Nicotinic receptors atlas in the adult human lung

Zania Diabasana[1], Jeanne-Marie Perotin[1,2], Randa Belgacemi[1], Julien Ancel[1,2], Pauline Mulette[1,2], Gonzague Delepine[3], Philippe Gosset[4], Uwe Maskos[5], Myriam Polette[1,6], Gaëtan Deslée[1,2], Valérian Dormoy[1]

[1]University of Reims Champagne-Ardenne, Inserm, P3Cell UMR-S1250, SFR CAP-SANTE, 51097 Reims, France

[2]CHU of Reims, Hôpital Maison Blanche, Department of respiratory diseases, 51092 Reims, France

[3]CHU of Reims, Hôpital Maison Blanche, Department of thoracic surgery, 51092 Reims, France

[4]University of Lille, CNRS UMR9017, Inserm U1019, CHRU Lille, Pasteur Institute of Lille, CIIL - Center for Infection and Immunity of Lille, 59000 Lille, France

[5]Pasteur Institute of Paris, Integrative Neurobiology of Cholinergic Systems, CNRS UMR 3571, Paris, France.

[6]CHU Reims, Hôpital Maison Blanche, Department of biopathology, 51092 Reims, France

Correspondence to:
Dr Valérian Dormoy
Inserm UMR-S 1250
University of Reims Champagne-Ardenne
CHU Maison Blanche
45 rue Cognacq-Jay
51092 Reims
Phone +33 (0)3 10 73 62 28; Fax +33 (0)3 26 06 58 61
e-mail: valerian.dormoy@univ-reims.fr

Short title: lung nicotinic receptors

Word count: 2595

ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels responsible for the rapid neural and neuromuscular signal transmission. Although it is well documented that 16 subunits are encoded by the human genome, their presence in airway epithelial cells (AEC) remains poorly understood, and contribution to pathology is mainly discussed in the context of cancer. We analysed nAChR subunit expression in the human lung of smokers and non-smokers using transcriptomic data for whole lung tissues, isolated large AEC, and isolated small AEC. We identified differential expressions of nAChRs in terms of detection and repartition in the three modalities. Smoking-associated alterations were also unveiled. Then, we identified a nAChR transcriptomic print at a single cell level. Finally, we reported the localizations of detectable nAChRs in bronchi and large bronchioles. Thus, we compiled the first complete atlas of pulmonary nAChRs to open new avenues to further unravel the involvement of these receptors in lung homeostasis and respiratory diseases.

Key words: Nicotinic receptors; Airway epithelial cells; Respiratory diseases

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated (cation-permeable) proteins expressed in the brain and in non-neuronal cells including lung AEC, macrophages, neutrophils, and muscle cells [1]. These receptors are composed of five subunits organized as homo- or hetero-pentamers forming a channel permeable to cations (predominantly Na^+, K^+ and Ca^{2+}) [1,2]. Each subunit shares a common structure comprising a large amino-terminal extracellular domain (about 200 residues), four transmembrane domains M1, M2, M3, and M4 (less than 30 residues), and a variable cytoplasmic domain (90 to 270 residues) [3–5]. There are 16 mammalian subunits, namely CHRNA1-A7, A9–A10, B1–B4, D, G and E [2]. Muscle-type nAChRs are generally assembled from 2(CHRNA1)/B1/G/D or 2(CHRNA1)/B1/E/D subtypes depending on muscle innervation [5–8]. Neuronal nAChRs are assembled from CHRNA2-A10 and CHRNB2-B4, and can either form homopentamers solely composed of CHRNA7 or CHRNA9 subunits, or heteropentamers constituted of CHRNA2-A6 and CHRNA10 combined with CHRNB2-B4 subunits [3,9–13]. All these subtypes confer different functional properties to the receptors [3,9,11].

In the lung, acetylcholine binding to nAChR leads to an allosteric conformational change permitting the channel opening, followed by ion fluxes that participate in cell survival, differentiation, and proliferation [14,15]. Nicotine, one of the components of cigarette smoke, acts as an agonist implicated in the inhibition of apoptosis and oxidative stress responses ultimately leading to lung impairments due to long-term exposure [5,16,17]. Previous studies have established that multiple single nucleotide nAChR polymorphisms are respectively associated with risks of lung cancer and chronic obstructive pulmonary disease (COPD), highlighting their potential implication in respiratory diseases [14,18,19]. In addition, it has been hypothesized that the nAChRs may play a role in coronavirus disease 2019 (COVID-19) and might represent a therapeutic target, particularly regarding their potential contribution in the regulation of angiotensin converting enzyme-2 (ACE-2), the main receptor for severe acute respiratory syndrome coronavirus (SARS-CoV-2) [20,21].

If the general expressions of muscle and neuronal nAChRs are well known, little information is available regarding their expression in the lung and particularly in different AEC [22]. It is of the utmost importance to provide an atlas of pulmonary nAChRs because of their potential implication in respiratory diseases. Therefore, we conducted a transcriptomic and a proteomic analysis of the localization and expression of all human nAChRs in the adult lung.

METHODS

Human subjects

Patients scheduled for lung resection for cancer (University Hospital of Reims, France) were prospectively recruited (n=10) following standards established and approved by the institutional review board of the University Hospital of Reims, France (IRB Reims-CHU 20110612). In addition, 10 patients who underwent a routine large airway fiberoptic bronchoscopy with bronchial brushings under local anaesthesia according to international guidelines were also recruited (5 non-smokers, 5 smokers) [23]. Informed consent was obtained from all the patients. Patients with chronic obstructive pulmonary disease, asthma, cystic fibrosis, bronchiectasis or pulmonary fibrosis were excluded. At inclusion, age, sex, smoking history, and pulmonary function tests results were recorded. Ex-smokers were considered for a withdrawal longer than 5 years.

Sample processing

Fresh airway epithelial cells (AEC) obtained from bronchial brushings (right lower lobe) were suspended within 30 min in RPMI (1% penicillin/streptomycin+ 10% BSA) before centrifugation (12,500 rpm x2 times). Cell pellet was dissociated in 1mL of Trypsine Versène®, centrifuged (12,500 rpm x2 times) and kept at -20°C until further steps.

RT-qPCR analyses

Total RNA from AEC bronchial brushings was isolated by High Pure RNA isolation kit (Roche Diagnostics) and 250ng was reverse transcribed into cDNA by Transcriptor First Stand cDNA Synthesis kit (Roche Diagnostics). Quantitative PCR reactions were performed with fast Start Universal Probe Master kit and UPL-probe system in a LightCycler 480 Instrument (Roche Diagnostics) as recommended by the manufacturer. Primers used are listed in ***Supplementary Table S1***. Results for all expression data regarding transcripts were normalized to the expression of the house-keeping gene GAPDH amplified with the following primers: forward 5′-ACCAGGTGGTCTCCTCTGAC-3′, reverse 5′-TGCTGTAGCCAAATTCGTTG-3′. We verified that GAPDH transcript detection levels were highly similar between non-smokers and smokers to validate the housekeeping gene (average Ct=25.54±0.17 in non-smokers vs 25.35±0.34 in smokers; p=0.64). Relative gene expression was assessed by the ΔΔCt method [24] and expressed as $2^{-\Delta\Delta Ct}$. To compare data generated via PCR with RNAseq analysis, we transformed the transcript expressions in a percentage scale considering the highest and lowest values per subunit for the detection or across all the subunits for the repartition.

Immunofluorescent staining and analyses

Immunohistochemistry was performed on formalin-fixed paraffin-embedded (FFPE) lung tissues distant from the tumour as previously described [25]. Only patients having no respiratory diseases were included (smokers and ex-smokers). Five µm sections were processed for H&E staining and observed on microscope (x20) to confirm the presence of bronchi and large bronchioles (pseudostratified epithelia). Bronchial epithelium was analysed on the entire slide including 2 to 7 units per patient. FFPE lung tissues sections slides were deparaffinised and blocked with 10% BSA in PBS for 30 min at room temperature. Tissue sections were then incubated with the primary antibodies as listed in ***Supplementary Table S2*** for one night at 4°C in 3% BSA in PBS. After PBS wash, a second primary antibody was used to highlight non-differentiated cells on epithelia for 2h at room temperature: p63 (AF1916, R&D Systems) or pan-cytokeratin (CK, E-AB-33599, Elabscience). Sections were washed with PBS and incubated with the appropriate secondary antibodies in 3% BSA in PBS for 30 min at room temperature. DNA was stained with DAPI during incubation with the secondary antibodies. Micrographs were acquired on a Zeiss AxioImageur (20x Ph) with ZEN software (8.1, 2012) and processed with ImageJ (National Institutes of Health) for analysis. For each patient, five random fields per section containing bronchi and large bronchioles were taken to evaluate the localization of nicotinic receptors on epithelial and stromal cells. We selected the most suitable primary antibodies directed against each subunit, considering external validations, identity and staining optimization to highlight the localization of nAChRs on bronchi and large bronchioles.

RNAseq data analysis

Gene expression of non-smoking and smoking subjects with no chronic respiratory diseases were collected from datasets available online (GEO database; http://www.ncbi.nlm.nih.gov/geo) including whole lung tissues samples in 153 subjects (42 non-smokers, 111 smokers; GSE103174, 76925 and 47460) or small airway bronchoscopic samples in 135 subjects (63 non-smokers, 72 smokers; GSE11784).

In order to compare transcriptomic data extracted from various datasets or PCR reactions, we formatted the absolute values to a percentage scale. Concerning the detection of genes, we first identified for each gene the highest and the lowest expression values in both non-smokers and smokers to set the maximal value at 100%. After proportionally expressing each of the single expression value for all the subunits, the average was calculated and plotted in a graph. To discuss the relative level of expressions, we arbitrarily categorized 4 groups: (1) very high

expressions, average percentage of expression is over 75% of the maximum; (2) high expressions, average percentage of expression is between 50 and 75% of the maximum; (3) moderate expressions, average percentage of expression is between 25 and 50% of the maximum; (4) and low expression, average percentage of expression is below 25% of the maximum.

Concerning the repartition, total expression of absolute values for all nAChR were summed for each patient of the considered data set to express the proportion of each subunit. The comparative average percentage of expression of each subunit for all patients was plotted in a pie chart.

Single cell sequencing

The published dataset can be found on lungcellatlas.org. We retained cell clustering based on the original studies and considered only subjects with no respiratory disease [26]. An Illumina Hiseq 4000 per 10x Genomics chip position was used (n=6; 2,000-5,000 cells/sample). Additional sequencing was performed to obtain coverage of at least mean coverage of 100,000 reads per cell.

Statistics

The data are expressed as mean values and percentages. Differences between groups were determined using the Student *t* test. A p-value <0.05 was considered significant.

RESULTS

Smoking-associated pulmonary nAChRs subunits transcripts expressions

Considering the whole lung containing all types of tissues (***Figure 1A***), the 16 nAChRs were detected among non-smoker subjects except for CHRNA7, that was consistent in both datasets containing non-smokers (***Figure 1B***). CHRNB1/E were very highly expressed; CHRNA6/A9/A10/B3/D were highly expressed. There was a significant increase of CHRNA1/A2/A7/B3/B4 transcript levels in smokers compared to non-smokers. Interestingly, CHRNA7 was only detected in smokers. On the contrary, CHRNA3/A4/A9/B2/D/G transcript levels were significantly decreased in smokers. The global repartition in non-smokers and smokers favoured CHRNA10/B1/E, representing almost half of all nAChRs expressed in lung tissues (***Figure 1C***). The differential repartitions of nAChRs between non-smokers and smokers matched their differential expressions.

In large AEC (LAEC), (***Figure 2A***) CHRNA1/A2/A4/B1/B3/D were not detected in non-smokers (***Figure 2B***). CHRNA5 was very highly expressed; CHRNA7/A10/E were highly expressed. Interestingly, CHRNB1/B4 were only detected in smokers. There was a significant decrease of CHRNA5/A10/E transcript levels in smokers when compared to non-smokers. The global repartition in non-smokers and smokers largely favoured CHRNE representing more than 85% of all nAChRs expressed in LAEC (***Figure 2C***). There were no significant differences in terms of nAChRs repartitions between non-smokers and smokers.

In small AEC (SAEC) (***Figure 3A***), the 16 nAChRs were detected among non-smokers with moderate or low expressions (***Figure 3B***). There was a significant increase of CHRNA5/A7/B2/B3 transcript levels in smokers compared to non-smokers. The global repartition in non-smokers and smokers favoured CHRNA7/A9/A10/B2 representing half of nAChR expressed in SAEC (***Figure 3C***). There was a significant increase of CHRNA7/B2/B3 and a significant decrease of CHRNA2/A9 in the repartition of nAChRs in smokers compared to non-smokers.

Differential pulmonary nAChRs transcripts expressions at a single cell scale

At the level of single cell transcriptomes (***Figure 4***), CHRNA5/A7/A9/A10/B1/E were highly expressed in most of the AEC populations including alveolar, basal, goblet, multiciliated and club cells. CHRNA1/A2/A4/A6/B3/D/G were showing low to no expression in AEC. Regarding non-epithelial cells, CHRNA1/A3/A5/A9/A10/B1/E were highly expressed in

specific populations of immune cells including macrophages, B cells, dendritic cells and mast cells. CHRNA5/B1/E were highly expressed in fibroblasts; CHRNA10/B1 in smooth muscle cells; CHRNA5/A7/A10/B1/E in endothelial cells. T and NK cells, and neutrophils did not expressed detectable levels of nAChRs transcripts.

Identification of nAChRs in bronchial and bronchiolar epithelia

To investigate nAChR localization in the lung, we selected commercially available validated primary antibodies displaying the antigenic sequences demonstrating the lowest percentage of identity with regard to cross-reactivity (***Supplemental Table S2 and S3, Supplemental Figure S1***). We focussed here on bronchi and large bronchioles as well as smooth muscle and blood vessels on FFPE tissues (***Figure 5A and B***). CHRNA1/A2/A4/B3/G were not detected. CHRNA3 seemed restricted to the apical side of differentiated cells. Surprisingly, CHRNA5 was systematically found in AEC nuclei and the apical side of differentiated AEC, while its pattern was consistent with membrane-bound receptors on smooth muscle cells. CHRNA6/A9 presented similar staining in differentiated AEC, such as CHRNA7/A10/B1/B2/D/E which in addition were found in non-differentiated AEC. Finally, CHRNB4 appeared in multiciliated cells only. When available, our observations were generally concordant with the data from the Human Protein Atlas (***Figure 5B***).

DISCUSSION

This is the first study showing transcript levels and localizations of all nAChRs in the human adult lung. Interestingly, we identified distinct variations in terms of nAChR transcript levels between whole lung tissues, LAEC and SAEC, as well as important changes between non-smokers and smokers. Since whole lung transcriptomics encompasses all pulmonary tissues, isolated cell studies represent the ideal strategy to unveil nAChR functions in airways. It has been successfully implemented in the context of AEC differentiation analysis, asthma and idiopathic pulmonary fibrosis [26–30]. As such, our comparative analysis points mainly towards CHRNA5/A7/A10/B2/B3/E to tackle the association of nAChR expression and smoking. Furthermore, the impact of smoking could be tie into the associated risks of respiratory diseases, provided complete clinical data are available.

We included in the analysis of nAChR expression levels 298 subjects in three distinct modalities (whole lung tissues, LAEC and SAEC) and performed a preliminary identification of single cell transcriptomic signature. Our immunostaining analyses provide important data regarding the sub-cellular localizations of nAChRs in bronchi and large bronchioles. Microscopic observations and transcriptomic analysis were generally concordant. Because of their modalities of association at the cell membrane and their high degree of amino acids identity, nAChRs immunostainings are generally sparse, rarely concordant, and performed on murine tissues in the literature [31–34]. Nonetheless, since we selected all our antibodies based on thorough sequence alignments of the antigenic sequences, we provide here a complete and robust description of all nAChRs in bronchi and large bronchioles.

Additional studies on larger cohorts are needed to complement and refine our analysis. Deciphering the cellular and molecular impact of the observed differences in transcript expressions in the context of smoking will be essential to understand nAChR-associated pathogenesis. It will be particularly insightful for at least three lung diseases where smoking may partly impact homeostasis: lung cancers, COPD, and COVID-19. (i) nAChRs single nucleotide polymorphisms (SNP) were associated to lung cancer cells [35,36] and nAChRs were shown to be involved in cancer cell proliferation and survival [37–40]. (ii) nAChRs SNPs were also associated with nicotine dependence and COPD [41–45]. (iii) Nicotine, the main ligand of nAChRs, and more generally smoking, have been shown *in vitro* and *in vivo* to modulate the expression of hACE2, the main receptor of SARS-CoV-2 spike S protein [46–49]. In addition, nicotine impairs the renin-angiotensin system (RAS) homeostasis [21], so

nicotine-induced imbalance of RAS via nAChRs is a strong candidate to understand hypertension, dysregulated cardiac remodelling, vascular dysfunction and chronic lung diseases associated with severe forms of COVID-19. Interestingly, CHRNA7 transcripts were correlated with hACE2 levels [50] feeding the possible connections between hACE2 and nAChRs localizations in airways [51,52].

We provide the first atlas of nAChRs in the lung and invite cartographers to complete the map in order to provide fundamental understanding of these crucial actors of homeostasis that may contribute to chronic and acute respiratory diseases.

Acknowledgments

We thank the members of the Inserm UMR-S 1250 unit and our collaborators for their helpful comments and insights. We thank the Platform of Cell and Tissue Imaging (PICT) for technical assistance.

Contributors

ZD performed experiments, analysed the data and wrote the manuscript; JMP collected samples and analysed the data; RB performed experiment; JA, PM and GoD collected samples; MP, PG and UM contributed to experimental analysis and interpretation of the results; GaD collected samples, analysed the data and supervised the experiments; VD designed the experiments, analysed the data and wrote the manuscript. All the authors contributed to the writing and critical appraisal of the manuscript.

Funding

This work was supported by Funding from University of Reims Champagne-Ardenne (URCA), the French National Institute of Health and Medical Research (Inserm) and a grant from the Research Institute in Public Health (IReSP) in association with the National Institute of Cancer (INCa). It was carried out in the framework of the Federative Research Structure *CAP-Santé* and benefited from the Project Research and Innovation in Inflammatory Respiratory Diseases (RINNOPARI).

Competing Interests

Dr. Deslée reports personal fees from Nuvaira, personal fees from BTG/PneumRx, personal fees from Chiesi, personal fees from Boehringer, personal fees from Astra Zeneca, outside the submitted work. Dr. Dormoy reports personal fees from Chiesi outside the submitted work.

Ethics approval

Subjects were recruited from the Department of pulmonary medicine at university hospital of Reims (France) and included in the cohort for Research and Innovation in Chronic Inflammatory Respiratory Diseases (RINNOPARI, NCT02924818). The study was approved by the ethics committee for the protection of human beings involved in biomedical research (CCP Dijon EST I, N°2016-A00242-49) and was conducted in accordance with the ethical guideline of Declaration of Helsinki.

REFERENCES

1. Zoli M, Pucci S, Vilella A, Gotti C. Neuronal and Extraneuronal Nicotinic Acetylcholine Receptors. Curr Neuropharmacol. 2018;16: 338–349. doi:10.2174/1570159X15666170912110450

2. Dani JA. Neuronal Nicotinic Acetylcholine Receptor Structure and Function and Response to Nicotine. Int Rev Neurobiol. 2015;124: 3–19. doi:10.1016/bs.irn.2015.07.001

3. Dani JA. Neuronal Nicotinic Acetylcholine Receptor Structure and Function and Response to Nicotine. Int Rev Neurobiol. 2015;124: 3–19. doi:10.1016/bs.irn.2015.07.001

4. Gündisch D, Eibl C. Nicotinic acetylcholine receptor ligands, a patent review (2006-2011). Expert Opin Ther Pat. 2011;21: 1867–1896. doi:10.1517/13543776.2011.637919

5. Grando SA. Connections of nicotine to cancer. Nat Rev Cancer. 2014;14: 419–429. doi:10.1038/nrc3725

6. Grassi F, Fucile S. Calcium influx through muscle nAChR-channels: One route, multiple roles. Neuroscience. 2019. doi:10.1016/j.neuroscience.2019.04.011

7. Conti-Tronconi BM, McLane KE, Raftery MA, Grando SA, Protti MP. The nicotinic acetylcholine receptor: structure and autoimmune pathology. Crit Rev Biochem Mol Biol. 1994;29: 69–123. doi:10.3109/10409239409086798

8. Rudolf R, Straka T. Nicotinic acetylcholine receptor at vertebrate motor endplates: Endocytosis, recycling, and degradation. Neuroscience Letters. 2019;711: 134434. doi:10.1016/j.neulet.2019.134434

9. Zoli M, Pucci S, Vilella A, Gotti C. Neuronal and Extraneuronal Nicotinic Acetylcholine Receptors. Curr Neuropharmacol. 2018;16: 338–349. doi:10.2174/1570159X15666170912110450

10. Crespi A, Colombo SF, Gotti C. Proteins and chemical chaperones involved in neuronal nicotinic receptor expression and function: an update. Br J Pharmacol. 2018;175: 1869–1879. doi:10.1111/bph.13777

11. Medjber K, Freidja ML, Grelet S, Lorenzato M, Maouche K, Nawrocki-Raby B, et al. Role of nicotinic acetylcholine receptors in cell proliferation and tumour invasion in broncho-pulmonary carcinomas. Lung Cancer. 2015;87: 258–264. doi:10.1016/j.lungcan.2015.01.001

12. Koukouli F, Rooy M, Changeux J-P, Maskos U. Nicotinic receptors in mouse prefrontal cortex modulate ultraslow fluctuations related to conscious processing. Proc Natl Acad Sci USA. 2016;113: 14823–14828. doi:10.1073/pnas.1614417113

13. Changeux J-P, Corringer P-J, Maskos U. The nicotinic acetylcholine receptor: From molecular biology to cognition. Neuropharmacology. 2015;96: 135–136. doi:10.1016/j.neuropharm.2015.03.024

14. Chen J, Cheuk IWY, Shin VY, Kwong A. Acetylcholine receptors: Key players in cancer development. Surg Oncol. 2019;31: 46–53. doi:10.1016/j.suronc.2019.09.003

15. Maouche K, Polette M, Jolly T, Medjber K, Cloëz-Tayarani I, Changeux J-P, et al. {alpha}7 nicotinic acetylcholine receptor regulates airway epithelium differentiation by controlling basal cell proliferation. Am J Pathol. 2009;175: 1868–1882. doi:10.2353/ajpath.2009.090212

16. Munakata S, Ishimori K, Kitamura N, Ishikawa S, Takanami Y, Ito S. Oxidative stress responses in human bronchial epithelial cells exposed to cigarette smoke and vapor from tobacco- and nicotine-containing products. Regul Toxicol Pharmacol. 2018;99: 122–128. doi:10.1016/j.yrtph.2018.09.009

17. Herman M, Tarran R. E-cigarettes, nicotine, the lung and the brain: multi-level cascading pathophysiology. J Physiol. 2020; JP278388. doi:10.1113/JP278388

18. Cui K, Ge X, Ma H. Four SNPs in the CHRNA3/5 alpha-neuronal nicotinic acetylcholine receptor subunit locus are associated with COPD risk based on meta-analyses. PLoS ONE. 2014;9: e102324. doi:10.1371/journal.pone.0102324

19. Maskos U. The nicotinic receptor alpha5 coding polymorphism rs16969968 as a major target in disease: functional dissection and remaining challenges. J Neurochem. 2020 [cited 4 May 2020]. doi:10.1111/jnc.14989

20. Sriram K, Insel PA. A hypothesis for pathobiology and treatment of COVID-19: the centrality of ACE1/ACE2 imbalance. Br J Pharmacol. 2020; bph.15082. doi:10.1111/bph.15082

21. Oakes JM, Fuchs RM, Gardner JD, Lazartigues E, Yue X. Nicotine and the renin-angiotensin system. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2018;315: R895–R906. doi:10.1152/ajpregu.00099.2018

22. Lam DC-L, Luo SY, Fu K-H, Lui MM-S, Chan K-H, Wistuba II, et al. Nicotinic acetylcholine receptor expression in human airway correlates with lung function. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2016;310: L232–L239. doi:10.1152/ajplung.00101.2015

23. Du Rand IA, Blaikley J, Booton R, Chaudhuri N, Gupta V, Khalid S, et al. British Thoracic Society guideline for diagnostic flexible bronchoscopy in adults: accredited by NICE. Thorax. 2013;68: i1–i44. doi:10.1136/thoraxjnl-2013-203618

24. Zuo W-L, Yang J, Strulovici-Barel Y, Salit J, Rostami M, Mezey JG, et al. Exaggerated BMP4 signalling alters human airway basal progenitor cell differentiation to cigarette smoking-related phenotypes. Eur Respir J. 2019;53. doi:10.1183/13993003.02553-2017

25. Perotin J-M, Coraux C, Lagonotte E, Birembaut P, Delepine G, Polette M, et al. Alteration of primary cilia in COPD. Eur Respir J. 2018;52: 1800122. doi:10.1183/13993003.00122-2018

26. Vieira Braga FA, Kar G, Berg M, Carpaij OA, Polanski K, Simon LM, et al. A cellular census of human lungs identifies novel cell states in health and in asthma. Nat Med. 2019;25: 1153–1163. doi:10.1038/s41591-019-0468-5

27. Reyfman PA, Walter JM, Joshi N, Anekalla KR, McQuattie-Pimentel AC, Chiu S, et al. Single-Cell Transcriptomic Analysis of Human Lung Provides Insights into the Pathobiology of Pulmonary Fibrosis. Am J Respir Crit Care Med. 2019;199: 1517–1536. doi:10.1164/rccm.201712-2410OC

28. Schiller HB, Montoro DT, Simon LM, Rawlins EL, Meyer KB, Strunz M, et al. The Human Lung Cell Atlas: A High-Resolution Reference Map of the Human Lung in Health and Disease. Am J Respir Cell Mol Biol. 2019;61: 31–41. doi:10.1165/rcmb.2018-0416TR

29. Zaragosi LE, Deprez M, Barbry P. Using single-cell RNA sequencing to unravel cell lineage relationships in the respiratory tract. Biochem Soc Trans. 2020;48: 327–336. doi:10.1042/BST20191010

30. Ruiz García S, Deprez M, Lebrigand K, Cavard A, Paquet A, Arguel M-J, et al. Novel dynamics of human mucociliary differentiation revealed by single-cell RNA sequencing of nasal epithelial cultures. Development. 2019. doi:10.1242/dev.177428

31. Moser N, Mechawar N, Jones I, Gochberg-Sarver A, Orr-Urtreger A, Plomann M, et al. Evaluating the suitability of nicotinic acetylcholine receptor antibodies for standard immunodetection procedures: Evaluation of nicotinic receptor antibodies. Journal of Neurochemistry. 2007;102: 479–492. doi:10.1111/j.1471-4159.2007.04498.x

32. Rommel FR, Raghavan B, Paddenberg R, Kummer W, Tumala S, Lochnit G, et al. Suitability of Nicotinic Acetylcholine Receptor α7 and Muscarinic Acetylcholine Receptor 3 Antibodies for Immune Detection: Evaluation in Murine Skin. J Histochem Cytochem. 2015;63: 329–339. doi:10.1369/0022155415575028

33. Lam DC-L, Luo SY, Fu K-H, Lui MM-S, Chan K-H, Wistuba II, et al. Nicotinic acetylcholine receptor expression in human airway correlates with lung function. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2016;310: L232–L239. doi:10.1152/ajplung.00101.2015

34. Lam DC -l., Girard L, Ramirez R, Chau W -s., Suen W -s., Sheridan S, et al. Expression of Nicotinic Acetylcholine Receptor Subunit Genes in Non-Small-Cell Lung Cancer Reveals Differences between Smokers and Nonsmokers. Cancer Research. 2007;67: 4638–4647. doi:10.1158/0008-5472.CAN-06-4628

35. Amos CI, Wu X, Broderick P, Gorlov IP, Gu J, Eisen T, et al. Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. Nat Genet. 2008;40: 616–622. doi:10.1038/ng.109

36. Hung RJ, McKay JD, Gaborieau V, Boffetta P, Hashibe M, Zaridze D, et al. A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. Nature. 2008;452: 633–637. doi:10.1038/nature06885

37. Sun P, Li L, Zhao C, Pan M, Qian Z, Su X. Deficiency of α7 Nicotinic Acetylcholine Receptor Attenuates Bleomycin-Induced Lung Fibrosis in Mice. Mol Med. 2017;23: 34–49. doi:10.2119/molmed.2016.00083

38. Sun H-J, Jia Y-F, Ma X-L. Alpha5 Nicotinic Acetylcholine Receptor Contributes to Nicotine-Induced Lung Cancer Development and Progression. Front Pharmacol. 2017;8: 573. doi:10.3389/fphar.2017.00573

39. Sun H, Ma X. α5-nAChR modulates nicotine-induced cell migration and invasion in A549 lung cancer cells. Experimental and Toxicologic Pathology. 2015;67: 477–482. doi:10.1016/j.etp.2015.07.001

40. Bordas A, Cedillo JL, Arnalich F, Esteban-Rodriguez I, Guerra-Pastrián L, de Castro J, et al. Expression patterns for nicotinic acetylcholine receptor subunit genes in smoking-related lung cancers. Oncotarget. 2017;8: 67878–67890. doi:10.18632/oncotarget.18948

41. Pillai SG, Ge D, Zhu G, Kong X, Shianna KV, Need AC, et al. A genome-wide association study in chronic obstructive pulmonary disease (COPD): identification of two major susceptibility loci. PLoS Genet. 2009;5: e1000421. doi:10.1371/journal.pgen.1000421

42. Pillai SG, Kong X, Edwards LD, Cho MH, Anderson WH, Coxson HO, et al. Loci Identified by Genome-wide Association Studies Influence Different Disease-related Phenotypes in Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med. 2010;182: 1498–1505. doi:10.1164/rccm.201002-0151OC

43. Cho MH, McDonald M-LN, Zhou X, Mattheisen M, Castaldi PJ, Hersh CP, et al. Risk loci for chronic obstructive pulmonary disease: a genome-wide association study and meta-analysis. The Lancet Respiratory Medicine. 2014;2: 214–225. doi:10.1016/S2213-2600(14)70002-5

44. The Tobacco and Genetics Consortium. Genome-wide meta-analyses identify multiple loci associated with smoking behavior. Nat Genet. 2010;42: 441–447. doi:10.1038/ng.571

45. Douaoui S, Djidjik R, Boubakeur M, Ghernaout M, Touil-boukoffa C, Oumouna M, et al. GTS-21, an α7nAChR agonist, suppressed the production of key inflammatory mediators by PBMCs that are elevated in COPD patients and associated with impaired lung function. Immunobiology. 2020;225: 151950. doi:10.1016/j.imbio.2020.151950

46. Cai G, Bossé Y, Xiao F, Kheradmand F, Amos CI. Tobacco Smoking Increases the Lung Gene Expression of ACE2, the Receptor of SARS-CoV-2. Am J Respir Crit Care Med. 2020; rccm.202003-0693LE. doi:10.1164/rccm.202003-0693LE

47. Zhang H, Rostami MR, Leopold PL, Mezey JG, O'Beirne SL, Strulovici-Barel Y, et al. Expression of the SARS-CoV-2 *ACE2* Receptor in the Human Airway Epithelium. Am J Respir Crit Care Med. 2020; rccm.202003-0541OC. doi:10.1164/rccm.202003-0541OC

48. Wang Q, Zhang Y, Wu L, Niu S, Song C, Zhang Z, et al. Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. Cell. 2020;181: 894-904.e9. doi:10.1016/j.cell.2020.03.045

49. Russo P, Bonassi S, Giacconi R, Malavolta M, Tomino C, Maggi F. COVID-19 and smoking: is nicotine the hidden link? Eur Respir J. 2020;55: 2001116. doi:10.1183/13993003.01116-2020

50. Leung JM, Yang CX, Sin DD. COVID-19 and nicotine as a mediator of ACE-2. Eur Respir J. 2020;55: 2001261. doi:10.1183/13993003.01261-2020

51. Farsalinos K, Angelopoulou A, Alexandris N, Poulas K. COVID-19 and the nicotinic cholinergic system. Eur Respir J. 2020; 2001589. doi:10.1183/13993003.01589-2020

52. Farsalinos K, Niaura R, Le Houezec J, Barbouni A, Tsatsakis A, Kouretas D, et al. Editorial: Nicotine and SARS-CoV-2: COVID-19 may be a disease of the nicotinic cholinergic system. Toxicology Reports. 2020;7: 658–663. doi:10.1016/j.toxrep.2020.04.012

Figure Legends

Figure 1. Evaluation of nAChR transcript levels in human lung tissues. (A). Illustrations depicting the origin of the samples. Whole lung tissues contained all the tissues present in the lung either in parenchyma or in/around bronchi and bronchioles **(B).** Histograms showing the detection of nAChRs in non-smokers (black) and smokers (red). **(C).** Pie charts showing the repartition of nAChRs in non-smokers (left) and smokers (right). *p<0.05; **p<0.01; ***p<0.001 non-smokers (n=42) vs smokers (n=111).

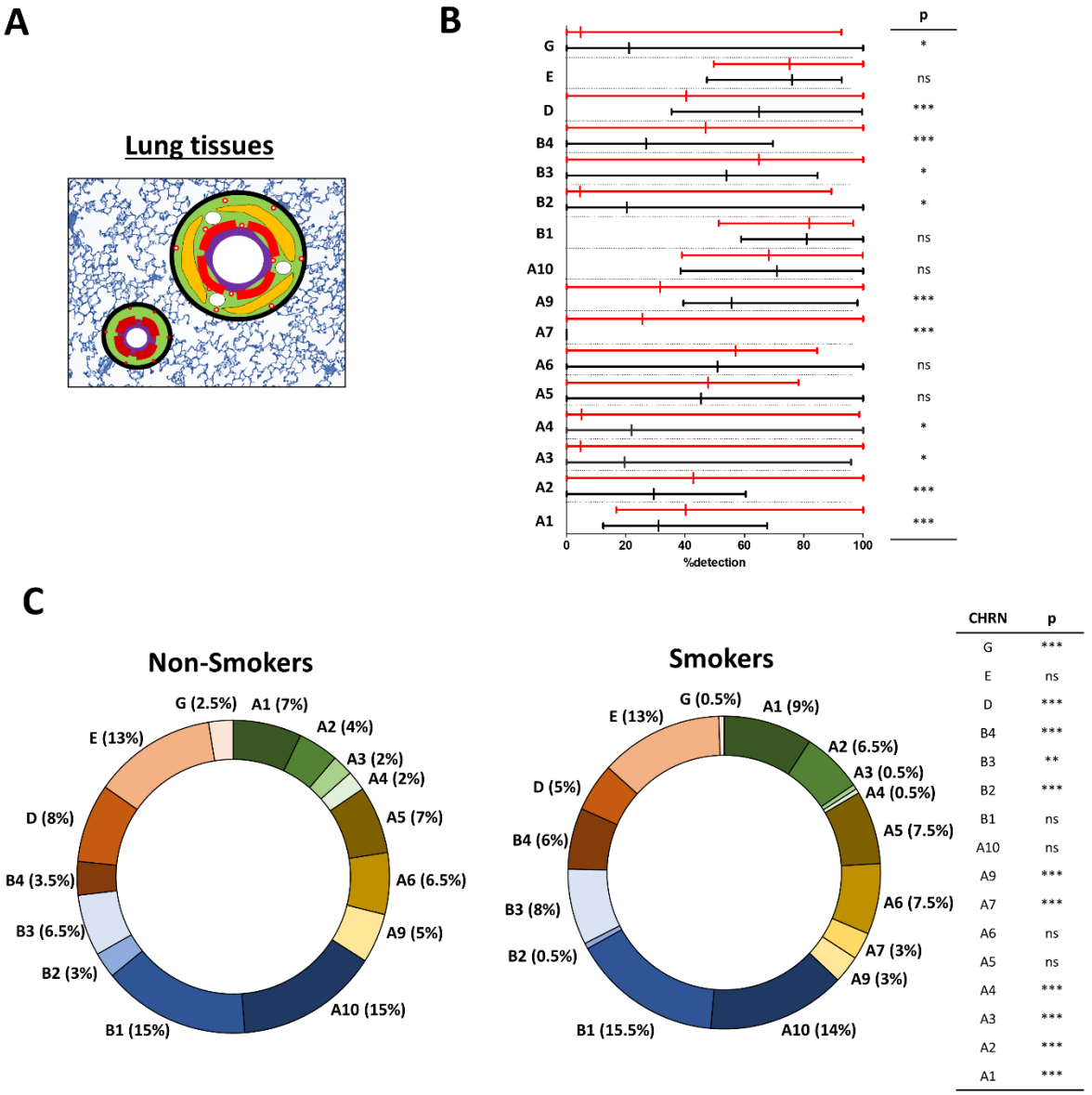
Figure 2. Evaluation of nAChR transcript levels in human large airway epithelial cells (LAEC). (A). Illustrations depicting the origin of the samples. Isolated AEC were collected from bronchi. Large airway epithelial cells (LAEC) are depicted in purple. **(B).** Histograms showing the detection of nAChRs in non-smokers (black) and smokers (red). **(C).** Pie charts showing the repartition of nAChRs in non-smokers (left) and smokers (right). *p<0.05 non-smokers (n=5) vs smokers (n=5).

Figure 3. Evaluation of nAChR transcript levels in human small airway epithelial cells (SAEC). (A). Illustrations depicting the origin of the samples. Isolated AEC were collected from bronchioles. Small airway epithelial cells (SAEC) are depicted in purple. **(B).** Histograms showing the detection of nAChRs in non-smokers (black) and smokers (red). **(C).** Pie charts showing the repartition of nAChRs in non-smokers (left) and smokers (right). *p<0.05; **p<0.01; ***p<0.001 non-smokers (n=63) vs smokers (n=72).

Figure 4. Evaluation of nAChR expressions in lung single cell populations. Coloured cases in the table depict gene expression levels associated with specific lung phenotypes according to the OMIM database (white, no detection). Raw expression values were normalized, log transformed and summarized by published cell clustering.

Figure 5. nAChR localizations in human respiratory epithelia. (A). Representative micrographs showing the bronchial epithelia on FFPE lung tissues stained for the nAChRs (all red); non-differentiated cells (p63 or pan-cytokeratin, green) and cell nuclei (DAPI, blue). Magnification corresponding to the selected area is shown. Cartoons depict the localization of each nAChR (in red). **(B).** Table summarizing nAChR cellular and sub-cellular localization, and the available microscopic data from the Human Protein Atlas (https://www.proteinatlas.org/). NA, not available; -, no detection.

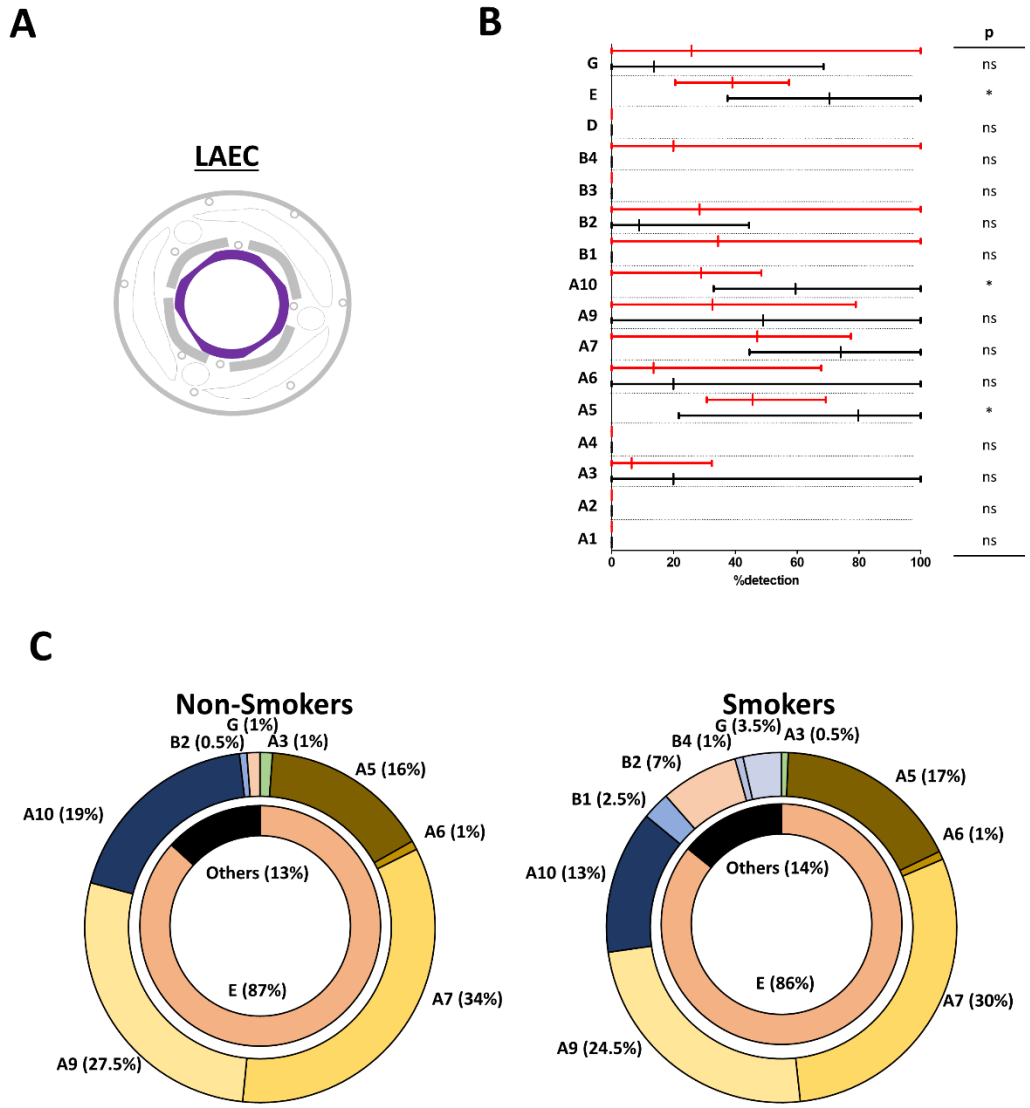
Figure 1


A
Lung tissues
B
p
G *
E ns
D ***
B4 ***
B3 *
B2 *
B1 ns
A10 ns
A9 ***
A7 ***
A6 ns
A5 ns
A4 *
A3 *
A2 ***
A1 ***
0 20 40 60 80 100
%detection
C
Non-Smokers
G (2.5%) A1 (7%) A2 (4%) A3 (2%) A4 (2%) A5 (7%) A6 (6.5%) A9 (5%) A10 (15%) B1 (15%) B2 (3%) B3 (6.5%) B4 (3.5%) D (8%) E (13%)
Smokers
G (0.5%) A1 (9%) A2 (6.5%) A3 (0.5%) A4 (0.5%) A5 (7.5%) A6 (7.5%) A7 (3%) A9 (3%) A10 (14%) B1 (15.5%) B2 (0.5%) B3 (8%) B4 (6%) D (5%) E (13%)
CHRN p
G ***
E ns
D ***
B4 ***
B3 **
B2 ***
B1 ns
A10 ns
A9 ***
A7 ***
A6 ns
A5 ns
A4 ***
A3 ***
A2 ***
A1 ***

1

2

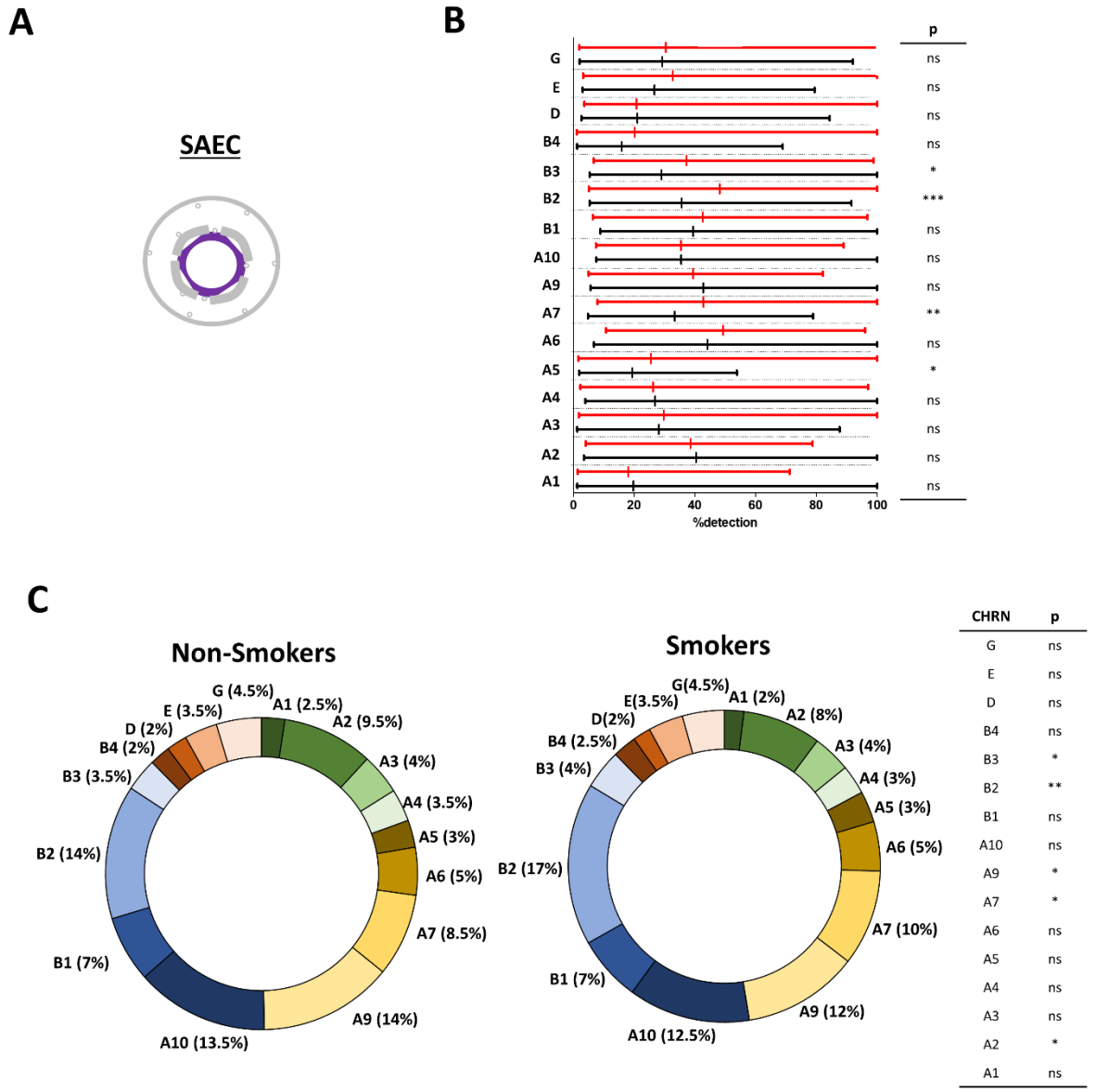
Figure 2


A
LAEC
B
p
G
E
D
B4
B3
B2
B1
A10
A9
A7
A6
A5
A4
A3
A2
A1
ns
*
0
20
40
60
80
100
%detection
C
Non-Smokers
G (1%)
B2 (0.5%)
A3 (1%)
A5 (16%)
A10 (19%)
A6 (1%)
Others (13%)
E (87%)
A7 (34%)
A9 (27.5%)
Smokers
G (3.5%)
B4 (1%)
A3 (0.5%)
B2 (7%)
A5 (17%)
B1 (2.5%)
A6 (1%)
A10 (13%)
Others (14%)
E (86%)
A7 (30%)
A9 (24.5%)

1

2

Figure 3


A
SAEC
B
p
G ns
E ns
D ns
B4 ns
B3 *
B2 ***
B1 ns
A10 ns
A9 ns
A7 **
A6 ns
A5 *
A4 ns
A3 ns
A2 ns
A1 ns
0 20 40 60 80 100
%detection
C
Non-Smokers
G (4.5%)
A1 (2.5%)
A2 (9.5%)
A3 (4%)
A4 (3.5%)
A5 (3%)
A6 (5%)
A7 (8.5%)
A9 (14%)
A10 (13.5%)
B1 (7%)
B2 (14%)
B3 (3.5%)
B4 (2%)
D (2%)
E (3.5%)
Smokers
G(4.5%)
A1 (2%)
A2 (8%)
A3 (4%)
A4 (3%)
A5 (3%)
A6 (5%)
A7 (10%)
A9 (12%)
A10 (12.5%)
B1 (7%)
B2 (17%)
B3 (4%)
B4 (2.5%)
D(2%)
E(3.5%)
CHRN p
G ns
E ns
D ns
B4 ns
B3 *
B2 **
B1 ns
A10 ns
A9 *
A7 *
A6 ns
A5 ns
A4 ns
A3 ns
A2 *
A1 ns

1

2

Figure 4

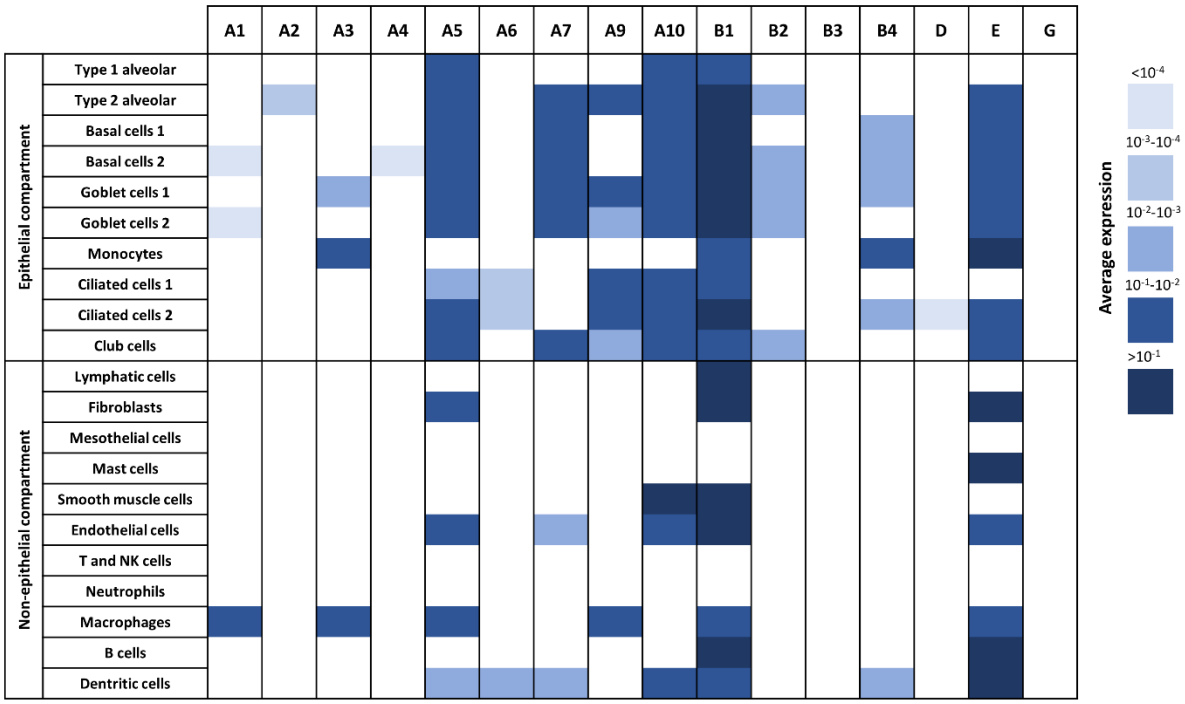
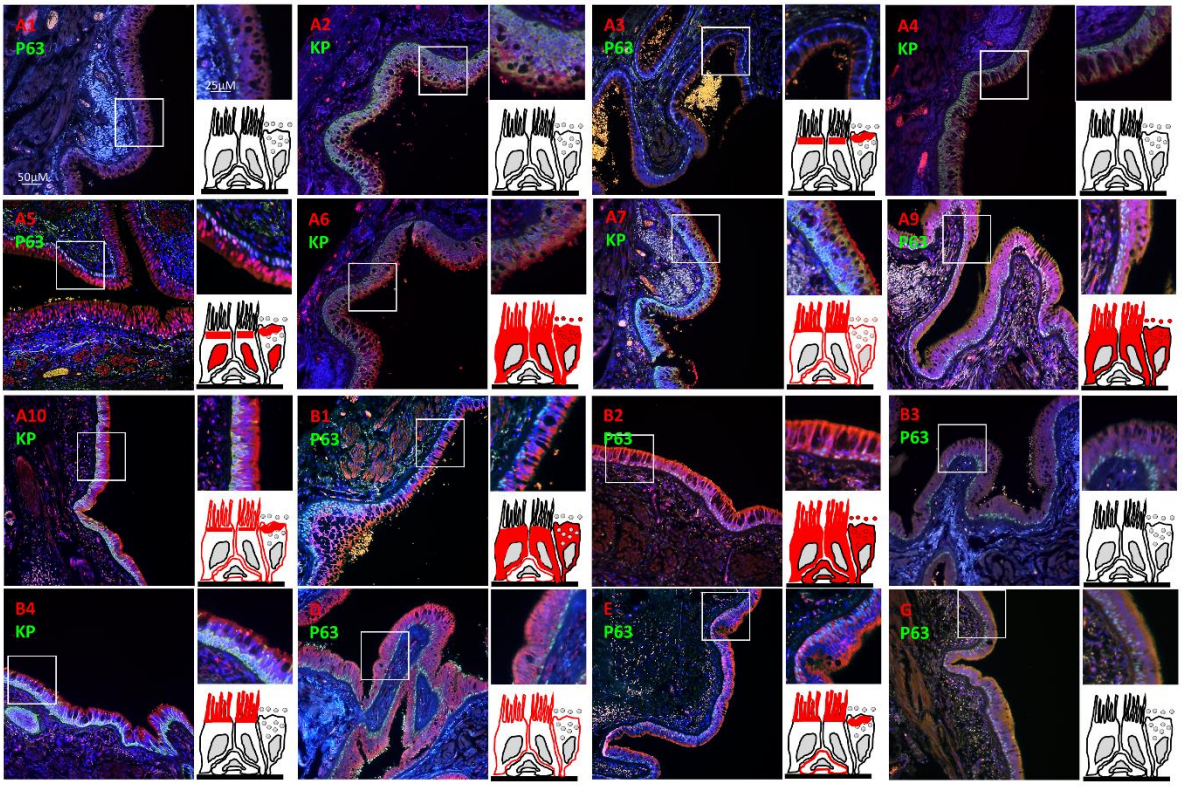

A1
A2
A3
A4
A5
A6
A7
A9
A10
B1
B2
B3
B4
D
E
G
Epithelial compartment
Type 1 alveolar
Type 2 alveolar
Basal cells 1
Basal cells 2
Goblet cells 1
Goblet cells 2
Monocytes
Ciliated cells 1
Ciliated cells 2
Club cells
Non-epithelial compartment
Lymphatic cells
Fibroblasts
Mesothelial cells
Mast cells
Smooth muscle cells
Endothelial cells
T and NK cells
Neutrophils
Macrophages
B cells
Dentritic cells
Average expression
<10-4
10-3-10-4
10-2-10-3
10-1-10-2
>10-1

Figure 5

A



B

CHRN	Lung localization (S, B, b*)	Cell population	Subcellular localization	Human protein atlas lung detection
A1	-	-	-	-
A2	-	-	-	NA
A3	B, b	Multiciliated and goblet cells	Sub-cilia, apical	Subciliary
A4	-	-	-	-
A5	S, B, b	Multiciliated, goblet and basal cells Smooth muscle	Epithelial cell nuclei, apical myoepithelial cell membrane	Muscle cell
A6	B, b	Multiciliated, goblet cells	Cilia, membrane, cytoplasm	NA
A7	S, B, b	Multiciliated, goblet and basal cells Smooth muscle	Cilia, membrane	Subciliary, cilia
A9	B, b	Multiciliated and goblet cells	Cilia, cytoplasm	NA
A10	S, B, b	Multiciliated, goblet and basal cells Smooth muscle cells	Cilia, membrane	NA
B1	S, B, b	Multiciliated, goblet and basal cells Blood vessels, Smooth muscles	Cytoplasm	Multiciliated and goblet cell
B2	B, b	Multiciliated, goblet and basal cells	Cilia, cell membrane	-
B3	-	-	-	-
B4	B, b	Multiciliated cells	Cilia	NA
D	B, b	Multiciliated and goblet cells	Cell membrane, cilia	NA
E	B, b	Multiciliated, goblet and basal cells	Cell membrane	NA
G	-	-	-	NA

* S, stroma; B, bronchi; b, large bronchioles