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Summary of the "take home" message

An alteration of primary and motile ciliogenesis is detected in mild/moderate COPD small airways and could be at the origin of the initiation of epithelial remodelling.

To the Editor,

Chronic obstructive pulmonary disease (COPD) is characterised by a progressive and irreversible airflow limitation due to airway obstruction and emphysema [1]. We and others showed that bronchial epithelial remodelling in COPD is characterised by alteration of ciliogenesis and cilia function [2,3], as well as a dysregulation of non-motile primary cilia (PC) [4]. In COPD, the main site of obstruction is located in small airways [5]. Considering that COPD is foremost a small airway disease (SAD) [6–8], we investigated the differentiation of bronchiolar epithelium in COPD, focusing on motile and primary ciliogenesis.

Bronchioles, defined and selected as small airways (maximum diameter ≈ 2 mm) devoid of any microscopically observable cartilage, were obtained from surgical resections of non-COPD (n=7, 100% men, mean age (interquartile range (IQR)) 70.6 years (63-87), FEV_1 (IQR) 87% (71-105)) and mild/moderate COPD (n=9, 88.9% men, mean age (IQR) 67.1 years (54-84), FEV_1 (IQR) 74% (68-82)) lungs. Bronchiolar epithelial cells were enzymatically dissociated from bronchioles flipped to expose inside-out, and used to establish air-liquid interface (ALI) cultures using a modified BEGM (Lonza, Walskerville, MD) medium [9]. All patients were ex-smokers (smoking cessation for more than 6 months) or current smokers, with no difference in smoking history between the groups (non-COPD: 6/7 ex-smokers, mean pack-years (IQR) 46 (35-60); COPD: 6/9 ex-smokers, mean pack-years (IQR) 43 (20-65)). Use of human tissues was authorized with the written consent of patients (Biological Collection DC-2012–1583, IRB 00003888 Inserm 21–775). Immunofluorescence staining was performed on ALI day (D) 25 cultures (methanol-fixed and formalin-fixed paraffin-embedded cultures), and lung tissues (non-COPD: n=8, 62.5% men, mean age (IQR) 72.9 (62-83); COPD: n=6, 83.3% men, mean age (IQR) 65.8 (56-80)) with antibodies anti-Arl13b [4], anti-β-tubulin [9], anti-MUC-5AC [9], anti-CK13 [9] and anti-SCGB1A1 (R&D Systems, Minneapolis, MN). Images were acquired using AxioImager Z1 microscope (Carl Zeiss, Oberkochen, Germany) or VS120 automated slide scanner (Olympus, Rungis, France). Cell proliferation was assessed using TOX8 kit (Sigma, St Louis, MO). Expression of ciliogenesis effectors in cultures (ALI D0, D7, D15 and D25) was assessed by RT-qPCR as previously described [3]. Specific primers (Eurogentec, Seraing, Belgium) were *P63* (forward-5'-CGCCATGCCTGTCTACAA-3', reverse-5'-TGACTAGGAGGGGCAATCTG-3'), *E2F4* (forward-5'-ATCAAGGCAGACCCCACA-3', reverse-5'-GGGCAAACACTTCTGAGGAC-3'), *GMNC* (forward-5'-ACGGAGACTTGGGTCTCTTTC-3', reverse-5'-TCCGGAAGAGGAAAATTTGA-3'),

GRHL2 (forward-5'-CTTTACCTGGGACGTGAATGA-3', reverse-5'-GGAAGTCCTTTCACCCCTTT-3'), *RFX3* (forward-5'-ACCTCAACCGTGTCGACTTT-3', reverse-5'-GCTGCTGAAGCATCTTGAAGT-3'), *SCGB1A1, FOXJ1, MUC-5AC, HEATR2, MCIDAS* and *RFX2*, all previously described [3,10]. Results were normalized to the housekeeping gene *GAPDH* expression [3,10]. Relative expression (ΔΔCt) was expressed as fold change from ALI D0 values of each gene in each group. Results were expressed as mean±SEM. Data were analysed using the Mann-Whitney test. Statistical significance was determined as $p<0.05$.

To decipher cellular mechanisms involved in SAD and airway remodelling in COPD, we studied the bronchiolar epithelium differentiation in ALI cultures. In the fully differentiated COPD epithelia, computer-assisted quantification (10 images/culture covering ≈ 80% of the total surface) showed a two-fold decrease in the number of β-tubulin-positive multiciliated cells (MCC) (1306±282 MCC/mm^2 vs 634±57 MCC/mm^2, COPD vs non-COPD) (Figure 1a). Although a slight increase in the number of CK13-positive basal cells, MUC-5AC-positive goblet cells and cells co-expressing MUC-5AC and SCGB1A1, as well as a small decrease in SCGB1A1-positive Club cells were detected in COPD cultures, these values did not differ significantly between non-COPD and COPD cultures, as previously reported in comparison between ex/current smokers with and without COPD [11]. Cell proliferation was not different between groups (not shown).

We therefore explored further the expression of ciliogenesis regulators [12] (Figure 1b) which was not significantly different at ALI D0 between non-COPD and COPD cultures. MCC differentiate from basal cells under the control of GMNC, master regulator of ciliated cell fate whose expression was found lower in COPD than in non-COPD differentiated cultures at ALI D25 (5.6±2.7 vs 24.1±13.4-fold change from ALI D0 values). GMNC activates MCIDAS forming the complex EDM containing E2F4 that activates genes required for centrioles biogenesis. Interestingly, we showed a lower expression of MCIDAS (93.2±59.1 vs 226.3±152) and E2F4 (1.5±0.1 vs 2.2±0.3) in COPD compared to non-COPD cultures at ALI D25. EDM also activates transcription factors implicated in multiciliogenesis later steps and required for the docking of basal bodies to the apical surface and the formation of motile cilia. Among them, FOXJ1, RFX2 and RFX3, that are believed to function together, were downregulated in ALI D25 COPD cultures (2545±664 vs 7800±1956, 12.8±2.8 vs 42.7±14.3, 7.3±1.2 vs 30.7±6.2, COPD vs non-COPD, respectively). Moreover, GRHL2, known to modulate MCIDAS and RFX2, was two-fold less expressed in COPD than in non-COPD differentiated cultures at ALI

D25 (3.6±0.7 vs 7.5±2.2), as well as HEATR2, involved in the initiation of dynein complex assembly (1.3±0.1 vs 2.8±0.4). Importantly, most of these regulators of ciliogenesis, namely GMNC, E2F4, FOXJ1, RFX2, RFX3 and HEATR2, showed expression that plateaued after ALI D15 in COPD cultures, unlike their expression in non-COPD cultures. MCC originate from primary ciliated cells (PCC) [10,13] and we previously reported a dysregulation of PC in COPD bronchial epithelium [3,4]. We therefore analysed primary ciliogenesis in bronchiolar epithelium. As in bronchi, PC were localised on undifferentiated cells at the basal part of the differentiated epithelia in cultures (Figure 1c) and tissues (Figure 1d). Double-blind manual counting (3 to 6 random fields per culture) revealed that the PCC number was decreased in COPD when compared to non-COPD cultures (5.5±0.7% vs 11±0.5% of total cells), reflecting observations in tissues (10.7±1.6 vs 17.7±2.9 PC/mm of epithelium).

Despite the crucial contribution of small airways to COPD pathogenesis [6], investigations on the bronchiolar epithelium biology remain sparse because of the complex isolation and culture of non-previously amplified primary cells. We circumvented this difficulty with the microdissection of small airways. This allowed us to harvest several million epithelial cells, thus avoiding prior amplification before ALI culture which leads to the loss of the primary characteristics of cells, in particular in terms of MCC differentiation capacity [9]. We were thus able to highlight for the first time an intrinsic impairment of the bronchiolar MCC differentiation in COPD. Our *in vitro* model being inflammation-free, and all patients being ex- or current smokers with no difference in smoking history, we demonstrated environment and tobacco-independent alterations of multiciliogenesis in small airways in mild/moderate COPD. This MCC defect was accompanied by downregulation of key multiciliogenesis regulators acting throughout cilia construction and by a decrease in the number of PCC. A correlation between the decreased number of PCC and that of MCC in COPD small airway epithelium could be expected. Indeed, it was previously reported that PC removal of airway undifferentiated cells led to impairment of multiciliogenesis [10] and that murine tracheal MCC originated from PCC [13]. However, in COPD bronchial epithelium, the number of PCC is markedly increased, especially in areas of epithelium remodelling, suggesting the involvement of PC in altered epithelial repair/regeneration processes in COPD [4]. It can be hypothesized that the small airway epithelium is less prone to environment-associated damage and consequently to regeneration than the large airway epithelium. The role of PC in the regulation of remodelling in small airways remains therefore to be elucidated. Based on our results, we

can suggest different roles of the PC in the complex molecular mechanisms regulating cell fate, depending on epithelial location and environmental stimuli.

Some limitations of our study are that the potential impact of COPD treatments is not taken into account in the phenomenon, and the low number of patients at the origin of bronchiolar epithelial cells. However, this last weakness also represents a strength. Indeed, the bronchiolar primary and motile ciliogenesis defect in COPD is convincingly evidenced with a cohort of small size, suggesting the robustness of the reported phenomenon.

In conclusion, we have shown both motile and primary impaired ciliogenesis in the small airways of mild/moderate COPD patients. These alterations could impact the mucociliary clearance with a potential involvement in the initiation of remodelling mechanisms in airway epithelium and/or the development of emphysema [14]. Data on severe COPD as well as longitudinal data could be needed in the future to provide the evidence that altered ciliogenesis represents an intrinsic abnormality of some smokers at-risk to develop COPD, or a feature seen in early disease and/or correlated to severity. The determinants and underlying mechanisms of this dysregulation of epithelial differentiation will have also to be determined to pave the way for the development of new therapeutic strategies for COPD patients.

FOOTNOTES

Contributors: Study concept: C. Coraux; study design: E. Luczka-Majérus and C. Coraux; acquisition data: E. Luczka-Majérus, A. Bonnomet, A. Germain, N. Lalun, C. Kileztky, J-M. Pérotin, G. Deslée, G. Delepine, V. Dormoy, C. Coraux; analysis and data interpretation: E. Luczka-Majérus, V. Dormoy, C. Coraux; revision of manuscript: M. Polette, V. Dormoy, C. Coraux; manuscript writing: E. Luczka-Majérus and C. Coraux

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Competing interests: None declared

FIGURE LEGENDS

Figure 1: Motile and primary ciliogenesis are altered in COPD bronchiolar epithelium.
a) Immunodetection and quantification of β-tubulin-positive (red) multiciliated cells (MCC) in non-COPD (n=7) and COPD (n=9) human bronchiolar cultures at ALI D25. Nuclei are stained with DAPI (blue). Results show means ± SEM, *p<0.05. **b)** Histograms representing the RT-qPCR assessment of fold-change at ALI D7, D15 and D25 (compared to ALI day 0) in the GAPDH-normalized expression of the ciliogenesis effectors GMNC, MCIDAS, E2F4, FOXJ1, RFX2, RFX3, GRHL2 and HEATR2, in non-COPD (n=7) and COPD (n=9) cultures. Results show means ± SEM, *p<0.05, ***p<0.001. **c)** Representative fluorescence acquisitions from non-COPD and COPD ALI D25 culture sections showing the presence of motile cilia and primary cilia (PC) stained using antibodies directed against Arl13b (red). Nuclei are stained with DAPI (blue). Boxed areas are shown as magnifications of PC (arrowhead). Histogram represents the quantification of cells with PC (% of total population). Results show means ± SEM, ***p<0.001. n=7 non-COPD cultures and n=9 COPD cultures. **d)** Representative micrographs showing motile cilia and PC, stained using antibodies directed against Arl13b (red), on fluorescence acquisitions of non-COPD (n=8) and COPD (n=6) sections of small airway tissues . Nuclei are stained with DAPI (blue). Boxed areas are shown as magnifications of PC (arrowhead). Histogram represents the quantification of cells with PC per mm of epithelium. Results show means ± SEM.

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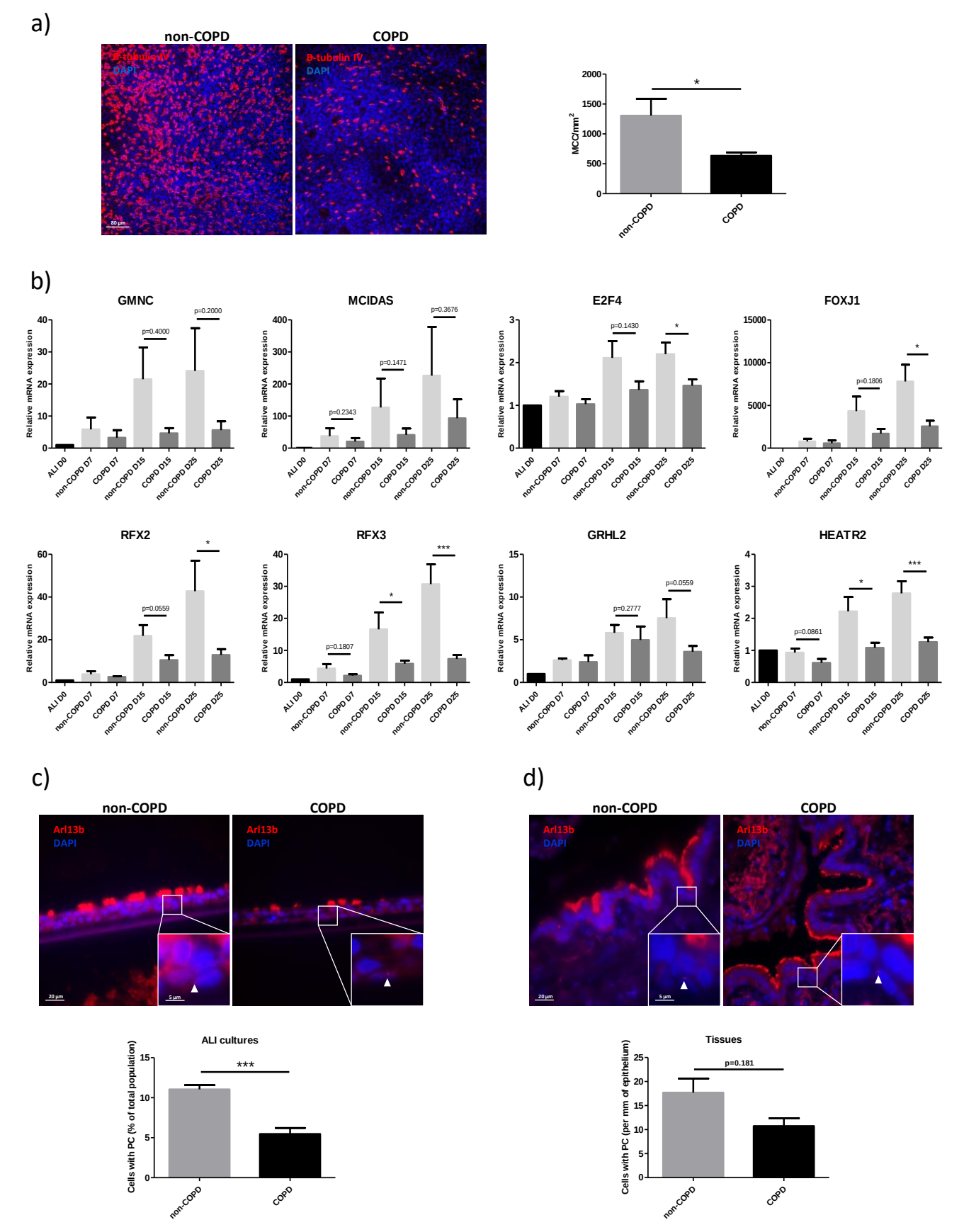
a)
non-COPD
COPD
β-tubulin IV
DAPI
80 µm
MCC/mm²
2000
1500
1000
500
0
*
non-COPD
COPD
b)
GMNC
MCIDAS
E2F4
FOXJ1
RFX2
RFX3
GRHL2
HEATR2
Relative mRNA expression
p=0.4000
p=0.2000
p=0.2343
p=0.3676
p=0.1471
p=0.1430
p=0.1806
p=0.0559
p=0.1807
p=0.2777
p=0.0861
ALI D0
non-COPD D7
COPD D7
non-COPD D15
COPD D15
non-COPD D25
COPD D25
c)
non-COPD
COPD
Arl13b
DAPI
20 µm
5 µm
ALI cultures
Cells with PC (% of total population)
***
d)
Tissues
Cells with PC (per mm of epithelium)
p=0.181