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

Journal:	*ERJ Open Research*
Manuscript ID	ERJOR-00423-2023.R2
Manuscript Type:	Original Research Article
Date Submitted by the Author:	19-Sep-2023
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Key Words:	COPD, autophagic stimulus, autophagy impairment, smoke, rehabilitation, Inflammation
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 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.

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To the Chief Editor
Prof. Barreiro
Hospital del Mar IMIM
CIBERES Group and CEXS
Pompeu Fabra University
Barcelona, Spain

Dear Professor Barreiro,

Please find enclosed the manuscript entitled "**Impaired autophagy in the lower airways and lung parenchyma in stable COPD**", which we would like to resubmit for publication after revision (R2) at ERJ Open Research. It appears that Reviewer 2 did not consider our responses to his comments. In any case, we added a new point-by-point response to his comments, with more explanations, and a revised version of the manuscript (R2) with adopted changes. The manuscript preparation guidelines were followed in the preparation of R2 text. All authors have read the R2 version of the manuscript and agree to its resubmission to the journal.

Looking forward to your response

Sincerely yours,

Di Stefano Antonino, PhD
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Point-by-point response

Date: September 19th, 2023

Manuscript ID: ERJOR-00423-2023

Type of manuscript: Article

Title: Impaired autophagy in the lower airways and lung parenchyma in stable COPD.

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Reviewer 1

Comments to the Author
The authors have answered my questions taking into account the limitations of using archival material.
These have been added to the limitations of the revised version. I have no further questions.

Reply: We thank the Reviewer.

Reviewer 2

Comments to the Author

Levra et al. have attempted to elucidate the participation of autophagic process during COPD pathogenesis at multiple levels by using human lung samples. RNA sequencing detected no clear difference in expression levels of transcripts of autophagic molecules between CNS, CS, and COPD, respectively. Immunohistochemical evaluation showed increased expression levels of autophagy-associated proteins in both control smokers and COPD. ELISA showed significant increase of p62 in COPD lungs tissue homogenates. Although this comprehensive evaluation of autophagic molecules can be potentially interesting for understanding the involvement of autophagy in COPD pathogenesis, the reviewer's enthusiasm was severely hampered due to the paucity of mechanistic insight and the preliminary nature of experimental results.

1. Immunohistochemical quantification in table 5 showed no difference between CS, and COPD, indicating that alteration of autophagic molecules is simply affected by smoking status but did not directly link to COPD pathogenesis. Are there any association between expression levels of autophagic molecules and smoking status or respiratory function?

   Reply: No difference between CS and COPD in autophagic molecules emerged by immunohistochemistry, by which we analysed bronchial epithelium, alveolar macrophages and alveolar septa separately. However, a difference emerged for p62 by ELISA test on lung tissue homogenate.
   In our study, no significant association emerged between expression levels of autophagic molecules and smoking history (pack years) or respiratory function, neither in bronchial

biopsies nor in peripheral lung. This result is at least in part in line with those previously reported. In particular, in the study of *Mercado et al.* (Mercado N, Colley T, Baker JR, *et al*. Bicaudal D1 impairs autophagosome maturation in chronic obstructive pulmonary disease. *FASEB BioAdvances* 2019; 1: 688–705) no correlation emerged between autophagic molecules and smoking history or respiratory function in smoking controls and patients with mild-moderate COPD. A correlation emerged only in patients with severe or very severe COPD, of whom we only had bronchial biopsies due to the process of samples collection. The following statement has been included in the text (line 229-230): "No significant association emerged between the expression levels of autophagic molecules in bronchial biopsies and smoking history or respiratory function". A similar statement was added concerning peripheral airways (line 248-250).

2. In table 6, ELISA was performed for only limited autophagic molecules. Other molecules included in immunohistochemistry should be performed. Furthermore, to further clarify the clinical implication of p62, COPD cases should be divided based on the severity as demonstrated in table 4.

Reply: In this study, lung parenchyma samples were obtained from patients undergoing resection surgery for a solitary peripheral neoplasm. Patients with severe or very severe COPD were not eligible for surgery, so lung samples were not available for useful comparison. We clarified this issue in the text (line 164-165).
ELISA tests were performed after immunohistochemistry, so we selected only autophagy molecules with significant differences between groups emerged from immunohistochemistry (added in the text, line 187-188). ELISA tests were performed for the most relevant molecules contributing to the autophagic flux in the lung parenchyma of CNS, CS and mild-moderate COPD patients. This experiment showed a clear increase of p62 in COPD patients compared to both CS and CNS (median values: 12.15 vs 0 vs 0 ng/ml, respectively for COPD, CS and CNS - Table 5). These findings, also confirmed by immunohistochemistry (particularly for alveolar macrophages - Table 4), clearly differentiate patients with COPD from control smokers. As stated in the manuscript, increased levels of autophagic molecules are frequently observed also in control smokers but impairment of autophagy (due to p62 increase) is only evident in COPD patients. As stated in the previous response, no significant associations emerged between the expression levels of autophagic molecules, included p62 protein, and smoking history or respiratory function.

3. Representative pictures of immunohistochemistry should be presented in all experiments.

Reply: In this study bronchial biopsies as well as peripheral lung specimens were used for immunohistochemistry, analysing 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. In view of the number of immunostainings, in the first version of the manuscript we decided to show an image of p62, the molecule most representative of autophagy impairment, together with a double staining for LC3B, the autophagy marker most expressed in alveolar macrophages. Under request of the Reviewer, in the revised version of the manuscript we have added a new figure showing bronchiolar epithelium, alveolar macrophages and alveolar septa of control non-smokers and COPD patients immunostained for identification of LC3A and LAMP1, all molecules significantly changed in the peripheral airways of our study. We believe that images included in the revised version of the paper (Figures 2 and 3 of the revised manuscript) well support our data showed in results section and tables.

In any case, we are available to show photomicrographs of the other molecules (cathepsin B and D, beclin-1 and TFEB) in case of specific request by the Editor.

4. Although authors concluded that autophagy failure may be a potential factor leading the COPD development, the presented results did not sufficiently support this conclusion. Based on the presented results, the meaning of autophagy failure is not clear.

Reply: We respectfully disagree with the opinion of this Reviewer. In the present study, as well as in others previously published by other Authors (Fujii S, Hara H, Araya J, *et al.* Insufficient autophagy promotes bronchial epithelial cell senescence in chronic obstructive pulmonary disease. *Oncoimmunology* 2012; 1: 630–641. Tran I, Ji C, Ni I, *et al.* Role of cigarette smoke-induced aggresome formation in chronic obstructive pulmonary disease-emphysema pathogenesis. *Am. J. Respir. Cell Mol. Biol.* 2015; 53: 159–173. Vij N, Chandramani-Shivalingappa P, Van Westphal C, *et al*. Cigarette smoke-induced autophagy impairment accelerates lung aging, copd-emphysema exacerbations and pathogenesis. *Am. J. Physiol. - Cell Physiol.* 2018; 314: C73–C87), accumulation of p62 is considered a representative indicator of insufficient autophagic clearance and thus of autophagy failure. Our conclusions were based on the integration of our findings together with information obtained by the already published studies.
We have reported autophagy failure in peripheral lung of patients with mild-moderate COPD in alveolar macrophages (Table 4) and in protein lung extracts (Table 5) by using two different technical approaches, immunohistochemistry and ELISA tests, applied to the same lung specimens. In our opinion, these data are well presented and discussed in the revised (R2) manuscript. In the present study we privileged the immuno-histological analysis of the autophagic flux in COPD and control subjects. This approach is original and not frequently adopted by other Authors. For this reason it may contribute to a deeper evaluation of the autophagic flux and its impairment in the lungs of COPD patients.
In response to the Reviewer's comment, we modified the conclusions in both the abstract and the main text of the revised manuscript (lines 326-327).

Sincerely,

Dr. Antonino Di Stefano

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

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Keywords

COPD, autophagic stimulus, autophagy impairment, smoke, rehabilitation, inflammation

Abbreviations

BECN1, beclin-1

CNS, control non-smokers

COPD, chronic obstructive pulmonary disease

CS, control smokers with normal lung function

CTSB, cathepsin B

CTSD, cathepsin D

ELISA, enzyme-linked immunosorbent assay

FEV_1, forced expiratory volume in one second

FVC, forced vital capacity

GOLD, Global Initiative for Chronic Obstructive Lung Disease

HBEC, human bronchial epithelial cell

LAMP1, lysosome-associated membrane protein 1

MAP1LC3 (LC3), microtubule-associated protein 1A/1B-light chain 3

MCOPD, mild/moderate chronic obstructive pulmonary disease

NUP62 (p62), nucleoporin 62

PI3K, phosphoinositide-3-kinase

SCOPD, severe/very severe chronic obstructive pulmonary disease

TFEB, transcription factor EB

VPS34, vacuolar protein sorting 34

Acknowledgements: this work was supported, in part, by Istituti Clinici Scientifici Maugeri, SpA, SB, IRCCS, Ricerca Corrente, and, in part, by Department of Clinical and Biological Sciences, University of Turin, Orbassano, Turin, Italy.

Author contributions: ADS, SL, UR and FLMR contributed in writing and revising the manuscript; IG, VC, FB, MP and PB, contributed to the production of the data and accuracy of the data analysis; AL and BB contributed to a critical revision of the manuscript.

Conflict of Interest: All authors declare absence of any conflict of interest concerning this study

Ethics approval and consent to participate

This study was approved by the Istituti Clinici Scientifici Maugeri (protocol p112) and by Ethical Committee of the San Luigi Gonzaga University Hospital (protocol n. 9544/2019) and performed following the Declaration of Helsinki. Written informed consent was received from all patients before inclusion in the study.

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 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 ~~be a potential factor leading to COPD development~~ characterise COPD.

Word count: ~~220~~ 250

1. 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 recognized: 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 localization 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 synthesized 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 the last years, evidence has emerged regarding autophagy activation in Chronic Obstructive Pulmonary Disease (COPD) [2,8,9], a heterogeneous lung condition characterized 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 hypothesized [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].

2. Methods

2.1 *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 Ethical Committee of the San Luigi Gonzaga University Hospital (protocol n. 9544/2019). All patients have signed an informed consent.

2.2 Lung function tests and volumes

Pulmonary function tests were performed in all subjects, according to current guidelines (see online supplement for details). The severity of the airflow obstruction in COPD patients was staged using GOLD criteria [www.goldcopd.org].

2.3 Samples collection

A fibreoptic bronchoscopy has been performed to collect bronchial biopsies from segmental and subsegmental airways (4th to 6th airway generation) of 55 subjects, 30 of whom with COPD (see online supplement for details). Thirty-four subjects undergoing lung resection surgery for a solitary peripheral neoplasm have been recruited, including 12 with COPD. All with mild or moderate disease severity, according to the eligibility criteria for surgery. Specimens of their lung parenchyma were frozen and used for analysis (see online supplement for details). 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.

2.4 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 (see online supplement for details). 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 (see online supplement for details).

2.5 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 (see online supplement for details). We considered the expression levels of MAP1LC3A, MAP1LC3B, CTSB, CTSD, BECN1, NUP62, LAMP1 and TFEB genes. The expression level of these genes was also evaluated in http://www.copdcellatlas.com/, a COPD-dedicated gene expression browser of single-cell RNA sequencing data [28].

2.6 ELISA tests in peripheral lung specimens

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

2.7 Statistical analysis

Group data were expressed as mean (standard deviation) for functional data or median (range) or interquartile range (IQR) for morphologic data. Differences between groups were analysed using analysis of variance (ANOVA) for functional data. ANOVA was followed by an unpaired t-test for comparison between groups. The Kruskal Wallis test was applied to the morphologic 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 for $p < 0.05$. Data analysis was performed using the Stat View SE Graphics program (Abacus Concepts Inc., Berkeley, CA-USA).

3. Results

3.1 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. Twelve of these had COPD, while the other 22 had normal lung function. Half of the control subjects were smokers.

3.2 Gene expression level in bronchial rings and lung parenchyma

We examined RNA-seq 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, CS and CNS samples. CTSD resulted to be the gene with the highest expression among the 8 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 into 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 1). Strikingly, CTSD resulted almost no expressed in single-cell data.

3.3 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 LC3B (Table 3). Specifically, the number of LC3B positive cells was significantly lower in the bronchial epithelium of non-smokers 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.

3.4 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,b), total LC3 ($p<0.01$) and LAMP1 ($p<0.01$) (Figure 2c,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,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,b). Double staining for LC3B (brown colour) and p62 protein (red colour), performed in 3 COPD patients and 3 CNS, showed a clear p62 immunoexpression in a large number of alveolar macrophages coexpressing LC3B in COPD lungs (Figure 3c). On the other hand, the number of cathepsin B positive cell 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.

3.5 ELISA tests for autophagic molecules in homogenized 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 3 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 p62 protein than CNS and CS ($p=0.02$), confirming data obtained by immunohistochemistry, particularly in alveolar macrophages.

4. 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 non-smokers, 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, on the other hand, 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].

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 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 proapoptotic 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 differentiates smokers, both COPD and control subjects, from non-smokers. This finding confirms the results obtained by Monick *et al.* in a previous study [31]. 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 hypothesized 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 non-smokers [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~~, identifying autophagy failure as a potential factor leading to COPD development~~. 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.

Tables

Table 1. Clinical characteristics of subjects for immunohistochemistry studies on the bronchial biopsies

Groups	n	Age (y)	M/F	Ex/current smokers	Pack years	FEV_1 (% pred) pre-β_2	FEV_1 (% pred) post-β_2	FEV_1/FVC (%)
Control non-smokers	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 mean ± standard deviation. Patients with COPD were classified according to the grades of severity of the Global Initiative for Chronic Obstructive Lung Disease 2011 (goldcopd.org) using only the severity of airflow obstruction. For patients with COPD, FEV1/FVC (%) are post-bronchodilator (β2) values. Abbreviations: COPD, chronic obstructive pulmonary disease; M, male; F, female, FEV1, forced expiratory volume in one second; FVC, forced vital capacity; ND, not determined. Statistical analysis: ANOVA test: #, $p<0.0001$, significantly different from control smokers with normal lung function and control never smokers; &, $p<0.0001$, significantly different from mild/moderate COPD.

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

Groups	n	Age (y)	M/F	Ex/Current smokers	Pack years	FEV_1 (% pred) pre-β_2	FEV_1 (% pred) post-β_2	FEV_1/FVC (%)
Control non-smokers	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 expressed as mean ± standard deviation. For patients with COPD, FEV1/FVC (%) are post-bronchodilator (β2) values. Abbreviations: COPD, chronic obstructive pulmonary disease; M, male; F, female; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; ND, not determined. Statistical analysis: ANOVA test: #, $p<0.0001$, significantly different from control smokers with normal lung function and control never smokers.

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

Localization	Control non smokers N=12	Control smokers N=13	Mild/Moderate COPD N=16	Severe/very severe COPD N=14	Kruskal Wallis (p value)
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/mm²					
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 median and range. Abbreviations: COPD, chronic obstructive pulmonary disease; LC3, microtubule-associated protein 1A/1B-light chain 3; TFEB, transcription factor EB; LAMP1, lysosome-associated membrane protein 1; ND, not determined. Statistical analysis: Mann Whitney U test: *, p=0.028 vs mild/moderate COPD; &, p=0.014 vs control non-smokers; §, p=0.0423 vs control non-smokers.

Table 4. Immunohistochemical quantification of autophagic molecules in the peripheral lung of patients with COPD, in control smokers and non-smoking subjects

Localization	Control non-smokers N=11	Control smokers N=11	Patients with COPD N=12	Kruskal Wallis (p value)
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 median and range. Abbreviations: COPD, chronic obstructive pulmonary disease; LC3, microtubule-associated protein 1A/1B-light chain 3; TFEB, transcription factor EB; LAMP1, lysosome-associated membrane protein 1; ND, not determined. Statistical analysis: ; Mann Whitney U test: *, $p<0.05$ vs control non-smokers; &, $p<0.01$ vs control non-smokers.

Table 5. ELISA tests for autophagic molecules in the lung parenchyma of control non-smokers, control smokers and patients with COPD.

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

Data are expressed as median and range. Abbreviations: COPD, chronic obstructive pulmonary disease; LC3, microtubule-associated protein 1A/1B-light chain 3; LAMP1, lysosome-associated membrane protein 1. Statistical analysis: ; Mann Whitney U test: *, $p<0.05$ vs control non-smokers; &, $p<0.05$ vs control smokers.

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Figure legends

Figure 1

Expression levels of selected autophagy genes obtained in bronchial rings (a) and lung parenchyma (b) of control non-smokers (CNS), control smokers (CS) patients with chronic obstructive pulmonary disease (COPD). The box plot showed the median and the distribution of expression values per gene. Abbreviations: TEFB, Transcription Factor EB; CTSB, Cathepsin B; CTSD, Cathepsin D; LAMP1, Lysosomal-associated membrane protein 1; MAP1LC3B, Microtubule Associated Protein 1 Light Chain 3 Beta; BECN1, Beclin 1; NUP62, Nucleoporin 62; MAP1LC3B, Microtubule Associated Protein 1 Light Chain 3 Alpha.

Figure 2

Photomicrographs showing bronchiolar epithelium, alveolar macrophages and alveolar septa of control non-smokers (a,c) and COPD patients (b,d) immunostained for identification of LC3A (a,b) and LAMP1 (c,d). Results for single stainings are representative of those from 11 non-smokers 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. CNS=Control Non Smokers, COPD=Chronic Obstructive Pulmonary Disease; Bars=30 μm**.**

Figure 3

Photomicrographs showing alveolar macrophages and alveolar septa (a,b) of control non-smokers (a) and COPD patients (b) immunostained for identification of p62 (a,b). Panel c shows double stained macrophages from a patient with COPD showing immunopositivity for p62 (red colour) and LC3B (brown colour), the most expressed autophagy marker in alveolar macrophages. Results for single

staining are representative of those from 11 non-smokers and 12 mild/moderate COPD patients. Double staining was performed in 3 control non-smokers and in 3 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 non-smokers. Bars=30 microns (a,b) and 15 microns (c).

Figure 4

Quantitation by ELISA tests of LC3A (a), LC3B (b), LC3A+LC3B (c), LAMP1 (d) and p62 (e) molecules in the lung protein extracts of control non-smokers (CNS, n=11), control smokers (CS, n=11) and patients with chronic obstructive pulmonary disease (COPD, n=12). LC3B and LC3A+LC3B showed higher values in COPD and CS compared to CNS (b,c). P62 was increased in COPD patients compared to CS and CNS (e). Data are expressed as mean ± standard errors. Mann Whitney was used test for comparison between groups.

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

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Keywords

COPD, autophagic stimulus, autophagy impairment, smoke, rehabilitation, inflammation

Abbreviations

BECN1, beclin-1

CNS, control non-smokers

COPD, chronic obstructive pulmonary disease

CS, control smokers with normal lung function

CTSB, cathepsin B

CTSD, cathepsin D

ELISA, enzyme-linked immunosorbent assay

FEV_1, forced expiratory volume in one second

FVC, forced vital capacity

GOLD, Global Initiative for Chronic Obstructive Lung Disease

HBEC, human bronchial epithelial cell

LAMP1, lysosome-associated membrane protein 1

MAP1LC3 (LC3), microtubule-associated protein 1A/1B-light chain 3

MCOPD, mild/moderate chronic obstructive pulmonary disease

NUP62 (p62), nucleoporin 62

PI3K, phosphoinositide-3-kinase

SCOPD, severe/very severe chronic obstructive pulmonary disease

TFEB, transcription factor EB

VPS34, vacuolar protein sorting 34

Acknowledgements: this work was supported, in part, by Istituti Clinici Scientifici Maugeri, SpA, SB, IRCCS, Ricerca Corrente, and, in part, by Department of Clinical and Biological Sciences, University of Turin, Orbassano, Turin, Italy.

Author contributions: ADS, SL, UR and FLMR contributed in writing and revising the manuscript; IG, VC, FB, MP and PB, contributed to the production of the data and accuracy of the data analysis; AL and BB contributed to a critical revision of the manuscript.

Conflict of Interest: All authors declare absence of any conflict of interest concerning this study

Ethics approval and consent to participate

This study was approved by the Istituti Clinici Scientifici Maugeri (protocol p112) and by Ethical Committee of the San Luigi Gonzaga University Hospital (protocol n. 9544/2019) and performed following the Declaration of Helsinki. Written informed consent was received from all patients before inclusion in the study.

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 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.

Word count: 250

1. 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 recognized: 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 localization 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 synthesized 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 the last years, evidence has emerged regarding autophagy activation in Chronic Obstructive Pulmonary Disease (COPD) [2,8,9], a heterogeneous lung condition characterized 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 hypothesized [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].

2. Methods

2.1 *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 Ethical Committee of the San Luigi Gonzaga University Hospital (protocol n. 9544/2019). All patients have signed an informed consent.

2.2 Lung function tests and volumes

Pulmonary function tests were performed in all subjects, according to current guidelines (see online supplement for details). The severity of the airflow obstruction in COPD patients was staged using GOLD criteria [www.goldcopd.org].

2.3 Samples collection

A fibreoptic bronchoscopy has been performed to collect bronchial biopsies from segmental and subsegmental airways (4th to 6th airway generation) of 55 subjects, 30 of whom with COPD (see online supplement for details). Thirty-four subjects undergoing lung resection surgery for a solitary peripheral neoplasm have been recruited, including 12 with COPD. All with mild or moderate disease severity, according to the eligibility criteria for surgery. Specimens of their lung parenchyma were frozen and used for analysis (see online supplement for details). 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.

2.4 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 (see online supplement for details). 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 (see online supplement for details).

2.5 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 (see online supplement for details). We considered the expression levels of MAP1LC3A, MAP1LC3B, CTSB, CTSD, BECN1, NUP62, LAMP1 and TFEB genes. The expression level of these genes was also evaluated in http://www.copdcellatlas.com/, a COPD-dedicated gene expression browser of single-cell RNA sequencing data [28].

2.6 ELISA tests in peripheral lung specimens

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

2.7 Statistical analysis

Group data were expressed as mean (standard deviation) for functional data or median (range) or interquartile range (IQR) for morphologic data. Differences between groups were analysed using analysis of variance (ANOVA) for functional data. ANOVA was followed by an unpaired t-test for comparison between groups. The Kruskal Wallis test was applied to the morphologic 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 for $p < 0.05$. Data analysis was performed using the Stat View SE Graphics program (Abacus Concepts Inc., Berkeley, CA-USA).

3. Results

3.1 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. Twelve of these had COPD, while the other 22 had normal lung function. Half of the control subjects were smokers.

3.2 Gene expression level in bronchial rings and lung parenchyma

We examined RNA-seq 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, CS and CNS samples. CTSD resulted to be the gene with the highest expression among the 8 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 into 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 1). Strikingly, CTSD resulted almost no expressed in single-cell data.

3.3 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 LC3B (Table 3). Specifically, the number of LC3B positive cells was significantly lower in the bronchial epithelium of non-smokers 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.

3.4 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,b), total LC3 ($p<0.01$) and LAMP1 ($p<0.01$) (Figure 2c,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,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,b). Double staining for LC3B (brown colour) and p62 protein (red colour), performed in 3 COPD patients and 3 CNS, showed a clear p62 immunoexpression in a large number of alveolar macrophages coexpressing LC3B in COPD lungs (Figure 3c). On the other hand, the number of cathepsin B positive cell 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.

3.5 ELISA tests for autophagic molecules in homogenized 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 3 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 p62 protein than CNS and CS ($p=0.02$), confirming data obtained by immunohistochemistry, particularly in alveolar macrophages.

4. 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 non-smokers, 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, on the other hand, 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].

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 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 proapoptotic 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 differentiates smokers, both COPD and control subjects, from non-smokers. This finding confirms the results obtained by Monick *et al.* in a previous study [31]. 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 hypothesized 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 non-smokers [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.

Tables

Table 1. Clinical characteristics of subjects for immunohistochemistry studies on the bronchial biopsies

Groups	n	Age (y)	M/F	Ex/current smokers	Pack years	FEV_1 (% pred) pre-β_2	FEV_1 (% pred) post-β_2	FEV_1/FVC (%)
Control non-smokers	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 mean ± standard deviation. Patients with COPD were classified according to the grades of severity of the Global Initiative for Chronic Obstructive Lung Disease 2011 (goldcopd.org) using only the severity of airflow obstruction. For patients with COPD, FEV1/FVC (%) are post-bronchodilator (β2) values. Abbreviations: COPD, chronic obstructive pulmonary disease; M, male; F, female, FEV1, forced expiratory volume in one second; FVC, forced vital capacity; ND, not determined. Statistical analysis: ANOVA test: #, $p<0.0001$, significantly different from control smokers with normal lung function and control never smokers; &, $p<0.0001$, significantly different from mild/moderate COPD.

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

Groups	n	Age (y)	M/F	Ex/Current smokers	Pack years	FEV_1 (% pred) pre-β_2	FEV_1 (% pred) post-β_2	FEV_1/FVC (%)
Control non-smokers	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 expressed as mean ± standard deviation. For patients with COPD, FEV1/FVC (%) are post-bronchodilator (β2) values. Abbreviations: COPD, chronic obstructive pulmonary disease; M, male; F, female; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; ND, not determined. Statistical analysis: ANOVA test: #, $p<0.0001$, significantly different from control smokers with normal lung function and control never smokers.

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

Localization	Control non smokers N=12	Control smokers N=13	Mild/Moderate COPD N=16	Severe/very severe COPD N=14	Kruskal Wallis (p value)
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/mm²					
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 median and range. Abbreviations: COPD, chronic obstructive pulmonary disease; LC3, microtubule-associated protein 1A/1B-light chain 3; TFEB, transcription factor EB; LAMP1, lysosome-associated membrane protein 1; ND, not determined. Statistical analysis: Mann Whitney U test: *, p=0.028 vs mild/moderate COPD; &, p=0.014 vs control non-smokers; §, p=0.0423 vs control non-smokers.

Table 4. Immunohistochemical quantification of autophagic molecules in the peripheral lung of patients with COPD, in control smokers and non-smoking subjects

Localization	Control non-smokers N=11	Control smokers N=11	Patients with COPD N=12	Kruskal Wallis (p value)
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 median and range. Abbreviations: COPD, chronic obstructive pulmonary disease; LC3, microtubule-associated protein 1A/1B-light chain 3; TFEB, transcription factor EB; LAMP1, lysosome-associated membrane protein 1; ND, not determined. Statistical analysis: ; Mann Whitney U test: *, $p<0.05$ vs control non-smokers; &, $p<0.01$ vs control non-smokers.

Table 5. ELISA tests for autophagic molecules in the lung parenchyma of control non-smokers, control smokers and patients with COPD.

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

Data are expressed as median and range. Abbreviations: COPD, chronic obstructive pulmonary disease; LC3, microtubule-associated protein 1A/1B-light chain 3; LAMP1, lysosome-associated membrane protein 1. Statistical analysis: ; Mann Whitney U test: *, $p<0.05$ vs control non-smokers; &, $p<0.05$ vs control smokers.

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Figure legends

Figure 1

Expression levels of selected autophagy genes obtained in bronchial rings (a) and lung parenchyma (b) of control non-smokers (CNS), control smokers (CS) patients with chronic obstructive pulmonary disease (COPD). The box plot showed the median and the distribution of expression values per gene. Abbreviations: TEFB, Transcription Factor EB; CTSB, Cathepsin B; CTSD, Cathepsin D; LAMP1, Lysosomal-associated membrane protein 1; MAP1LC3B, Microtubule Associated Protein 1 Light Chain 3 Beta; BECN1, Beclin 1; NUP62, Nucleoporin 62; MAP1LC3B, Microtubule Associated Protein 1 Light Chain 3 Alpha.

Figure 2

Photomicrographs showing bronchiolar epithelium, alveolar macrophages and alveolar septa of control non-smokers (a,c) and COPD patients (b,d) immunostained for identification of LC3A (a,b) and LAMP1 (c,d). Results for single stainings are representative of those from 11 non-smokers 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. CNS=Control Non Smokers, COPD=Chronic Obstructive Pulmonary Disease; Bars=30 μm**.**

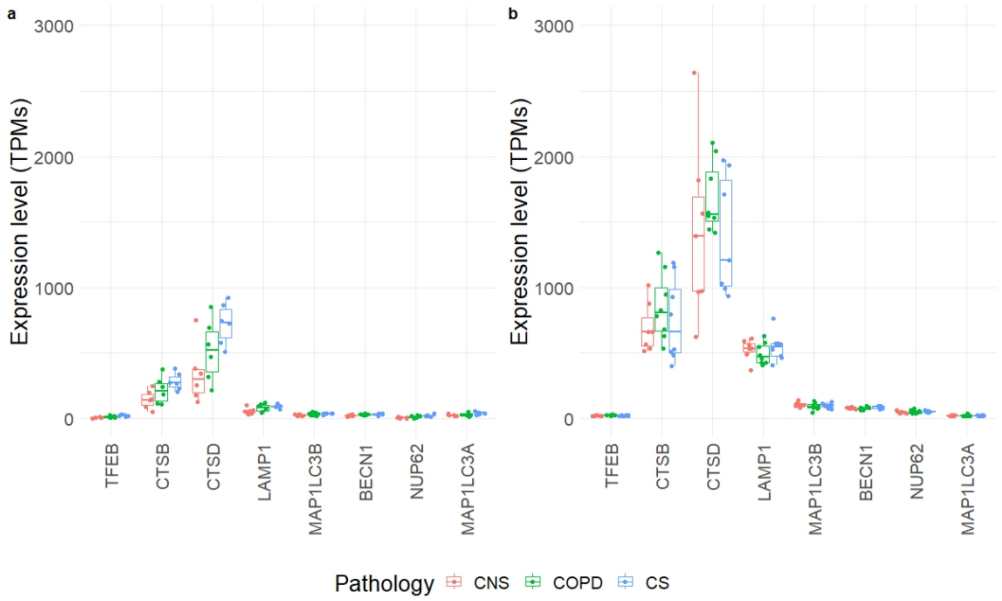
Figure 3

Photomicrographs showing alveolar macrophages and alveolar septa (a,b) of control non-smokers (a) and COPD patients (b) immunostained for identification of p62 (a,b). Panel c shows double stained macrophages from a patient with COPD showing immunopositivity for p62 (red colour) and LC3B (brown colour), the most expressed autophagy marker in alveolar macrophages. Results for single

staining are representative of those from 11 non-smokers and 12 mild/moderate COPD patients. Double staining was performed in 3 control non-smokers and in 3 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 non-smokers. Bars=30 microns (a,b) and 15 microns (c).

Figure 4

Quantitation by ELISA tests of LC3A (a), LC3B (b), LC3A+LC3B (c), LAMP1 (d) and p62 (e) molecules in the lung protein extracts of control non-smokers (CNS, n=11), control smokers (CS, n=11) and patients with chronic obstructive pulmonary disease (COPD, n=12). LC3B and LC3A+LC3B showed higher values in COPD and CS compared to CNS (b,c). P62 was increased in COPD patients compared to CS and CNS (e). Data are expressed as mean ± standard errors. Mann Whitney was used test for comparison between groups.



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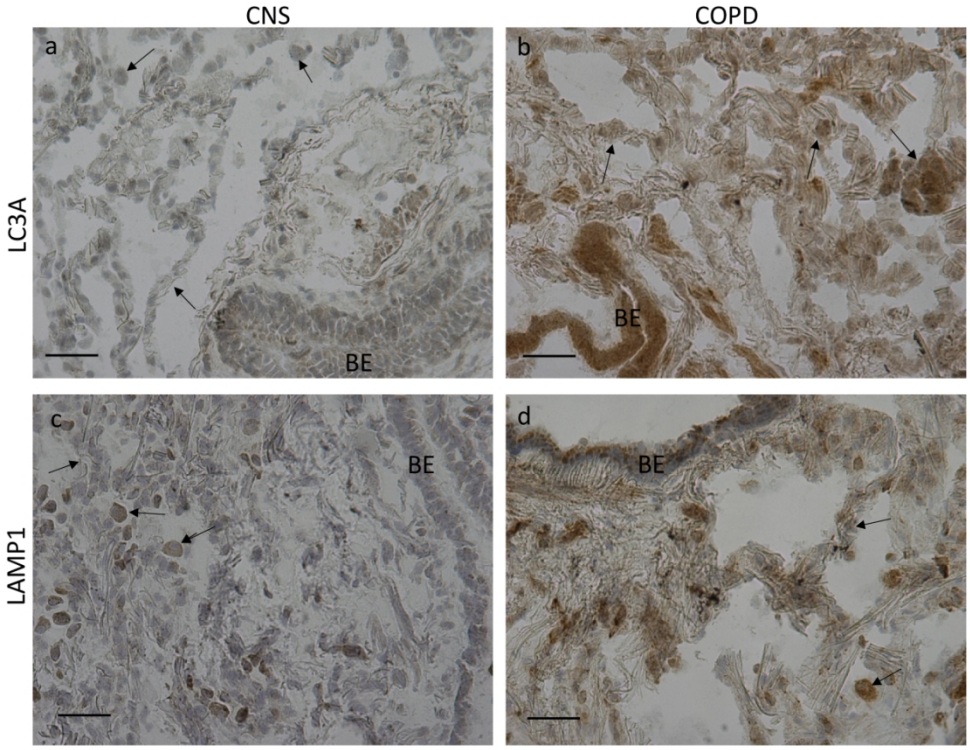

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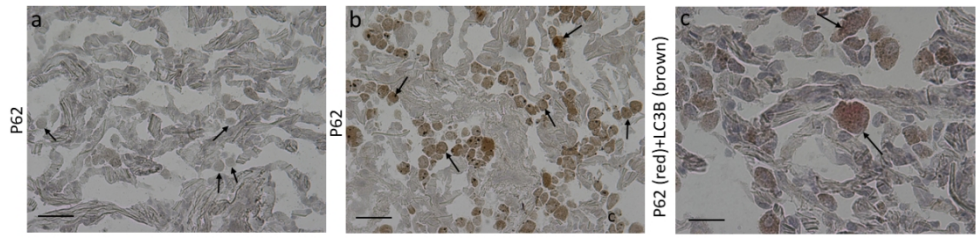

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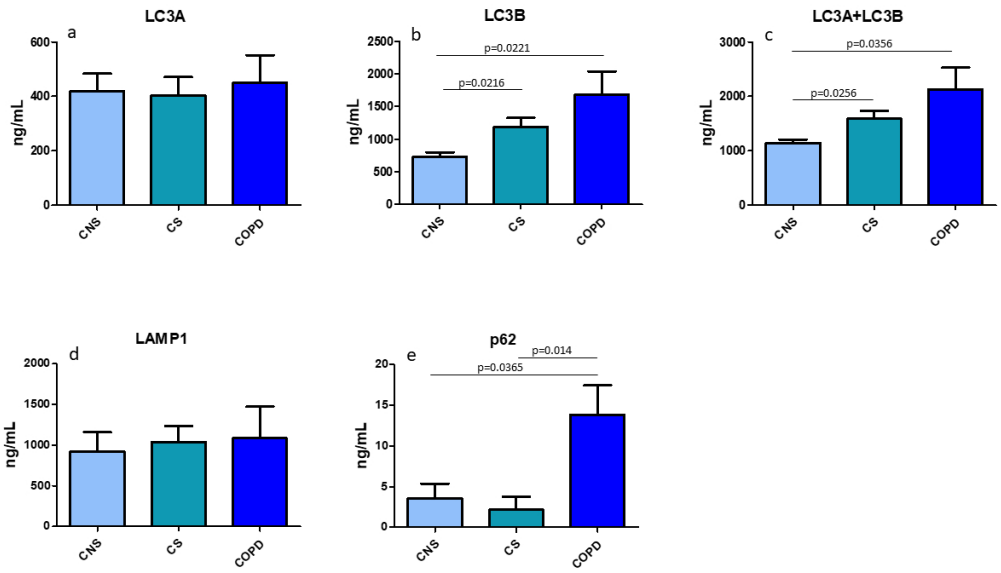

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24x14mm (1200 x 1200 DPI)

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

Stefano Levra MD[1], Umberto Rosani PhD[2], Isabella Gnemmi[3], Paola Brun PhD[4], Andrea Leonardi MD[5], Vitina Carriero PhD[1], Francesca Bertolini PhD[1], Bruno Balbi MD[6], Mirella Profita PhD[7], Fabio Luigi Massimo Ricciardolo MD[1,7,8]*, Antonino Di Stefano PhD[3]*.

Methods

Subjects

All COPD patients and healthy control 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). Archival material was used in the present study [1]. We obtained bronchial biopsies from 55 subjects for the immunohistochemical study of bronchial tissue. Thirty-four subjects undergoing lung resection for a solitary peripheral neoplasm were recruited for the immunohistochemical study of peripheral lung tissue and transcriptomic analysis of bronchial rings and lung tissue. 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 lung resection surgery.

Lung function tests and volumes

Pulmonary function tests included measurements of FEV_1 and FEV_1/FVC under baseline conditions in all the subjects examined (6200 Autobox Pulmonary Function Laboratory; Sensormedics Corp., Yorba Linda, CA). In order to assess the reversibility of airflow obstruction and post bronchodilator functional values, the measurements in the groups of subjects with $FEV_1/FVC \leq 70\%$ were repeated 20 min after the inhalation of 0.4 mg of salbutamol.

Fibreoptic bronchoscopy, collection and processing of bronchial biopsies

We obtained bronchial biopsies from 55 subjects for the immunohistochemical study of bronchial tissue, including 30 with COPD (Table 1 of the main manuscript). Subjects attended the bronchoscopy suite at 8.30 AM after having fasted from midnight and were pre-treated with atropine (0.6 mg IV) and midazolam (5-10 mg IV). Oxygen (3 l/min) was administered via nasal prongs throughout the procedure and oxygen saturation was monitored with a digital oximeter. After local

anaesthesia with lidocaine (4%) to the upper airways and larynx, a fibreoptic bronchoscope (Olympus BF10 Key-Med, Southend, UK) was passed into the trachea through the nose. Further lidocaine (2%) was sprayed into the lower airways, and four bronchial biopsy specimens were taken from segmental and subsegmental airways (4^{th} to 6^{th} airway generation) of the right lower and upper lobes using size 19 cupped forceps. Bronchial biopsies for immunohistochemistry were gently extracted from the forceps and processed for light microscopy [1]. At least two samples were embedded in Tissue Tek II OCT (Miles Scientific, Naperville, IL), frozen within 15 min in isopentane pre-cooled in liquid nitrogen, and stored at –80°C. The best frozen sample was then oriented and 6µm thick cryostat sections were cut for immunohistochemical light microscopy analysis and processed as described below.

Collection and processing of the peripheral lung tissue

Thirty-four subjects undergoing lung resection surgery for a solitary peripheral neoplasm were recruited. Eleven were non-smokers with normal lung function, 11 were smokers with normal lung function and 12 were smokers with COPD (Table 2 of the main manuscript). All former smokers had stopped smoking for more than one year. All subjects did not undergo preoperative chemotherapy and/or radiotherapy and had not been treated with bronchodilators, theophylline, antibiotics, antioxidants and/or glucocorticoids in the month prior to surgery. Lung tissue processing was performed as previously described [1]. Two to four randomly selected tissue blocks and one-two bronchial rings were taken from the lung obtained at surgery, avoiding areas grossly invaded by neoplasia. Samples were fixed in 4% formaldehyde in phosphate-buffered saline at pH 7.2 or frozen in liquid nitrogen. Fixed specimens, after dehydration, were embedded in paraffin wax. Serial sections 6µm thick from frozen specimens were first cut and stained with haematoxylin-eosin (H&E) in order to visualize the morphology and to exclude the presence of microscopically evident tumour

infiltration. Frozen tissue specimens were used in this study. Specimens were then cut for immunohistochemical analysis and were placed on charged slides as previously reported [1].

Immunohistochemistry on OCT-embedded bronchial biopsies

Sections from each sample were stained with antibodies specific for autophagy markers and proteins (Table 1). Briefly, after blocking non-specific binding sites with serum derived from the same animal species as the secondary antibody, the primary antibody was applied at optimal dilutions in TRIS-buffered saline (0.15 M saline containing 0.05 M TRIS-hydrochloric acid at pH 7.6) and incubated 1hr at room temperature in a humid chamber. Antibody binding was detected with secondary anti-mouse (Vector, BA 2000), anti-rabbit (Vector, BA 1000) antibodies followed by ABC kit AP AK5000, Vectastain and fast-red substrate (red colour) or ABC kit HRP Elite, PK6100, Vectastain and diaminobenzidine substrate (brown colour). Nasal polyp sections were used as positive controls. For the negative control, normal mouse (sc-2025) or rabbit (sc-2027) non-specific immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at the same protein concentration as the primary antibody.

Immunohistochemistry in peripheral lung tissue

Immunostaining of frozen peripheral lung tissue was performed as previously described [1]. Endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide (H_2O_2) in phosphate-buffered saline (PBS) followed by washing in PBS. Cell membranes were permeabilized adding 0.1% saponin to the PBS. Non-specific labelling was blocked by coating with blocking serum (5% normal specie-specific serum) for 20 minutes at room temperature. After washing in PBS the sections were incubated with anti-primary antibodies used for bronchial biopsies (Supplementary table 1). Control slides were included in each staining run using human normal tonsils or nasal polyps as a positive control for all the immunostaining performed. Slides were then incubated with

chromogen-fast diaminobenzidine (DAB) as chromogenic substance. After which they were counterstained in haematoxylin and mounted on permanent mounting medium. Double staining was performed for identification of alveolar macrophages positively stained for LC3B (brown colour) coexpressing also p62 (red colour) protein in three control non-smokers and in 3 patients with COPD. Chromogens used were the fast-red (red colour) and DAB (brown colour) substrates for identification of double marked alveolar macrophages.

Scoring system for immunohistochemistry in the bronchial biopsies

Light-microscopic analysis was performed at a magnification of 630x. The immunostaining for all the antigens studied was scored (range: 0 = absence of immunostaining to 3 = extensive intense immunostaining) in the intact bronchial epithelium. The final result was expressed as the average of all scored fields performed in each biopsy.

Immunostained cells in the bronchial lamina propria were quantified 100μm beneath the epithelial basement membrane in several non-overlapping high-power fields until the whole specimen was examined. The final result was expressed as the number of positive cells per square millimetre.

Scoring system for immunohistochemistry in the peripheral lung tissue

All bronchioles, alveolar macrophages and alveolar septa observed in each lung section specimen were analysed for each immunostained section. The immunopositivity was scored (0 = absence of immunostaining, 1 = 33% of immunostained cells, 2 = 66% of immunostained cells, 3 = almost all positive cells). Intensity of immunopositivity was considered adding a 0.5 score point to the established score applied on the basis of number of positive cells in the bronchiolar epithelium, bronchiolar lamina propria, alveolar macrophages, and alveolar septa [1].

RNA extraction and sequencing from bronchial rings and peripheral lung specimens

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. RNA extraction was performed with the RNAeasy micro kit (Qiagen, Hilden, Germany) following manufacturer instructions, with a DNA removal step using 500 units of RNase-free DNase (Qiagen) at room temperature for 15 minutes. Total RNA was resuspended in RNase-free water (Thermo Fisher, Carlsbad, US) and the RNA/DNA concentrations in each sample were quantified using the Qubit RNA and DNA high-sensitivity Assay Kit (Thermo Fisher). RNA qualities were assessed with an Agilent Bioanalyzer 2100 equipped with a RNA nano 6000 kit (Agilent, Santa Clara, CA, USA). Due to the low RIN values obtained for lung parenchyma samples, RNA-sequencing libraries for these samples were prepared following a 3'-end sequencing procedure using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria). Differently, no quality issues were encountered for the bronchial ring samples and, therefore, standard Illumina library preparation were performed. Consequently to the different library preparation methods, lung parenchyma libraries were sequenced using an Illumina NextSeq500 with a 75 single end read layout, whereas bronchial ring libraries were sequenced with a 150 paired-end read layout (Cribi, UniPD, Padova, Italy).

Data analysis of RNA-seq data

The raw Illumina reads were trimmed for quality using *fastp* [2], setting a minimal Phred quality of 25 and removing the sequencing adaptors. Raw Illumina datasets have been submitted to the NCBI Short Read Archive (SRA) under the project ID PRJNA80144. FASTQ files were imported in the CLC Genomic Workbench v.21 (Qiagen, Hilden, Denmark) and analysed as follows. The trimmed reads were mapped on the human reference genome (hg19, Ensembl v.99) applying the following parameters: mismatch cost = 2; insertion cost = 3; deletion cost = 3; length fraction = 0.8; similarity fraction = 0.8 and setting a strand-specific mapping. Expression values were counted as Read Per Kilobase of Mapped reads (RPKM). To identify differentially expressed genes (DEGs), a Baggerley

test with false discovery rate (FDR) p-value correction was applied, setting a cut-off of a minimal 2-Fold changes (FC) and a maximal 0.05 of FDR p-value. Limited to the gene of interest, MAP1LC3A, MAP1LC3B, CTSB, CTSD, BECN1, NUP62, LAMP1 and TFEB, the expression levels were extracted from the overall dataset and discussed (supplementary Table 2). The expression level of these genes was also evaluated in http://www.copdcellatlas.com/, a COPD-dedicated gene expression browser of single-cell RNA sequencing data [3].

ELISA tests in the peripheral lung tissue homogenates

LC3A (MyBioSource, Cat. N. MBS760439, lower detection limit, 0.156ng/ml), LC3B (MyBioSource, Cat.N. MBS7254329, lower detection limit, 0.10 ng/ml), LAMP1 (MyBioSource, Cat.N. MBS2023492, lower detection limit, 0.055 ng/ml) and p62 (MyBioSource, Cat.N. MBS008191, lower detection limit, 0.10 ng/ml) proteins quantification was performed in the lung tissue homogenates obtained from frozen tissue specimens used also for immunohistochemical analysis. ELISA kits were used according to the manufacturer's instructions.

Figure legend

Supplementary Figure 1

The figure depicts the expression levels of seven selected genes (BECN, CTSB, CTSD, LAMP1, MAP1LC3A, MAP1LC3B and TFEB) in different cell types (epithelial, endothelial, stromal and immune cells) of patients with COPD and control subjects obtained from the COPD Cell Atlas (http://www.copdcellatlas.com/). The expression level is represented by a colour-scale, whereas the fraction of the cells expressing the given gene is represented by the size of the dot.

Tables

Supplementary Table 1. Primary antibodies and immunohistochemical conditions used for identification of autophagic molecules and proteins in bronchial biopsies and lung parenchyma

Target	Supplier	Cat.#[a]	Source	Dilution	Positive Control
LC3A	LS Bio	LS-B7938	Rabbit	1:100	Nasal polyp
LC3B	Novus Bio	NB100-2220	Rabbit	1:1000	Nasal polyp
TFEB total	Santa Cruz	Sc-166736	Mouse	1:100	Nasal polyp
Cathepsin B	Santa Cruz	Sc-365558	Mouse	1:100	Nasal polyp
Cathepsin D	Santa Cruz	Sc-13148	Mouse	1:200	Nasal polyp
Beclin-1	Novus Bio	NB500-249	Rabbit	1:800	Nasal polyp
P62	Santa Cruz	Sc-28359	Mouse	1:100	Nasal polyp
LAMP1	Invitrogen	PA1-654A	Rabbit	1:20000	Nasal polyp

Abbreviations: LC3, microtubule-associated protein 1A/1B-light chain 3; TFEB, transcription factor EB; LAMP1, lysosome-associated membrane protein 1.

Supplementary Table 2. Transcriptomic data for autophagic molecules obtained in bronchial rings and lung parenchyma of COPD, control non-smokers and control smoker patients.

Gene ID	Gene description	COPD - Mean	CNS - Mean	CS - Mean	COPD - Mean	CNS - Mean	CS - Mean
		Bronchial rings			Lung parenchyma		
MAP1LC3A (LC3A)	Microtubule Associated Protein 1 Light Chain 3 Alpha	29.1	23.9	41.4	22.5	21.9	21.2
MAP1LC3B (LC3B)	Microtubule Associated Protein 1 Light Chain 3 Beta	33.9	23.6	35.3	92.1	106.8	97.2
CTSB	Cathepsin B	216.2	144.8	281.9	852.9	690.4	748.0
CTSD	Cathepsin D	519.5	338.4	723.5	1685.3	1425.3	1613.3
BECN1	Beclin 1	29.6	20.6	30.2	76.2	77.5	85.7
NUP62 (p62)	Nucleoporin 62	12.4	5.1	21.2	50.0	47.3	52.5
LAMP1	Lysosomal-associated membrane protein 1	81.4	56.9	91.0	493.6	525.9	543.8
TFEB	Transcription Factor EB	12.7	5.3	20.6	25.0	20.5	21.1

Abbreviations: CNS, control non-smokers; CS, control smokers; COPD, chronic obstructive pulmonary disease.

References

1. Di Stefano A, Caramori G, Barczyk A, *et al.* Innate immunity but not NLRP3 inflammasome activation correlates with severity of stable COPD. *Thorax* 2014; 69: 516–524.
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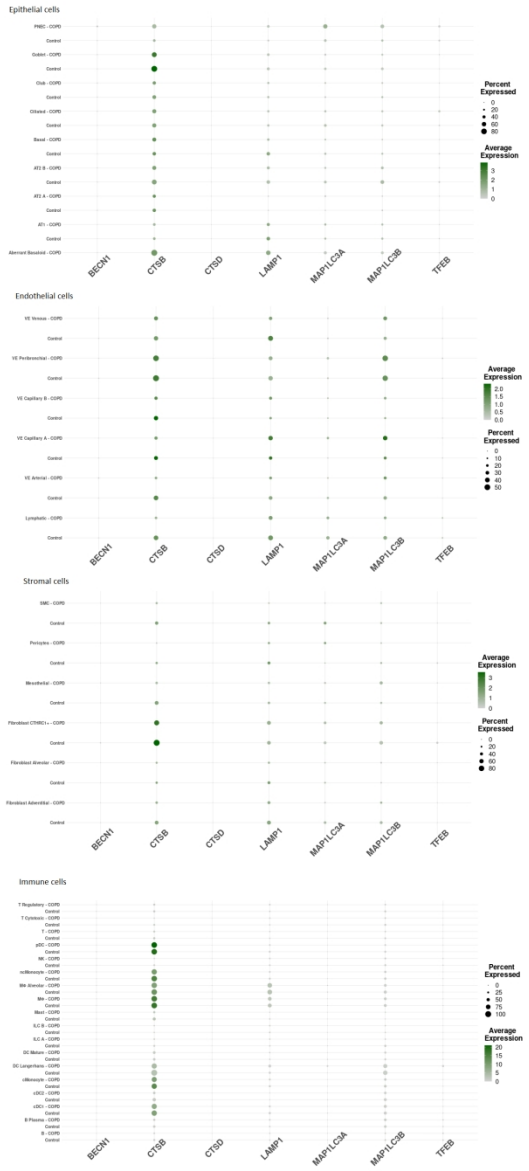

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31x60mm (1200 x 1200 DPI)