



COVID-19 and smoking: is nicotine the hidden link?

To the Editor:

LEUNG *et al.* [1] have recently published, in the *European Respiratory Journal*, a paper on the expression of angiotensin-converting enzyme II (ACE-2) in the small airway epithelia of smokers and COPD patients, discussing its effects on the risk of severe coronavirus disease 2019 (COVID-19). The authors found an increased expression of the ACE-2 gene in the airways of subjects with COPD and in current smokers. Indeed, a recent systematic review reporting data on the smoking habits of patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), concluded that smoking may be associated with a negative progression of the disease and with the adverse outcome [2]. These conclusions were challenged in a correspondence by CAI [3] on the basis that a reliable mechanism explaining this association was missing. The need for these results to be supported by additional studies is quite clear, but we believe that a robust mechanistic explanation exists. Nicotine has a known influence on the homeostasis of the renin-angiotensin system (RAS) up-regulating the angiotensin-converting enzyme (ACE)/angiotensin (ANG)-II/ANG II type 1 receptor axis, and down-regulating the compensatory ACE-2/ANG-(1-7)/Mas receptor axis, contributing in turn to the development of cardiovascular and pulmonary diseases [4]. Different airway cells, such as bronchial epithelial cells, type II alveolar epithelial cells and interstitial lung fibroblasts, express nicotinic acetylcholine receptors (nAChR), specifically the $\alpha 7$ subtype [5]. All these cells express components of the RAS [4]. In addition, nicotine increases the expression and/or activity of ACE in the lung [4], an increase which has been found also in the serum of smokers, and that required at least 20 min to return to control level [4]. ACE-2 serves as a physiologically relevant cellular entry receptor for SARS-CoV, for the human respiratory coronavirus NL63, and probably for SARS-CoV-2 [6]. ACE binds the SARS-CoV-2 S protein, and through its tissutal expression mediates the localisation and the efficiency of the infection [6]. Moreover, nicotine induces the epithelial-mesenchymal transition (EMT) [5, 7], a mechanism sufficient to allow “normal” differentiated cells to acquire the stem cell-like characteristics and properties. We planned experiments on human bronchial epithelial cells (HBEpC), obtained from Cell Applications Inc. (www.cellapplications.com/product number 502K-05a). Cells were maintained as adherent monolayer in complete bronchial/tracheal epithelial cell growth medium (www.cellapplications.com/product) at 37°C in 95% air/5% CO₂, seeded at an initial density of 7.5×10^4 cells·cm⁻², and sub-cultured with a 0.25% trypsin-1 mM EDTA solution (Sigma-Aldrich, Milan, Italy) when cultures reached 80% confluence. HBEpC are derived from the surface epithelium of normal human bronchi non-diseased (*i.e.* asthma, COPD or type 2 diabetes). The morphology is consistent with epithelial origin, and is positive for epithelial cell marker cytokeratin 18. Semi-confluent HBEpC at fourth passage (7.5×10^4 cells·cm⁻²) were treated: 1) for 1 h with zero or 1.0×10^{-7} M nicotine (Sigma-Aldrich, Milan, Italy) dissolved in saline in complete medium; 2) with 1.0×10^{-6} M α -Bungarotoxin (α -BTX; Sigma-Aldrich, Milan, Italy) dissolved in saline, in the continued presence of nicotine at zero or 1.0×10^{-7} M for 1 h; 3) treated continuously with nicotine for additional passages, 1 passage every 48 h for a total of 16 passages. We showed, for the first time, that nicotine at 1×10^{-7} M (the concentration present on the alveolar lining fluids after one cigarette is in the range 6×10^{-6} to 6×10^{-5} M [5]) is able to increase ACE-2 (figure 1a) in HBEpC. Treatment with nicotine induces phospho-S6 ribosomal protein (Ser235/236), Akt1, phospho-Akt (Ser473), phospho-Akt (Thr308) and phospho-p44/42 MAPK (Thr202/Tyr204) (figure 1b). To verify the hypothesis that ACE-2 is induced by nicotine through $\alpha 7$ -nAChR, HBEpC, at fourth passage, in the exponential growth phase, plated at a density of 1×10^6 cells·mL⁻¹, were incubated with $\alpha 7$ -nAChR siRNA (0.1 μ g) diluted in 100 μ L of siRNA transfection medium. Transfection was performed as described by LI *et al.* [8], who transfected, successfully, HBE16 human airway epithelial cell line (unaffected cells). A clone of transfected HBEpC that did not express $\alpha 7$ -nAChR proteins, also after



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Nicotine via alpha7-nicotinic receptor induces ACE-2 overexpression in human bronchial epithelial cells (HBEpC) <https://bit.ly/3eJ5b35>

Cite this article as: Russo P, Bonassi S, Giacconi R, *et al.* COVID-19 and smoking: is nicotine the hidden link? *Eur Respir J* 2020; 55: 2001116 [<https://doi.org/10.1183/13993003.01116-2020>].

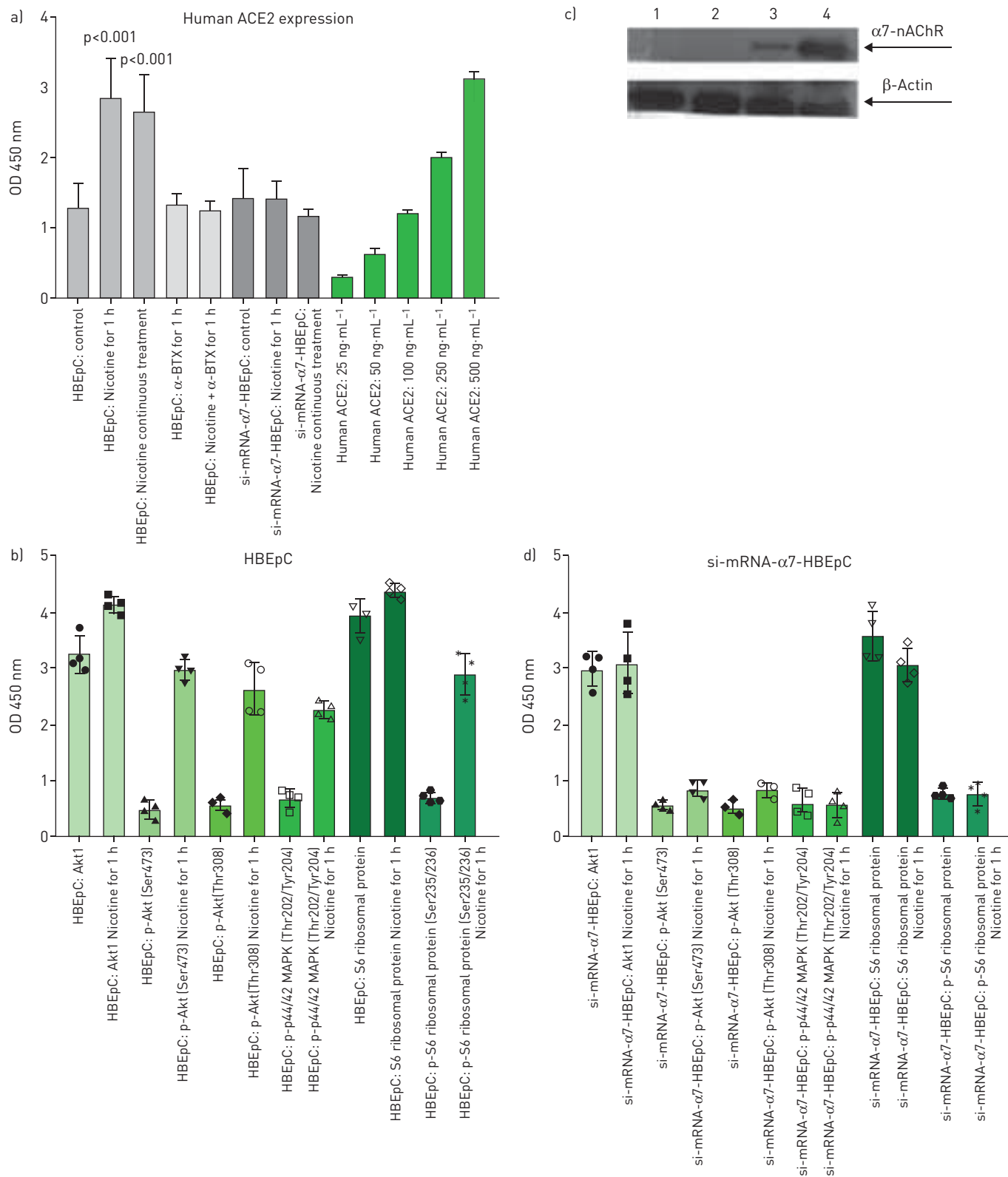


FIGURE 1 Effect of nicotine on human bronchial epithelial cells (HBEpC) or si-mRNA- α 7-HBEpC. a) Angiotensin-converting enzyme II (ACE-2) detection. ACE-2 was measured with human ACE-2 ELISA kit ab235649 (www.abcam.com/human-ace2-elisa-kit-ab235649.html) according to the manufacturer's instructions. Data are mean \pm SEM; p-value was evaluated using t-test. The green plots are the human ACE-2 standard. Experiments were performed twice in triplicate. b) Induction of phospho-S6 ribosomal protein (Ser235/236), Akt1, phospho-Akt (Ser473), phospho-Akt (Thr308)

and phospho-p44/42 MAPK [Thr202/Tyr204] in HBEpC. Data were obtained using PathScan® cell growth Multi-target Sandwich ELISA kit n.7239 [Cell Signaling] PathScan® Cell Growth Multi-Target Sandwich ELISA Kit is a solid phase sandwich ELISA that combines the reagents necessary to detect endogenous levels of S6 ribosomal protein, phospho-S6 ribosomal protein [Ser235/236], Akt1, phospho-Akt [Ser473], phospho-Akt [Thr308] and phospho-p44/42 MAPK [Thr202/Tyr204]. Data are mean \pm SEM; p-value was evaluated using t-test. Experiments were performed twice in duplicate. c) α 7-nAChR protein detection. Western blotting was performed as described previously [11]. Human α 7-nAChR antibody NBP1-49348 was purchased from Novus Biologicals (www.novusbio.com). 1–2 si-mRNA- α 7-HBEpC treated with zero (lane 1) or 1.0×10^{-7} M nicotine (lane 2) for 1 h. 3–4 HBEpC treated with zero (lane 3) or 1.0×10^{-7} M nicotine (lane 4) for 1 h. Experiments were performed twice. d) as in panel b, but treated cells are si-mRNA- α 7-HBEpC.

treatment with nicotine (figure 1c), and is not able to induce phospho-S6 ribosomal protein (Ser235/236), Akt1, phospho-Akt (Ser473), phospho-Akt (Thr308) and phospho-p44/42 MAPK (Thr202/Tyr204) after nicotine treatment (figure 1d), was selected for further experiments. Nicotine did not induce ACE-2 in this clone (si-mRNA- α 7-HBEpC) (figure 1a). This observation supports the hypothesis that ACE-2 increase is specifically mediated by α 7-nAChR. Moreover, when HBEpC were incubated simultaneously with nicotine and α -BTX, an α 7 nicotine antagonist [9], no induction of ACE-2 was observed (figure 1d). Importantly, treatment with nicotine, α -BTX or with the combination is not cytotoxic (data not shown). On these bases, we suggest that smoking may promote cellular uptake mechanisms of SARS-CoV-2 through α 7-nAChR signalling. A possible α 7-nAChR down-stream mechanism may be the induction of phospho-Akt and phospho-p44/42 MAPK. This mechanism was hypothesised, partially, by OLDS and KABBANI [10] on their schematic model explaining how nicotine exposure increases the risk of SARS-CoV-2 entry into lung cells. α 7-nAChR is present both in neuronal and non-neuronal cells (*i.e.* lung, endothelial, lymphocyte); consequently, smoking may impact COVID-19 pathophysiology and clinical outcome in several organ systems, including the brain.

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Received: 10 April 2020 | Accepted after revision: 16 April 2020

Conflict of interest: None declared.

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COVID-19 and nicotine as a mediator of ACE-2

From the authors:

We recently reported that current smokers and those with COPD had higher airway epithelial cell expression of the angiotensin-converting enzyme II (ACE-2) viral entry receptor [1]. We thus read with great interest the work of P. Russo and co-workers, which proposes a mechanism for this finding, namely that this upregulation is mediated by nicotine exposure specifically through the $\alpha 7$ subtype of nicotine acetylcholine receptors ($\alpha 7$ -nAChR). While exposure to increasing concentrations of nicotine caused epithelial cells to increase ACE-2 levels, subsequent gene silencing of $\alpha 7$ -nAChR appeared to significantly dampen this response. A secondary transcriptome sequencing analysis of our cohort (consisting of 42 subjects who underwent bronchoscopy for epithelial cell brushings [1]) reveals evidence in support of this hypothesis. We found that airway epithelial cell expression of *CHRNA7*, encoding $\alpha 7$ -nAChR, was significantly correlated with the expression of *ACE2* (Pearson $r=0.54$, $p=2.31\times 10^{-8}$) (figure 1). There was significantly higher *CHRNA7* expression in those with COPD (2.75 ± 0.73 versus 2.14 ± 0.43 in those without COPD; $p=1.47\times 10^{-4}$), with a trend towards higher expression in current smokers compared to former and never smokers (2.86 ± 0.92 in current smokers, 2.35 ± 0.57 in former smokers, and 2.27 ± 0.45 in never smokers; $p=6.16\times 10^{-2}$). *CHRNA7* was also negatively correlated with forced expiratory volume in 1 s percent predicted (Pearson $r=-0.37$, $p=2.83\times 10^{-4}$). Interestingly, *CHRNA7* was positively if weakly correlated with body mass index (Pearson $r=0.14$, $p=6.31\times 10^{-3}$), raising the intriguing possibility that nicotine receptor mediation of ACE-2 may also be related to why obese individuals have made up a considerable proportion of coronavirus disease 2019 (COVID-19) cases [2].

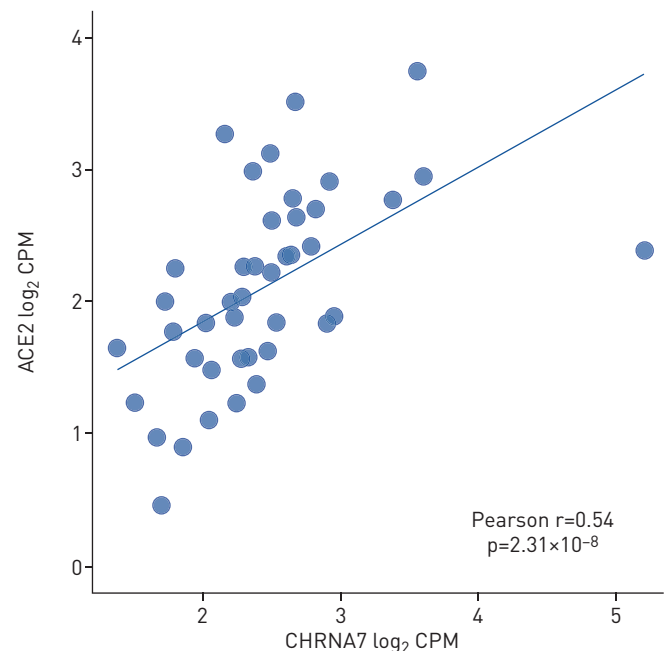


FIGURE 1 Transcriptome profiles generated through RNA-Seq of airway epithelial cells demonstrated a significant positive correlation between *ACE2* and *CHRNA7* expression.

Together, these data further help to characterise the connections between airway epithelial ACE-2, and $\alpha 7$ -nAChR, and the unique vulnerability of patients with COPD to severe COVID-19. $\alpha 7$ -nAChR's

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 $\alpha 7$ -nAChR may upregulate ACE-2 <https://bit.ly/2xS0cfT>

Cite this article as: Leung JM, Yang CX, Sin DD. COVID-19 and nicotine as a mediator of ACE-2 *Eur Respir J* 2020; 55: 2001261 [<https://doi.org/10.1183/13993003.01261-2020>].

widespread abundance in the human body, from neuronal tissue to immune cells to the lung and digestive tract, and its various roles in diseases such as schizophrenia [3], Alzheimer's disease [4] and Parkinson's disease [5] has meant that considerable work has already been done to target $\alpha 7$ -nAChR as a therapeutic modality. As an example, $\alpha 7$ -nAChR antagonists for the purpose of smoking cessation have long been proposed [6] and the idea of potentially repurposing these compounds for a pandemic with few therapeutic options currently available is certainly appealing. Whether $\alpha 7$ -nAChR-selective antagonists, such as methyllycaconitine [7] and α -conotoxin [8], can meaningfully alter ACE-2 expression to prevent severe acute respiratory syndrome coronavirus 2 entry into the airway epithelium seems the next logical investigation in our furious pursuit for better therapeutics.

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Received: 18 April 2020 | Accepted after revision: 20 April 2020

Conflict of interest: J.M. Leung has nothing to disclose. C.X. Yang has nothing to disclose. D.D. Sin reports grants from Merck, personal fees for advisory board work from Sanofi-Aventis and Regeneron, grants and personal fees for lectures from Boehringer Ingelheim and AstraZeneca, personal fees for lectures and advisory board work from Novartis, outside the submitted work.

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