Impaired autophagy in the lower airways and lung parenchyma in stable COPD

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Abstract

Background There is increasing evidence of autophagy activation in COPD, but its role is complex and probably regulated through cell type-specific mechanisms. This study aims to investigate the autophagic process at multiple levels within the respiratory system, using different methods to clarify conflicting results reported so far.

Methods This cross-sectional study was performed on bronchial biopsies and peripheral lung samples obtained from COPD patients (30 and 12 per sample type, respectively) and healthy controls (25 and 22 per sample type, respectively), divided by smoking history. Subjects were matched for age and smoking history. We analysed some of the most important proteins involved in autophagosome formation, such as LC3 and p62, as well as some molecules essential for lysosome function, such as lysosome-associated membrane protein 1 (LAMP1). Immunohistochemistry was used to assess the autophagic process in both sample types. ELISA and transcriptomic analysis were performed on lung samples.

Results We found increased autophagic stimulus in smoking subjects, regardless of respiratory function. This was revealed by immunohistochemistry through a significant increase in LC3 ($p<0.01$) and LAMP1 ($p<0.01$) in small airway bronchiolar epithelium, alveolar septa and alveolar macrophages. Similar results were obtained in bronchial biopsy epithelium by evaluating LC3B ($p<0.05$), also increased in homogenate lung tissue using ELISA ($p<0.05$). Patients with COPD, unlike the others, showed an increase in p62 by ELISA ($p<0.05$). No differences were found in transcriptomics analysis.

Conclusions Different techniques, applied at post-transcriptional level, confirm that cigarette smoke stimulates autophagy at multiple levels inside the respiratory system, and that autophagy failure may characterise COPD.

Introduction

Autophagy is a pivotal process in cellular homeostasis, contributing to the regulation of intracellular component processing and recycling [1]. There are several forms of autophagy, but three are classically recognised: macroautophagy, microautophagy and chaperone-mediated autophagy [1].

Macroautophagy is the main form of autophagy and starts with the creation of transient double-membrane vesicles called autophagosomes. The process requires the formation of an isolated membrane by a class III phosphoinositide-3-kinase (PI3K) complex, which includes proteins such as beclin-1 and vacuolar protein sorting 34 (VPS34) [2, 3]. The PI3K complex also induces nuclear localisation of the transcription factor

EB (TFEB), which activates several lysosomal and autophagy-related genes [2]. The isolated membrane then elongates to form a double crescent-shaped membrane called the phagophore. Further elongation and closure of the phagophore to form the autophagosome vesicle requires the presence of a protein called microtubule-associated protein 1A/1B-light chain 3 (MAP1LC3 or LC3) [4]. It is initially synthesised in a form called pro-LC3, which is converted to a proteolytically processed form called LC3A and finally modified through lipidation into LC3B [5]. Another protein deemed necessary to autophagosome formation and proper function is p62 (sequestosome-1 (SQSTM1)), which binds to LC3 and also to ubiquitinated proteins and organelles designated for degradation [2, 6]. Through this mechanism, the cargo is incorporated into the autophagosome vesicle for transport to the lysosome, resulting in the degradation of its contents by lysosomal acid hydrolases. Several proteins essential for the proper functioning of the lysosome have been identified, including cathepsins and the lysosome-associated membrane protein 1 (LAMP1), which is also required for the proper fusion of lysosomes with autophagosomes [7].

In recent years, evidence has emerged regarding autophagy activation in COPD [2, 8, 9], a heterogeneous lung condition characterised by chronic respiratory symptoms and persistent airflow obstruction [10]. This activation is deemed to be mainly due to cigarette smoking, as induced autophagy is considered fundamental in maintaining cellular homeostasis in adverse environments. Indeed, it can preserve lung function through several mechanisms, such as the inhibition of cellular senescence, the degradation of damaged organelles, the suppression of myofibroblast differentiation and the elimination of pathogens [11–15]. Nevertheless, autophagy is a highly dynamic process and if excessive can become harmful [13, 16]. Activated autophagy has been in fact linked to aberrant inflammatory response, mucus hyperproduction, mucociliary clearance impairment and cell death [17–21].

Data on autophagy based on human bronchial epithelial cells (HBECs) and lung tissues from COPD patients are conflicting, and there is no unifying explanation for these discrepancies. The role of autophagy in COPD is considered complex and probably regulated *via* cell type-specific mechanisms [13, 14, 17, 22, 23]. Being both beneficial and harmful, autophagy can be considered as a double-edged sword [24]. A prognostic role has also been attributed to autophagy in COPD patients and the use of drugs able to modulate the process has been hypothesised [19, 25, 26]. However, a better understanding of changes in the autophagic process in response to cigarette smoke is deemed necessary before considering this process as a therapeutic target in COPD [16].

Considering the above, the purpose of this study is to investigate the autophagic process at different levels within the respiratory system, comparing data obtained by multiple methods to achieve greater robustness and try to better understand the conflicting results reported so far. A deeper knowledge of the mechanisms of activation and dysregulation of autophagy in different areas of the respiratory system and in different cell types could lead to the use of new specific drugs that can regulate the autophagic process in a targeted manner to achieve optimal levels [2].

Methods

Subjects

Archival material from COPD patients and healthy controls was used in the present study [27]. All subjects were recruited from the Respiratory Medicine Unit of the Istituti Clinici Scientifici Maugeri (Veruno, Italy) and the Department of Clinical and Biological Sciences of the San Luigi Gonzaga University Hospital (Orbassano, Italy). The study complies with the Declaration of Helsinki and has been approved by the institutional review boards of Istituti Clinici Scientifici Maugeri (protocol p112) and by the ethical committee of the San Luigi Gonzaga University Hospital (protocol n. 9544/2019). All patients have signed an informed consent.

Lung function tests and volumes

Pulmonary function tests were performed in all subjects, according to current guidelines (supplementary material). The severity of the airflow obstruction in COPD patients was staged using Global Initiative for Chronic Obstructive Lung Disease criteria using only the levels of bronchial obstruction [10].

Sample collection

Fibreoptic bronchoscopy was performed to collect bronchial biopsies from segmental and subsegmental airways (4th to 6th airway generation) of 55 subjects, 30 of whom had COPD (supplementary material). 34 subjects undergoing lung resection surgery for a solitary peripheral neoplasm were recruited, including 12 with COPD. All subjects had mild or moderate disease severity, according to the eligibility criteria for surgery. Specimens of their lung parenchyma were frozen and used for analysis (supplementary material).

All COPD patients were stable and had not been treated with theophylline, antibiotics, antioxidants, mucolytics and/or glucocorticoids in the month prior to bronchoscopy or surgery.

Immunohistochemistry in bronchial and peripheral lung tissue

Bronchial biopsies as well as peripheral lung specimens were used for immunohistochemical analysis. Sections from each sample were stained with antibodies specific for autophagy markers and proteins (supplementary material). In particular for LC3A, LC3B, cathepsin B, cathepsin D, beclin-1, p62, LAMP1 and TFEB. The immunostaining for all the antigens studied was scored in the intact bronchial epithelium, as well as in the lamina propria. All bronchioles, alveolar macrophages and alveolar septa observed in each lung section specimen were also analysed (supplementary material).

RNA extraction, sequencing and analysis from bronchial and peripheral lung tissue

Frozen lung parenchymal tissues used for immunohistochemical analysis and bronchial rings from the same patients were also used for RNA extraction, sequencing and gene expression analysis (supplementary material). We considered the expression levels of MAP1LC3A, MAP1LC3B, CTSB, cathepsin D (CTSD), BECN1, NUP62, LAMP1 and TFEB genes. The expression level of these genes was also evaluated in www.copdcellatlas.com, a COPD-dedicated gene expression browser of single-cell RNA sequencing data [28].

ELISA tests in peripheral lung specimens

LC3A, LC3B, LAMP1 and p62 protein quantification was performed in the lung tissue homogenates obtained from frozen tissue specimens used also for immunohistochemical and RNA analysis (supplementary material). The choice of molecules analysed by ELISA was made on the basis of the differences revealed by immunohistochemistry.

Statistical analysis

Group data were expressed as mean±SD for functional data or median (range) or interquartile range for morphological data. Differences between groups were analysed using ANOVA for functional data. ANOVA was followed by an unpaired t-test for comparison between groups. The Kruskal–Wallis test was applied to the morphological data and followed, in case of a significant result, by a Mann–Whitney U-test for comparison between groups. Correlation coefficients were calculated using the Spearman rank method. Results were considered statistically significant at $p < 0.05$. Data analysis was performed using the StatView SE Graphics programme (Abacus Concepts, Berkeley, CA, USA).

Results

Population of the study

Bronchial biopsies were obtained from 30 COPD patients and 25 control subjects, 13 of whom had a significant smoking history. The characteristics of all these subjects are reported in table 1. Peripheral lung samples were obtained from lung resections of 34 subjects, whose characteristics are shown in table 2. 12 of these had COPD, while the other 22 had normal lung function. Half of the control subjects were smokers.

TABLE 1 Clinical characteristics of subjects for immunohistochemistry studies on the bronchial biopsies								
	Subjects	Age years	Male/ female	Ex-smokers/ current smokers	Smoking pack-years	Pre-bronchodilator FEV$_1$ % pred	Post-bronchodilator FEV$_1$ % pred	FEV$_1$/FVC %
Control nonsmokers	12	63±13	8/4	0	0	117±18	ND	86±10
Control smokers	13	60±11	11/2	1/12	41±31	101±14	ND	81±6
COPD grades I and II (mild/ moderate)	16	72±6	11/5	7/9	50±29	63±11****	67±14	57±10****
COPD grades III and IV (severe/ very severe)	14	68±18	13/1	11/3	61±44	38±5****,####	42±7	43±10****,####

Data are expressed as n or mean±SD. Patients with COPD were classified according to the grades of severity of the Global Initiative for Chronic Obstructive Lung Disease [10] using only the severity of airflow obstruction. For patients with COPD, forced expiratory volume in 1 s (FEV$_1$)/forced vital capacity (FVC) are post-bronchodilator (β$_2$) values. ND: not determined. ****: $p < 0.0001$, significantly different from control smokers with normal lung function and control never-smokers (ANOVA); ####: $p < 0.0001$, significantly different from mild/moderate COPD (ANOVA).

TABLE 2 Clinical characteristics of subjects for immunohistochemistry studies on the peripheral lung tissue

|	Subjects	Age years	Male/ female	Ex-smokers/ current smokers	Smoking pack-years	Pre-bronchodilator FEV$_1$ % pred	Post-bronchodilator FEV$_1$ % pred	FEV$_1$/ FVC %
Control nonsmokers	11	71±10	5/6			115±15	ND	80±5
Control smokers	11	67±6	7/4	7/4	36±14	96±10	ND	74±4
Patients with COPD	12	69±6	11/1	10/2	51±39	72±16****	79±14	59±9****

Data are presented as n or mean±SD. For patients with COPD, forced expiratory volume in 1 s (FEV$_1$)/forced vital capacity (FVC) are post-bronchodilator (β$_2$) values. ND: not determined. ****: p<0.0001, significantly different from control smokers with normal lung function and control never-smokers (ANOVA).

Gene expression level in bronchial rings and lung parenchyma

We examined RNA-sequencing expression data of 18 samples of bronchial rings and 23 lung parenchyma samples, which were obtained from frozen blocks adjacent to the specimens used for immunohistochemical analysis and from frozen bronchial rings (figure 1). Considering MAP1LC3A, MAP1LC3B, CTSB, CTSD, BECN1, NUP62, LAMP1 and TFEB genes, no significant differences emerged in their expression levels between COPD, control smoker (CS) and control nonsmoker (CNS) samples. CTSD was the gene with the highest expression among the eight selected ones in both tissues, with increasing expression in CS and COPD compared to CNS. Similarly, CTSB also showed considerable expression levels in both tissues, although the increasing trend is less evident. LAMP1 showed moderate expression levels in lung parenchyma only, whereas the expression of MAP1LC3A, MAP1LC3B, BECN1, NUP62 and TFEB were lower. By browsing single-cell RNA-sequencing datasets, we confirmed the high expression of CTSB in both COPD- and control-derived lung cells, whereas the other genes appeared less expressed (supplementary figure S1). Strikingly, CTSD results showed almost no expression in single-cell data.

Immunohistochemistry of autophagic molecules in bronchial biopsies

No differences in the expression of LAMP1, LC3A, p62, TFEB, beclin-1 and cathepsin B/D were found in the bronchial epithelium of COPD patients compared to CNS and CS, but some differences emerged for
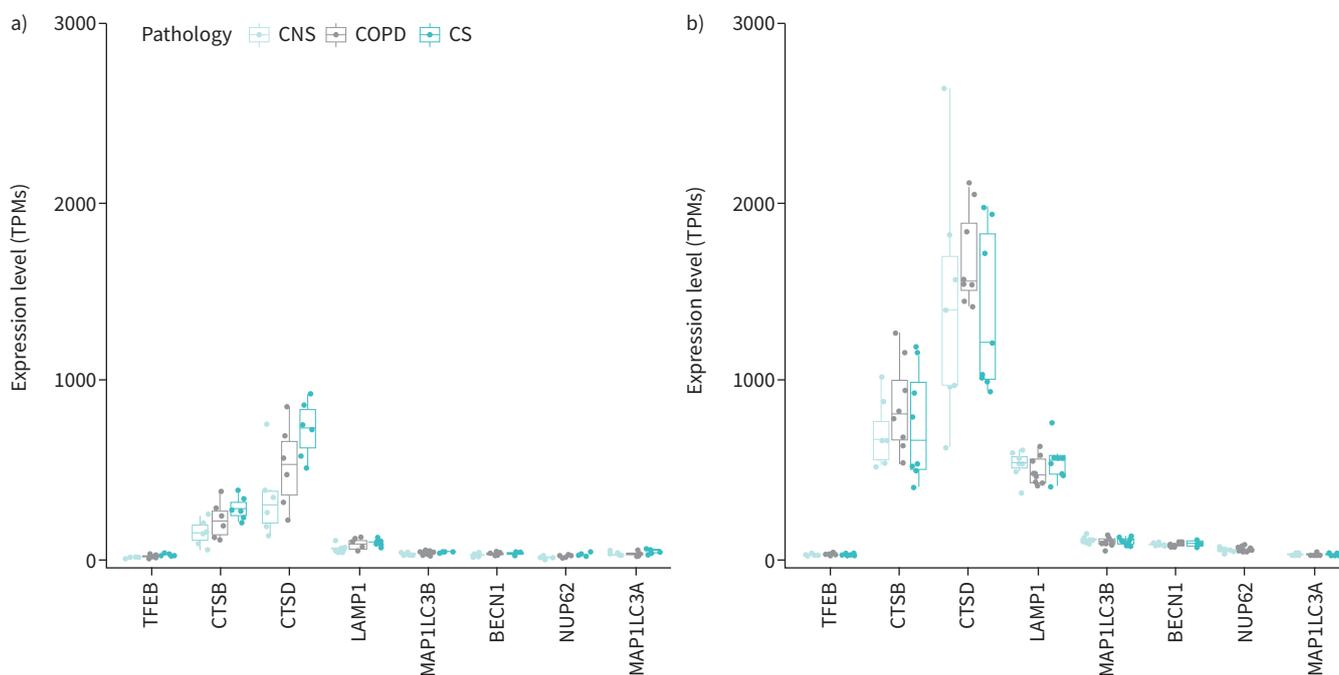
FIGURE 1 Expression levels of selected autophagy genes obtained in a) bronchial rings and b) lung parenchyma of control nonsmokers (CNS), control smokers (CS) and patients with COPD. The box plots show the median and the distribution of expression values per gene. TPM: transcript per million; TFEB: transcription factor EB; CTSB: cathepsin B; CTSD: cathepsin D; LAMP1: lysosomal-associated membrane protein 1; MAP1LC3B: microtubule associated protein 1 light chain 3β; BECN1: beclin 1; NUP62: nucleoporin 62; MAP1LC3A: microtubule associated protein 1 light chain 3α.

LC3B (table 3). Specifically, the number of LC3B-positive cells was significantly lower in the bronchial epithelium of nonsmokers than in CS (p=0.04) and in patients with severe/very severe COPD (p=0.01). The level of LC3B-positive cells in patients with severe/very severe COPD was also significantly higher than in patients with mild/moderate disease (p=0.03). The number of cells positive for LAMP1, LC3A, LC3B, TFEB, beclin-1, p62 and cathepsin B/D were similar in the bronchial lamina propria of COPD patients of different severity compared to CNS and CS (table 3). No significant association emerged between the expression levels of autophagic molecules in bronchial biopsies and smoking history or respiratory function.

Immunohistochemistry of autophagic molecules in peripheral airways and lung parenchyma

As shown in table 4, no differences in the expression of LC3B, p62, TFEB, beclin-1 and cathepsin B/D were found in the bronchiolar epithelium of peripheral airways of COPD patients compared to CNS and CS. In comparison to CS and COPD patients, CNS expressed significantly lower levels of LC3A (p<0.01 and p<0.05, respectively) (figure 2a and b), total LC3 (p<0.01) and LAMP1 (p<0.01) (figure 2c and d). Similar results were found also in the analysis of alveolar macrophages and alveolar septa (table 4). Specifically, alveolar macrophages of CS and COPD patients expressed higher levels of LC3A (p<0.01) and total LC3 (p<0.01) in comparison to CNS. A higher level of LAMP1 was found in CS in comparison to CNS (p<0.01), but not to COPD patients. Concerning alveolar septa, CNS expressed lower levels of LC3A (p<0.01) (figure 2a and b), total LC3 (p<0.01) and LAMP1 (p<0.01) in comparison to both CS and COPD patients (table 4). Importantly, CNS showed also a lower number of p62 immunostained alveolar macrophages in comparison to CS and COPD patients (p<0.01 and p<0.05, respectively) (table 4, figure 3a and b). Double staining for LC3B (brown colour) and p62 protein (red colour), performed in three COPD patients and three CNS subjects, showed a clear p62 immunoexpression in a large number of alveolar macrophages coexpressing LC3B in COPD lungs (figure 3c). The number of cathepsin B-positive cells in alveolar septa was slightly but significantly higher in CNS than in the other two groups (p<0.05). Finally, the level of LC3B was higher in the alveolar septa of CNS than in CS (p<0.05). No significant association emerged between the expression levels of autophagic molecules and smoking history or respiratory function.

ELISA tests for autophagic molecules in homogenised peripheral lung tissue

As shown in table 5 and figure 4, we found no differences in the concentration of LAMP1 and LC3A in the lung tissue homogenates of the three groups. CNS showed a reduced level of LC3B in comparison to CS and COPD patients (p=0.02). Importantly, patients with COPD were found to have a higher level of

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TABLE 3 Immunohistochemistry of autophagic molecules in the bronchial biopsies of patients with COPD and control subjects

	Control nonsmokers	Control smokers	Mild/moderate COPD	Severe/very severe COPD	Kruskal–Wallis p-value
Subjects	12	13	16	14	
Epithelium score (0–3)					
LC3A	0.25 (0.25–0.5)	0 (0–0.75)	0.5 (0–1)	0.25 (0–1)	0.066
LC3B	1.5 (0.5–3)	2.5 (1.5–2.5)[#]	1.75 (1–2.5)	2.5 (1.5–3)[¶,+]	0.020
TFEB total	0.0 (0.0–0.5)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.925
Cathepsin B	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	ND
Cathepsin D	0.87 (0.75–1.25)	1 (0.5–1.5)	1.5 (0.75–2)	1.37 (0.75–1.5)	0.131
Beclin-1	1.5 (1.5–1.5)	1.5 (1–2)	1.5 (1.5–2)	1.5 (1.5–2.5)	0.742
P62	0.37 (0–0.5)	0.75 (0.25–1.5)	0.5 (0.25–1)	0.5 (0.25–1)	0.359
LAMP1	1.0 (0.5–1.5)	0.75 (0.25–1)	0.75 (0.25–1.5)	0.75 (0.5–1)	0.377
Lamina propria cells·mm^{-2}					
LC3A	8 (0–13)	4 (0–26)	9.5 (0–55)	13 (0–24)	0.373
LC3B	70 (32–200)	69 (21–97)	78 (16–118)	71 (29–156)	0.794
TFEB total	0.0 (0.0–34.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.953
Cathepsin B	4 (0–8)	5 (0–13)	0 (0–24)	0 (0–32)	0.494
Cathepsin D	12.5 (8–48)	47.5 (9–97)	41.5 (11–64)	38 (12–97)	0.256
Beclin-1	39 (9–81)	19.5 (0–92)	42 (0–87)	11 (5–124)	0.729
P62	32 (5–74)	35 (16–118)	13 (0–56)	20 (0–90)	0.098
LAMP1	216 (142–322)	200 (165–226)	208 (78–274)	193 (134–240)	0.564

Data are expressed as n or median (range). LC3: microtubule-associated protein 1A/1B-light chain 3; TFEB: transcription factor EB; LAMP1: lysosome-associated membrane protein 1; ND: not determined. [#]: p=0.0423 *versus* control nonsmokers; [¶]: p=0.028 *versus* mild/moderate COPD; [+]: p=0.014 *versus* control nonsmokers (Mann–Whitney U-test).

TABLE 4 Immunohistochemical quantification of autophagic molecules in the peripheral lung of patients with COPD, in control smokers and nonsmoking subjects

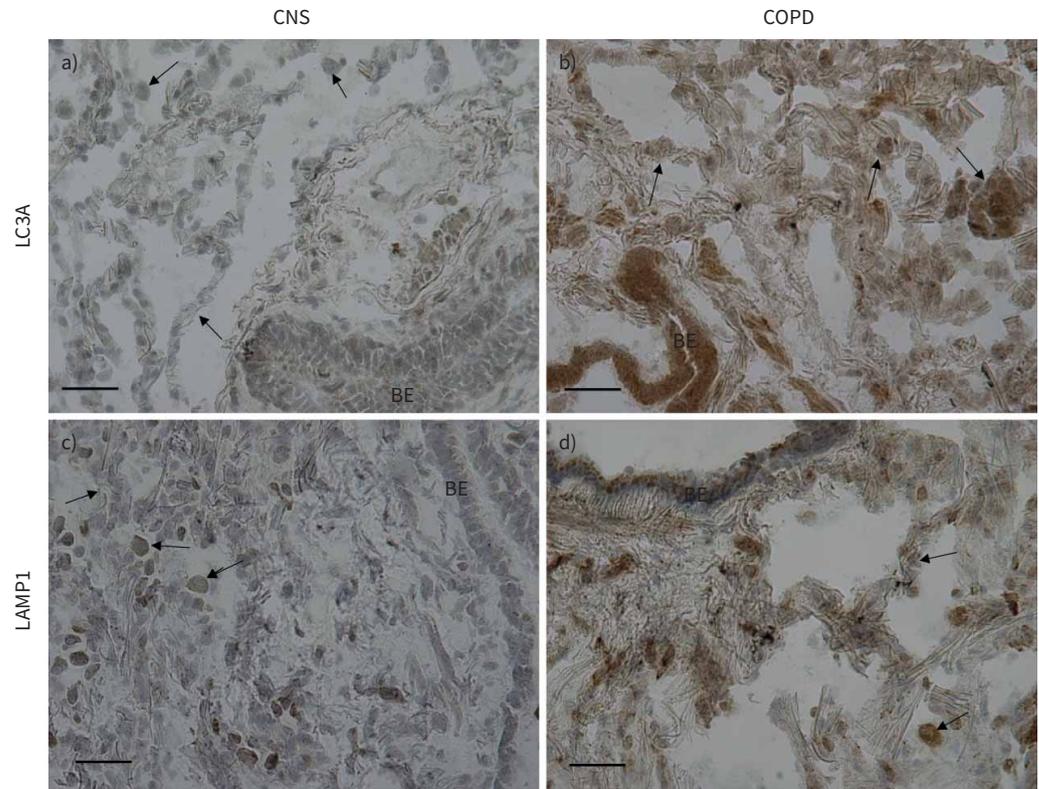|	Control nonsmokers	Control smokers	Patients with COPD	Kruskal–Wallis p-value
Subjects	11	11	12	
Bronchiolar epithelium (score 0–3)				
LC3A	0.40 (0.25–1.0)	1.50 (0.75–2.0)**	1.25 (0.5–2.0)*	**0.0013**
LC3B	2.50 (1.9–2.75)	2.75 (2.0–3.0)	2.75 (2.5–3.0)	0.287
LC3 (A+B)	2.75 (2.15–3.75)	3.95 (2.75–4.75)**	4 (3.24–4.5)**	**0.028**
TFEB total	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	ND
Cathepsin B	0.12 (0–0.50)	0.0 (0.0–0.50)	0.0 (0.0–0.12)	0.218
Cathepsin D	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	ND
Beclin-1	2.32 (2.0–2.5)	2.50 (1.5–2.75)	2.5 (1.5–2.75)	0.214
P62	0.0 (0.0–0.0)	0.0 (0.0–0.12)	0.0 (0.0–0.0)	0.715
LAMP1	0.12 (0.0–0.25)	1.0 (0.37–1.50)**	0.55 (0.5–0.75)**	**<0.0001**
Alveolar macrophages (score 0–3)				
LC3A	0.5 (0.25–1.0)	1.5 (1.0–2.0)**	1.25 (1.0–1.25)**	**0.0001**
LC3B	2.0 (1.5–2.5)	2.0 (1.75–2.5)	2.0 (2–2.5)	0.237
LC3 (A+B)	2.5 (1.75–3)	3.5 (3–4.5)**	3.25 (3–375)**	**0.0004**
TFEB total	0.0 (0.0–1.4)	0.05 (0.0–1.6)	0.0 (0.0–1.5)	0.7342
TFEB nuclear	0.0 (0.0–0.35)	0.0 (0.0–0.27)	0.0 (0.0–0.24)	0.6924
TFEB cytoplasmic	0.0 (0.0–1.05)	0.05 (0.0–1.5)	0.0 (0.0–1.5)	0.7260
Cathepsin B	0.12 (0.12–0.75)	0.50 (0.0–1.75)	0.31 (0.12–1.5)	0.640
Cathepsin D	0.0 (0.0–0.25)	0.0 (0.0–0.50)	0.10 (0.0–0.50)	0.980
Beclin-1	1.5 (1.25–2.0)	1.75 (1.25–2.0)	1.62 (1.25–2.0)	0.272
P62	0.0 (0.0–0.12)	0.50 (0.5–1.0)**	0.50 (0.0–1.0)*	**0.0009**
LAMP1	1.5 (0.75–2.0)	2.0 (1.5–2.5)**	1.5 (1.5–2.0)	**0.021**
Alveolar septa (score 0–3)				
LC3A	0.0 (0.0–0.5)	1.0 (0.75–1.25)**	1.0 (1.0–1.0)**	**<0.0001**
LC3B	2.0 (1.5–2.5)	1.75 (1.5–2.0)*	2.0 (1.75–2.0)	**0.022**
LC3 (A+B)	2.5 (1.5–2.5)	3 (2.25–3)**	3 (2.75–3)**	**0.0007**
TFEB total	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	ND
Cathepsin B	0.12 (0.0–0.50)	0.0 (0.0–0.12)*	0.0 (0.0–0.5)*	**0.041**
Cathepsin D	0. (0.0–0.12)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.890
Beclin-1	1.5 (1.25–2.0)	1.5 (1.25–2.0)	1.62 (1.5–1.75)	0.722
P62	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	ND
LAMP1	0.75 (0.25–1.0)	1.0 (1.0–1.5)**	1.0 (1.0–1.0)**	**0.0021**

Data are expressed as n or median (range). Bold type represents statistical significance. LC3: microtubule-associated protein 1A/1B-light chain 3; TFEB: transcription factor EB; LAMP1: lysosome-associated membrane protein 1; ND: not determined. *: $p<0.05$ *versus* control nonsmokers; **: $p<0.01$ *versus* control nonsmokers (Mann–Whitney U-test).

p62 protein than CNS and CS (p=0.02), confirming data obtained by immunohistochemistry, particularly in alveolar macrophages.

Discussion

With this study, we sought to evaluate through multiple techniques the autophagic process in COPD patients in different areas of the respiratory tract, comparing it with that of healthy subjects with or without smoking history.

Our data point out that autophagy is more stimulated in smokers than in nonsmokers, regardless of the presence of COPD. We found this stimulation in the bronchial mucosa as well as in small airways and lung parenchyma. As for the bronchial mucosa, this process was highlighted by an increase in LC3B-positive cells in smoking controls and severe/very severe COPD patients compared with non-smokers. In peripheral airways and lung parenchyma, stimulation of the autophagic process in smokers was indicated by increased LC3A, total LC3 and LAMP1 in bronchiolar epithelium, alveolar septa and alveolar macrophages. ELISA tests performed on lung tissue homogenate confirmed these data, showing a higher concentration of LC3B in both COPD patients and smoking subjects compared with CNS. Taken together, these findings strongly confirm previous data and point out that autophagy represents a response to smoke exposure [4, 25, 29, 30]. Of note, the increase in LAMP1 together with LC3 supports a real boost in autophagic flux and confirms the proper functioning of the lysosomal system [4, 5, 29, 30].

FIGURE 2 Photomicrographs showing bronchiolar epithelium, alveolar macrophages and alveolar septa of a, c) control nonsmokers (CNS) and b, d) COPD patients immunostained for identification of a, b) microtubule associated protein 1 light chain 3 (LC3A) and c, d) lysosomal-associated membrane protein 1 (LAMP1). Results for single stainings are representative of those from 11 nonsmokers and 12 mild/moderate COPD patients. An increased immunopositivity for LC3A and for LAMP1 was observed in bronchiolar epithelium (BE), alveolar macrophages (arrows) and alveolar septa (arrows) in COPD patients. Scale bars=30 µm.

Intriguingly, the increase in autophagy seems to be independent of lung function deterioration, as it was evident (and comparable) in both CS and COPD patients. However, an impaired autophagic flux emerged in patients with COPD when compared with controls. This difference was only highlighted by ELISA test as an increased concentration in p62 level in the lung tissue homogenate and in alveolar macrophages by
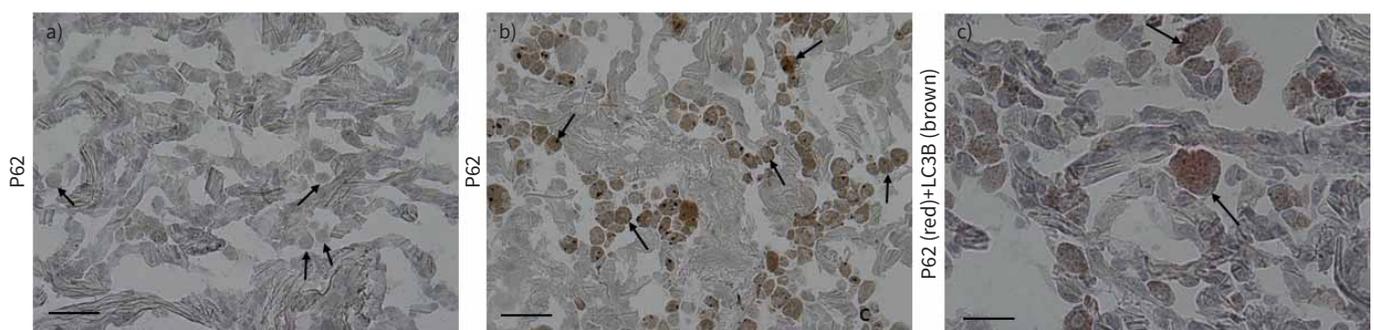
FIGURE 3 Photomicrographs showing alveolar macrophages and alveolar septa of a) control nonsmokers and b) COPD patients immunostained for identification of p62. Panel c) shows double-stained macrophages from a patient with COPD showing immunopositivity for p62 (red) and LC3B (brown), the most expressed autophagy marker in alveolar macrophages. Results for single staining are representative of those from 11 nonsmokers and 12 mild/moderate COPD patients. Double staining was performed in three control nonsmokers and in three patients with COPD. Arrows indicate single- or double-immunostained alveolar macrophages. An increased immunopositivity for p62 antigen is shown in COPD patients compared to control nonsmokers. Scale bars=30 µm (a, b) and 15 µm (c).

TABLE 5 ELISA tests for autophagic molecules in the lung parenchyma of control nonsmokers, control smokers and patients with COPD

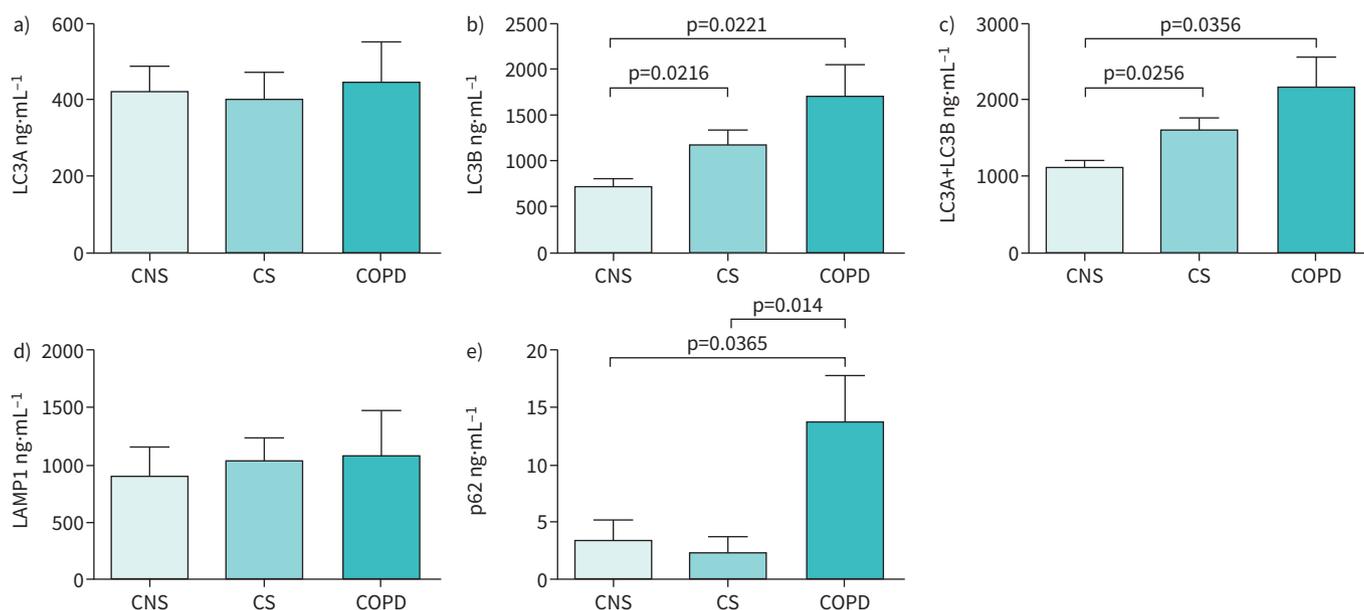|	Control nonsmokers	Control smokers	Patients with COPD	Kruskal–Wallis p-value
Subjects	11	11	10	
Proteins				
LC3A ng·mL^{-1}	334 (188.5–835.5)	438 (25.50–700)	405.5 (63.50–1048)	0.9976
LC3B ng·mL^{-1}	720 (405–1105)	1235 (545–1985)*	1200 (490–3625)*	**0.0230**
LC3A+LC3B ng·mL^{-1}	1184 (820–1520)	1586 (816–2564)*	1768 (716–4470)*	**0.0333**
LAMP1 ng·mL^{-1}	679 (223–2752)	966.5 (230.5–2687)	705.5 (179–4352)	0.5941
P62 ng·mL^{-1}	0 (0.00–15.85)	0 (0.00–18.55)	12.15 (0.00–33.45)*,#	**0.0231**

Data are expressed as n or median (range). Bold type represents statistical significance. LC3: microtubule-associated protein 1A/1B-light chain 3; LAMP1: lysosome-associated membrane protein 1. *: p<0.05 *versus* control nonsmokers; #: p<0.05 *versus* control smokers (Mann–Whitney U-test).

immunohistochemistry. The increase of p62 in lung tissue of patients with COPD has been reported previously and has been interpreted as a potential mechanism of impaired autophagy and proteostasis dysfunction, leading to COPD–emphysema development [15, 25, 31–33]. Indeed, this autophagy defect, induced by cigarette smoke, but not present in smokers with a normal lung function, could play a pro-apoptotic function and underlie the loss of peripheral lung tissue, which is a feature of pulmonary emphysema [31–34]. Our data further support these hypotheses. The absence of differences in LAMP1 concentration, despite the increase in LC3B, also suggests an impairment in the lysosomal system.

On the other hand, transcriptomics analyses conducted on both bronchial tissue and lung parenchyma showed no significant differences in the expression of the mRNAs studied among the three groups. Supporting these results is the lack of differences in immunohistochemical analysis of TFEB, considered a tool for monitoring transcriptional regulation connected with autophagy [5]. This lack of differences might suggest that both stimulation and impairment of autophagic flux are primarily post-transcriptional, but further studies are needed to confirm these findings.

Also of note, the increased positivity of alveolar macrophages for p62 is able to differentiate smokers, both COPD and control subjects, from nonsmokers. This finding confirms the results obtained by MONICK *et al.* [31]

FIGURE 4 Quantitation by ELISA tests of a) microtubule associated protein 1 light chain 3 (LC3)A, b) LC3B, c) LC3A+LC3B, d) lysosomal-associated membrane protein 1 (LAMP1) and e) p62 molecules in the lung protein extracts of control nonsmokers (CNS; n=11), control smokers (CS; n=11) and patients with COPD (n=12). b, c) LC3B and LC3A+LC3B showed higher values in COPD and CS compared to CNS. e) p62 was increased in COPD patients compared to CS and CNS. Data are presented as mean±SE. Mann–Whitney U-test was used for comparison between groups.

in a prior study. Their data indicated an altered autophagic process at the level of alveolar macrophages, induced by a defect in the delivery of autophagosomes to the lysosome. However, as in our study, this defect was associated with an increased stimulus for autophagy, which they highlighted by an increased number of autophagosomes in smoking patients compared with controls. This could be explained by an increased macrophage loading of poorly digestible material, which may lead to a defect in autophagy/lysosomal function [35]. This finding seems particularly interesting because impairment of autophagy in alveolar macrophages could also impair xenophagy, the autophagy involved in pathogen clearance [36]. As highlighted in previous studies, impairment of xenophagy in macrophages could be one of the factors that explain the increased incidence of respiratory infections in smokers [31, 37], regardless of respiratory function.

Our results can be analysed in light of other studies where bronchial epithelial cells were stimulated with cigarette smoke [18, 25, 30]. In these studies, stimulation with cigarette smoke led to activation of autophagy followed by accumulation of p62 and ubiquitinated proteins, which was interpreted as impairment of the process and a precursor to cellular senescence [15]. Therefore, it can be hypothesised that different stimuli are capable of activating autophagy in HBECs (inflammation, smoking exposure), but that only cigarette smoke is capable of leading to an impairment of the autophagic process with accumulation of p62 and ubiquitinated proteins, precursor of cellular senescence. Indeed, it has been proposed that the increased baseline autophagy found in patients with COPD is mainly due to oxidative stress and results in a reduced reserve of autophagy flux activation in the bronchial epithelial cells of these patients. Confirming this, a significant decrease in autophagy induction in response to cigarette smoke exposure was found in bronchial epithelial cells isolated from COPD patients when compared to those from nonsmokers [15].

This study has some strengths and limitations. The main strength is the use of multiple methods simultaneously to reinforce the results obtained at protein level quantitation, and to study different stages of the autophagic process. Another strength is the analysis of these elements at different levels of the respiratory system including different cell types, so that a more complete view of an extremely complex system can be obtained. In contrast, one of the main limitations of the study is the absence of *in vitro* tests, dedicated to specific mechanistic actions, which could have further strengthened our findings. Another limitation is the use of archival material, which sometimes did not allow a more detailed analysis or an equal distribution of the population within the groups for certain variables, such as gender, inhaled therapy and time since smoking cessation.

In conclusion, our study confirms by multiple techniques, all focused on mRNA and protein quantitation, that cigarette smoke stimulates autophagy in different manners inside the respiratory system. The increase in the autophagic flux seems to be independent of lung function deterioration, but an impairment of the process emerged in COPD patients. A reduced reserve of autophagy flux activation due to oxidative stress may be one of the factors differentiating smokers with normal lung function from COPD patients, but this hypothesis needs to be confirmed. The same process could make smokers more prone to respiratory infections due to xenophagy impairment in alveolar macrophages. Both stimulation of autophagy and its deficiency appear primarily post-transcriptional, but further studies are needed to confirm these findings.

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