepoint chosen for assessing the effects of TA on sputum cell gene expression was not optimal for the detection of any effect of TA on ACE2 or TMPRSS2 gene expression in lung cells.

To provide a comparison for our analysis SARS-CoV2-related genes in sputum cells in the SARP-3 cohort, we included analysis of the expression of the human rhinovirus (HRV) receptor, ICAM. HRV airway infections are a common cause of asthma exacerbations(25), and most HRV-A, and all HRV-B strains bind ICAM-1 on airway epithelial cells(26). Unlike SARS-CoV2-related genes which were not differentially expressed in asthma, ICAM-1 expression was increased in asthma. Non-asthma related factors, such as male gender, African American race, and metabolic disease (diabetes mellitus), may be more important and more generalizable risks for susceptibility to SARS-CoV2 infection and more severe COVID19 morbidity.

We do note limitations to our approach. First, regarding studies of gene expression in induced sputum, this biospecimen comprises a mix of multiple cell types, including structural airway cells (e.g. epithelial cells and squamous cells) and multiple immune cells (e.g. macrophages and granulocytes). The cellular components of sputum generally reflect cells originating in the oropharynx and upper airway. Although sputum provides a valuable window into gene expression in the airways and lungs, variance in

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

sputum cell gene expression reflects between both differences in per cell expression and proportion of different cell types in the sample. Therefore caution is urged in biological interpretations. Second, for the corticosteroid results reported here, the data were not generated in a randomized, prospective, or placebo-controlled study. Third, it is not confirmed that mRNA expression reflects protein levels, which need to be validated. Finally, our findings were generated in patients with asthma and should not be extrapolated to non asthma patients without further study.

In summary, in a large cohort of well-characterized patients with asthma, we report higher sputum cell expression of ACE2 and TMPRSS2 in males, African Americans, and patients with diabetes mellitus and lower expression in patients taking inhaled corticosteroids. These findings can inform prospective study of COVID19 outcomes in specific asthma subgroups, including subgroups taking ICS in different dose strengths.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

REFERENCES

1. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China. JAMA Intern Med. 2020 Mar 13;

2. Yan R, Zhang Y, Li Y, Xia L, Guo Y, Zhou Q. Structural basis for the recognition of the SARS-CoV-2 by full-length human ACE2. Science. 2020 Mar 4;

3. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell. 2020 Mar 4;

4. Guan W-J, Ni Z-Y, Hu Y, Liang W-H, Ou C-Q, He J-X, et al. Clinical Characteristics of Coronavirus Disease 2019 in China. N Engl J Med. 2020 Feb 28;

5. Teague WG, Phillips BR, Fahy JV, Wenzel SE, Fitzpatrick AM, Moore WC, et al. Baseline Features of the Severe Asthma Research Program (SARP III) Cohort: Differences with Age. J Allergy Clin Immunol Pract. 2018 Apr;6(2):545-554.e4.

6. Basnet S, Palmenberg AC, Gern JE. Rhinoviruses and Their Receptors. Chest. 2019;155(5):1018–25.

7. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. EurRespirJ. 2014;43(2):343–73.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

8. Phipatanakul W, Mauger DT, Sorkness RL, Gaffin JM, Holguin F, Woodruff PG, et al. Effects of Age and Disease Severity on Systemic Corticosteroid Responses in Asthma. Am J Respir Crit Care Med. 2016 Dec 14;

9. Gershman NH, Wong HH, Liu JT, Mahlmeister MJ, Fahy JV. Comparison of two methods of collecting induced sputum in asthmatic subjects. EurRespirJ. 1996;9(12):2448–53.

10. Peters MC, Mekonnen ZK, Yuan S, Bhakta NR, Woodruff PG, Fahy JV. Measures of gene expression in sputum cells can identify TH2-high and TH2-low subtypes of asthma. JAllergy ClinImmunol. 2014;133(2):388–94.

11. Peters MC, Kerr S, Dunican EM, Woodruff PG, Fajt ML, Levy BD, et al. Refractory airway type 2 inflammation in a large subgroup of asthmatic patients treated with inhaled corticosteroids. J Allergy Clin Immunol. 2019 Jan;143(1):104-113.e14.

12. Skewer: a fast and accurate adapter trimmer for next-generation sequencing paired-end reads | BMC Bioinformatics | Full Text [Internet]. [cited 2020 Mar 26]. Available from: https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-15-182

13. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods. 2015 Apr;12(4):357–60.

14. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015 Jan 15;31(2):166–9.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

15. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol [Internet]. 2014 [cited 2016 Sep 7];15(12). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4302049/

16. McCulloch, Charles E. Chapter 4: Generalized linear mixed models (GLMMs). Generalized Linear Mixed Models, 28--33. Beechwood OH and Alexandria VA: Institute of Mathematical Statistics and American Statistical Association; 2003.

17. McNeish D, Kelley K. Fixed effects models versus mixed effects models for clustered data: Reviewing the approaches, disentangling the differences, and making recommendations. Psychol Methods. 2019 Feb;24(1):20–35.

18. Peters MC, Ringel L, Dyjack N, Herrin R, Woodruff PG, Rios C, et al. A Transcriptomic Method to Determine Airway Immune Dysfunction in T2-High and T2-Low Asthma. Am J Respir Crit Care Med. 2018 Oct 29;

19. Santos RAS, Sampaio WO, Alzamora AC, Motta-Santos D, Alenina N, Bader M, et al. The ACE2/Angiotensin-(1-7)/MAS Axis of the Renin-Angiotensin System: Focus on Angiotensin-(1-7). Physiol Rev. 2018 01;98(1):505–53.

20. Kuba K, Imai Y, Penninger JM. Angiotensin-converting enzyme 2 in lung diseases. Curr Opin Pharmacol. 2006 Jun;6(3):271–6.

21. Liu J, Ji H, Zheng W, Wu X, Zhu JJ, Arnold AP, et al. Sex differences in renal angiotensin converting enzyme 2 (ACE2) activity are 17β-oestradiol-dependent and sex chromosome-independent. Biol Sex Differ. 2010 Nov 5;1(1):6.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

22. Zhu X, Chang Y-PC, Yan D, Weder A, Cooper R, Luke A, et al. Associations between hypertension and genes in the renin-angiotensin system. Hypertension. 2003 May;41(5):1027–34.

23. Henderson SO, Haiman CA, Mack W. Multiple Polymorphisms in the renin-angiotensin-aldosterone system (ACE, CYP11B2, AGTR1) and their contribution to hypertension in African Americans and Latinos in the multiethnic cohort. Am J Med Sci. 2004 Nov;328(5):266–73.

24. Kumar A, Li Y, Patil S, Jain S. A haplotype of the angiotensinogen gene is associated with hypertension in african americans. Clin Exp Pharmacol Physiol. 2005 Jun;32(5–6):495–502.

25. Busse WW, Lemanske RF, Gern JE. Role of viral respiratory infections in asthma and asthma exacerbations. Lancet. 2010 Sep 4;376(9743):826–34.

26. Bianco A, Sethi SK, Allen JT, Knight RA, Spiteri MA. Th2 cytokines exert a dominant influence on epithelial cell expression of the major group human rhinovirus receptor, ICAM-1. Eur Respir J. 1998 Sep;12(3):619–26.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

Figure Legends:

Figure 1: Sputum Gene expression at the initial study visit in asthma participants (n=330) and healthy participants (n=79). **A,B)** No difference in SARS-Co-V2-related genes, ACE2 and TMPRSS2, between asthma participants and healthy participants **C)** Gene expression for the rhinovirus binding protein ICAM1 was higher in asthma participants compared to healthy participants.

Figure 2: Sputum Gene expression of ACE2 and TMPRSS2 is strongly correlated in both A) healthy participants and B) asthma participants. Best fit line was fitted using a cubic smoothing spline.

Figure 3: Multivariate mixed effects models displaying the effect of demographic features and co-morbidities on sputum gene expression. The multivariate models include each demographic or co-morbidity feature, and is also controlled for the three asthma control factors (FEV1% predicted, number of asthma exacerbations in the past year, and asthma symptoms as measured by the score on the asthma control test [ACT]). The figure shows estimates for the mean effect (regression coefficient) of each demographic or co-morbidity feature on sputum gene expression expressed on a $\log_{10}$ change. The effect size displayed is per 10-year change for age and per 10 unit change for BMI. *The reference group for race is white. Two subjects with AIAN race were not included in the race analysis. Data is presented as mean difference (circles) and 95% CI (whiskers).

Figure 4: A) Multivariate mixed effects models displaying the effect of inhaled corticosteroid use on sputum gene expression. Models incorporate the same

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

demographic features in figure 3 and the three asthma control factors. ACE2 and TMPRSS2 are statistically significantly lower in patients on inhaled corticosteroids compared to the control group of subjects not on inhaled corticosteroids. Data presented as mean effect (circles) and 95% CI (whiskers). B) No change in sputum gene expression in asthma subjects (n=158) before (Baseline) and 2 weeks after a 40 mg injection of triamcinolone (Post-CS). Comparison of means were performed using a matched paired t-test.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

TABLE 1

Characteristic	Healthy (n=79)	Asthma (n=330)	p-value
Age (years)	40.6 (14.5)	48.5 (13.8)	<0.001
Female sex - no. (%)	52 (66)	230 (69)	0.62
Race - no. (%)*			0.001
American Indian and Alaska Native (AIAN)	0 (0)	2 (1)	
Asian	20 (26)	13 (4)	
African American	9 (12)	77 (23)	
Caucasian	43 (57)	217 (66)	
Native Hawaiian or other Pacific Islander	0 (0)	0 (0)	
Mixed race	4 (5)	23 (7)	
BMI (kg/m^2)	29.1 (7.8)	32.4 (8.7)	0.002
Spirometry			
FEV1 (% predicted)	95.7 (9.9)	72.8 (19.3)	<0.001
FVC (% predicted)	97.0 (12.9)	85.2 (17.0)	0.001
FEV1%/FVC%	98.6 (5.3)	84.5 (12.1)	<0.001

* AIAN patients are not included in the mixed effects models because only 2 patients identified as AIAN. 3 healthy control subjects did not answer the race questionnaire.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

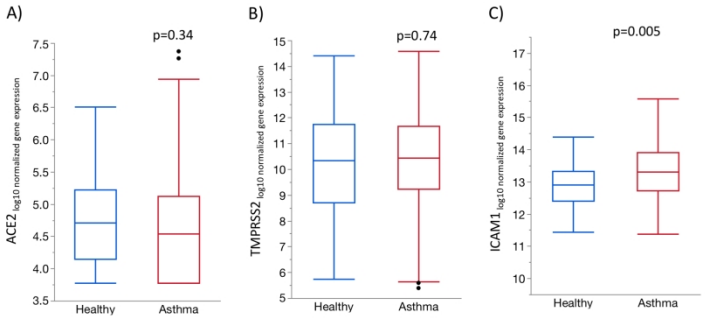
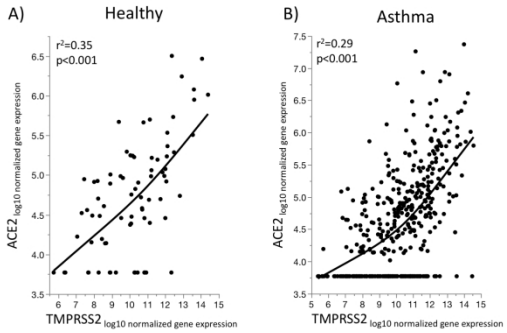


Figure 1: Sputum Gene expression at the initial study visit in asthma participants (n=330) and healthy participants (n=79). A,B) No difference in SARS-Co-V2-related genes, ACE2 and TMPRSS2, between asthma participants and healthy participants C) Gene expression for the rhinovirus binding protein ICAM1 was higher in asthma participants compared to healthy participants.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC



Sputum Gene expression of ACE2 and TMPRSS2 is strongly correlated in both A) healthy participants and B) asthma participants. Best fit line was fitted using a cubic smoothing spline.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

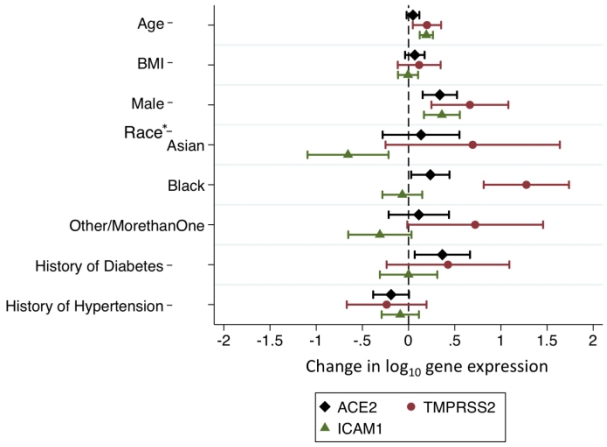


Figure 3: Multivariate mixed effects models displaying the effect of demographic features and co-morbidities on sputum gene expression. The multivariate models include each demographic or co-morbidity feature, and is also controlled for the three asthma control factors (FEV1% predicted, number of asthma exacerbations in the past year, and asthma symptoms as measured by the score on the asthma control test [ACT]). The figure shows estimates for the mean effect (regression coefficient) of each demographic or co-morbidity feature on sputum gene expression expressed on a log10 change. The effect size displayed is per 10-year change for age and per 10 unit change for BMI. *The reference group for race is white. Two subjects with AIAN race were not included in the race analysis. Data is presented as mean difference (circles) and 95% CI (whiskers).

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

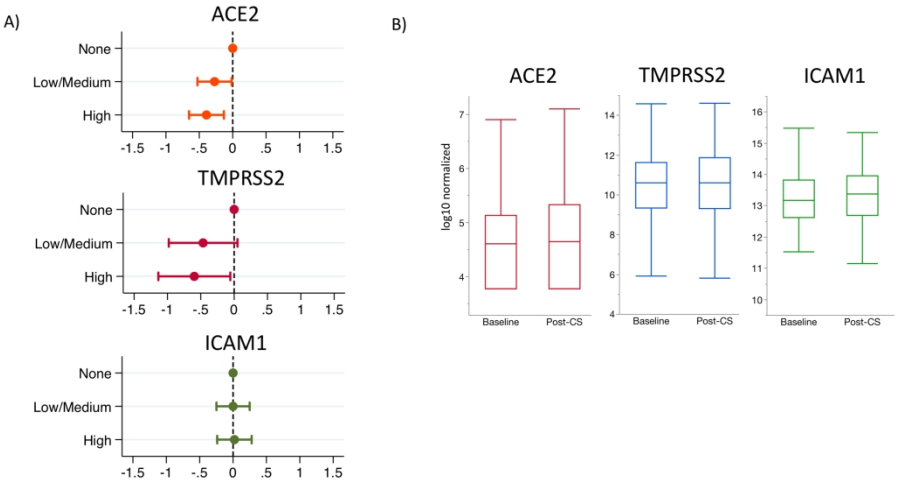


Figure 4: A) Multivariate mixed effects models displaying the effect of inhaled corticosteroid use on sputum gene expression. Models incorporate the same demographic features in figure 3 and the three asthma control factors. ACE2 and TMPRSS2 are statistically significantly lower in patients on inhaled corticosteroids compared to the control group of subjects not on inhaled corticosteroids. Data presented as mean effect (circles) and 95% CI (whiskers). B) No change in sputum gene expression in asthma subjects (n=158) before (Baseline) and 2 weeks after a 40 mg injection of triamcinolone (Post-CS). Comparison of means performed using a matched paired t-test.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

COVID-19 Related Genes in Sputum Cells in Asthma: Relationship to Demographic Features and Corticosteroids

Michael C. Peters M.D.[1], Satria Sajuthi, Ph.D.[2], Peter Deford, Ph.D.[2], Stephanie Christenson M.D.[1], Cydney L. Rios[2], Michael T. Montgomery[2], Prescott G. Woodruff, M.D.[1], David T. Mauger, Ph.D.[3], Serpil C. Erzurum, M.D.[4], Mats W. Johansson Ph.D.[5], Loren C. Denlinger M.D. Ph.D.[6], Nizar N. Jarjour M.D.[6], Mario Castro, M.D.[7], Annette T. Hastie Ph.D.[8], Wendy Moore M.D. [8], Victor E. Ortega M.D. Ph.D[8], Eugene R. Bleecker M.D.[9], Sally E. Wenzel M.D.[10], Elliot Israel, M.D.[11], Bruce D. Levy, M.D.[11], Max A. Seibold, Ph.D.[2,12,13] *, John V. Fahy, M.D. M.Sc.[1]*, and National Heart, Lung, and Blood Institute Severe Asthma Research Program-3 Investigators.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

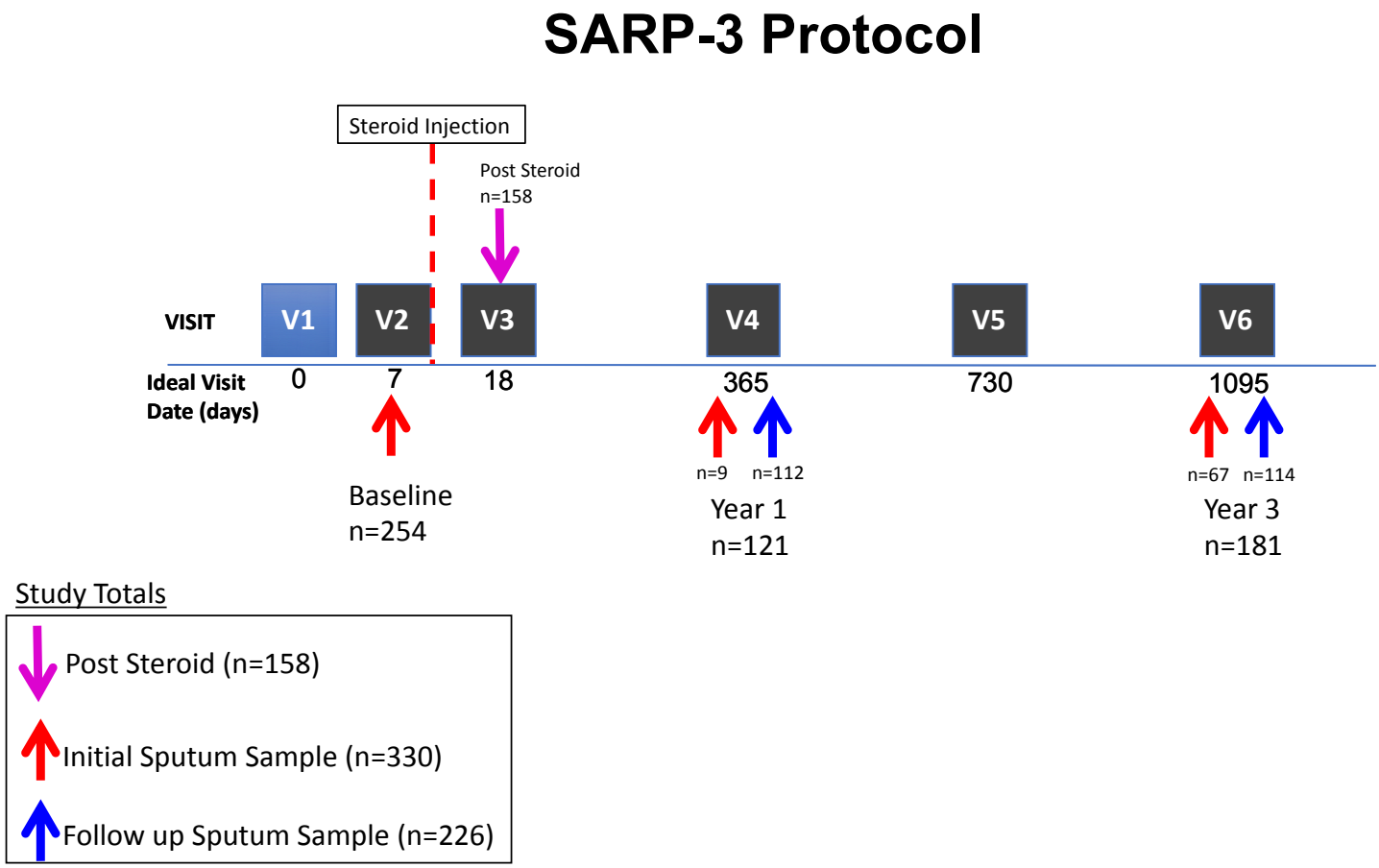


Figure S1: Structure of the SARP Sputum RNA-sequencing biobank.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

Table S1: Multivariate Models for the Effect of Demographic Characteristics on Sputum ACE2 gene expression*

Characteristic	Mean (95% CI)	p-value
Age	0.05 (-0.02 to 0.12)	0.18
BMI	0.07 (-0.04 to 0.17)	0.21
Male	0.34 (0.15 to 0.52)	<0.001
Race#		
White	ref	ref
Asian	0.14 (-0.28 to 0.55)	0.53
Black	0.24 (0.28 to 0.44)	0.03
More than One	0.11 (-0.22 to 0.43)	0.51
History of Diabetes	0.37 (0.07 to 0.67)	0.02
History of Hypertension	-0.19 (-0.38 to 0.00)	0.06
ICS Dose		
None	ref	ref
Low/Medium	-0.27 (-0.53 to -0.02)	0.04
High	-0.39 (-0.65 to -0.13)	0.003

*Multivariate Models controlled for the effect of FEV1% predicted, asthma control test scores, and number of asthma exacerbations. Data displays the estimates for the mean effect of each demographic feature on a log10 change in sputum gene expression. The effect size for age is per 10 years and for BMI is per 10 units. #The reference group for race is white. 2 subjects with race AIAN were not included in the race analysis secondary to small sample sizes.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

Table S2: Multivariate Models for the Effect of Demographic Characteristics on Sputum TMPRSS2 Gene Expression*

Characteristic	Mean (95% CI)	p-value
Age	0.20 (0.05 to 0.35)	0.01
BMI	0.12 (-012 to 0.35)	0.33
Male	0.66 (0.25 to 1.08)	0.002
Race#		
White	ref	ref
Asian	0.69 (-0.25 to 1.63)	0.15
Black	1.28 (0.82 to 1.73)	<0.001
More than One	0.72 (-0.01 to 1.46)	0.06
History of Diabetes	0.43 (-0.24 to 1.09)	0.21
History of Hypertension	-0.24 (-0.67 to 0.19)	0.28
ICS Dose		
None	ref	ref
Low/Medium	-0.46 (-0.98 to 0.05)	0.08
High	-0.60 (-1.13 to -0.06)	0.03

*Multivariate Models controlled for the effect of FEV1% predicted, asthma control test scores, and number of asthma exacerbations. Data displays the estimates for the mean effect of each demographic feature on a log10 change in sputum gene expression. The effect size for age is per 10 years and for BMI is per 10 units. #The reference group for race is white. 2 subjects with race AIAN were not included in the race analysis secondary to small sample sizes.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

Table S3: Multivariate Models for the Effect of Demographic Characteristics on Sputum ICAM1 Gene Expression*

Characteristic	Mean (95% CI)	p-value
Age	0.19 (0.12 to 0.26)	<0.001
BMI	-0.00 (-0.11 to 0.12)	0.97
Male	0.37 (0.18 to 0.56)	<0.001
Race#		
White	ref	ref
Asian	-0.73 (-1.17 to 0.29)	<0.001
Black	-0.07 (-0.28 to 0.15)	0.53
More than One	-0.29 (-0.63 to 0.05)	0.10
History of Diabetes	0.01 (-0.31 to 0.32)	0.96
History of Hypertension	-0.09 (-0.29 to 0.11)	0.39
ICS Dose		
None	ref	ref
Low/Medium	0.00 (-0.24 to 0.25)	0.99
High	0.02 (-0.23 to 0.28)	0.88

*Multivariate Models controlled for the effect of FEV1% predicted, asthma control test scores, and number of asthma exacerbations. Data displays the estimates for the mean effect of each demographic feature on a log10 change in sputum gene expression. The effect size for age is per 10 years and for BMI is per 10 units. #The reference group for race is white. 2 subjects with race AIAN were not included in the race analysis secondary to small sample sizes.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC

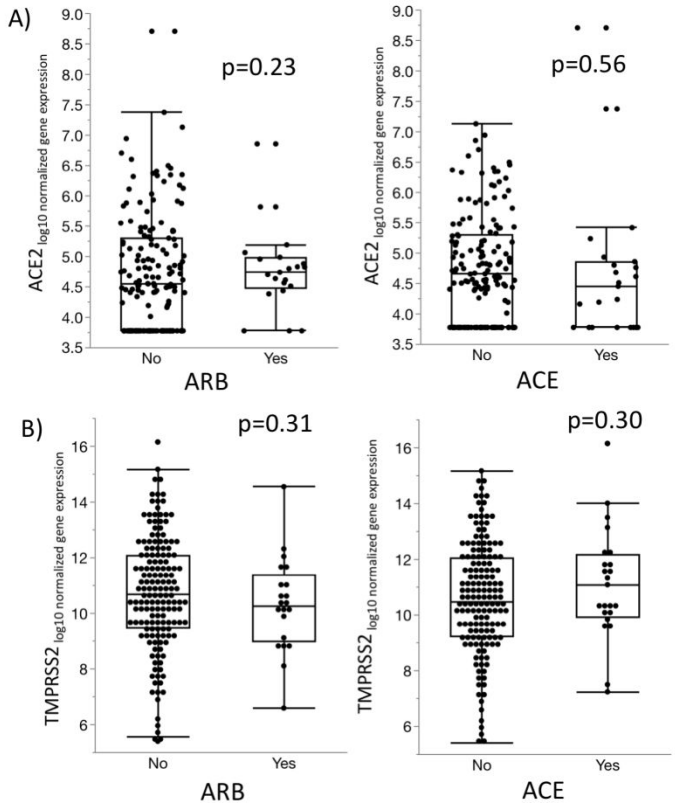


Figure S2: No difference in ACE2 (A) or TMPRSS2 (B) sputum gene expression at Visit 6 between participants taking angiotensin receptor blockers (ARB) (n=21) to participants not on ARB (n=159) or participants taking ACE inhibitors (ACE) (n=23) to participants not on ACE (n=157). Comparisons made using wilcoxon rank sums.

AJRCCM Articles in Press. Published April 29, 2020 as 10.1164/rccm.202003-0821OC