Expression of *ACE2*—a Key SARS-CoV-2 Entry Factor—Is Not Increased in the Nasal Mucosa of People with Cystic Fibrosis

To the Editor:

Mutations in *CFTR* (cystic fibrosis transmembrane regulator) lead indirectly to impaired innate defense of the respiratory tract, and people with cystic fibrosis (PwCF) develop a host of bacterial and fungal infections. The role of viruses in cystic fibrosis (CF) pulmonary disease has gained increased attention, and multiple studies have shown that viral infections are common antecedents for pulmonary exacerbations. Specifically, influenza, respiratory syncytial virus, and rhinovirus, all RNA viruses, are the most common and are sometimes associated with precipitous, unrecoverable loss of lung function in PwCF (1–5). Less well understood is whether PwCF are predisposed to developing specific viral infections or are more susceptible to severe complications once infected.

The global coronavirus disease (COVID-19) pandemic has been caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a highly contagious RNA virus that has caused millions of deaths worldwide. SARS-CoV-2 requires ACE2 (angiotensin-converting enzyme 2) and priming protease TMPRSS2 (transmembrane serine protease 2) for entry into epithelial cells, and studies published early in the pandemic have shown that inflammation, particularly IFN responses to viruses, can lead to increased *ACE2* expression (6, 7). It has been demonstrated that *ACE2* expression is highest in the nasal epithelium, which is a primary site of SARS-CoV-2 infection, and gradually decreases into the distal airways and parenchyma (8). As PwCF often have mucus accumulation and neutrophilic inflammation of the upper and lower airways, we wished to evaluate whether they have increased *ACE2* nasal expression. Potentially mitigating this risk is the finding that chronic inflammation can also induce the expression of a short form of ACE2 that lacks a receptor-binding domain and may lead to decreased susceptibility to infection (9). We therefore tested whether expression of *ACE2* and the inflammatory response are different in the nasal epithelium of PwCF compared with healthy volunteers (HVs). We reanalyzed our previously published pilot and validation data sets, for which the study methods and demographics of the recruited cohort have already been reported (10). (Detailed code for analysis and reproducibility is available at https://github.com/NUPulmonary/2021_CF_ACE2.) We separated PwCF into two cohorts, one consisting of PwCF who were F508del homozygotes and the second of those who were F508el compound heterozygotes.

To test whether *ACE2* and *TMPRSS2* are differentially expressed, we first performed direct pairwise comparison in our pilot cohort, which consisted of six HVs and seven PwCF, and did not detect differentially expressed genes between PwCF and HVs (data not shown). In our larger validation cohort, we compared samples from HVs (11 subjects) and PwCF homozygous (13 subjects) and heterozygous (10 subjects) for F508del. We found 709 genes upregulated in HVs compared with 2,299 genes upregulated in PwCF homozygous for F508del (false discovery rate [FDR], $q < 0.05$) and 607 genes upregulated in HVs compared with 2,277 genes upregulated in PwCF compound heterozygous for F508del (FDR $q < 0.05$). Although *ACE2* was not among the differentially expressed genes in any comparison, expression of *TMPRSS2* was decreased in PwCF heterozygous for F508del but not in PwCF homozygous for F508del (Figure 1A; also *see* Figures E1B and E1C and Tables E1 and E2 in the data supplement).

As gene expression profiles in bulk RNA-sequencing (RNA-seq) samples can be affected by the sample cell composition, we performed *k*-means clustering on the basis of highly variable genes (ANOVA-like test in DESeq2 [11]) and hierarchical clustering on subjects to better understand sources of variation in our data sets. This analysis identified three clusters (Figures 1B and E1A and Tables E3 and E4). We further investigated the larger data set and found that cluster A included HVs (11 of 11 subjects) and both homozygous (7 of 13 subjects) and compound heterozygous (3 of 10 subjects) PwCF. Subject cluster A was characterized by increased expression of genes related to secretory and ciliated epithelial cells, such as *BPIFA1* (BPI fold containing family A member 1), *CAPS* (calcyphosine), and *TPPP3* (tubulin polymerization promoting protein family member 3) (Figure 1C). Subject clusters B and C contained both homozygous and compound heterozygous PwCF but no HVs and were characterized by genes restricted to neutrophils and genes involved in immune responses, such as *CSF3R* (colony stimulating factor 3 receptor), *FCGR3B* (Fc γ receptor IIIb), and *ITGA2* (integrin subunit α 2) (Figures 1B and 1C). As expected, *ACE2* was not among highly variable genes. *TMPRSS2* belonged to gene cluster 1. To better understand the cellular composition of samples, we then performed *in silico* deconvolution of bulk RNA-seq signature using the AutoGeneS algorithm and publicly available single-cell RNA-seq data of nasal epithelium as a reference (12, 13). This analysis demonstrated that cluster A was dominated by an abundance of secretory and ciliated epithelial cells (Figure 1D; and Table E5). Cluster C in addition to epithelial cells also contained neutrophils, whereas cluster B was dominated by neutrophils. To ensure that the sample composition did not influence our analysis of *ACE2* or *TMPRS2* expression, we then performed a pairwise comparison using only samples from cluster A (epithelial cluster), which contained samples from HVs ($n = 11$) and PwCF homozygous ($n = 7$) and heterozygous ($n = 3$) for F508del. This analysis identified 379 genes upregulated in HVs and 146 genes upregulated in PwCF homozygous for F508del, and enrichment analysis for gene ontology biological processes demonstrated that these genes were related to T-cell activation (*see* Tables E6 and E7). When we performed a comparison between HVs and PwCF heterozygous for F508del with similar cell type composition, only 44 and 6 genes were upregulated in HVs and PwCF heterozygous for F508del, respectively (FDR, $q < 0.05$) (Figure 1E and Table E8). *ACE2* and *TMPRS2* were not differentially expressed in either comparison of cluster A. A similar analysis in the pilot data set did not detect differential gene expression for *ACE2* and

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This letter has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

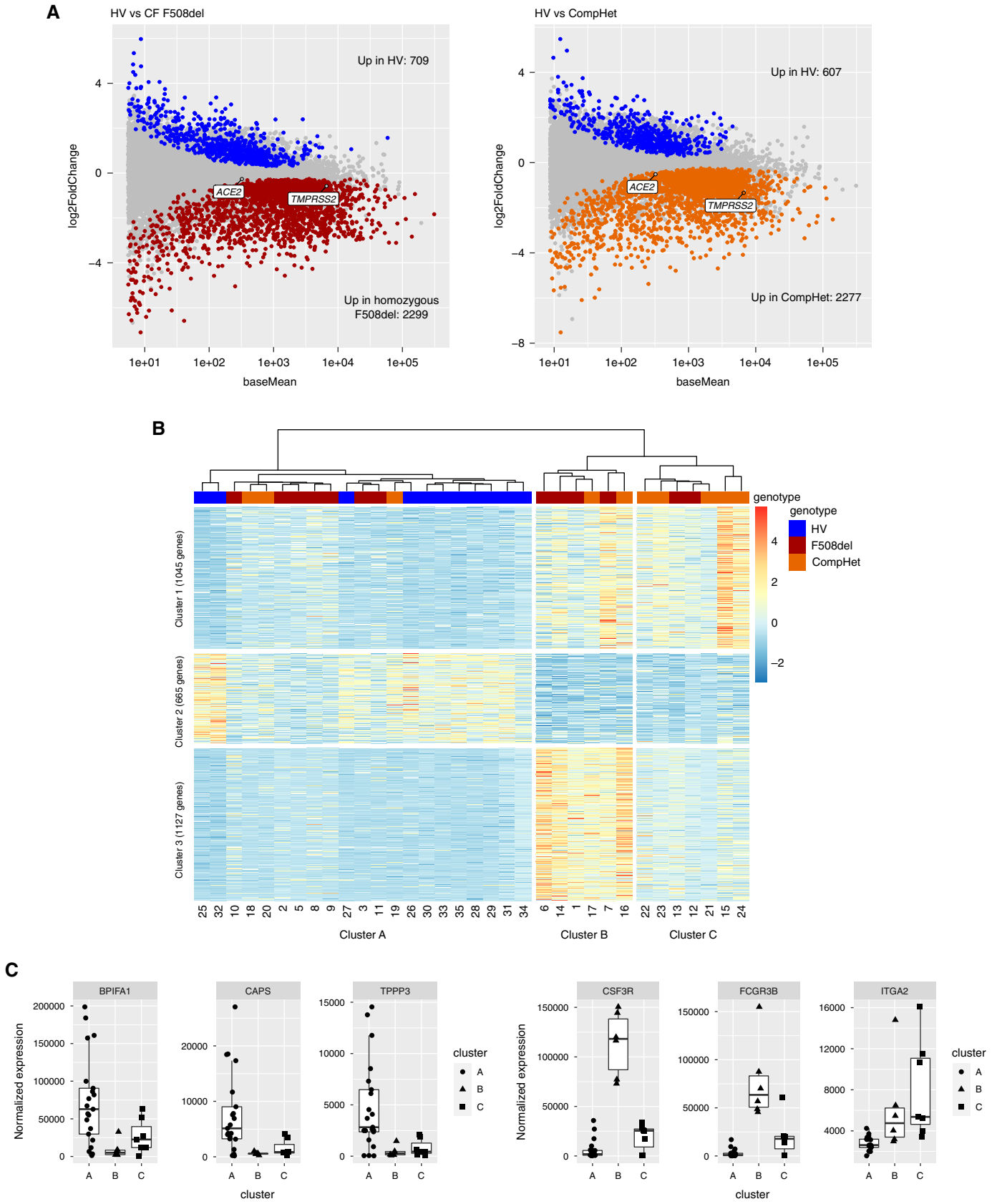

Figure 1. Expression of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry factor *ACE2* (angiotensin-converting enzyme 2) in the nasal epithelium in healthy volunteers (HVs) and people with cystic fibrosis (CF) (validation cohort). (*A*) MA plot demonstrating differentially expressed genes (FDR, $q < 0.05$) in the nasal epithelium between HVs (blue dots) and people with CF (PwCF) homozygous for F508del

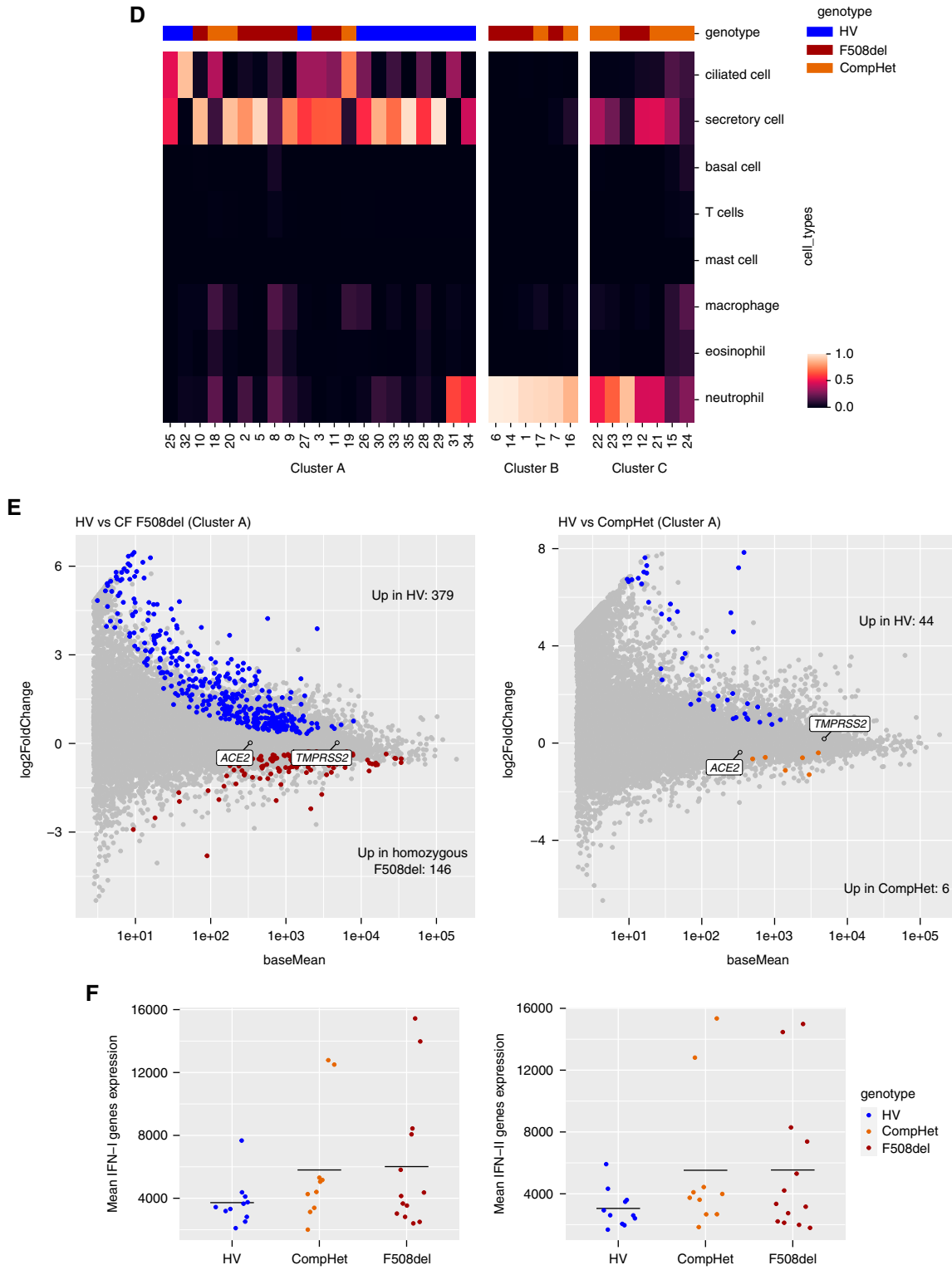

Figure 1. (*Continued*). mutation (F508del; red dots) or compound heterozygotes (CompHet; orange dots). (*B*) Heatmap demonstrating *k*-means clustering of highly variable genes (FDR, $q < 0.05$, ANOVA-like test in DESeq2, clusters 1–3). Subjects were hierarchically clustered (clusters A–C). Column numbers correspond to subjects' numbers from Sala and colleagues (10). (*C*) Box plots demonstrating expression of cell type–specific genes in clusters A–C. (*D*). Heatmap demonstrating estimated abundance of cell types in the clusters from *B*. Values are normalized across columns. (*E*) MA plot demonstrating differentially expressed genes (FDR, $q < 0.05$) in the nasal epithelium between samples from HVs (blue dots) and PwCF from epithelium-rich cluster A: homozygous for F508del mutation (red dots) or CompHet (orange dots).

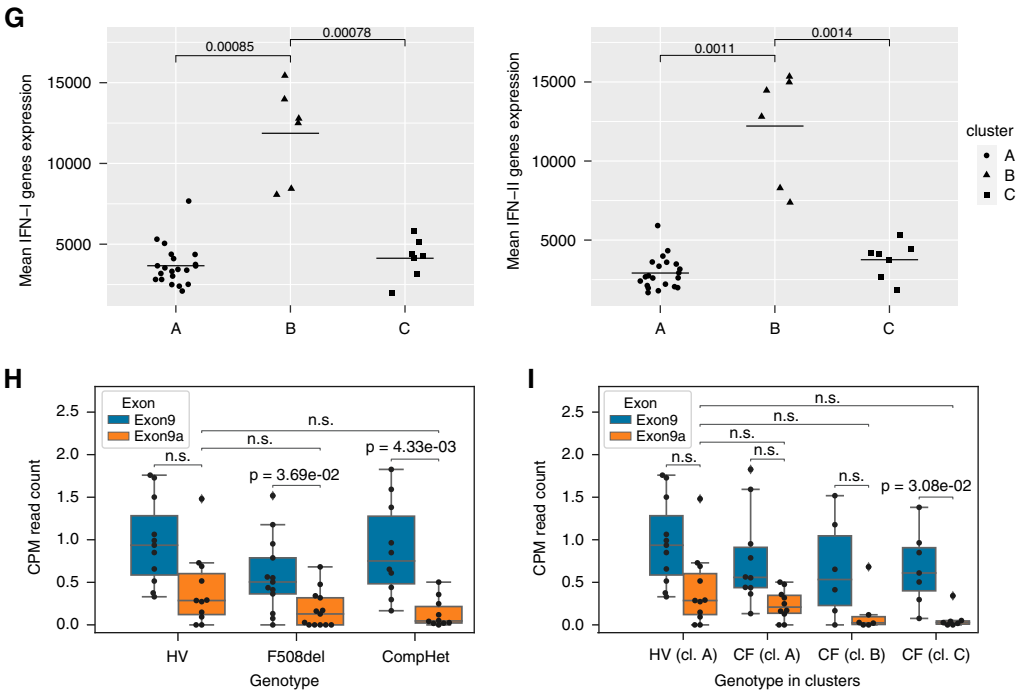

Figure 1. (*Continued*). (*F*) Mean expression of type I IFN-response genes (left panel; HALLMARK_INTERFERON_α_RESPONSE M5911) and type II IFN-response genes (right panel; HALLMARK_INTERFERON_GAMMA_RESPONSE M5913). Differences in expression between genotypes are not significant (*t* test). (*G*) Mean expression of type I IFN-response genes (left panel; HALLMARK_INTERFERON_α_RESPONSE M5911) and type II IFN-response genes (right panel; HALLMARK_INTERFERON_GAMMA_RESPONSE M5913) stratified according to clusters from *B* (*t* test). (*H*) Counts per million (CPM) reads mapped to exon 9 and exon 9a (corresponding to short *ACE2* isoform) between different genotypes (*t* test with Bonferroni multiple-test correction). Differences in reads mapped to exon 9a of *ACE2* are not significant between subject genotypes. (*I*) CPM of reads mapped to exon 9 and exon 9a (corresponding to short *ACE2* isoform) between HVs and PwCF (F508del and CompHet combined), stratified between clusters from *B* (*t* test with Bonferroni multiple-test correction). Differences in reads mapped to exon 9a of *ACE2* are not significant between genotypes and clusters. BPIFA1 = BPI fold containing family A member 1; CAPS = calcyphosine; cl. = cluster; CSF3R = colony stimulating factor 3 receptor; FCGR3B = Fc γ receptor IIIb; FDR = false discovery rate; ITGA2 = integrin subunit α 2; MA = log ratio–mean average; n.s. = not significant; TMPRSS2 = transmembrane serine protease 2; TPPP3 = tubulin polymerization promoting protein family member 3.

TMPRS2 in HVs compared with PwCF heterozygous for F508del (*see* Figure E1D). Expression of IFN-response genes was not different between HVs and PwCF homozygous and heterozygous for F508del (Figure 1F) or between HVs and the combined PwCF cohort (*see* Figures E1E and E1F). However, when we performed this comparison with adjustment for sample composition, expression of IFN-response genes was significantly increased in cluster B, which is characterized by an increased proportion of neutrophils (Figure 1G). On the basis of a recent report that a short form of ACE2, which lacks SARS-CoV-2 spike high-affinity binding sites, can be induced in respiratory epithelia by both IFN and RNA viruses, we assessed expression of the short *ACE2* isoform in our study cohorts (9). Although we detected reads mapping to an alternative exon 9a across all three groups, its abundance was not different among the groups (Figure 1H). Last, as samples from cluster B had evidence of increased IFN signaling, we also compared expression of the short *ACE2* isoform between samples from HVs (cluster A) and from PwCF from clusters A, B, and C (Figure 1I) and again found no difference in expression of the short *ACE2* isoform in samples with an increased IFN-response signature.

A recent analysis demonstrated that risk of severe COVID-19 was increased in patients with chronic obstructive pulmonary disease and pulmonary fibrosis, supporting concerns that PwCF also might be at increased risk (14). Despite initial fears, as the pandemic has progressed, there have been a number of reports from around the world highlighting the relatively low incidence of SARs-CoV-2 infection or severe complications in PwCF (15, 16). Our analysis provides evidence that expression of *ACE2*, a key entry factor on epithelial cells for SARS-CoV-2, is not different in the nasal epithelium between HVs and PwCF. Other groups have recently assessed ACE2 expression in the airways of PwCF (17–18). Hou and colleagues performed single-molecule fluorescence *in situ* hybridization for *ACE2* and *TMPRSS2* in lungs explanted from PwCF with end-stage disease and control donor lungs but did not quantify *ACE2* expression or assess nasal epithelium (19). In contrast to our findings, reanalysis of publicly available data sets from PwCF by Stanton and colleagues demonstrated increased expression of *ACE2* and decreased expression of *TMPRSS2*, but the original studies were performed on cultured airway cells rather than on primary cells obtained from PwCF (18). Using quantitative PCR and oropharyngeal swabs, Bitossi and colleagues reported decreased expression of ACE2 in PwCF (17). It is important to note that analysis in both studies was done without adjustment for sample composition, which, as we have shown in the case of *TMPRSS2*, can

affect expression results. Thus, our data set is one of the few to date to provide a direct assessment of ACE2 and TMPRSS2 in the nasal mucosa of PwCF and control subjects. Moreover, our data suggest that expression of *ACE2* or its short isoform may not be affected by IFN signaling *in vivo*, which differs from the two previous studies showing that IFNs can increase ACE2 expression *in vitro* (6, 17). Nevertheless, an additional validation study using orthogonal techniques would be required to confirm our findings. In addition, we also demonstrate a framework for interpretable analysis of bulk transcriptomic data from clinical samples containing multiple cell types. Using deconvolution of bulk RNA-seq data and comparing transcriptomic signatures only across samples with similar cellular composition (i.e., epithelial cell–rich samples), rather than by predefined clinical groups alone, we were able to improve the statistical power and biological significance of our analysis. Using this approach, we demonstrate that after adjustment for sample composition expression of *TMPRSS2*, another important factor mediating SARS-CoV-2 entry, was also not different across groups, which prevented us from drawing the erroneous conclusion that it is downregulated in the nasal epithelium of PwCF who are heterozygous for F508del.

One limitation of our work is the relatively low read counts for ACE2, although this is mitigated to a degree by similar findings in both the pilot and validation cohorts. A potential confounder of our work is that a higher proportion of PwCF were prescribed inhaled nasal steroids, though we cannot ascertain which patients were actually using them. However, the presence of a neutrophil signature in clusters B and C was not associated with higher *ACE2* or *TMPRSS2* expression in PwCF, suggesting that the presence or absence of inflammatory cells did not have an impact. In addition, our CF cohort did not include any patients who underwent transplantation, although we did include patients with severe pulmonary disease (10). In addition, we did not measure ACE2 protein expression directly, though previous data have demonstrated a correlation between mRNA and protein expression (20).

In conclusion, there are likely multiple reasons why PwCF have had a relatively low incidence of severe SARS-CoV-2 infection, including young age, familiarity of socially distancing strategies and mask wearing, and low incidence of obesity and cardiovascular disease. Our data suggest that an additional reason may be that PwCF do not differentially express factors that expose them to higher risk for SARS-CoV-2 acquisition. This is consistent with multiple reports of patients with asthma who also did not have elevated *ACE2* expression compared with control subjects (21–25). However, in contrast to most asthma studies, we sampled nasal mucosa, which has the highest density of *ACE2* expression, and patients with CF have neutrophilic mucosal inflammation, which is seen infrequently in patients with asthma. ■

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Marc A. Sala, M.D.*
Nikolay S. Markov, M.S.*
Yuliya Politanska, B.S.
Hiam Abdala-Valencia, Ph.D.
Alexander V. Misharin, M.D., Ph.D.
Northwestern University
Chicago, Illinois

Manu Jain, M.D., M.Sc.‡
Northwestern University
Chicago, Illinois
and
Lurie Children's Hospital
Chicago, Illinois

ORCID ID: 0000-0003-1534-5629 (M.J.).

*These authors contributed equally to this work and are co–first authors.
‡Corresponding author (e-mail: m-jain@northwestern.edu).

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