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Smoking and COVID-19: Similar bronchial ACE2 and TMPRSS2 expression and higher TMPRSS4 expression in current versus never smokers

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Abstract

Uncertainties remain concerning the pathophysiology, epidemiology, and potential therapeutics for COVID-19. Among unsettled controversies is whether tobacco smoking increases or protects from severe COVID-19. Several epidemiological studies reported reduced COVID-19 hospitalizations among smokers, while other studies reported the opposite trend. Some authors assumed that smokers have elevated airway expression of ACE2, the cell recognition site of the SARS-Cov-2 spike protein, but this suggestion remains unverified. We therefore performed data mining of two independent NCBI GEO genome-wide RNA expression files (GSE7894 and GSE994) and report that in both data sets, current smokers and never smokers have, on average, closely similar bronchial epithelial cell mRNA levels of *ACE2*, as well as *TMPRSS2*, coding for a serine protease priming SARS-Cov-2 for cell entry, and *ADAM17*, coding for a protease implicated in ACE2 membrane shedding. In contrast, the expression levels of *TMPRSS4*, coding for a protease that primes SARS-CoV-2 for cell entry similarly to *TMPRSS2*, were elevated in bronchial epithelial cells from current smokers compared with never smokers, suggesting that higher bronchial *TMPRSS4* levels in smokers might put them at higher SARS-Cov-2 infection risk. The effects of smoking on COVID-19 severity need clarification with larger studies. Additionally, the postulated protective effects of nicotine and nitric oxide, which may presumably reduce the risk of a "cytokine storm" in infected individuals, deserve assessment by controlled clinical trials.

KEYWORDS

ACE2, nicotine, nitric oxide, TMPRSS2, TMPRSS4

1 | INTRODUCTION

Since the outbreak of the SARS-Cov-2 pandemic, epidemiological studies have analyzed infection risk factors, as well as health comorbidities and lifestyle aspects relevant for disease course. Early on, it became apparent that old age is the key risk factor, along with secondary risk factors of respiratory disorders, diabetes, and male sex (Wang et al., 2020). As tobacco smoking is a key risk for respiratory disorders (Rigotti, 2013), and is by far more common in men than in women in China (Parascandola & Xiao, 2019), attention was given to the smoking history of patients, with the assumption that smokers are more susceptible to severe COVID-19 requiring intensive care hospitalization. However, early epidemiological studies from China indicated the opposite: the percent of smokers among hospitalized COVID-19 patients was lower compared with the general population. Farsalinos et al. identified 13 PubMed listed studies examining the clinical characteristics of hospitalized COVID-19 patients (N = 5,960) in China with known smoking status, and concluded, based on their

pooled analysis, that only 6.5% of hospitalized patients were current smokers, that is, one quarter compared with the 26.6% smoking prevalence in China (Farsalinos, Barbouni, & Niaura, 2020). Based on their epidemiological findings, and considering the well-known anti-inflammatory properties of nicotine, these authors suggested that nicotine in cigarette smoke protects COVID-19 patients from "cytokine storm" and might therefore have a therapeutic potential. The percentage of smokers among hospitalized COVID-19 patients was also smaller than in the general population according to a report from the United States, albeit, no conclusions were made due to the small cohorts (CDC COVID-19 Response Team, 2020). Among the earlier COVID-19 studies from China, Zheng et al. reported lower proportion of smokers in the poor outcome group of COVID-19 patients (Zheng et al., 2020), and another Chinese study (Cen et al., 2020) also indicated that current or past smoking was protective against severe COVID-19 progression (HR 0.56, 95% CI 0.34–0.91).

At the same time, others raised concerns that smokers are at increased risk of SARS-CoV-2 infection due to the established respiratory risks of chronic smoking; some authors suggested that smoking leads to elevated bronchial expression of ACE2 (Engin, Engin, & Engin, 2020; Wilson, 2020), the well-established cell recognition site of the SARS-Cov-2 spike protein (Hoffmann et al., 2020). An immunostaining study demonstrated ACE2 expression in resected lung tissues of smokers, while being entirely absent in lung tissues from heathy nonsmokers (Brake et al., 2020). In agreement, two meta-analysis studies reported higher risk for severe COVID-19 in smokers (Karanasos et al., 2020; Patanavanich & Glantz, 2020). Another recent review concluded that there is a significant association between COVID-19 and current or ever smoking, while the analysis remains limited due to the low quality of primary data (Grundy, Suddek, Filippidis, Majeed, & Coronini-Cronberg, 2020). However, the largest web-based survey published so far (Adorni et al., 2020; N = 171,310 responders), while not considering COVID-19 severity, found that current smokers were less prevalent among the Italian responders with a positive SARS-CoV-2 nasopharyngeal swab test compared with never or past smokers. At time of writing this article, the effect of smoking on COVID-19 severity—an increased or decreased risk—remains highly controversial (Gallus, Lugo, & Gorini, 2020).

In an attempt to clarify this controversy, in particular with regard to the effects of tobacco smoking on the bronchial expression levels of genes affecting SARS-CoV-2 infection, we studied published mRNA expression data sets from bronchial epithelial cells. We compared samples from current smokers and never smokers, and analyzed the expression *ACE2*, coding for the SARS-CoV-2 spike protein cell surface receptor; *TMPRSS2*, coding for a serine protease priming SARS-Cov-2 for cell entry (Hoffmann et al., 2020); and *ADAM17*, coding for a protease that cleaves ACE2 and causes its membrane shedding (Lambert et al., 2005), thereby tentatively reducing SARS-CoV-2 infectivity. We also studied the mRNA levels of *TMPRSS4*, coding for a serine protease reported to prime SARS-CoV-2 for cell entry similarly to *TMPRSS2* (Zang et al., 2020).

2 | METHODS

We searched the website of the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO; https://www.ncbi.nlm.nih.gov/geo/) for data sets comparing bronchial epithelial cells biopsied from tobacco smokers and never-smokers. We identified two data sets with >20 adult never smokers and >20 current smokers in general good health (no known chronic illness), GSE7895 and GSE994 (for details see Figures 1 and 2). Comparing SARS-CoV-2 relevant genes in bronchial epithelial cells of current smokers with never smokers was an important consideration, as chronic smoking has lasting effects on lung physiology and pathology for many years following smoking cessation (Muscat & Wynder, 1995). Both GSE files were analyzed using GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/), a free tool available at the NCBI website which allows comparisons of user selected cohorts and calculates fold-change (FC) and P values adjusted for the number of detected genes (Padj values) for each queried gene.

3 | RESULTS AND DISCUSSION

We first analyzed the mRNA expression levels of *ACE2*, *ADAM17*, *TMPRSS2*, and *TMPRSS4* in human bronchial epithelial cells from the NCBI GEO data set GSE7895 (Beane et al., 2007). As outlined above, the proteins coded by *ACE2*, *TMPRSS2*, and *TMPRSS4* take part in viral binding and entry to their host epithelial cells, while *ADAM17* codes for a protease implicated in membrane shedding of ACE2 and thereby also playing a role in SARS-CoV-2 infectivity. Figure 1 presents the expression levels of the above genes in bronchial epithelial cells obtained via bronchoscopy brushings from 21 never smokers and 52 current smokers (GSE7895) as described by Beane et al., 2007. As shown, similar mRNA expression levels of *ACE2*, *ADAM17*, and *TMPRSS2* (Padj = 0.99 for these three genes) were found when comparing bronchial epithelial cells from never smokers and current smokers. In contrast, bronchial epithelial cells of current smokers had two-fold higher *TMPRSS4* mRNA compared with never smokers (Padj = 7.25e-06). Analysis of GSE994 (Figure 2; Spira et al., 2004) also showed lack of significant differences in the expression levels of *ACE2*, *ADAM17*, and *TMPRSS2* in bronchial epithelial cells of current smokers compared with never smokers; while it indicated increased *TMPRSS2* expression in bronchial samples from current smokers (albeit with smaller FC and larger Padj values compared with findings from GSE7895). The top genes showing differential mRNA levels in bronchial epithelial cells of current and never smokers (with Padj<1e-04) are shown in Table S1 (GSE7895) and Table S2 (GSE994). Many shared genes were detected with differential expression in bronchial epithelial cells from current smokers compared with never smokers in both GSE7895 and GSE994 (Padj<1e-07; the shared genes are highlighted in light blue in Tables S1 and S2). This further supports the validity of our findings, as these data sets represent two independent studies (Beane et al., 2007 and Spira et al., 2004, respectively). Notably, many of the genes listed in Table 1 with elevated bronchial

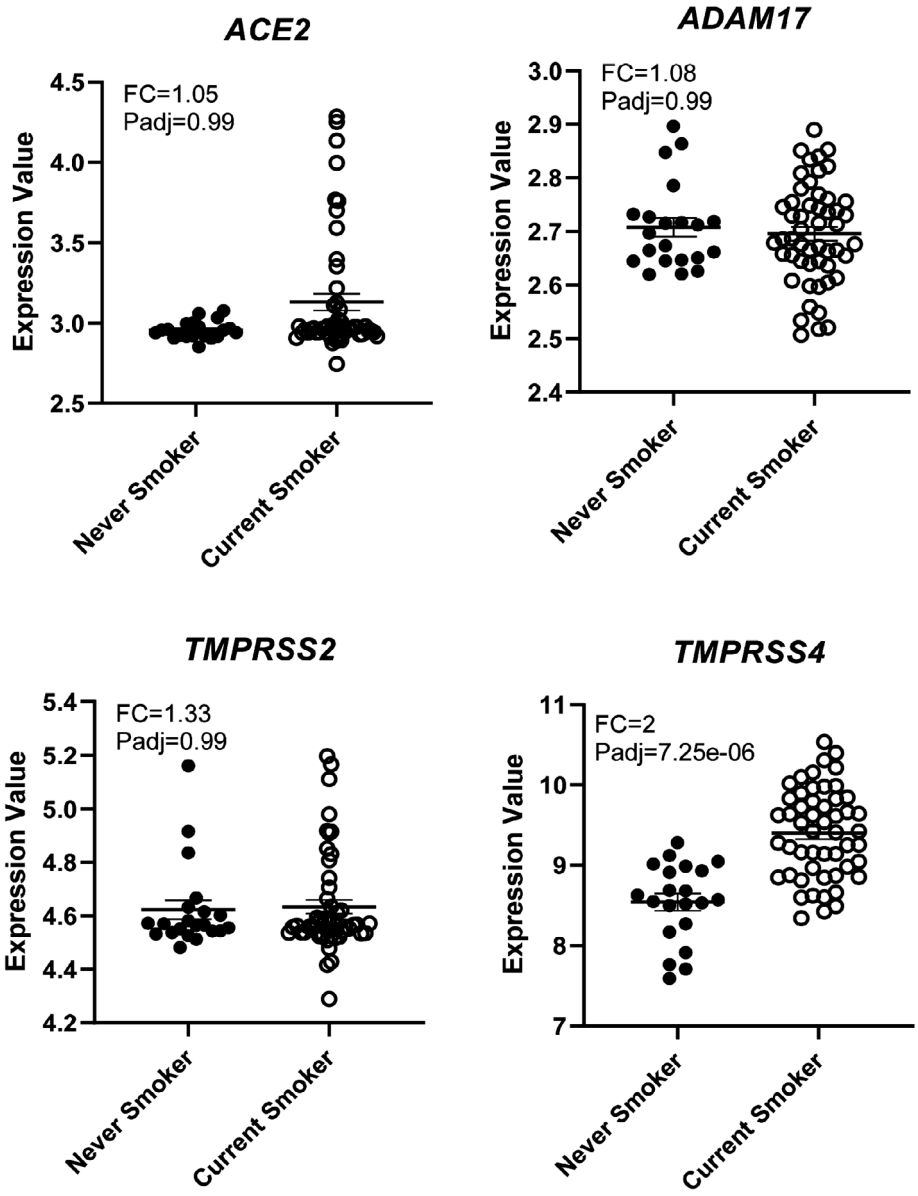

FIGURE 1 Analysis of GSE7895 expression levels of *ACE2*, *ADAM17*, *TMPRSS2*, and *TMPRSS4* in human bronchial epithelial cells from never smokers and current smokers. Data set GSE7895 available from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) was analyzed by GEO2R for comparing mRNA expression levels (log2 scale; Affymetrix U133A microarrays) of selected genes in human bronchial epithelial cells obtained via bronchoscopy brushings. Samples were from 21 never smokers and 52 current smokers (average age 32.3 ± 10.7 and 48.6 ± 15.2 years, respectively) as described by Beane et al. (2007). Padj and FC values were generated by GEO2R. Figures were prepared using GraphPad Prism v.8.4.2

epithelial cell expression in current smokers compared with never smokers are implicated in immunity. The genes upregulated in bronchial epithelial cells from smokers in both GSE7895 and GSE994 include NAD(P)H quinone dehydrogenase 1 (*NQO1*; Table 1; Padj = 1.52e-16) for which knockout mice exhibited reduced blood B cells (Iskander, Li, Han, Zheng, & Jaiswal, 2006) and glutathione peroxidase 2 (*GPX2*; Table 1; Padj = 1.82e-16), a component of the immune system (Spallholz, 1990). This suggests elevated immune function in bronchial epithelial cells of chronic smokers, which might explain the reports on fewer smokers among hospitalized COVID-19 patients compared with the general population. While the controversy on smoking and COVID-19 remains unsettled, these findings further support interpretations of a protective effects of smoking and suggestions that smokers have a reduced tendency for a "cytokine storm" following infection by SARS-CoV-2. Further implication of immune system effects of smoking on the transcriptome of bronchial epithelial cells are shown in Table S3, which presents a GeneMANIA analysis (https://genemania.org/) for functions and pathways of the genes listed in Table 1.

We must nevertheless keep in mind that cigarette smoking is a leading health risk: indeed, another gene dramatically elevated in bronchial epithelial cells of smokers, aldehyde dehydrogenase 3 family member A1 (*ALDH3A1*; Table 1; Padj = 1.82e-16), was recently reported with increased expression in both melanoma and lung cancer (Terzuoli et al., 2019). This further highlights tentative double-edge effects of smoking on human health.

TMPRSS4 codes for a membrane bound serine protease closely similar to *TMPRSS2*, which codes for the well-established serine protease priming SARS-Cov-2 for cell entry by cleaving its spike protein (Hoffmann et al., 2020). Notably, *TMPRSS4* (transmembrane serine protease 4) was recently shown to similarly recognize the SARS-Cov-2 spike protein and prime it for cell entry (Zang et al., 2020). Remarkably, both *TMPRSS2* and *TMPRSS4* are co-expressed in numerous tissues, including the entire digestive tract, kidney, and bladder,

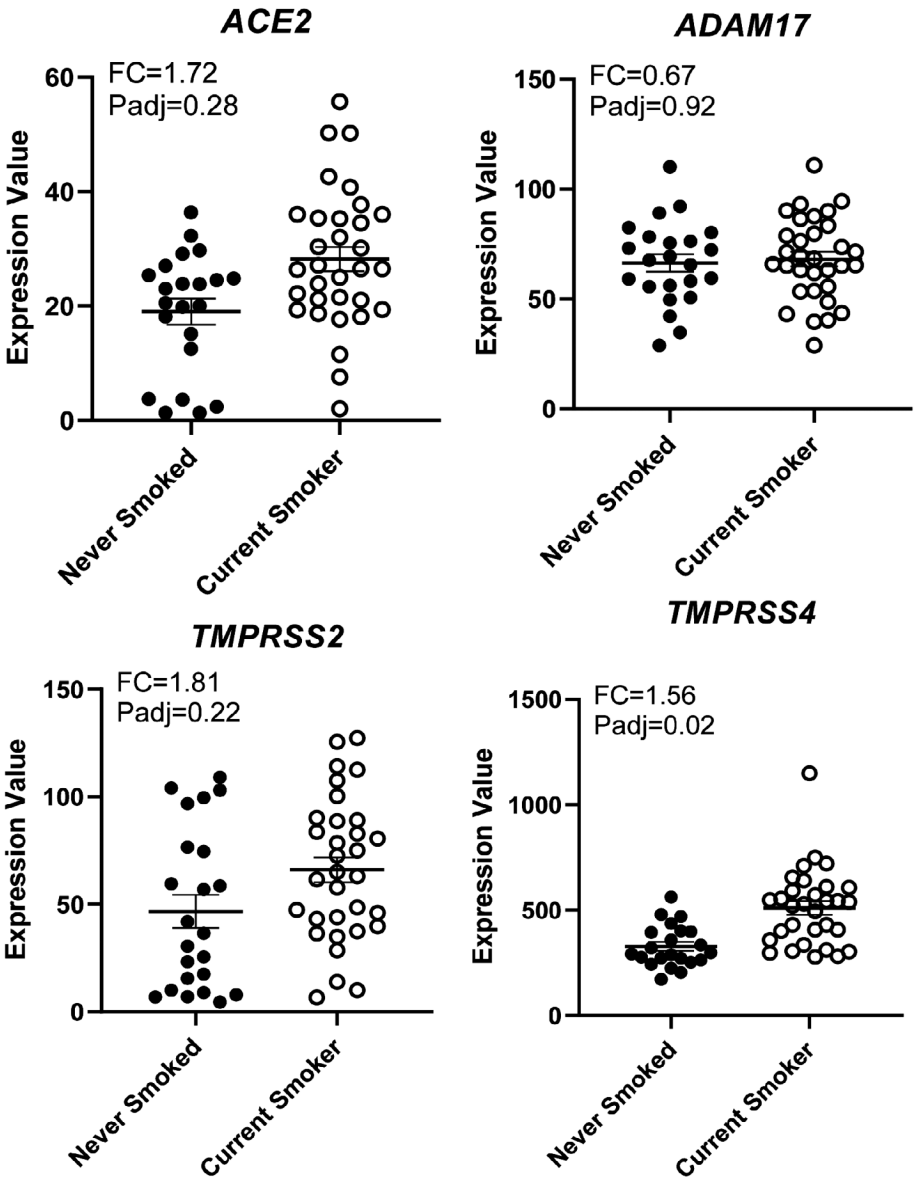

FIGURE 2 Analysis of GSE944 expression levels of *ACE2*, *ADAM17*, *TMPRSS2*, and *TMPRSS4* in human bronchial epithelial cells from never smokers and current smokers. NCBI GEO data set GSE944 was analyzed by GEO2R for comparing mRNA expression levels (log2 scale; Affymetrix HG-U133A microarrays) of selected genes in human bronchial epithelial cells obtained via bronchoscopy brushings. Samples were from 23 never smokers and 32 current smokers (average age 32.30 ± 11.72 and 43.59 ± 12.23 years, respectively) as described by Spira et al. (2004). Padj and FC values were generated by GEO2R. Figures were prepared using GraphPad Prism v.8.4.2

and are both implicated in the activation and cell entry of H3N2 influenza A and influenza B viruses, so that TMPRSS4 allows cell infection in the albescence of the host *TMPRSS2* gene (Bertram et al., 2010; Chaipan et al., 2009; Harbig et al., 2020; Hayashi et al., 2018; Kühn et al., 2016; Zmora et al., 2017). Both TMPRSS2 and TMPRSS4 play a role in lung cancer metastasis (Martin & List, 2019); high levels of TMPRSS4 were observed in several malignancies and were associated with epithelial to mesenchymal cell transition, invasion and metastasis (de Aberasturi & Calvo, 2015).

Notably, the serine protease inhibitor camostat, which inhibits both *TMPRSS2* and *TMPRSS4*, was shown to reduce influenza virus replication and cytokine production in primary cultures of human tracheal epithelial cells (Yamaya et al., 2015). Indeed, camostat (as its mesylate salt) was proposed as COVID-19 therapeutic (Uno 2020) and is under clinical trials in COVID-19 patients; as of June 18, 2020 six clinical trials with camostat mesylate were listed in the U.S. National Library of Medicine ClinicalTrials.gov website. Additional serine protease inhibitors capable of inhibiting both *TMPRSS2* and *TMPRSS4* are also being considered as COVID-19 therapeutics (Yamamoto et al., 2020).

The elevated expression of *TPMRSS4* in lung epithelial cells of current smokers may result from effects of chronic tobacco smoking, which include exposure to many compounds in tobacco smoke, including nicotine (Benowitz, 1988), acetaldehyde (Seeman, Dixon, & Haussmann, 2002) as well as tar deposition (Burns, 1991), the latter resulting in bronchial oxidative stress and inflammation (Lim et al., 1990; Siew, Wu, Ying, & Corrigan, 2017; Son et al., 2020).

3.1 | Protection by nicotine or nitric oxide?

It has been suggested that owing to the well-established immunosuppressive effects of nicotine, tobacco smoking has beneficial effects on the course of COVID-19 (Piao et al. 2009). Based on the yet

TABLE 1 Shared top genes showing differential expression levels in bronchial epithelial cells between current and never smokers for GSE7895 and GSE994

Padj value	Log FC	Gene symbol	Gene name
1.52e-16	−2.09758	**NQO1**	NAD(P)H quinone dehydrogenase 1
1.82e-16	−3.2188	**ALDH3A1**	Aldehyde dehydrogenase 3 family member A1
1.82e-16	−3.01961	**GPX2**	Glutathione peroxidase 2
2.79e-15	−2.17533	AKR1C2	Aldo-keto reductase family 1 member C2
5.34e-14	−2.15202	PIR	Pirin
7.03e-11	−2.4561	MUC5AC	Mucin 5AC
1.00e-09	−2.3736	ME1	Malic enzyme 1
2.30e-09	−2.3222	CLDN10	Claudin 10
2.86e-09	1.57608	CX3CL1	C-X3-C motif chemokine ligand 1
3.86e-09	−1.131	TXN	Thioredoxin
4.26e-09	0.84758	PPP1R16B	Protein phosphatase 1 regulatory subunit 16B
1.42e-08	−1.0327	FAM114A1	Family with sequence similarity 114 member A1
1.64e-08	−2.0664	SRPX2	Sushi repeat containing protein, X-linked 2
6.06e-08	−0.9326	S100A10	S100 calcium binding protein A10
7.85e-08	−1.2819	TRIM16	Tripartite motif containing 16
7.95e-08	−1.4009	UGT1A1	UDP glucuronosyltransferase 1A

Note: The list includes shared genes with differential expression between bronchial epithelial cells from current and never smokers with Padj < 1e-07 in GSE7895, and in addition showing differential expression between bronchial epithelial cells from current and never smokers in GSE994. For the latter GSE data set: Padj < 1e-05 and log FC in the same direction. GSE files were analyzed by GEO2R (see Figures 1 and 2 for details). Genes are listed by increasing Padj values. Genes in bold letters indicate immune system function genes mentioned in the text. A GeneMANIA analysis (https://genemania.org/) for functions and pathways of the 16 genes listed below is shown in Table S3.

controversial epidemiological evidence for fewer COVID-19 hospitalizations among smokers it has been suggested that nicotine has a beneficial therapeutic value, presumably due to its anti-inflammatory action which may calm the "cytokine storm" in some COVID-19 patients. It was proposed that nicotine reduces inflammation via the nicotinic acetylcholine receptor (nAChR) α7 subunit on macrophages (Farsalinos, Barbouni, et al. 2020; Farsalinos, Niaura, et al., 2020; Kloc, Ghobrial, & Kubiak, 2020). Cholinergic regulation of antibody response and immunosuppression via α7 nAChR has been demonstrated (Koval, Kalashnyk, Lykhmus, & Skok, 2018), and activation of these receptors was shown to ameliorate atherosclerosis in a mouse model (Ulleryd et al., 2019). Nicotine agonists may also reduce inflammation. For example, Encenicline, an α7 nAChR partial agonist, reduced colon immune cell infiltration and improved experimental colitis in model mice (Salaga et al., 2016). Chronic nicotine administration attenuated the inflammatory response and mortality in mice infected with influenza A virus (Sopori et al., 1998). Yet, it was proposed that smokers are at higher SARS-CoV-2 infection risk as nicotine itself may increase the expression of ACE2 (Olds & Kabbani, 2020), apparently via the activation of α7 nAChR on bronchial epithelial cells (Leung, Yang, & Sin, 2020; Russo et al., 2020).

In order to examine the effects of nicotine on the expression levels of the same four genes we analyzed in the GSE7895 and GSE994 data sets, we next examined the GSE42172 data set which includes information on mRNA levels in NCI-H460 human lung carcinoma cells following 24 h exposure to nicotine (no data sets for longer nicotine exposure of cultured cells were found in the NCBI GEO website). Analysis with the GEO2R tool indicated that mRNA levels of the same four genes remained unchanged following 24 h nicotine treatment (not shown). These findings suggest that the elevated *TPMRSS4* mRNA we now report in lung epithelial cells from current smokers versus never smokers (Figures 1 and 2) unlikely represent a direct short-term effect of nicotine on lung cells. Studies are required for directly assessing the effects of chronic nicotine administration on the cellular components implicated in SARS-CoV-2 infection, viral spread outside the respiratory tract, and COVID-19 progression.

Cigarette smoke includes hundreds of chemicals besides nicotine. Among them, nitric oxide (NO), present at concentrations of 250–1,350 ppm in cigarette smoke, was also proposed to reduce the risk of severe COVID-19 in smokers (Hedenstierna, Chen, Hedenstierna, Lieberman, & Fine, 2020). NO is an established pulmonary vasodilator and applied in some severe respiratory distress patients along with oxygen (Stewart, Vogel, Jarrett, & Potenziano, 2018). Albeit, others proposed that excessive NO might compromise immunity and thereby increase SARS-CoV-2 infection risk, and that assuring normative NO tone by therapeutics and dietary supplements should be considered for COVID-19 patients (Stefano, Esch, & Kream, 2020). Meanwhile, beneficial effects of inhaled NO in spontaneously breathing COVID-19 patients were reported (Parikh et al., 2020). Thus, in case of an eventual conclusion on a protective effect of smoking against severe COVID-19, this could reflect (at least in part) an effect of NO in inhaled cigarette smoke, possibly in synergy

with the immune-modulatory effects of nicotine. At time of writing, ClinicalTrials.gov lists 17 clinical trials with inhaled nitric oxide in COVID-19 patients.

3.2 | Limitations

A key limitation of our study is that it is based on data mining of only two GSE files identified for bronchial epithelial cell mRNA levels from current and never smokers in the NCBI GEO data base (GSE7895 and GSE994). Studies with larger cohorts, and applying the more comprehensive RNA-sequencing technology, would yield more robust findings and in addition provide information for effects of chronic smoking on miRNAs and lncRNAs expression in bronchial epithelial cells. Addressing the ongoing controversy on smoking as risk or protection for COVID-19 severity is of utmost urgency, as it may offer clues for treating patients (regardless of their smoking status). Our findings that bronchial epithelial cells from current and never smokers have similar *ACE2*, *TMPRSS2*, and *ADAM17* mRNA expression levels, while not solving the controversy on smoking and COVID-19 severity, suggest that explanations for the effects of smoking on disease risk (either increased or reduced) may reflect pathways other than viral cell entry *per se*. Most notably, the effects of smoking on the immune system need further studies in this context. In particular, suggestions that nicotine or NO might have a therapeutic potential for COVID-19 patients need to be explored.

4 | CONCLUSIONS AND FORWARD LOOK

Our findings indicate that current and never smokers have similar bronchial epithelial cell expression levels of *ACE2*, the major SARS-CoV-2 cell surface binding site, as well as *ADMA17*, implicated in ACE2 membrane shedding. Our analysis also shows that the bronchial epithelial cell expression levels of *TPMRSS2*, coding for a protease required for priming SARS-CoV-2 for cell entry, are also similar in current and never smokers. The mRNA expression levels of the above genes thus seem unlikely to assist in solving the controversy on COVID-19 hospitalizations among current versus never smokers. Our observations on elevated bronchial epithelial cell *TPMRSS4* mRNA in smokers are also puzzling, as they appear to suggest a higher risk of SARS-CoV-2 infection due to the higher availability of a protease priming the virus for epithelial cell entry. Whether TMPRSS4 can effectively replace TMPRSS2 in priming SARS-CoV-2 for cell entry requires further studies.

At time of writing this article, only a single clinical trial for nicotine in COVID-19 patients is listed on ClinicalTrials.gov ("Impact of smoking and nicotine on the risk of being Infected with COVID-19"; NCT04429815; listed on June 12th, 2020 and sponsored by Central Hospital, Nancy, France). However, as its name implies, this clinical trial will explore the potential of nicotine to protect from infection – while according to our hypothesis, the aim of such clinical trials should rather be to examine a tentative protection from deterioration to severe, life-threatening COVID-19. Larger epidemiology studies, possibly followed by controlled clinical trials, should address this intriguing concept.

Note added in proof: On June 30th 2020 the World Health Organization (WHO) has issued a Scientific Brief on "Smoking and COVID-19" (https://www.who.int/news-room/commentaries/detail/smoking-and-covid-19). It concludes with the following statement: "At the time of this review, the available evidence suggests that smoking is associated with increased severity of disease and death in hospitalized COVID-19 patients. Although likely related to severity, there is no evidence to quantify the risk to smokers of hospitalization with COVID-19 or of infection by SARS-CoV-2 was found in the peer-reviewed literature. Population-based studies are needed to address these questions." The authors recommend to closely follow this and future WHO recommendations on the COVID-19 pandemic.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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SUPPORTING INFORMATION

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