Department of BIOMORF

University of Messina

Translational Molecular Medicine and Surgery
Cycle XXXVIII

ROLE OF CELLULAR SENESCENCE AND IMPAIRED INTRACELLULAR DEGRADATION PATHWAYS IN THE PATHOGENESIS OF CHRONIC OBSTRUCTIVE PULMUNARY DISEASE (COPD)

By

Francesco Nucera, MD, PhD Fellow

Supervisor: Prof. Antonio Ieni, University of Messina

PHD THESIS OF DR. FRANCESCO NUCERA FOR THE XXXVIII° CYCLE OF THE INTERNATIONAL PhD TRANSLATIONAL MOLECULAR MEDICINE AND SURGERY IN THE UNIVERSITY OF MESSINA

Title: ROLE OF CELLULAR SENESCENCE AND IMPAIRED INTRACELLULAR DEGRADATION PATHWAYS IN THE PATHOGENESIS OF CHRONIC OBSTRUCTIVE PULMUNARY DISEASE (COPD)

Index

Introduction

Physiological pulmonary aging

In never smokers without any lung diseases, forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) reach their peak ~ 25 years of age and then slowly decline over the following years (Bowdish 2019).

There is the hypothesis that suggests how these flow-volume curve changes are correlated with the progressive obstruction of the small airways, the decrease in lung elasticity and the enlargement of the alveolar spaces (improperly termed senile "emphysema"), with a consequent slight decrease in the alveolar gas exchange.

During the physiological lung aging process there could be an increased susceptibility to oxidative stress damage induced by environmental factors such as cigarette smoke, and decreased antioxidant factors (Aghali 2022, Barnes 2022, Hecke 2018).

It is also hypothesized that during physiological pulmonary aging there is decreased chronic inflammation in the small airways and in the lung parenchyma (Ascher 2017), correlated with increased susceptibility to pulmonary infections mediated by impaired mucociliary clearance and a decreased innate and adaptive immune response (immunosenescence) (Yanagi 2017).

COPD as a disease of accelerated pulmonary aging

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity and mortality worldwide, affecting over 350 million people, with 44 million cases in Europe alone [http://www.europeanlung.org/en/], whose management requires enormous healthcare expenditure, particularly in elderly patients (Barnes 2015, 2019, Burney 2015, US burden of disease collaborators 2018).

COPD is characterized by chronic inflammation and remodelling of the lower airways [sometimes associated with destruction of the alveolar septa (pulmonary emphysema)] inducing chronic, non-reversible airflow limitation (obstruction) and an accelerated decrease in FEV1 compared to non-smokers without any lung diseases (Figure 1, Figure 2). In patients with COPD, the primary site of airflow limitation is localized in the small airways (Ito 2009, Mercado 2015, Caramori 2016, Hogg 2017, Suzuki 2017).

Some COPD patients do not develop a significant degree of pulmonary emphysema, and patients with severe pulmonary emphysema can have normal lung function (Caramori 2015, Janssen 2019).

The clinical diagnosis of chronic non-obstructive bronchitis is associated with long-term mortality, particularly in smokers, but it has not yet been demonstrated whether treatments other than smoking cessation are clinically effective in these subjects (Balte 2020).

The aetiology of COPD is represented by complex interactions between environmental factors (cigarette smoke) and genetic factors.

The existence of a genetic predisposition correlated to the development of COPD has been hypothesized as only a minority of subjects chronically exposed to cigarette smoke develop the disease.

The results of many pangenomic association studies (GWAS) have demonstrated that there are hundreds of gene polymorphisms are associated with an increased risk of developing COPD, and/or the level of lung function decline and/or the risk of addiction exposure to nicotine, and/or the risk of pulmonary emphysema development, and/or the risk of developing chronic bronchitis symptoms (Silverman 2020).

However, the only currently well characterized and treatable genetic factor is the severe hereditary deficiency of serum α1-antitrypsin (A1AT) [serpin-1 protease inhibitor (PI)], that is a risk factor, almost exclusively in cigarette smokers, for the development of COPD (usually associated with severe pulmonary emphysema mainly of the upper lobes), present in less than 1% of all COPD patients (Balbi 2019; Strnad 2020).

Currently, the inflammatory mediators involved in COPD have not been fully defined, but it is well known that several lipid mediators, inflammatory peptides, oxidant agents, chemokines, cytokines, proteases, and growth factors are involved in orchestrating the complex inflammatory process leading to fibrosis of the small airways, mucus hypersecretion and alveolar destruction (Barnes 2004, Barnes 2013, 2017, Caramori 2013, 2016, Lo Bello 2020, Nucera 2021).

However, the functional relevance of each individual mediator and receptor and the complex interactions between the latter and their receptors and intracellular signal transduction pathways are largely unknown (Caramori 2015, 2016, Nucera 2021).

COPD is currently hypothesized to be a disease of accelerated lung aging as the cells of the lower airways of these patients show several features of cellular aging: for example shortening of telomeres and damage to DNA (deoxyribonucleic acid), cellular senescence, activation of Intracellular signalling pathway mediated by phosphatidyl-inositol-3-kinase and the mammalian target of rapamycin (PI3K-mTOR), autophagy impairments, mitochondrial disorders, stem cell depletion, microRNA profile impairment, immunosenescence, chronic inflammation (inflammaging), associated with a decrease in the expression of anti-aging molecules such as sirtuins (SIRTs).

Many of these processes are activated by oxidative stress (Barnes 2017, 2019).

Oxidative stress and pathogenesis of COPD

<u>Oxidative/nitrosative stress in lower airway cells of normal subjects</u>

Atoms contain a nucleus, and electrons move around it in pairs.

A free radical is any atom or molecule that contains one or more unpaired electrons.

Unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than its non-radical counterpart.

Oxygen free radicals (ROS) are highly reactive species, and although generally with a short half-life (<1 second), they are able to reach the lower airways. They include: superoxide anion ($O2^-$), hydroxyl radical (OH^-), hydroperoxide radical (HOO^-), hydrogen peroxide (H_2O_2), also called hydrogen peroxide.

Free radicals of nitrogen (RNS) include: nitric oxide (NO), nitrogen dioxide ($NO2^-$) and the radical peroxynitrite ($ONOO^-$) produced by the reaction between O2- and nitric oxide (NO). Peroxynitrite can then be decomposed into hydroxyl radical and nitrogen dioxide.

The reaction that leads to the transformation of oxygen into superoxide anion ($O2^-$) is an important stage in the formation of oxidizing substances.

The main cellular sources of O_2^- are $NADPH^-$ oxidase, cytochrome P-450, the cytoplasmic xanthine oxidase system and mitochondrial respiration (mitochondrial electron transport chain).

Superoxide anion is converted, either spontaneously or by the superoxide dismutase family of enzymes (see below), to hydrogen peroxide (H_2O_2).

Both O_2^- and H_2O_2 are not strong oxidizing agents, but are essential for the formation of potent cytotoxic free radicals through their interaction with other molecules.

H2O2 in the presence of Cl- or Br- can form a hypohalogen acid [hypochlorous acid (HClO) or hypobrompous acid (HBrO)], this reaction can be catalyzed by lysosomal enzymes, myeloperoxidase (MPO) of neutrophils and monocytes/macrophages, which catalyze the oxidation of halides (Cl^-, Br) by H_2O_2 to form HClO or HBrO. MPO mainly produces hypochlorous acid. The production of hypohalogen acids is important for host defense mechanisms against infectious agents, but hydroxyl radical (OH^-), a potent oxidant, is also evolved during this reaction.

MPO-derived ROS can interact with nitrites (NO_2^-) and H_2O_2 leading to the formation of RNS (Barnes 2022, Nucera 2022, Psarras 2005).

Cigarette smoke is the main exogenous source of oxidants, it is an aerosol composed of ~92% vaporized agents and ~8% particulate matter suspended in a gaseous medium. The inhaled gaseous component of cigarette smoke can contain up to 1014-1016 free radicals per inhalation.

Cigarette smoke contains about 500 ppm of nitric oxide (NO), members of the quinone family and polyphenolic agents (catechol) generated by the combustion of tobacco and capable of generating superoxide anion and hydrogen peroxide.

Oxidative/nitrosative stress leads to oxidation (with consequent functional alterations that can alter normal cellular homeostasis) of proteins, lipids, carbohydrates and cellular DNA and to alteration of the mitochondrial electron transport chain with consequent possible cell death (necrosis or apoptosis).
In addition to cellular toxicity, oxidants participate (for example by acting on sensitive cysteine residues within the molecules involved in signalling pathways) in the normal intracellular signal transduction pathways which have the task of promoting cell growth and proliferation, but also the release of pro-inflammatory mediators (Barnes 2022, 2020, Kirkham 2013, Nucera 2022, Psarras 2005).
For example, the accumulation of reactive carbonyls and the subsequent carbonylation of proteins (it is not an enzymatic process and affects specific amino acid residues: lysine, arginine, cysteine and histidine and can modify the function of the proteins themselves) is called carbonylative stress, and is associated with the development of chronic diseases related to aging.

Antioxidant defenses cells of the lower airways of normal subjects

Under physiological conditions, oxidative stress is counteracted by endogenous and exogenous antioxidants (taken with the diet).
The lung is constantly exposed to exogenous and endogenous sources of oxidative stress; therefore, several effective antioxidant strategies were developed including reduced glutathione (GSH) plays a fundamental role.
Up to 20% of the glutathione is located within the mitochondria to neutralize the production of ROS produced by cellular metabolism.
Exposure of airway epithelial cells of healthy subjects to acute oxidative stress triggers an increase in GSH synthesis by regulating the expression and activity of a key enzyme of GSH synthesis, glutamate-cysteine ligase.
Other GSH-dependent antioxidant enzymes are glutathione-S-transferase pi isoenzyme (GST-pi), GST-M1, and glutathione peroxidase.
The superoxide dismutase (SOD) family consists of three isoforms: SOD1 mainly localized in cytoplasm (and for this reason also known in the past as cSOD) that requires copper and zinc as cofactors (and for this reason also known in the past as Cu -ZnSOD); SOD2 with mitochondrial localization (and for this reason also

known in the past as mSOD) that requires manganese as a cofactor (and for this reason also known formerly as MnSOD); SOD3 mainly localized in the extracellular matrix linked to type 1 collagen fibers (and for this reason also known in the past as EC-SOD) which requires iron as a cofactor.

The nuclear transcription factor erythroid-2 (Nrf2) is a transcription factor capable of positively regulating the expression of many antioxidant genes, which have been shown in animal models *in vitro*, to protect the cell from oxidative stress and induced inflammation from cigarette smoke (Barnes 2022, Caramori 2013, Nucera 2022). NRF2 also regulates apoptosis, autophagy, cellular stress response and DNA repair (Dodson 2019).

Over 200 antioxidant and detoxifying cellular enzymes are under the control of Nrf2.

Oxidative/nitrosative stress and antioxidant defenses in the lower airways of smokers with normal lung function and in patients with COPD

One of the main triggers of accelerated cellular aging is represented by exogenous and endogenous oxidative/nitrosative stress (see above) that can mediate shortening of telomeres and DNA damage, activation of the intracellular signalling pathway mediated by phosphatidyl- inositol-3-kinase and the mammalian target of rapamycin (PI3K-mTOR), autophagy impairment, mitochondrial impairment, stem cell exhaustion, microRNA profiling impairment, immunosenescence, chronic inflammation (inflammaging), associated with a decrease in the expression of anti-aging molecules.

Oxidative stress may therefore be involved in individual susceptibility to COPD and may also have a role in mucus hypersecretion, small airway damage, destruction of lung parenchyma, inflammation, impaired response to glucocorticoid drugs, and carcinogenesis. pulmonary disease, all known features of patients with COPD.

An increase in exogenous and endogenous oxidative stress has been observed in the lower airways of COPD patients induced by chronic exposure to oxidizing substances present in cigarette smoke, and by activation by pro-inflammatory mediators (e.g. cytokines, chemokines, proteases, growth factors) of both

inflammatory cells (alveolar macrophages and neutrophils) and lower airway structural cells (e.g. epithelial cells and airway smooth muscle cells) (Barnes 2022, Kirkham 2013, Nucera 2022).

These oxidants are mainly produced by NADPH-oxidase, cytochrome P-450, the cytoplasmic xanthine oxidase (XO) system and mitochondrial respiration (mitochondrial electron transport chain) (Kirkham 2013).

This process is present both in smokers with normal lung function and in patients with COPD, and oxidative stress levels correlate with disease severity (Barnes 2022,2020, Kirkham 2013, Nucera 2022).

Increased lipid peroxidation and increased oxidative DNA damage (8-hydroxyguanine formation) were observed in the lower airways of smokers with normal respiratory function compared to the lungs of non-smokers (Caramori 2011, Nucera 2022).

In the lower airways of smokers with normal respiratory function, there is increased expression of the enzyme inducible nitric oxide synthetase (NOS2) resulting in additional production of NO and $ONOO^-$ as a product of the reaction of NO with O_2^-.

In the lower airways of COPD patients, the further increase in oxidative stress, compared to smokers with normal respiratory function, can therefore lead to an increased expression of pro-inflammatory genes, insensitivity to glucocorticoids, a reduced ability to induce endogenous antioxidant defences and accelerated lung aging during COPD (Kirkham 2013, Nucera 2022).

Oxidative stress can activate nuclear factor-κB (NF-κB) mediating increased pro-inflammatory status at several expression levels, and its increased activation of the NF-κB pathway in COPD is related to flux limitation plane (Figure 3) (Osoata 2009, Nucera 2022).

The resolution of the inflammatory response is equally important as its induction and the removal by phagocytosis of apoptotic cells plays a fundamental role in this process. In COPD patients there is an impaired phagocytosis of alveolar macrophages [mediated by carbonylation of tissue proteins that compete for the same innate immunity receptors (PRRs) of alveolar macrophages which thus fail to distinguish between carbonylated proteins and apoptotic cells] and failure to

remove apoptotic cells can cause necrosis contributing to prolong and maintain lower airway inflammation and increased susceptibility to lower airway infections. Carbonylation and nitrosylation reduce the activity and expression of histone deacetylase 2 (HDAC2), essential for the suppression of activated inflammatory genes, as well as the loss of HDAC2 activity, as occurs in COPD (Figure 3) (Ito 2005).

Oxidative stress mediates decreased expression and activity of SIRT1, resulting in cellular senescence (Barnes 2020).

It has also been hypothesized that chronic oxidative stress is not properly balanced by the antioxidant mechanisms present in COPD patients.

Telomere shortening and the pathogenesis of COPD

Human telomeres are composed by many kilobases (kb) of repeating nucleotide sequences (TTAGGG)n, associated with various proteins, localized at the ends of all chromosomes mediating a dual function such as preventing fusion between the ends of chromosomes during mitosis and preserving and protecting the ends of chromosomes from damage and rearrangement in the course of semi-conservative DNA replication.

At each cycle of cell division, during the S phase, the telomeres undergo a shortening of about 50-200 base pairs due to incomplete DNA replication, therefore when, after a certain number of mitoses, they reach a level of shortening critically, the cell cycle is blocked and the cell is initiated towards apoptosis.

Under physiological conditions, the progressive shortening of the telomeres is counterbalanced by the activity of telomerase, an enzymatic complex that can maintain the length of the telomere which adds new repeating nucleotide sequences (TTAGGG)n.

This enzyme complex is composed by telomerase reverse transcriptase (TERT) that has a catalytic activity, an RNA fragment called telomerase RNA component (TERC) which functions as a template for the addition of new repeating nucleotide sequences, and the nucleolar protein dyskerin which associates with TERC and is essential for the proper functioning of telomerase.

In most cells, telomerase activity is insufficient to balance the progressive shortening of telomeres, thus after a certain number of mitoses, the cell may undergo cellular senescence (replicative senescence) and/or programmed death by apoptosis (Alder 2022, Armanios 2013).
Oxidative stress impairs telomerase activity (Amsellen 2011).
In alveolar macrophages (Katsuyuki 2010) and blood leukocytes (Lee 2012, Houben 2009, Savale 2009) of COPD patients it has been demonstrated that there is increased telomerase shortening, compared to smokers with normal lung function (Barnes 2019ab, Birch 2018) (Figure 3).

Cell senescence in the pathogenesis of COPD

Cellular senescence is defined as a state characterized by irreversible cell cycle arrest in cells that are still metabolically active but losing the ability to replicate (Munoz-Espin 2014).
Cellular senescence is a feature of aging and occurs after repeated cell divisions, following the progressive shortening of telomeres which triggers a DNA damage response and subsequent activation of tumour suppressor protein 53 (p53) (replicative senescence).
Alternatively, cellular senescence can be induced by cellular stress (e.g. by oxidative stress), resulting in activation of the cyclin-dependent kinase inhibitor p16INK4 (stress senescence). These two pathways can interact with each other of them and both activate the cyclin-dependent kinase inhibitor p21CIP1 which causes cell cycle arrest (Tchkonia 2013, Munoz-Espin 2014) (Figure 3).
However, senescent cells are still metabolically active and the set of their secreted proteins characterizes the senescence-associated secretory cell phenotype (SASP) (Salama 2014, Correia-Melo 2014).
The SASP response is activated by p21CIP1, which inhibits cell apoptosis through the activation of Janus kinases (JAK) and p38 mitogen-activated protein kinases (p38MAPK). This also leads to the activation of NF-κB and the consequent secretion of pro-inflammatory cytokines: interlukin-1β (IL-1β), IL-6, tumour necrosis factor-α (TNF-α), factors growth factors [vascular-endothelial

growth factor (VEGF), TGF-β], chemokines (CXCL1, CXCL8, CCL2) and metalloproteases (MMP-2, MMP-9) (Figure 3).

CXCL8 binds to the chemokine receptor CXCR2, and induces cellular senescence, while blockade of CXCR2, with the use of specific antagonists, reduces both replicative senescence and stress-induced senescence (Acosta 2008).

Activation of the p16INK4a pathway also activates the enzyme nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase, resulting in increased oxidative stress that further activates NF-κB (Takahashi 2006).

Plasminogen activator inhibitor-1 (PAI-1) is another protein characteristic of the SASP response (Barnes 2019).

It has been hypothesized that cellular senescence has a protective function against neoplasms, however, in animals, the accumulation of senescent cells during aging causes tissue dysfunction, age-related diseases and shortening of their life span (van Deursen 2014). The senescence of both adaptive and innate immune cells (immunosenescence) can compromise the immune response and increase the susceptibility to infections, the development of neoplasms and autoimmune processes (Sadighi Akha 2018, Barnes 2017).

In an animal model of accelerated aging, the elimination of senescent cells expressing p16INK4 produces a significant increase (> 30%) of their life span, thanks to a decreased incidence of organ pathologies related to aging (Baker 2016).

Oxidative stress is thought to be an important cause of cellular senescence in COPD, as it causes an increase in senescence markers in lower airway cells (Birch 2015,2018).

Bronchial and alveolar epithelial cells, alveolar macrophages and senescent endothelial cells are present in increased numbers in the lower airways of patients with stable COPD compared to smokers with normal respiratory function (Tomita 2002, Tsuji 2006, Amsellem 2011, Rutten 2016).

In stable COPD patients compared to smokers with normal lung function there is also an increased activation of NF-κB and the secretion of pro-inflammatory mediators characteristic of the SASP response such as CXCL1, CXCL8, CCL2,

IL-6, MMP -2, MMP-9, TGF-β, (Barnes 2019, Di Stefano 2002 and 2018, Caramori 2013 and 2014, Nucera 2021).

PAI-1 expression is increased in sputum and alveolar macrophages of stable COPD patients (To 2013).

Cellular senescence may also be linked to an increased risk of lung cancer in COPD patients (Koo 2016).

Intracellular autophagy and the pathogenesis of COPD

Intracellular autophagy [micro-macroautophagy and chaperone-mediated autophagy (CMA)] is a finely regulated physiological process for the maintenance of cellular homeostasis through the removal, within lysosomes, of abnormal protein aggregates, RNA bodies, droplets lipids and abnormally functioning cellular organelles [including mitochondria (mitophagy)] (Murrow 2013).

Under physiological conditions, autophagy plays a key role in cell adaptation to stress by acting as an essential cytoprotective mechanism (Douglas 2011), and inhibition of intracellular autophagy increases cell susceptibility to oxidative damage and apoptosis (Murrow 2013).

The molecules to be sent towards intracellular autophagy are recognized by selective receptors for autophagy (SARs), which can be either anchored to the cell membrane or localized in the cytoplasm. The best-known SAR is the p62 protein (or sequestroma-1) (Birgisdottir 2020, Sabbieti 2022).

The stages of autophagy are (Figure 4 and Table 1):

a) **initiation and nucleation**: during this phase there is the beginning of the formation of the phagophore, a structure with a crescent-shaped double membrane.

For example, in fasting, amino acid deficiency, or inactivation of the MTORC1 kinase activates the formation of the complex with serine/threonine kinase activity called Unc-51-like kinase [Unc-511-like kinase (ULK)], formed by the isoforms ULK-1 and ULK-2, from autophagy proteins (ATG)13 and ATG101, and from the 200 kDa FAK (focal adhesion kinase) family interacting protein, called FIP200 (or RB1CC1) (Birgisdottir 2020).

The ULK complex phosphorylates by activating several components of PI3K type III complex 1 (PI3KC3-C1), which consists of the proteins PI3KC3 (called in yeast Vps34), PI3KR4 (or subunit p150 or in yeast Vps15), beclin 1, and ATG14. Another variant of this complex is called PI3KC3-C2 and instead of ATG14 it contains the protein associated with resistance to ultraviolet radiation [UV radiation resistance associated (UVRAG)] (Birgisdottir 2020).

After ULK-mediated activation, PI3KC3-C1 produces local accumulations of phosphatidyl-inositol-3-phosphate (PI3P) which will become the assembly site of the phagophore (specific subdomains of the endoplasmic reticulum rich in PI3P called omegasomes due to their similarity to the letter Greek omega) by recruiting PI3P binding proteins.

b) **expansion and closure**: In this phase the newly formed phagophore membrane begins to expand and surround the cytoplasmic target, forming the autophagic vacuole (autophagosome) (Birgisdottir 2020, Lamb 2013, Maeda 2019, Valverde 2019).

Phagophore expansion is mediated by 2 ubiquitin-like conjugation systems, including the ATG5-ATG12 conjugation system and in particular the ATG8 system (Birgisdottir 2020).

There are 2 different subfamilies of ATG8: the GABA-type A protein-associated receptor (GABARAP) (Birgisdottir 2020, Shpilka 2011, Wan 2020), and the most representative one in humans, the complex of microtubule-associated proteins and ATG8 protein 1 light 3 (LC3, or MAP1LC3) which in turn comprises 2 isoforms including LC3A, and LC3B.

In particular, LC3B is localized on the membranes of phagophores by mediating their elongation, whereas LC3A is mainly localized in the cytoplasm and can be converted into LC3B when the autophagy process is necessary.

LC3 is produced as a precursor called “Pro-LC3”, this peptide is truncated by the proteolytic activity of ATG4 (which in turn includes the -A; -B; -C and -D isoforms) forming the LC3-I fragment, which interacts, through the activity of ATG3 and ATG7, with phosphatidylethanolamine (PE) producing the LC3-II complex through the "lipidation" reaction

(Fu 2019, Martens 2020, Maruyana 2018, Perez-Perez 2021).

LC3-II is the active form of LC3 and is localized both on the external and internal surfaces of the phagophore membranes and determines their elongation, expansion and closure (Fu 2019, Martens 2020, Birgisdottir 2020, Mizushima 2011, Nguyen 2016, Perez-Perez 2021).

c) maturation and fusion: the maturation of autophagosomes to degrading autolysosomes occurs through their fusion with endolysosomal compartments. Before fusion the autophagic machinery detaches from the outer membrane of the autophagosome (Birgisdottir 2020, Fu 2019, Maruyana 2018, Zhao 2019, Perer-Perez 2021).

In this phase, LC3-II is converted back to LC3-I by ATG4 itself through its delipidation activity, resulting in the detachment of LC3 from the autophagosome membrane; this stage represents a key step both in the recycling of LC3-I and in allowing the closure of the autophagosome and its subsequent fusion with lysosomes through the "microtubule organizing center" (MTCO) which mediates the movement of closed autophagosomes and mature at the point where fusion with lysosomes is to occur (forming autophagolysosomes); lysosomal enzymes degrade their content which is finally released into the cytoplasm to be recycled in other pathways (He 2009, Kuwano 2016, Racanelli 2018, Qian 2017, Xie 2007).

The role of autophagy/mitophagy alterations in lower airway cells of COPD patients has not yet been well defined, as they could have both protective and harmful effects (Jiang 2019).

Acute exposure to oxidative stress causes an increase in autophagy with the aim of removing damaged cellular components and ensuring cell survival (Jiang 2019). Autophagy also regulates the inflammatory response through the modulation of inflammatory cell survival (Tan 2019, Qian 2017).

If there is a decrease in autophagy and/or mitophagy in lower airway cells of COPD patients it could increase the survival of cells with damaged cellular components. The accumulation of non-functioning mitochondria could cause an increase in the production of endogenous oxidants of mitochondrial origin with a consequent increase in oxidative/nitrosative stress, thus creating a vicious circle,

in which the cell is subjected to chronic stress, thus favouring cellular senescence (Jian 2019).

Role of SERPINS protease family in the pathogenesis of COPD

SERPINs are mainly protease inhibitors (Kelly-Robinson 2021). In humans, 4/5 of the 37 identified SERPINs are inhibitory in function and are involved in many biological processes, including tissue remodelling, blood coagulation cascade, thrombosis, and inflammatory responses (Kelly-Robinson 2021). Non-inhibitory functions have also been reported, which include hormone transport (SERPINA6, SERPINA7), blood pressure regulation (SERPINA8), and tumor suppression (SERPINB5) (Kelly-Robinson 2021). SERPINA1 (AAT) deficiency and its polymerization may cause emphysema and cirrhosis (Carrel 1994). Most SERPINs, separated into nine clades (A–I) in humans, are extracellular molecules, while intracellular ones, usually belonging to clade B, tend to be tissue-specific and participate in cellular events (Kelly-Robinson 2021). Considering functionally the selected SERPINs and proteases included in this study, SERPINA3 (alpha-1-anti-chymotrypsin) inhibits chymotrypsin, mast cell chymases, kallikreins 2 and 3 and cathepsin G (Baker 2007); SERPINA6 (corticosteroid-binding globulin), a non-inhibitory serine protease inhibitor, is the primary cortisol-binding protein involved in the regulation of acute, severe, and chronic inflammation (Meyer 2016); SERPINB2, known as plasminogen activator inhibitor-2 (PAI-2) is a major intracellular inhibitor of tissue and urokinase plasminogen activator (tPA, uPA) (Stasinopoulos 2010); SERPINB3 inhibits cysteine proteases, cathepsins K, L, S and papain (Sun 2017, Schick 1998), promotes epithelial cell proliferation, and inhibits inflammation. An antiangiogenic activity for SERPINB5 has been reported; however, its functions need to be better explored (Kelly-Robinson 2021, Silverman 2004). SERPINB11 is a non-inhibitory serine protease for which functions related to host-pathogen interactions to fight infectious diseases have been proposed (Seixsas 2021). SERPINB13 inhibits cathepsins K and L inhibiting lysosomal cysteine proteinases (Jayakumar 2003). The activity of thrombin in the vasculature is controlled by SERPINC1 (antithrombin) and SERPIND1 (heparin cofactor II). In exacerbated COPD a

reduced SERPINC1 activity (Zhang 2022), associated with coagulation activation and hypoxia (Sabit 2012) was reported, whereas the levels and actions of SERPIND1 were not reported (Corral 2004) in COPD. SPINK1 (serine peptidase inhibitor Kazal type 1) is a trypsin inhibitor with diverse physiological functions such as promoting cell proliferation and migration (Chang 2017) while preventing cell apoptosis. A direct relationship between SPINK1 with COPD has not been reported. PLAUR (UPAR, urokinase-type plasminogen activator receptor) contributes to plasminogen activation, chemotaxis, cell adhesion; and immune cell activation (Desmedt 2017); it is increased in COPD and correlated with the severity of the disease (Bocskei 2019). Tissue-plasminogen activator (tPA) is involved in dissolving blood clots (Ismail 2021) and, after plasmin activation, may have pro-angiogenic (Bootle-Wilbraham 2001) activity. Cathepsin K and L are lysosomal cysteine proteases involved in protein degradation (Dickinson 2002). Elastinolytic activity *"in vivo"* has been reported for cysteine cathepsins involved in atherosclerosis and pulmonary emphysema (Novinec 2007). Cathepsins have been shown to modulate innate immunity by degrading antimicrobial peptides (Andrault 2015) and to inactivate secretory leuco-protease inhibitor (SLPI) and its anti-neutrophil elastase capacity (Taggart 2001). Cathepsin L activity and its mRNA levels were increased in the alveolar macrophages of smokers compared to nonsmokers (Takahashi 1993). Conflicting results are reported for inflammasome activation via the "canonical pathway" involving caspase 1 functions in stable COPD patients (Di Stefano 2014, Markelic 2022), which were found to be upregulated by some authors (Markelic 2022) or not changed by others (Di Stefano 2015). A progressive reduction in the numbers of peripheral airways has been reported in patients with COPD (Hogg 2009, Vasilescu 2020). These molecular alterations are also present in smokers with near-normal lung function (Hogg 2009, Vasilescu 2020). Proteases have been directly implicated in these structural alterations developing in COPD of increasing severity and in emphysema (Kelly-Robinson 2021, Vasilescu 2020, Di Stefano 2025). The inhibitory and non-inhibitory SERPINS are summarized in table 2 e and 3.

Mitochondrial alterations and pathogenesis of COPD

Mitochondria are involved in a large number of processes fundamental in maintaining cellular homeostasis and in the adaptation of the cell to oxidative stress.

At the mitochondrial level, the production of adenosine triphosphate (ATP) takes place through the electron transport chain and of oxidized nicotinamide adenine dinucleotide phosphate (NADP+) and the regulation of calcium metabolism. Mitochondria can also activate and regulate innate and adaptive immune responses (Xie 2020) and contribute to pro-inflammatory responses for example through the synthesis and release of oxidants and peptides derived from mitochondrial DNA (Mendelson 2018). During the cellular aging process there is a gradual accumulation of mutations at the level of mitochondrial DNA and their decreased resistance to oxidative stress and in aging individuals defects of mitochondrial structure and function have been described (Zheng 2012, Osiewacz 2013).

The mitochondria of the epithelial cells of the lower airways of ex-smokers with COPD show alterations both at a structural level with loss of the ridges, lengthening and swelling of the body; both at a functional level with an increase in the expression and activity of the SOD2 enzyme, and of the transcription factors of the coactivator 1α (PGC-1α) of the gamma receptor activated by peroxisome proliferators (PPAR-γ), of the VF1α complex (Hoffmann 2013).

In vitro in bronchial smooth muscle cells obtained from COPD patients, compared to those obtained from control subjects, there is mitochondrial dysfunction with decreased reserve capacity of cellular respiration and increased production of oxidants (Wiegman 2015).

In COPD patients this increased production of mitochondrial oxidants may contribute to the increased expression of nitrotyrosine in the lower airways of these patients (Ricciardolo 2005) through for example interaction of superoxide anion with the increased concentration of NO present due to increased expression of mitochondrial oxidants. nitric oxide synthetase enzymes and the formation of peroxynitrite. NO also modulates the expression of the enzyme cytochrome C oxidase (also called mitochondrial electron transport chain complex IV) which is the primary site of cellular oxygen consumption and is critical

for oxidative phosphorylation and ATP synthesis. Moreover, in the alveolar macrophages of COPD patients there is an increased production, compared to control subjects, of mitochondrial oxidants which could represent a mechanism that contributes to the destruction inside the phagolysosomes of phagocytosed infectious agents (for example bacteria). However, at the same time, particularly if associated with the decreased concentration of the antioxidant enzymes SOD2 and heme oxygenase-1 (whose production is regulated by the transcription factor Nrf2) present in the lower airways of COPD patients, it can lead to apoptosis, with consequent decreased phagocytosis, and cellular senescence with activation of pro-inflammatory transcription factors, such as NF-κB and increased synthesis of inflammatory mediators (Belchamber 2019).

Some proteins, such as humanin, encoded by mitochondrial DNA, have cytoprotective functions helping to preserve mitochondrial vitality and functionality (Mendelsohn 2018).

For example, humanin stimulates CMA-mediated autophagy which helps to direct oxidized proteins towards lysosomes (Gong 2018) protecting against senescence as an endogenous senolytic agent and its decrease could therefore contribute to the pathogenesis of diseases related to accelerated aging such as COPD (Mendelsohn 2018, Kim 2018).

Immunosenescence and pathogenesis of COPD

During the aging process the immune system shows an altered immune response to new antigens with a greater susceptibility to infections and an increased risk of developing autoimmune diseases.

Immunosenescence involves both innate and adaptive immunity, resulting in impaired cell migration and recognition processes through toll-like receptors (TLRs), decreased phagocytosis of macrophages and neutrophils, loss of naïve B and T lymphocytes, due both to the involution of the thymus which occurs physiologically with aging, and to the shortening of the telomeres, with consequent defective immune response to the new antigens.

TGF-β1 has a key role in regulating the immune tolerance to self and the decrease of its expression present in the small airways of patients with stable

COPD could contribute to the pathogenesis of the autoimmune response present in many of these patients with the progression of the their disease (Caramori 2018, Di Stefano 2018) and this seems to contribute to the low-grade chronic inflammatory response (inflammaging) present in their lower airways by creating a local microenvironment favourable to the appearance of autoimmune responses (Barnes 2019).

This hypothesis is also in agreement with the significant increase in the expression in the bronchial mucosa of patients with stable-phase COPD of BMP-membrane binding inhibitor activin (BAMBI), a TGF-β pseudo-receptor which, in animal models, appears to stimulate the autoimmune responses through an increase in the Th17/Treg ratio (imbalance that is present in the lower airways of COPD patients; Caramori 2016).

Aim of the research project and applied methodologies

Working hypothesis

Our working hypothesis was that accelerated aging of lower airway cells and particularly impaired intracellular degradation pathways can have a key role in the pathogenesis of COPD and in the progression of this disease.

Aim of the research project

The aim of this research project was to investigate the localization and expression of novel or poor known cellular senescence markers in the small airways and lung parenchyma obtained from patients with stable COPD compared to smokers with normal respiratory function and non-smoker subjects matched by age, gender and smoking habit (control group).

In particular, although there are several potential senescence markers potentially involved in COPD pathogenesis, we started to evaluate the autophagy intracellular pathways.

This decision was founded by the presence of previous robust data on autophagy in COPD published by a study group with we already collaborated in the past and with whom we shared this research (Levra 2023).

This study has evaluated just the LC3 protein (Levra 2023) that represent the core of autophagy machine, for these reasons we decided to further evaluate this topic analysing all the ATG proteins involved in this intracellular pathway.
Then, we decided to analyse also the SERPINS family performing a large evaluation on each member of this protease's family.
We have chosen these markers because these are involved in the physiological cellular processes of phagocytosis and intracellular recycling processes. In addition, currently there are very few data regarding these makers in cellular senescence and COPD patients and to our knowledge we performed the first global analysis of these molecules in COPD.
As the large number of members composing SERPINS family, we decided to perform a genomic analysis to screen the proteins to select the SERPINS members to subsequently evaluate through IHC method. Finally, to further confirm the data regarding SERPINS family, founding with IHC, we have also performed an ELISA analysis on samples.

Experimental plan of the research project and applied methodologies

Subjects recruited in the research project

Three groups of subjects, both over fifty years of age, undergoing lung resection for a solitary peripheral carcinoma will be recruited for this research project:
1) 13 patients with mild to moderate COPD in a stable phase;
2) 14 cigarette smokers with normal lung function;
3) 12 non-smoker subjects
COPD patients and the control groups were be comparable in age and sex and their clinical phenotype will be characterized very precisely as described previously (Kirkham 2011). Ex-smokers must have stopped smoking for at least a year.
COPD patients were in stable phase (for at least 3 months) and none of the subjects must have received glucocorticoids (systemic or inhaled), theophylline, antibiotics or antioxidants in the month before surgery.

All subjects must not have undergone pre-operative chemotherapy and/or radiotherapy and must not have received other immunosuppressive drugs. Within 30 days prior to surgery, each patient had an accurate medical history (including smoking habits, long-term home drug therapy taken and the presence or absence of chronic bronchitis) and was subjected to spirometry with acute bronchodilation test, and routine chest computed tomography (usually with contrast), as previously described (Marwick 2010, 2009).

All patients have signed an informed consent.

COPD diagnosis was posed as an exclusion diagnosis in subjects > 40 years with a smoking history > 20 p-y and persistent airflow obstruction (FEV1/FVC ratio < 0.7) after the exclusion of the many other known causes of persistent airflow limitation such as bronchial asthma with persistent airflow limitation, bronchiectasis, previous TB, ILDs, occupational exposures (coal mine dust, silica, welding fume, textile dust, agricultural dust, cadmium fume), cryptogenic organizing pneumonia, pulmonary Langherans' cell histiocytosis, and lymphangioleiomyomatosis.

Approval has already been obtained until 2027 (non-profit experimental study 55-17, resolution CS n.270 of 10.18.2017 and subsequent additions, scientific director Prof. Gaetano Caramori) from the local Ethics Committee for this type of studies that will follow the recommendations of the National Committee for Biosafety, Biotechnology and Life Sciences (Collection of biological samples for research purposes, informed consent, 2009:
http://www.governo.it/bioetica/gruppo_misto/Consenso_Informato_annex_Petrini_2009.pdf

Lung function tests and volumes

Pulmonary function tests were performed as previously described (Maruyama 2017, Vestbo 2013) according to published guidelines (Di Stefano 2014). Pulmonary function tests included measurements of FEV1 and FEV1/FVC under baseline conditions in all the subjects examined (6200 Autobox Pulmonary Function Laboratory; Sensormedics Corp., Yorba Linda, CA, USA). Pulmonary

function tests were performed during the pre-operative surgical visit for the removal of the pulmonary nodule. All subjects involved in the study performed pulmonary function tests and patients performed long-term inhaled therapy they suspended inhalation therapy for at least one day before performing the pulmonary function tests. In order to exclude the reversibility of airflow obstruction (suggesting asthma) and post-bronchodilator function, values of the FEV1 and FEV1/FVC% measurements in both COPD and CS groups (but not in the CNS group) prebronchodilator were repeated 20 min after the inhalation of 0.4 mg of salbutamol. The airflow obstruction was defined as reversible when the post-BD FEV1 values were increased compared to the pre-BD FEV1 values of at least 200 mL and 12%. The data regarding the subjects involved in this project has reported in Table 4.

Collection, processing and molecular biology investigations of the peripheral lung

Non-neoplastic peripheral lung, obtained from patients with COPD and control subjects, were collected during scheduled surgical lung resection for a lung neoplasm and then processed as previously described (Kirkham 2011); of this, four to six selected portions were taken from the subpleural lung parenchyma (Balte 2020) of the resected lobe during the operation, avoiding the areas affected by the tumour, as previously described (Kirkham 2011).

During surgery, frankly pathological lung tissue was removed with a more or less large quantity (depending on the type of surgery necessary) of macroscopically undamaged lung tissue around it. The pathological lung tissue was immediately sent to the Anatomy-Pathological Unit of the AOU Policlinico G. Martino of Messina for the necessary anatomo-pathological investigations for diagnostic purposes. In parallel, the same Anatomy-Pathological Unit selected, as per current clinical practice, a very small fraction of the non-neoplastic lung tissue as far away as possible from the lung lesion resected during surgery. This material will then be immediately fixed in formalin/paraffin (and then stored at room temperature) or frozen in liquid nitrogen and then stored at -80°C (using the methods already described using the methods already described (Ito 2005). This

procedure did not lead to any delay in sending the pathological lung tissue to the Anatomy-Pathological Unit of the AOU Policlinico G. Martino of Messina, Italy and did not in any way compromise the quality of the subsequent anatomy-pathological investigations for diagnostic purposes.
From these pieces of non-pathological lung tissue processed for research purposes was
subsequently cut small sections and/or extracted the total proteins following already validated protocols (Ito 2005).

Expression profile and sequencing of messenger RNAs (mRNAs) obtained from frozen lung tissues

Frozen lung parenchymal tissue used for the immunohistochemical analysis; bronchial rings from the same patients were also used for RNA extraction, sequencing and gene expression data analysis. RNA extraction was performed with the RNAeasy micro kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A DNA removal step was applied using 500 units of RNase–free DNase (Qiagen) at room temperature for 15 min. Total RNA was resuspended in RNase-free water (Thermo Fisher, Carlsbad, CA, USA) 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 an 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). Consequently, lung parenchyma libraries were sequenced using an Illumina NextSeq500 (Cribi, UniPD, Padova, Italy) with a 75-single-end read layout. In contrast, no quality issues were encountered for the bronchial ring samples; therefore, standard Illumina library preparation methods were performed. Bronchial ring libraries were then sequenced with a 150-paired-end read layout (Cribi).

Immunohistochemistry (IHC) for specific markers of cellular senescence

Immunostaining of paraffin-embedded peripheral lung tissue was performed as previously described (Taraseviciene-Stewart 2012).

For immunohistochemical analysis, 5-micron thick sections obtained from corresponding tissue blocks were deparaffinized, then washed in descending alcohol scale, treated with 3% hydrogen peroxide for 10 min, washed again in deionized water three times, and incubated with normal sheep serum to prevent unspecific adherence of serum proteins for 30 min at room temperature. Subsequently, sections were washed with deionized water and incubated for 30 min at 37 °C with commercially obtained primary anti-human antisera against different ATGs and SERPINS (see Table 5 and Table 6). Specifically, the primary antibody working dilution ratio able to affect the intensity of immunoreactivity has been determined after an accurate scalar dilution test in order to obtain the best specific results without any stained background. Next, the sections were washed three times with PBS and incubated in a Ventana BenchMark Ultra (Roche Diagnostics, Rotkreuz, Switzerland). An UltraView Universal DAB detection kit (Roche Diagnostics) was used in accordance with the manufacturer's instructions. Slides were then removed from the Autostainer Ventana BenchMark Ultra (Roche Diagnostics, Rotkreuz, Switzerland), counterstained with Mayer's Hematoxylin, mounted with Permount (Fisher Chemical, Rangeland Parkway, FL, USA), and coverslipped. Negative controls were obtained by omitting the specific primary antisera, replacing them with PBS or normal rabbit serum. In this way, no immunoreaction was revealed. By contrast, the positive control was represented by gastric cancer cells, since they were tested and stained by ATGs, whereas we have used human tonsils or nasal polyps as positive control for SERPINS as reported in a previous study with similar results to those elsewhere reported (Ieni 2019).

The immunoreactivity of ATGs was evaluated according to the intensity and percentage of positively stained cells, as elsewhere reported (Masuda 2016). The cytoplasmic immunostaining intensity was rated as follows: 0, negative; 1, weak; and 2, strong. The percentage of positively stained cells was graded as follows: grade 0, 0–5%; grade 1, >5–25%; grade 2, >25–50%; grade 3, >50–75%; and grade 4, >75–100% for all antibodies. The immunohistochemical staining

samples were independently scored by two pathologists, who were blinded to patient outcomes and other clinical findings, using a Zeiss Axioskop microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) at 40× objective magnification. The interobserver agreement for immunohistochemistry staining had a kappa value ranging from 0.73–0.80 (substantial agreement) for the antisera.

The immunoreactive score was calculated by adding the staining intensity score and the percentage score of positively stained cells. Lung tissue compartments with an immunoreactive score of 0–3 were classified as negative, and those with a score of >3 were classified as positive. Autophagy was defined when samples were positive for at least two out of the three protein expressions (Masuda 2016).

ELISA Tests in the Peripheral Lung Tissue Homogenates

SERPINA6 (MyBioSource, San Diego, CA, USA Cat. N. MBS700017, lower detection limit, 20 pmol/mL), SERPINB3 (Antibodies-online, Aachen, Germany Cat. N. ABIN6574275, lower detection limit, 33 pg/mL), SERPINB5 (MyBioSource, San Diego, CA, USA, Cat. N. MBS9426741, lower detection limit, 0.78 ng/mL), SERPINB11 (MyBioSource, San Diego, CA, USA Cat. N. MBS9313345, lower detection limit, 0.1 ng/mL), SERPINB13 (MyBioSource, San Diego, CA, USA Cat. N. MBS1607013, lower detection limit, 9.33 ng/L), TPA (MyBioSource, San Diego, CA, USA Cat. N. MBS161723, lower detection limit, 0.05 ng/mL), caspase1 (Antibodies-online, Aachen, Germany Cat. N. ABIN 6574284, lower detection limit, 0.113 ng/mL), cathepsin L (Antibodies-online, Aachen, Germany Cat. N. ABIN 6954461, lower detection limit, 0.122 ng/mL) protein quantification was performed in the lung tissue homogenates obtained from the frozen tissue specimens that were also used for the immunohistochemical analysis. All ELISA kits were used according to the manufacturer's instructions.

Statistical analysis for the research project

Data Analysis of RNA-Seq Data

The raw Illumina reads were trimmed for quality using fastp (Chen 2018), setting a minimal Phred quality of 25 and removing the sequencing adaptors. Raw

Illumina datasets were submitted to the NCBI Short Read Archive (SRA) under the project ID PRJNA1041288. FASTQ files were imported using CLC Genomic Workbench, v. 21 (Qiagen, Hilden, Denmark) and analysed as follows. To identify differentially expressed genes (DEGs), 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 strand-specific mapping. Expression values were counted as transcripts per millions (TPMs). A Baggerley test with a false discovery rate (FDR) p-value correction was applied to identify differentially expressed genes (DEGs), setting a cutoff of 2 logarithmic fold changes (FC) and 0.01 of the p-value. Limited to the gene of interest, the expression levels were extracted from the overall dataset and further discussed.

Statistical Analysis Applied to Functional and Morphological Data

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, followed by the Mann–Whitney U-test for comparison between groups. Correlation coefficients were calculated using the Spearman rank method. Probability values of $p < 0.05$ were considered significant. Data analysis was performed using the Stat View SE Graphics program (Abacus Concepts Inc., Berkeley, CA, USA).

Results

IHC for ATGs in the Peripheral Airways and Lung Parenchyma

All significant positive data are summarized in Table 7. The immunoreactivity was analysed in its distribution, either nuclear or cytoplasmic, mainly concerning the bronchial epithelium as well as the alveolar lining. In detail, a statistically significant increased expression of ATG4A ($p = 0.0047$) (Figure 5A), ATG4D ($p = 0.018$) (Figure 5B), and ATG5 ($p = 0.019$) (Figure 5C) was documented in the

bronchiolar epithelium in patients with stable COPD compared to CS and CNS groups (Figure 5D).

Similar statistically significant values were encountered in the immunoexpression of ATG4A ($p = 0.0036$, Figure 6A), ATG4B ($p = 0.0054$, Figure 6B), ATG4C ($p = 0.0064$, Figure 6C), ATG4D ($p = 0.0084$, Figure 6D), ATG5 ($p = 0.0088$, Figure 6E), and ATG7 ($p = 0.018$, Figure 6F) in alveolar lining of stable COPD compared to CS and CNS patients.

By contrast, no significant differences in other compartments of lower airways including lung smooth muscle cells, alveolar capillary, endothelial cell veins, and alveolar macrophages were found in terms of immunoexpression of ATG4A, ATG4B, ATG4C, ATG4D, ATG5, and ATG7. Moreover, any further significant differences in the expression of ATG2A, ATG2B, ATG3, ATG10, ATG12, ATG14 (Thr429), ATG14, and ATG16L were found in all other cellular compartments of COPD patients compared to CNS and CS.

Gene Expression Level of SERPIN-Signalling Molecules in Lung Parenchyma

We examined whole-transcriptome RNA-sequencing expression datasets obtained from the lung parenchyma (CNS, n = 6; COPD, n = 7, CS, n = 5) used for immunohistochemical analysis. After a preliminary analysis of 57 molecules (Table 8), we extracted the expression levels of 15 selected SERPIN-signalling molecules (Figure 7).

Overall, we observed two gene expression conditions, with the first including genes with higher expression levels such as caspase 1, cathepsins, tissue-type plasminogen activator (PLAT), urokinase plasminogen activator surface receptor (PLAUR) and a single SERPIN gene (SERPINA3). These genes are characterized by expression levels ranging generally over 20 TPMs and reaching almost 300 TPMs as SERPINA3 in one CNS sample. Despite their considerable expression levels, which appeared detectable in all the sample types (Figure 1b), these genes were poorly regulated among the tested conditions. A second group

of genes included most of the considered SERPINs and serine protease inhibitor Kazal-type 1 and are characterized by lower expression levels, generally below 10 TPMs, with a relevant number of samples showing no detected expression levels (TPMs < 3), particularly considering the COPD and CNS samples (Figure 7).

IHC for SERPIN-Signalling Proteins in the Peripheral Airways and Lung Parenchyma

In bronchiolar epithelium, decreased levels of SERPINA6 immunopositivity was observed in CS compared to CNS (Table 9).

In COPD, this value tended to be decreased as well. SERPINB5 increased in CS compared to CNS and COPD (Table 9). TPA increased in CS and COPD compared to CNS (Table 9). In bronchiolar lamina propria, SERPINA6 levels decreased in CS and COPD compared to CNS (Table 9). SERPINB13 increased in CS and COPD compared to CNS (Table 9). Caspase 1 decreased in CS compared to CNS (Table 9). In alveolar macrophages, SERPINB5 increased in CS compared to CNS (Table 9).

SERPINB13 was increased in CS and COPD compared to CNS (Table 9). PLAUR (UPAR) increased in CS compared to CNS and COPD (Table 9). Cathepsin L tended to be increased in CS and COPD compared to CNS (Table 9). In alveolar septa, SERPINB3 tended to be decreased in CS compared to CNS (Table 9, Figure 8).

SERPINB5 and B13 was increased in CS and COPD compared to CNS (Table 9). SERPINB11 was decreased in CS compared to CNS and COPD (Table 9). Cathepsin L was increased in COPD compared to CNS (Table 9). Caspase 1 was decreased in CS compared to CNS (Table 9). In lung vessel endothelium, SERPINA6 tended to be decreased in CS compared to CNS (Table 9). SERPINB11 was decreased in CS compared to CNS and COPD (Table 9). SERPINB13 and tPA (endothelium and smooth muscle) was increased in CS and COPD compared to CNS (Table 9). Endothelial caspase 1 was decreased in CS and COPD compared to CNS (Table 9). In all lung compartments studied, the

least expressed SERPINs were SERPINA3, SERPINB2 (PAI-2) and SERPIND1 (Table 9).

ELISA Tests for SERPIN-Signalling Proteins in Homogenized Peripheral Lung Tissue

As shown in Figure 9, we found significant differences in the concentration of SERPINA6, which was decreased in the total lung protein extracts of mild moderate COPD compared to CNS, in accordance with the IHC data.

Caspase 1 was increased in mild-moderate COPD compared to both CNS and CS subjects, at variance with the IHC localized quantifications performed in the bronchioles and alveolar septa. SERPINs B3, B5, B11, B13, tPA and cathepsin L did not change significantly among groups.

Principal Correlations Between Clinical Parameters, SERPINs, Proteases and ATGs in Lung Parenchyma

In COPD patients, there was a significant positive correlation between SERPINA6 in bronchiolar epithelium and the predicted post-bronchodilator FEV1% ($R = 0.610$, $p = 0.046$, Figure 10); in alveolar septa, SERPINA6 was positively correlated to SERPINB3 ($R = 0.902$, $p < 0.0001$, Figure 10), and SERPINB3 was inversely correlated to tPA values ($R = -0.755$, $p = 0.0072$, Figure 10).

However, no significant correlation between lung tissue ATGs expression and clinical parameters was found.

Discussion

The intracellular pathways involved in physiological senescence are numerous and currently are not fully clear still now. In the other hand, in the COPD pathology are involved several intracellular pathways correlated to chronic inflammation, impaired immune system and increased oxidative stress. Many of these intracellular pathways are shared with these involved in the physiological cellular senescence. In addition, although there are several evidences that both COPD can accelerate the cellular senescence and that lung cells of COPD patients can

show several signs of accelerated senescence, it is not fully clear still now which intracellular signalling pathways are more prominent in the correlation between COPD and cellular senescence. Furthermore, it is not fully clear if blocking the intracellular pathways cellular senescence-correlated can modify the pathogenesis of COPD and vice versa (Barnes 2019)

The impaired mechanisms involved in the intracellular degradation protecting from infections or recycling old or damaged cellular organelles, such as the intracellular autophagy and the SERPINS signalling pathways have a central role in both COPD pathogenesis and cellular senescence correlating with increased oxidative stress, decreased effectiveness of immunity system and enhanced pro-inflammatory status that all features of these two conditions (Nucera 2022).

The role of autophagy in both cellular senescence and chronic inflammatory diseases including COPD is still now controversial [18], although it is well known that autophagic flux is impaired during cellular senescence, this may change during chronic inflammation, with contrasting data concerning either an increase or decrease of the autophagic cascade in COPD (Choi 2013, Racanelli 2020, Xia 2021). However, growing evidence suggest that autophagy is induced by cigarette smoke, but it tends to be impaired during COPD showing that in stable COPD patients impaired macroautophagy can play a key role in maintaining the pro-inflammatory state of the lungs, in damaging and inducing apoptosis in lung cells, leading to pulmonary emphysema and worsening of the disease (Vij 2018), mediating activation of the same intracellular pathway in cellular senescence. Furthermore, impaired autophagy in alveolar macrophages is correlated with their altered phagocytosis and killing capacity and consequently with decreased defence against several pathogens, such as bacteria and viruses (Pehote 2017) that are features shared by immune cells of COPD patients and in older people characterized the so termed immunosenescence. In particular, autophagy in pulmonary myeloid cells, including alveolar macrophages, is known to prevent excessive immune responses and inflammation under pathological conditions, such as endotoxemia, cystic fibrosis, and hemorrhagic shock (Kanayama 2015). On the other hand, the role of ATGs in tissue-resident cells, such as muscle cells, endothelial cells, and fibroblasts should be not emphasized since no significant

differences have been observed in these compartments in relation to all classes of ATGs. Therefore, it may be suggested that autophagy may determine any increased susceptibility to infections and consequently any increased risk of COPD exacerbation, worsening the quality of life and prognosis of COPD patients.

Recently, these data have been confirmed by analysing through several molecular methods the lower airways of stable COPD patients with different grades of severity, showing increased expression of LC3A + LC3B in both smokers with normal lung function and in COPD patients compared to non-smoker subjects, suggesting thus an enhanced autophagic flux (Levra 2023). Moreover, an increase of p62 was found in COPD patients compared to controls, suggesting that the autophagic flux is also ineffective in COPD (Levra 2023).

Our investigation has extensively analysed the immunoexpression of a larger panel of ATGs in order to verify if a further increased expression of them may be present in COPD patients. Although some biases are present in this analysis, such as the size of the analysed cohort as well as the use of an exclusive immunohistochemical approach, and the absence of molecular/genetic data of patients, data are in accordance with several studies that have previously demonstrated this kind of alteration in autophagic flux in other chronic inflammatory lung diseases, including also lung cancer that is strictly correlated to COPD representing an independent risk factor for lung cancer development (Eapen 2019, He 2022, Perez-Perez 2021).

Interestingly, we extended previously reported data (Levra 2023), showing an increased expression of selected AGTs such as AGT5, ATG7, and in particular, the ATG4 protein family that has a key role in the process of elongation, cargo recruitment, and closure of phagophore through both the transformation of the pro-LC3 in active LC3, but also in the delipidation and recycling of LC3 (He 2022, Xia 2021) [18,24]. These data are consistent with the previous ones appeared in literature and they could explain both the increased expression of LC3 in COPD as well as the ineffective autophagic flux characterizing COPD since the increased expression of these ATGs (such as ATG5, ATG7, and ATG4 protein family) involved in activation and recycling of LC3 can occur in an accelerated,

uncoordinated and impaired autophagic flux. Secondly, we also demonstrated that the most involved ATGs in COPD are represented by several ATG4 isoforms. It is well known that during physiological conditions, the most important ATG4 is represented by ATG4B, whereas the other isoforms are poorly expressed, and not effective compared to ATG4B. However, it is known that the other isoforms can replace the ATG4B absence of inactivation [18,25] and this could enforce the present results, suggesting that the impaired autophagic flux can be mediated by the increased several ATG4 isoforms, which are less effective compared to the ATG4B isoform. Thirdly, it can be hypothesized that the impaired expression of the ATG4 isoforms could be related to oxidative stress that has a key role in cellular senescence. In fact, is noteworthy that oxidative stress is increased in COPD and has a key role in the development and evolution of this disease. Moreover, the ATGs, and particularly the ATG4 isoforms, can be inactivated by oxidative stress (He 2022, Perez-Perez 2021). This furtherly enhances the hypothesis that the increased expression of several ATG4 isoforms could be a compensatory mechanism during COPD, which, however, results in ineffective autophagy.

In addition, to our knowledge, this is the first study that has comprehensively analysed the gene and protein expression of all members of the SERPIN family in different lung compartments in COPD, in comparison to two control groups including CS and CNS. The SERPIN family is a large group of antiproteases involved in several biological functions and is composed of 37 members. To avoid the analysis of SERPINs not expressed in lung tissue, we first analysed all members of the SERPIN family through a transcriptomic methodological approach to evaluate the mRNA levels for each SERPIN amongst all groups. We found significant different expressions in terms of mRNA levels for 15 members of the SERPIN family. These molecules were then analysed through both IHC and ELISA.

Specifically, one of the relevant results is the decreased expression of SERPINA6 in CS (which also tends to decrease in the COPD group) compared to that in CNS. This molecule, also termed corticosteroid-binding globulin (CBG), is usually an extracellular protein that can bind and transport glucocorticoids (Lee 2024).

Impaired expression of SERPINA6 is correlated with cardiovascular and metabolic diseases (Bankier 2023, Lee 2024). A decreased expression of SERPINA6 in COPD patients was correlated with impaired expression of SERPINA1 (Meyer 2016, Nenke 2016). Our result confirms these data, showing that the expression of this molecule is decreased in COPD, differing from other inflammatory diseases such as asthma (Nucera 2022). Our result was found in several lung compartments, as was also confirmed through ELISA analysis. In addition, we also found a positive correlation between CBG expression and lung function (FEV1%), reinforcing the notion of a CBG decrement in relation to the severity of the COPD. These data may relate to clinical reports on the decreased effectiveness of ICS, particularly in COPD patients who continue to smoke.

Regarding the clade B of SERPINs, we found impaired expression of several isoforms including B13, B5, B3 and B11.

Increased SERPINB13 expression was found in COPD/CS patients compared to the CNS group in several lung compartments, including alveolar septa and lung vessels. It is known that SERPINB13 has a role in cell proliferation and differentiation. Moreover, an association between SERPINB13 gene expression and squamous cell carcinoma was found (Janciauskiene 2024, Yuan 2020). Our data may represent another link between the presence of COPD and lung cancer development (Perrotta 2022).

This hypothesis is also reinforced by another result found in our analysis showing increased expression of SERPINB5, specifically in the CS group. Although the role of this molecule is largely unknown, SERPINB5 seems to act as a tumor suppressor through the inhibition of cancer cell migration, invasion capability, and angiogenesis, suggesting a compensatory mechanism in CS and COPD patients in the relationship between COPD and lung cancer (Cagnin 2024, Nucera 2024, Perrotta 2022, Yuan 2020).

We found no significant differences in SERPINB3 expression amongst all groups in the major part of the analysed lung compartments; we found it was decreased in the alveolar septa of COPD/CS patients compared to the CNS group. Recent data have shown that SERPINB3 has a protective role in mediating tissue repair, especially during hypoxic conditions, through its proliferative action (Park 2021),

suggesting a role in the development of pulmonary emphysema through the decreased repair capability in lung tissue of these patients.
We found increased cathepsin L both at the protein and mRNA levels. The IHC analysis showed increased levels in alveolar septa and alveolar macrophages in COPD/CS patients compared to the CNS group. Cathepsins, in particular, cathepsin L expression, is regulated by the SERPINB group, and particularly by SERPINB3; thus, the decreased expression of this molecule in alveolar septa could be related to the increased expression of cathepsin L, which is able to modulate the lysosome-mediated intracellular protein degradation. Cathepsin L has a key role in phagocytosis and intracellular protein degradation; this can be related to both pulmonary emphysema development and the impaired alveolar macrophages functions reported in COPD (Nucera 2024).

Another important finding is the increased expression of SERPINB11 in the alveolar septa and lung vessels in COPD patients compared to the CS group. Although the role of SERPINB11 is still now largely unclear, there is some evidence that this molecule is increased in several types of cancer, correlating with poor patient prognosis and therapy response (Park 2021).
We also found an increased expression of PLAUR in the alveolar macrophages of both COPD and CS groups, which is in line with other data in the literature (Bocskei 2019). Although PLAUR has an important fibrinolytic role similar to that of tPA, this molecule plays a key role in inflammation, cell adhesion, and chemotaxis; pre-clinical data demonstrated that decreased levels of PLAUR are correlated to decreased lung inflammation (van Zoelen 2009).
Finally, both tPA and PLAUR, which were shown to have increased in several lung compartments in this study, are modulated and inhibited by SERPINB2. The lack of an increase in SERPINB2, in contrast to the tPA and PLAUR activity, may have a role in increased inflammation in COPD patients.
We did not find any significant variations in SERPINC1 and SERPIND1 in the different lung compartments analysed by IHC, which is in contrast to the reduced SERPINC1 activity (Zhang 2022) previously reported in exacerbated COPD. This may be due to the different disease states of the patients studied.

We found a discrepancy between some IHC and ELISA data; in fact, not all IHC data were confirmed by ELISA. We speculate that this may be due to the different methodological approaches; ELISA tests represent the total amount of lung proteins extracts while IHC results are obtained specifically in different lung compartments.

Finally, our transcriptomic analysis showed a clear prevalence of proteases expression in the lungs of all patients together with an imbalance in SERPINs, mainly observed at the post-transcriptional level. This is in line with our experience, as there are frequently discrepancies between transcriptomic and protein data (Vallese 2015).

In different lung compartments of COPD patients, we reported decreased levels of SERPINB3, unchanged levels of SERPINB2 concomitant to increased levels of some related proteases such as cathepsin L, PLAUR and tPA. This study highlights the most important SERPINs and related proteases involved in the pathogenesis of stable COPD of increasing severity.

Interestingly, these data enforce the correlation between COPD and cellular senescence, although there are few data in literature, the impaired expression of several SERPIN in different lung compartments in lung tissue COPD patients are strictly correlated with many cellular senescence such as cellular proliferation, differentiation and defence from carcinogenesis (B13, B5, B3 and B11). Moreover, other SERPINs are involved in a chronic pro-inflammatory and pro-thrombotic status that are other features of cellular senescence. Finally, important data are regarding Cathepsin L and the correlated SERPINs, this enforce our data regarding autophagy showing that impaired intracellular degradation has a central role in both COPD patients and cellular senescence and this is an important link between these two conditions.

Conclusions

The pathogenesis of COPD is currently not full clear, similarly, although several molecular mechanisms involved in cellular senescence was already found, this represents still now and important research field. The link between cellular senescence and COPD is well-known, as several molecular mechanisms and

intracellular pathways are shared between these two conditions and both senescent lung cells and COPD lung cells show similar features such as pro-inflammatory status, increased oxidative stress, impaired cell cycling, increased genetic alterations.

The impairing of several intracellular alteration pathways such as autophagy and/or activity of SERPINs are still now known, but our data show that these have a key role in these two conditions. The alterations of this mechanism can correlate with features shared by senescence cells and COPD cells and can play a key role in the pathogenesis of these conditions, such as increasing oxidative stress and pro-inflammatory status and decreasing immune system effectiveness.

Although our study show several limitations, to our knowledge we have performed for the first time a large and systemic analysis of the molecules involved in these intracellular pathways (ATGs and SERPINs) representing a solid base to further studies to enlarge the knowledge in this field that is poor known still now and that could found in the future novel targets to both evaluate and treat these two conditions, in particular COPD that is a diffuse pathological condition correlated still now with high mortality and morbidity.

References

Acosta JC, O'Loghlen A, Banito A, et al.Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell 2008;133:1006-1018.

Aghali A, Ngassie MLK, Pabelick CM, Prakash YS. Cellular Senescence in Aging Lungs and Diseases. Cells. 2022 May 29;11(11):1781.

Alder JK, Armanios M. Telomere-mediated Lung Disease. Physiol Rev. 2022. Online ahead of print.

Amsellem V, Gary-Bobo G, Marcos E et al. Telomere dysfunction causes sustained inflammation in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2011;184:1358-1366.

Andrault PM, Samsonov SA, Weber G, Coquet L, Nazmi K, Bolscher JG, Lalmanach A-C, Jouenne T, Brömme D, Pisabarro MT, et al. Antimicrobial Peptide LL-37 Is Both a Substrate of Cathepsins S and K and a Selective Inhibitor of Cathepsin L. Biochemistry. 2015;54:2785–2798.

Arbeev KG, Verhulst S, Steenstrup T, Kark JD, Bagley O, Kooperberg C, Reiner AP, Hwang SJ, Levy D, Fitzpatrick AL, et al. Association of leukocyte telomere length with mortality among adult participants in 3 longitudinal studies. JAMA Netw Open 2020;3: e200023.

Armanios M. Telomeres and age-related disease: how telomere biology informs clinical paradigms. J. Clin. Investig. 2013;123:996–1002.

Ascher K, Elliot SJ, Rubio GA, Glassberg MK. Lung diseases of the elderly: cellular mechanisms. Clin Geriat Med 2017; 33: 473-490.

Baker DJ, Childs BG, Durik M, et al. Naturally occurring p16Ink4a-positive cells shorten healthy lifespan. Nature 2016;530:184-189.

Baker C, Belbin O, Kalsheker N, Morgan K. SERPINA3 (aka alpha-1-antichymotrypsin) Front. Biosci. 2007;12:2821–2835.

Balbi B, Sangorgi C, Gnemmi I, Ferrarotti I, et al. Bacterial load and inflammatory response in sputum of alpha-1 antitrypsin deficiency patients with COPD. Int J Chron Obstruct Oukmon Dis 2019;14:1879-1893.

Balte PP, Chaves PHM, Couper DJ, et al. Association of Nonobstructive Chronic Bronchitis With Respiratory Health Outcomes in Adults. JAMA Intern Med 2020;180:676-86.

Bankier S, Wang L, Crawford A, Morgan RA, Ruusalepp A, Andrew R, Björkegren JLM, Walker BR, Michoel T. Plasma cortisol-linked gene networks in hepatic and adipose tissues implicate corticosteroid-binding globulin in modulating tissue glucocorticoid action and cardiovascular risk. Front. Endocrinol. 2023;14:1186252.

Barnes PJ. Oxidative Stress in Chronic Obstructive Pulmonary Disease. Antioxidants (Basel). 2022;11:965.

Barnes PJ, Baker J, Donnelly LE. Cellular senescence as a mechanism and target in chronic lung diseases. Am J Respir Crit Care Med 2019;200:556-564.

Barnes PJ, Burney PGJ, Silverman EK, Celli BR, Vestbo J, et al. Chronic obstructive pulmonary disease. Nat. Rev. Primers 2015;1:1–21.

Barnes PJ. Cellular and molecular mechanisms of asthma and COPD. Clin Sci (Lond) 2017;131:1541-1558.

Barnes PJ. Cellular and molecular mechanisms of chronic obstructive pulmonary disease. Clin Chest Med 2014;35:71-86.

Barnes PJ. Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. J. Allergy Clin. Immunol. 2013;131:636–645.

Barnes PJ. Oxidative stress-based therapeutics in COPD. Redox Biol 2020;33: 101544.

Barnes PJ. Mechanisms of development of multimorbidity in the elderly. Eur Respir J 2015;45:790-806.

Belchamber KBR, Singh R, Batista CM, Whyte MK, Dockrell DH, et al. Defective bacterial phagocytosis is associated with dysfunctional mitochondria in COPD macrophages. Eur Respir J 2019;54:1802244.

Birch J, Anderson RK, Correia-Melo C, et al.. DNA damage response at telomeres contributes to lung aging and chronic obstructive pulmonary disease. Am J Physiol Lung Cell Mol Physiol 2015;309:L1124-1137.

Birch J, Barnes PJ, Passos JF. Mitochondria, telomeres and cell senescence: Implications for lung ageing and disease. Pharmacol Ther 2018;183:34-49.

Birgisdottir AB, Johansen T. Autophagy and endocytosis - interconnections and interdependencies. J Cell Sci 2020;133: jcs228114.

Bocskei RM, Benczur B, Losonczy G, Illyes M, Cziraki A, Muller V, Bohács A, Bikov A. Soluble Urokinase-Type Plasminogen Activator Receptor and Arterial Stiffness in Patients with COPD. Lung. 2019;197:189–197.

Bootle-Wilbraham CA, Tazzyman S, Thompson WD, Stirk CM, Lewis CE. Fibrin fragment E stimulates the proliferation, migration and differentiation of human microvascular endothelial cells in vitro. Angiogenesis. 2001;4:269–275.

Bowdish DME. The aging lung: Is lung health good health for older adults? Chest 2019; 155: 391-400.

Brandsma CA, Van den Berge M, Hackett TL, Brusselle G, Timens W. Recent advances in chronic obstructive pulmonary disease pathogenesis: from disease mechanisms to precision medicine. J Pathol 2020;250:624-635.

Burney P, Jarvis D, Perez-Padilla R. The global burden of chronic respiratory disease in adults. Int J Tuberc Lung Dis 2015;19:10-20.

Byrne AJ, Powell JE, O'Sullivan BJ, Ogger PP, Hoffland A, Cook J, Bonner KL, Hewitt RJ, Wolf S, Ghai P, et al. Dynamics of human monocytes and airway macrophages during healthy aging and after transplant. J Exp Med 2020;217:e20191236.

Cannavò MF, Coppolino I, Monaco F, Caramori G. Overview of current management of COPD. In: Encyclopedia of Respiratory Medicine, 2nd edition. Elsevier,
London(2022)(https://www.sciencedirect.com/referencework/9780081027240/encyclopedia-of respiratory-medicine).

Caramori G, Casolari P, Adcock I. Role of transcription factors in the pathogenesis of asthma and COPD. Cell Commun Adhes 2013;20:21-40.

Caramori G, Casolari P, Barczyk A, Durham AL, Di Stefano A, Adcock I. COPD immunopathology. Semin Immunopathol 2016;38:497-515.

Caramori G, Coppolino I, Cannavò MF, Nucera F, Proietto A, Mumby S, Ruggeri R, Adcock IM. Transcription inhibitors and inflammatory cell activity. Curr Opin Pharmacol 2019;46:82-89.

Caramori G, Kirkham P, Barczyk A, Di Stefano A, Adcock I. Molecular pathogenesis of cigarette smoking-induced stable COPD. Ann N Y Acad Sci 2015;1340:55-64.

Carrell RW, Whisstock J, Lomas DA. Conformational changes in serpins and the mechanism of alpha 1-antitrypsin deficiency. Pt 2Am. J. Respir. Crit. Care Med. 1994;150:S171.

Chang C, Zhao W, Luo Y, Xi L, Chen S, Zhao C, Wang G, Guo J, Xu C. Serine peptidase inhibitor Kazal type I (SPINK1) promotes BRL-3A cell proliferation via p38, ERK, and JNK pathways. Cell Biochem. Funct. 2017;35:339–348.

Chen D, Liu J, Meng J, Li D, Zhao P, Duan Y, Wang J. Integrative analysis of long non-coding RNAs (lncRNAs), miRNAs, and mRNA-associated ceRNA network in lung tissue of aging mice and changes after treatment with codonopsis pilosula. Med Sci Monit 2020;26: e921580.

Chen S, Zhou Y, Chen Y, Gu J. fastp: An ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34:i884–i890.

Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. N. Engl. J. Med. 2013;368:1845–1846.

Corral J, Aznar J, Gonzalez-Conejero R, Villa P, Minano A, Vaya A, Carrell RW, Huntington JA, Vicente V. Homozygous deficiency of heparin cofactor II: Relevance of P17 glutamate residue in serpins, relationship with conformational diseases, and role in thrombosis. Circulation. 2004;110:1303–1307.

Correia-Melo C, Hewitt G, Passos JF. Telomeres, oxidative stress and inflammatory factors: partners in cellular senescence? Longev Healthspan 2014;3:1.

Costa-Machado LF, Martin-Hernandez R, Sanchez-Luengo MA et al. Sirt1 protects from K-Rasdriven lung carcinogenesis. EMBO Rep 2018;19.

Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. Nat Protoc. 2009;4(12):1798-806.

Desmedt S, Desmedt V, Delanghe JR, Speeckaert R, Speeckaert MM. The intriguing role of soluble urokinase receptor in inflammatory diseases. Crit. Rev. Clin. Lab. Sci. 2017;54:117–133.

Dickinson DP. Cysteine peptidases of mammals: Their biological roles and potential effects in the oral cavity and other tissues in health and disease. Crit. Rev. Oral Biol. Med. 2002;13:238–275.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A. 1995 Sep 26;92(20):9363-7.

Di Stefano A, Nucera F, Rosani U, Brun P, Gnemmi I, Maniscalco M, D'Anna SE, Leonardi A, Carriero V, Bertolini F, Freni J, Ieni A, Gangemi S, Ruggeri P, Ricciardolo FLM. Impaired SERPIN-Protease Balance in the Peripheral Lungs of Stable COPD Patients. Int J Mol Sci. 2025 Mar 21;26(7):2832.

Di Stefano A, Caramori G, Barczyk A, Vicari C, Brun P, Zanini A, Cappello F, Garofano E, Padovani A, Contoli M, et al. Innate immunity but not NLRP3 inflammasome activation correlates with severity of stable COPD. Thorax. 2014;69:516–524.

Dodson M, de la Vega MR, Cholanians AB, et al. Modulating NRF2 in Disease: Timing Is Everything. Annu. Rev. Pharmacol. Toxicol. 2019;59, 555-575.

Douglas RG, Galluzzi L, Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. Science 2011;333:1109-1112.

Eapen MS, McAlinden KD, Myers S, Lu W, Sohal SS. microRNAs are key regulators in chronic lung disease: exploring the vital link between disease progression and lung cancer. J Clin Med 2019;8:1986.

García-Nieto PE, Morrison AJ, Fraser HB. The Somatic mutation landscape of the human body. Genome Biol 2019;20:298.

Garth J, Easter M, Harris ES, Sailland J, Kuenzi L, Chung S, Dennis JS, Baumlin N, Adewale AT, Rowe SM, King G, Faul C, Barnes JW, Salathe M, Krick S. The effects of the anti-aging protein klotho on mucociliary clearance. Frot Med (Lausanne) 2020;6:339.

Global Initiative for Chronic Obstructive Lung Disease. Global Strategy for the Diagnosis, Management and Prevention of Chronic Obstructive Pulmonary Disease. NHLBI/WHO workshop report. Bethesda, National Heart, Lung and Blood Institute, April 2001; NIH Publication No 2701:1-100. Last update 2019. http://www.goldcopd.com.

Gong Z, Tasset I. Humanin enhances the cellular response to stress by activation of chaperone-mediated autophagy. Oncotarget 2018;9:10832–10833.

Hansel C, Jendrossek V, Klein D. Cellular senescence in the lung: the central role of senescent epithelial cells. Int J Mol Sci 2020;21:3279.

Hashimoto Y, Niikura T, Tajima H, Yasukawa T, Sudo H, Ito Y, Kita Y, Kawasumi M, Kouyama K, Doyu M, Sobue G, et al. A rescue factor abolishing neuronal cell death by a wide spectrum of familial Alzheimer's disease genes and Abeta. Proc. Natl. Acad. Sci. U. S. A. 2001;98:6336–6341.

He J, Dong L, Luo L, Wang K. Redox Regulation of Autophagy in Cancer: Mechanism, Prevention and Therapy. Life. 2022;13:98.

Hecker L. Mechanisms and consequences of oxidative stress in lung disease: therapeutic implications for an aging populace. Am J Physiol Lung Cell Mol Physiol 2018; 314: L642-l653.

Hodge G, Tran HB, Reynolds PN, Jersmann H, Hodge S. Lymphocyte senescence in COPD is associated with decreased sirtuin 1 expression in steroid resistant pro-inflammatory lymphocytes. Ther Adv Respir Dis 2020;14:1753466620905280.

Hodge G, Jersmann H, Tran HB, Roscioli E, Holmes M, et al. 2015. Lymphocyte senescence in COPD is associated with decreased histone deacetylase 2 expression by pro-inflammatory lymphocytes. Respir. Res. 2015;16:130.

Hodge G, Mukaro V, Reynolds PN, Hodge S. Role of increased CD8/CD28(null) T cells and alternative co-stimulatory molecules in chronic obstructive pulmonary disease. Clin Exp mmunol 2011;166:94-102.

Hoffmann RF, Zarrintan S, Brandenburg SM, Kol A, de Bruin HG, Jafari S, Dijk F, et al. Prolonged cigarette smoke exposure alters mitochondrial structure and function in airway epithelial cells. Respir Res 2013;14:97.

Hogg JC, Parè PD, Hackett TL. The contribution of small airway obstruction to the pathogenesis of chronic obstructive pulmonary disease. Physiol Rev 2017;97:529-552.

Hogg JC, McDonough JE, Sanchez PG, Cooper JD, Coxson HO, Elliott WM, Naiman D, Pochettino M, Horng D, Gefter WB, et al. Micro-computed tomography measurements of peripheral lung pathology in chronic obstructive pulmonary disease. Proc. Am. Thorac. Soc. 2009;6:546–549.

Höhn A, Jung T, Grimm S, Grune T. Lipofuscin-bound iron is a major intracellular source of oxidants: role in senescent cells. Free Radic Biol Med. 2010 Apr 15;48(8):1100-8.

Hou J, Sun Y. Role of regulatory T cells in disturbed immune homeostasis in patients with chronic obstructive pulmonary disease. Front Immunol 2020;11:723.

Houben JM, Mercken EM, Ketelslegers HB, Bast A,Wouters EF, et al. Telomere shortening in chronic obstructive pulmonary disease. Respir. Med. 2009;103:230–236.

Houssaini A, Breau M, Kebe K, et al. mTOR pathway activation drives lung cell senescence and emphysema. JCI Insight 2018;3.

Hughes MJ, McGettrick HM, Sapey E. Shared mechanisms of multimorbidity in COPD, atherosclerosis and type-2 diabetes: the neutrophil as a potential inflammatory target. Eur Respir Rev 2020;29:190102.

Idda ML, McClusky WG, Lodde V, Munk R, Abdelmohsen K, Rossi M, Gorospe M. Survey of senescent cell markers with age in human tissues. Aging (Albany NY) 2020;12:4052-4066.

Ieni A, Cardia R, Giuffre G, Rigoli L, Caruso RA, Tuccari G. Immunohistochemical Expression of Autophagy-Related Proteins in Advanced Tubular Gastric Adenocarcinomas and Its Implications. Cancers. 2019;11:389.

Ismail AA, Shaker BT, Bajou K. The Plasminogen-Activator Plasmin System in Physiological and Pathophysiological Angiogenesis. Int. J. Mol. Sci. 2021;23:337.

Ito K, Barnes PJ. COPD as a disease of accelerated lung aging. Chest 2009;135:173-180.

Jiang S, Sun J, Mohammadtursun N, Hu Z, et al. Dual role of autophagy/mitophagy in chronic obstructive pulmonary disease. Pulm Pharmacol Ther 2019;56:116-125.

Jayakumar A, Kang Y, Frederick MJ, Pak SC, Henderson Y, Holton PR, Mitsudo K, Silverman G, El-Naggar AK, Brömme D, et al. Inhibition of the cysteine

proteinases cathepsins K and L by the serpin headpin (SERPINB13): A kinetic analysis. Arch. Biochem. Biophys. 2003;409:367–374.

Janciauskiene S, Lechowicz U, Pelc M, Olejnicka B, Chorostowska-Wynimko J. Diagnostic and therapeutic value of human serpin family proteins. Biomed Pharmacother. 2024 Jun;175:116618.

Jung T, Bader N, Grune T. Lipofuscin: formation, distribution, and metabolic consequences. Ann N Y Acad Sci 2007;1119:97–111.

Kanayama M., He Y.W., Shinohara M.L. The lung is protected from spontaneous inflammation by autophagy in myeloid cells. J. Immunol. 2015;194:5465–5471.

Kang MJ. Recent advances in molecular basis of lung aging and its associated diseases. Tuberc Respir Dis (Seoul) 2020;83:107-115.

Katsuyuki T, Caramori G, Kazuhiro I, Lim S, Sano H, Tohda Y, Adcock IM, Barnes PJ. Telomere shortening in alveolar macrophages of smokers and COPD Patients. The Open Pathology Journal 2010;4:23-29.

Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. Immunol Lett. 2006;107:102-108.

Kelly-Robinson GA, Reihill JA, Lundy FT, McGarvey LP, Lockhart JC, Litherland GJ, Thornbury KD, Martin SL. The Serpin Superfamily and Their Role in the Regulation and Dysfunction of Serine Protease Activity in COPD and Other Chronic Lung Diseases. Int. J. Mol. Sci. 2021;22:6351.

Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu. Rev. Pharmacol. Toxicol. 2007;47:89–116.

Kim SJ, Mehta HH, Wan J, et al. Mitochondrial peptides modulate mitochondrial function during cellular senescence. Aging 2018;10:1239–1256.

Kirkham PA, Barnes PJ. Oxidative stress in COPD. Chest 2013;144:266–73.

Koo HJ, Do KH, Lee JB, Alblushi S, Lee SM. Lung cancer in combined pulmonary fibrosis and emphysema: a systematic review and meta-analysis. PLoS One 2016;11:e0161437.

Lee WH, Larsson SC, Wood A, Di Angelantonio E, Butterworth AS, Burgess S, Allara E. Genetically predicted plasma cortisol and common chronic diseases: A Mendelian randomization study. Clin. Endocrinol. 2024;100:238–244.

Lee J, Sandford AJ, Connett JE, Yan J, Mui T, et al. The relationship between telomere length and mortality in chronic obstructive pulmonary disease (COPD). PLOS ONE 2012;7:e35567.

Levra S, Rosani U, Gnemmi I, Brun P, Leonardi A, Carriero V, Bertolini F, Balbi B, Profita M, Ricciardolo FLM, Di Stefano A. Impaired autophagy in the lower airways and lung parenchyma in stable COPD. ERJ Open Res. 2023 Dec 18;9(6):00423-2023.

Livieri F, Albertini MC, Orciani M, Ceka A, Cricca M, Procopio AD, Bonafè M. DNA damage response (DDR) and senescence: shuttled inflamma-miRNAs on the stage of inflamm-aging. Oncotarget 2015;6:35509–35521.

Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell 2013;153: 1194-1217.

Malhotra D, Thimmulappa R, Navas-Acien A, Sandford A, Elliott M, et al. 2008. Decline in NRF2 regulated antioxidants in COPD lungs due to loss of its positive regulator DJ-1. Am. J. Respir. Crit. Care Med. 2008;178:592–604.

Markelic I, Hlapcic I, Ceri A, Radic Antolic M, Samarzija M, Popovic-Grle S, Dugac AV, Rumora L. Activation of NLRP3 inflammasome in stable chronic obstructive pulmonary disease. Sci. Rep. 2022;12:7544.

Martens S, Fracchiolla D. Activation and targeting of ATG8 protein lipidation. Cell Discov 2020;6:23.

Maruyama T, Noda NN. Autophagy-regulating protease Atg4: Structure, function, regulation and inhibition. J. Antibiot. 2017;71:72–78.

Masuda GO, Yashiro M, Kitayama K, Miki Y, Kasashima H, Kinoshita H, Morisaki T, Fukuoka T, Hasegawa T, Sakurai K, et al. Clinicopathological Correlations of Autophagy-related Proteins LC3, Beclin 1 and p62 in Gastric Cancer. Anticancer Res. 2016;36:129–136.

Mendelsohn AR, Larrick JW. Mitochondrial-derived peptides exacerbate senescence. Rejuvenation Res 2018;21:369-373.

Mercado N, Ito K, Barnes PJ. Accelerated ageing in chronic obstructive pulmonary disease: new concepts. Thorax 2015;70:482–89.

Mercado N, Thimmulappa R, Thomas CM, Fenwick PS, Chana KK, et al. 2011. Decreased histone deacetylase 2 impairs Nrf2 activation by oxidative stress. Biochem. Biophys. Res. Commun. 2011;406:292–98.

Meyer EJ, Nenke MA, Rankin W, Lewis JG, Torpy DJ. Corticosteroid-Binding Globulin: A Review of Basic and Clinical Advances. Horm. Metab. Res. 2016;48:359–371.

Mikawa R, Sato T, Suzuki Y, Baskoro H, Kawaguchi K, Sugimoto M. p19^{Arf} exacerbates cigarette smoke-induced pulmonary dysfunction. Biomolecules 2020;10:462.

Mizumura K, Cloonan SM, Haspel JA, Choi AM. The emerging importance of autophagy in pulmonary diseases. Chest 2012;142:1289–1299.

Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature 2008;451:1069–1075.

Monick MM, Powers LS, Walters K, Lovan N, Zhang M, et al. Identification of an autophagy defect in smokers' alveolar macrophages. J. Immunol. 2010;185:5425–5435.

Munoz-Espin D, Serrano M. Cellular senescence: from physiology to pathology. Nature Rev Mol Cell Biol 2014;15:482-496.

Murrow L, Debnath J. Autophagy as a stress-response and quality-controlmechanism: implications for cell injury and human disease. Annu. Rev. Pathol. Mech. Dis. 2013;8:105–137.

Nakamaru Y, Vuppusetty C, Wada H, et al. A protein deacetylase SIRT1 is a negative regulator of metalloproteinase-9. FASEB J 2009;23:2810-2819.

Nenke MA, Holmes M, Rankin W, Lewis JG, Torpy DJ. Corticosteroid-binding globulin cleavage is paradoxically reduced in alpha-1 antitrypsin deficiency: Implications for cortisol homeostasis. Clin. Chim. Acta. 2016;452:27–31.

Novinec M, Grass RN, Stark WJ, Turk V, Baici A, Lenarcic B. Interaction between human cathepsins K, L, and S and elastins: Mechanism of elastinolysis and inhibition by macromolecular inhibitors. J. Biol. Chem. 2007;282:7893–7902.

Nucera F, Di Stefano A, Ricciardolo FLM, Gnemmi I, Pizzimenti C, Monaco F, Tuccari G, Caramori G, Ieni A. Role of ATG4 Autophagy-Related Protein Family in the Lower Airways of Patients with Stable COPD. Int. J. Mol. Sci. 2024;25:8182.

Nucera F, Mumby S, Paudel KR, Dharwal V, Di Stefano A, Casolaro V, Hansbro PM, Adcock IM, Caramori G. Role of oxidative stress in the pathogenesis of COPD. Minerva Med. 2022; Online ahead of print.

Osiewacz HD, Bernhardt D. Mitochondrial quality control: impact on aging and life span – a mini-review. Gerontology 2013;59:413-420.

Osoata GO, Hanazawa T, Brindicci C, Ito M, Barnes PJ, et al. Peroxynitrite elevation in exhaled breath condensate of COPD and its inhibition by fudosteine. Chest 2009;135:1513–20.

Park SJ, Lim W, Mun J, Paik H, Park S, Lim H, Kim J, Lee EJ, Yim GW, Lee N, et al. SERPINB11 Expression Is Associated With Prognosis of High-grade Serous and Clear Cell Carcinoma of the Ovary. In Vivo. 2021;35:2647–2653.

Paschalaki K, Zampetaki A, Baker J,et al. Downregulation of microRNA-126 augments DNA damage response in cigarette smokers and COPD patients. Am J Respir Crit Care Med 2018;197:665-668.

Pehote G, Bodas M, Brucia K, Vij N. Cigarette Smoke Exposure Inhibits Bacterial Killing via TFEB-Mediated Autophagy Impairment and Resulting Phagocytosis Defect. Mediat. Inflamm. 2017;2017:3028082.

Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference rna targets in normal and cancerous human solid tissues. RNA 2008;14:844-852.

Perez-Perez ME, Lemaire SD, Crespo JL. The ATG4 protease integrates redox and stress signals to regulate autophagy. J Exp Bot 2021;72:3340-51.

Perrotta F, D'Agnano V, Scialo F, Komici K, Allocca V, Nucera F, Salvi R, Stella GM, Bianco A. Evolving concepts in COPD and lung cancer: A narrative review. Minerva Med. 2022;113:436–448.

Podolsky MJ, Yang CD, Valenzuela CL, Datta R, Huang SK, Nishimura SL, Dallas SL, et al. Age-dependent regulation of cell-mediated collagen turnover. JCI Insight 2020;5: e137519.

Psarras S, Caramori G, Contoli M, Papadopoulos N, Papi A. Oxidants in asthma and in chronic obstructive pulmonary disease (COPD). Curr Pharm Des 2005;11:2053-2062.

Racanelli AC, Choi AMK, Choi ME. Autophagy in chronic lung disease. Prog. Mol. Biol. Transl. Sci. 2020;172:135–156

Racanelli AC, Kikkers SA, Choi AMK, Cloonan SM. Autophagy and inflammation in chronic respiratory disease. Autophagy 2018;14:221-32.

Rezwan FI, Imboden M, Amaral AFS, Wielscher M, Jeong A, Triebner K, Gómez Real F, Jarvelin MR, Jarvis D, Probst-Hensch NM, Holloway JW. Association of adult lung function with accelerated biological aging. Aging (Albany NY) 2020;12:518-542.

Rutten EP, Gopal P, Wouters EF, et al. Various Mechanistic Pathways Representing the Aging Process Are Altered in COPD. Chest 2016;149:53-61.

Sabbieti MG, Marchegiani A, Sufianov AA, Gabai VL, Shneider A, Agas D. P62/SQSTM1 beyond Autophagy: Physiological Role and Therapeutic Applications in Laboratory and Domestic Animals. Life (Basel). 2022;12(4):539.

Sabit R, Thomas P, Shale DJ, Collins P, Linnane SJ. The effects of hypoxia on markers of coagulation and systemic inflammation in patients with COPD. Chest. 2010;138:47–51.

Sadighi Akha AA. Aging and the immune system: An overview. J Immunol Meth 2018;463:21-26.

Salama R, Sadaie M, Hoare M, Narita M. Cellular senescence and its effector programs. Genes Dev 2014;28:99-114.

Savale L, Chaouat A, Bastuji-Garin S, Marcos E, Boyer L, et al. Shortened telomeres in circulating leukocytes of patients with chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 2009;179:566–71.

Schulte H, Mühlfeld C, Brandenberger C. Age-related structural and functional changes in the mouse lung. Front Physiol 2019;10:1466.

Schick C, Pemberton PA, Shi GP, Kamachi Y, Cataltepe S, Bartuski AJ, Gornstein ER, Brömme D, Chapman AHA, Silverman GA. Cross-class inhibition of the cysteine proteinases cathepsins K, L, and S by the serpin squamous cell carcinoma antigen 1: A kinetic analysis. Biochemistry. 1998;37:5258–5266.

Seixas S, Ivanova N, Ferreira Z, Rocha J, Victor BL. Loss and gain of function in SERPINB11: An example of a gene under selection on standing variation, with implications for host-pathogen interactions. PLoS ONE. 2012;7:e32518.

Shaw AC, Goldstein DR, Montgomery RR. Age-dependent dysregulation of innate immunity. Nat. Rev. Immunol. 2013;13:875–87.

Silverman G.A., Whisstock J.C., Askew D.J., Pak S.C., Luke C.J., Cataltepe S., Irving J., Bird P. Human clade B serpins (ov-serpins) belong to a cohort of evolutionarily dispersed intracellular proteinase inhibitor clades that protect cells from promiscuous proteolysis. Cell Mol. Life Sci. 2004;61:301–325.

Singh CK, Chhabra G, Ndiaye MA, Garcia-Peterson LM, Mack NJ, Ahmad N. The Role of Sirtuins in Antioxidant and Redox Signaling. Antioxid Redox Signal 2018;28:643-61.

Sorheim IC, DeMeo DL, Washko G, Litonjua A, Sparrow D, et al. Polymorphisms in the superoxide dismutase-3 gene are associated with emphysema in COPD. COPD 2010;7:262–68.

Stasinopoulos S, Mariasegaram M, Gafforini C, Nagamine Y, Medcalf RL. The plasminogen activator inhibitor 2 transcript is destabilized via a multi-component 3’ UTR localized adenylate and uridylate-rich instability element in an analogous manner to cytokines and oncogenes. FEBS J. 2010;277:1331–1344.

Strnad P, McElvaney NG, Lomas DA. Alpha 1-antitrypsin deficiency: N Engl J Med 2020;382:1443-1455.

Sun Y, Sheshadri N, Zong WX. SERPINB3 and B4: From biochemistry to biology. Semin. Cell Dev. Biol. 2017;62:170–177.

Sureshbabu A, Bhandari V. Targeting mitochondrial dysfunction in lung diseases: emphasis on mitophagy. Front. Physiol. 2013;4:384.

Suzuki M, Sze MA, Campbell JD et al. The cellular and molecular determinants of emphysematous destruction in COPD, Sci Rep 2017;7:9562.

Taggart CC, Lowe GJ, Greene CM, Mulgrew AT, O'Neill SJ, Levine RL, McElvaney NG. Cathepsin B, L, and S cleave and inactivate secretory leucoprotease inhibitor. J. Biol. Chem. 2001;276:33345–33352.

Takahashi A, Ohtani N, Yamakoshi K, Iida S, Tahara H, Nakayama K, Nakayama KI, Ide T, Saya H, Hara E. Mitogenic signalling and the p16INK4a-Rb pathway cooperate to enforce irreversible cellular senescence. Nature Cell Biol 2006;8:1291-1297.

Takahashi H, Ishidoh K, Muno D, Ohwada A, Nukiwa T, Kominami E, Kira S. Cathepsin L activity is increased in alveolar macrophages and bronchoalveolar lavage fluid of smokers. Pt 1Am. Rev. Respir. Dis. 1993;147:1562–1568.

Tanno A, Fujino N, Yamada M, Sugiura H, Hirano T, Tanaka R, Sano H, Suzuki S, Okada Y, Ichinose M. Decreased expression of a phagocytic receptor Siglec-1 on alveolar macrophages in chronic obstructive pulmonary disease. Respir Res 2020;21:30.

Taraseviciene-Stewart L, Voelkel NF. Oxidative stress-induced antibodies to carbonyl-modified protein correlate with severity of chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 2012;185:1026.

Tchkonia T, Zhu Y, van Deursen J, Campisi J, Kirkland JL. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. J Clin Invest 2013;123:966-972.
therapeutics. J Clin Invest 2013; 123: 980-989.

To M, Takagi D, Akashi K, Kano I, Haruki K, Barnes PJ, Ito K. Sputum PAI-1 elevation by oxidative stress-dependent NF-kappaB activation in chronic obstructive pulmonary disease. Chest 2013;144:515-521.

To Y, Ito K, Kizawa Y, Failla M, et al. Targeting phosphoinositide-3-kinase-delta with theophylline reverses corticosteroid insensitivity in chronic obstructive pulmonary disease. Am J Resp Crit Care Med 2010; 182:897-904.

Tomita K, Caramori G, Lim S, et al. Increased p21CIP1/WAF1 and B cell lymphoma leukemiaxL expression and reduced apoptosis in alveolar macrophages from smokers. Am J Respir Crit Care Med 2002;166:724-731.

Tsuji T, Aoshiba K, Nagai A. Alveolar cell senescence in patients with pulmonary emphysema. Am J Respir Crit Care Med 2006;174:886-893.

US burden of disease collaborators, Mokdad AH, Ballestros K, Echko M, et al. The State of US Health, 1990-2016: burden of diseases, injuries, and risk factors among US States. JAMA 2018;319:1444-1472.

Vallese D, Ricciardolo FL, Gnemmi I, et al. Phospho-p38 MAPK expression in COPD patients and asthmatics and in challenged bronchial epithelium. Respiration 2015;89:329-42.

van Deursen JM. The role of senescent cells in ageing. Nature 2014;509:439-446.

van Zoelen MAD, Schouten M, de Vos AF, Florquin S, Meijers JCM, Nawroth PP, Bierhaus A, van der Poll T. The receptor for advanced glycation end products impairs host defense in pneumococcal pneumonia. J. Immunol. 2009;182:4349–4356.

Vasilescu DM, Hackett TL, Martinez FJ, Curtis JL, Hogg JC, Han MK. Reply to Janssen and Wouters: Loss of Alveolar Attachments as a Pathomechanistic Link between Small Airway Disease and Emphysema. Am. J. Respir. Crit. Care Med. 2020;201:879–880.

Vestbo J, Hurd SS, Agusti AG, Jones PW, Vogelmeier C, Anzueto A, Barnes PJ, Fabbri LM, Martinez FJ, Nishimura M, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am. J. Respir. Crit. Care Med. 2013;187:347–365.

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.

Watz H, Barnacle H, Hartley BF, Chan R. Efficacy and safety of the p38 MAPK inhibitor losmapimod for patients with chronic obstructive pulmonary disease: a randomised, double-blind, placebo-controlled trial. Lancet Respir Med 2014;2:63-72.

Weng T, Karmouty-Quintana H. Crystal deposits in macrophages and distal lung remodeling: a tale of aging in SFTPC-deficient mice. Am J Respir Cell Mol Biol 2020;62:405-406.

Worby CA, Dixon JE. PTEN. Annu Rev Biochem 2014;83:641-669.

Wu X, Huang Q, Javed R, Zhong J, Gao H, Liang H. Effect of tobacco smoking on the epigenetic age of human respiratory organs. Clin Epigenetics 2019;11:183.

Xia F, Liu P, Li M. The regulatory factors and pathological roles of autophagy-related protein 4 in diverse diseases: Recent research advances. Med. Res. Rev. 2021;41:1644–1675.

Xia S, Zhou C, Kalionis B, Shuang X, Ge H, Gao W. Mesenchymal stem cell treatment: a possible therapeutic direction in elderly patients with chronic obstructive pulmonary disease. Aging Dis 2020;11:129-140.

Yamagishi Y, Hashimoto Y, Niikura T, Nishimoto I. Identification of essential amino acids in Humanin, a neuroprotective factor against Alzheimer's disease-relevant insults. Peptides 2003;24:585–595.

Yanagi S, Tsubouchi H, Miura A, Matsuo A, Matsumoto N, Nakazato M. The impacts of cellular senescence in elderly pneumonia and in age-related lung diseases that increase the risk of respiratory infections. Int J Mol Sci 2017; 18.

Yousefzadeh MJ, Zhao J, Bukata C, Wade EA, McGowan SJ, Angelini LA, Bank MP, Gurkar AU, McGuckian CA, Calubag MF, Kato JI, Burd CE, Robbins PD, Niedernhofer LJ. Tissue specificity of senescent cell accumulation during physiologic and accelerated aging of mice. Aging Cell 2020;19:e13094.

Yuan F, Lu L, Zou Q. Analysis of gene expression profiles of lung cancer subtypes with machine learning algorithms. Biochim. Biophys. Acta Mol. Basis Dis. 2020;1866:165822

Zhang S, Li X, Ma H, Zhu M, Zhou Y, Zhang Q, Peng H. Relationship between Antithrombin III Activity and Mortality in Patients with Acute Exacerbation of Chronic Obstructive Pulmonary Disease. COPD. 2022;19:353–364.

Zhao K, Dong R, Yu Y, Tu C, Li Y, Cui Y, Bao L, Ling C. Cigarette smoke-induced lung inflammation in COPD mediated via CCR1/JAK/STAT /NF-κB pathway. Aging (Albany NY) 2020;12:9125-9138.

Zheng S,Wang C, Qian G, Wu G, Guo R, et al. Role of mtDNA haplogroups in COPD susceptibility in a southwestern Han Chinese population. Free Rad. Biol. Med. 2012;53:473–81

Tables

Table 1

List of autophagic proteins and their effect on the autophagosome

Autophagic proteins	
ATG3	Involved in the elongation of the phagophore membrane
ATG4 (-A, -B, -C, -D)	Involved in the conversion and recycling of LC3-I (inactive form) into LC3-II (active form) and vice versa
ATG5	Required for elongation of the phagophore membrane
ATG7	Required for elongation of the phagophore membrane
ATG8 (LC3 o MAP1LC3)	Promotes the elongation of the phagophore membrane until the formation of the autophagosome, which is crucial for union with the lysosome

ATG9A	Located on vesicles coming from the Golgi apparatus
ATG12	Mediates the elongation of the phagophore membrane
ATG13	Component of the ULK complex
ATG14	Component of the PI3KC3-C1 complex, it is crucial for the formation of the phagophore and promotes the fusion of the autophagosome with lysosomes
ATG16L1	Mediates the elongation of the phagophore membrane
ATG10	Component of the ULK complex
Beclin 1	Component of the PI3KC3-C1 complex fundamental for the formation of the phagophore
FIP200 (or RB1CC1)	Component of the ULK complex
PIK3C3 (VPS34)	Component of PI3KC3-C1 fundamental for the formation of the phagophore
PIK3R4 (subunit p150 or VPS15)	Component of PI3KC3-C1 fundamental for the formation of the phagophore
ULK	Complex with serine/threonine kinase activity essential for the formation of the phagophore

UVRAG	Component of PI3KC3-C2 involved in the maturation of the autophagosome and its fusion with lysosomes
WIPI2	Recruits ATG16L1 to specific sites of the ATG8 conjugation system

Data obtained from (Birgisdottir 2020, Sabietti 2002, Martens 2020, Perez-Perez 2021, Racanelli 2018).

ATG: autophagy protein; PI3K: phosphatidyl-inositol-3-phosphate; PI3KC3-C1: type III PI3K complex 1; FIP200: 200 kDa protein interacting with the FAK family, ULK: Unc-51-like kinase, UVRAG: UV radiation resistance associated; WIPI2: PI3P effector WD repeat domain phosphoinositide interacting protein

Table 2. Inhibitory SERPINS

SERPIN	**Clade**	**Localization**	**Biological function and associated disease**
SERPINA1	A	Extracellular	Inhibitor of neutrophil elastase and other serine, cysteine, and metalloproteases. Risk for lung and/or liver diseases due to AAT deficiency
SERPINA2	A	Extracellular	Inhibitor of serine-type peptidase. Associated with emphysema.
SERPINA3	A	Extracellular	Inhibitor of chymotrypsin, cathepsin G, chymases, kallikreins 2 and 3, and pancreatic cationic elastase. Associated with lung and liver diseases, Parkinson's and Alzheimer's diseases, cancer, and

			atherosclerosis; potential biomarker of renal fibrosis and inflammation.
SERPINA4	A	Extracellular	Inhibitor of tissue kallikrein. Associated with abdominal aortic aneurysm; diagnostic marker for chronic liver diseases
SERPINA5	A	Extracellular	Inhibitor of activated protein C factor, coagulation factors and fibrinolytic enzymes; inactivates serine proteases implicated in the reproductive system; binds retinoic acid and plays a role as a hormone carrier. Inhibits u-PA-dependent tumor cell invasion and metastasis; associated with angioedema.
SERPINA9	A	Extracellular	Responsible for maintenance of naive B cells; enables serine-type endopeptidase inhibitor activity. Predictive factor of CD5-positive diffuse large B-cell lymphoma (CD5+ DLBCL).
SERPINA10	A	Extracellular	Inhibitor of coagulation factors Xa and Xia. Deficiency is linked to venous thromboembolism.
SERPINA11	A	Extracellular	Suppressor of hepatocellular carcinoma; predicted to be involved in a negative regulation of endopeptidase activity; not well characterized.
SERPINA12	A	Extracellular	Involved in lipid metabolism, gluconeogenesis and regulation of endopeptidase; exerts anti-

			inflammatory effects. Associated with cardiovascular, metabolic, endocrine, and neoplastic diseases.
SERPINA13	A	Extracellular	Predicted to be involved in a negative regulation of endopeptidase activity. Altered expression in brain of patients with Parkinson's disease.
SERPINB1	B	Intracellular	Inhibitor of serine elastase-like and chymotrypsin-like proteases. Involved in inflammatory process and the regulation of the innate immune response. Promotes beta-cell proliferation via its protease inhibitory function.
SERPINB2	B	Intracellular	Major intracellular inhibitor of tPA and uPA, affecting fibrinolysis.
SERPINB3	B	Intracellular	Inhibitor of cysteine proteases cathepsin K, L, S and papain. Involved in autocrine, paracrine signalling and the regulation of metabolic processes.
SERPINB4	B	Intracellular	Inhibitor of cathepsin G, mast cell chymase and granzyme M. Highly expressed in many tumor cells. Associated with chronic inflammation and psoriasis.
SERPINB6	B	Intracellular	Inhibitor of cathepsin G, kallikrein-8 and thrombin; co-localizes with cathepsin G in monocytes and granulocytes. Associated with non-syndromic progressive hearing loss.

SERPINB7	B	Intracellular	Inhibitor of lysine-specific proteases; involved in megakaryocyte maturation and is increased in IgA nephropathy. Mutations cause palmoplantar keratoderma, Nagashima
SERPINB8	B	Intracellular	Inhibitor of furin and is involved in platelet functions, epithelial desmosome-mediated cell-cell adhesion and cell-cell adhesion in the skin. Its defects are associated with an autosomal-recessive form of exfoliative ichthyosis
SERPINB9	B	Intracellular	Inhibitor of granzyme B, stored in granules within cytotoxic lymphocytes and natural killer cells. The overexpression may prevent cytotoxic T-lymphocytes from eliminating tumor cells.
SERPINB10	B	Intracellular	Inhibitor of trypsin and thrombin. Regulates the expression of chemokine C-C motif ligand 26 in allergic airway eosinophilic inflammation; may increase a risk of prostate cancer.
SERPINB11	B	Intracellular	Inhibitor of endopeptidase activity. Involved in kidney impairment and liver malfunction (ischemia, hemolysis, and endothelial damage in the hepatic vessels).
SERPINB12	B	Intracellular	Inhibitor of trypsin, hepsin and plasmin; part of collagen-containing

			extracellular matrix; predicted to act upstream of or within hematopoietic progenitor cell differentiation; plays a role in cell differentiation and epithelial cell protection.
SERPINB13	B	Intracellular	Inhibitor of cathepsins L and K; plays a role in the proliferation or differentiation of keratinocytes. Downregulated in many types of cancer.
SERPINC1	C	Extracellular	Inhibitor of thrombin and factor Xa (FXa), IXa, XIa, XIIa, PKa, tissue plasminogen activator, urokinase, and plasmin matriptase-3/TMPRSS7; its inhibitory activity is enhanced by heparin. Low ATIII levels are related with a risk of deep vein thrombosis, pulmonary embolism and ischemic stroke
SERPIND1	D	Extracellular	Inhibitor of thrombin and chymotrypsin; peptides at the N-terminal of HC-II have chemotactic activity for monocytes and neutrophils. Prevents excessive blood clotting
SERPINE1	E	Extracellular	Inhibitor of plasminogen activation and thrombin. Associated with abnormal bleeding and idiopathic pulmonary arterial hypertension
SERPINE2	E	Extracellular	Inhibitor of trypsin-like serine proteases, trypsin, thrombin, urokinase, plasminogen activators

			and plasmin; present in platelet granules; expresses strong antithrombotic and antifibrinolytic properties. Associated with emphysema, thoracic aortic aneurysms and atherosclerosis
SERPINE3	E	Extracellular	Inhibitor of multiple proteases; not well characterized. Influences age-related diseases and human eye phenotypes; candidate biomarker of breast cancer progression.
SERPINF2	F	Extracellular	Inhibitor of plasmin and trypsin, inactivates matriptase-3/TMPRSS7 and chymotrypsin. Mutations result in inhibitor deficiency characterized by severe hemorrhagic diathesis
SERPING1	G	Extracellular	Inhibitor of C1s and C1r from the classical complement pathway, plasma kallikrein and activated factor XII. Associated with angioedema and complement component 4 deficiency
SERPINI1	I	Extracellular	Inhibitor of tPA, uPA and plasmin; plays a role in the regulation of axonal growth, synaptic plasticity, and neuron protection. Associated with neurological diseases, familial encephalopathy with neuroserpin inclusion bodies (FENIB)
SERPINI2	I	Extracellular	Inhibitor of cancer metastasis, coagulation, fibrinolysis, development, and inflammation.

			Downregulated during pancreatic carcinogenesis.

Abbreviations used in the table: AMPK - 5 ′ AMP-activated protein kinase; COPD - chronic obstructive pulmonary disease; NE – neutrophil elastase; NF-κB - nuclear factor kappa B; u-PA - urokinase-type plasminogen activator; tPA - tissue-type plasminogen activator
Data obtained: Janciauskiene 2024

Table 3. Non-inhibitory SERPINS

SERPIN	**Clade**	**Localization**	**Biological function and associated disease**
SERPINA6	A	Extracellular	Major transporter for blood glucocorticoids and progestins; acts as an acute phase “negative” protein. Linked to Corticosteroid-binding Globulin Deficiency
SERPINA7	A	Extracellular	Major thyroid hormone transport protein; binds and transports triiodothyronine (T3) and thyroxine (T4). Associated with thyroxine-binding globulin deficiency
SERPINA8	A	Extracellular	Precursor of angiotensin peptides in the renin-angiotensin system, pivotal protein in blood pressure regulation. Linked to hypertension and renal tubular dysgenesis
SERPINB5	B	Intracellular	Enables serine-type endopeptidase inhibitor activity; involved in extracellular matrix organization and epithelial cell proliferation. Acts as a

			tumor suppressor by inhibiting cancer cell migration, invasion, and angiogenesis; biomarker of hepatocellular carcinoma.
SERPINF1	F	Extracellular	Exhibits tumor suppressive properties, angiostatic activity and neurotrophic capabilities. Increased in the lungs of IPF patients; associated with angiogenesis and congenital osteogenesis imperfecta
SERPINH1	H	Intracellular	Enhances effects of heparin on thrombin, involved in blood clot regulation and collagen biosynthesis. Associated with osteogenesis imperfecta and preterm birth caused by premature rupture of membranes. May play a role in rheumatoid arthritis, cancer, and aortic stenosis/insufficiency.

Abbreviations used in the table: IPF - Idiopathic Pulmonary Fibrosis.
Data obtained: Janciauskiene 2024

Table 4. Clinical characteristics of subjects involved in the study

Groups	**Control non-Smokers**	**Control Smokers**	**Patients with COPD**
N°	**12**	**14**	**13**
Age (y)	**66**±4.6	**67**±1.6	**70**±1.7
M/F	**5/7**	**9/5**	**11/2**
Ex/Current Smokers	**---**	**9/5**	**10/3**
Pack Years	**---**	**37**±4.8	**54**±11

FEV_1(% pred) pre-β2	**113**±4.2	**97**±2.9	**73**±4.7#
FEV_1(% pred) post-β2	**ND**	**ND**	**82**±5.2
FEV_1/FVC (%) pre-β2	**81**±1.4	**73**±1.0	**58**±2.4#
TLC (%)	**105.2**±3.5	**102.7**±2.1	**114.6**±3.9*
RV (%)	**109.0**±6.5	**114.6**±3.7	**150.7**±8.7#
DLCO (%)	**ND**	**90.4**±3.6	**80.6**±5.5

Data expressed as mean±SE. Patients with chronic obstructive pulmonary disease (COPD) were classified according to GOLD 2024 (goldcopd.org) grades of severity using only the severity of airflow obstruction. For COPD patients FEV_1/FVC (%) are post-bronchodilator values. Abbreviations: M, male; F, female, FEV_1: 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; *, $p<0.010$ significantly different from control smokers with normal lung function. TLC%, RV%, DLCO% all values at baseline.

Table 5. Characteristics of primary antibodies utilized to identify autophagy signaling pathway components in the peripheral lung

Primary antibody specificity	**Company manufacturer**	**Catalogue number**	**Source/ Host**	**Working Dilution**	**Positive control**
ATG2A	Atlas	HPA038715	Rabbit	1:700	Tubular gastric adenocarcinomas
ATG2B	Atlas	HPA001427	Rabbit	1:50	Tubular gastric adenocarcinomas
ATG3	Atlas	HPA040471	Rabbit	1:300	Tubular gastric adenocarcinomas
ATG4A	Atlas	HPA036374	Rabbit	1:300	Tubular gastric adenocarcinomas

ATG4B	Invitrogen	PA5-30462	Rabbit	1:500	Tubular gastric adenocarcinomas
ATG4C	Atlas	HPA007049	Rabbit	1:50	Tubular gastric adenocarcinomas
ATG4D	Atlas	HPA067683	Rabbit	1:50	Tubular gastric adenocarcinomas
ATG5	Atlas	HPA042973	Rabbit	1:100	Tubular gastric adenocarcinomas
ATG7	Atlas	HPA007639	Rabbit	1:300	Tubular gastric adenocarcinomas
ATG10	Atlas	HPA044163	Rabbit	1:100	Tubular gastric adenocarcinomas
ATG12	Invitrogen	PA5-32180	Rabbit	1:500	Tubular gastric adenocarcinomas
ATG14 (Thr429)	Invitrogen	PA5-105670	Rabbit	1:200	Tubular gastric adenocarcinomas
ATG14	Invitrogen	PA5-78833	Mouse	1:500	Tubular gastric adenocarcinomas
ATG16L	Atlas	HPA063900	Rabbit	1.300	Tubular gastric adenocarcinomas

Table 6 Primary antibodies and immunohistochemical conditions used for identification of Serpins signaling proteins in peripheral lung

Target	**Supplier**	Cat.#[a]	**Source**	**Dilution**	**Positive control**
Serpin A3	ThermoFisher	PA5-120834	Rabbit	1:800	Nasal polyp, human tonsil

Serpin A6	LSBio	LS-B10686	Rabbit	1:50	Nasal polyp, Human tonsil
Serpin B2 (PAI-2)	ThermoFisher	PA5-27857	Rabbit	1:200	Nasal polyp, human tonsil
Serpin B3	ThermoFisher	PA5-30164	Rabbit	1:200	Nasal polyp, human tonsil
Serpin B5	LSBio	LS-C164042	Rabbit	1:50	Nasal polyp, human tonsil
Serpin B11	LSBio	LS-C770539	Rabbit	1:50	Nasal polyp, human tonsil
Serpin B13	BiossAntibodies	BS-8324R	Rabbit	1:100	Nasal polyp, human tonsil
Serpin C1	ThermoFisher	PA5-13674	Rabbit	1:100	Nasal polyp, human tonsil
Serpin D1	Santa Cruz	SC-69784	Mouse	1:500	Nasal polyp, Human tonsil

SPINK1	BiossAntibodies	BS-2916R	Rabbit	1:50	Nasal polyp, Human tonsil
PLAUR (UPAR)	BiossAntibodies	BS-1927R	Rabbit	1:100	Nasal polyp, Human tonsil
tPA	ThermoFisher	PA5-95629	Rabbit	1:250	Nasal polyp, Human tonsil
Cathepsin K	ThermoFisher	PA5-102483	Rabbit	1:100	Nasal polyp, Human tonsil
Cathepsin L	ThermoFisher	PA5-119012	Rabbit	1:100	Nasal polyp, Human tonsil
Caspase 1	ThermoFisher	MA5-16215	Mouse	1:100	Nasal polyp, Human tonsil

[a]Cat#, catalogue number

Table 7 Immunohistochemical quantification of autophagic molecules in the peripheral lung of patients with COPD, in control smokers and non-smoking subjects

Localization	Control non smokers with normal lung function	Control smokers with normal lung function	COPD patients	Kruskal Wallis (p value)
Bronchiolar epithelium (score 0-6)				
ATG4A	1.6 (0-4)	0.90 (0-3)	3.5 (2-6) #*	0.0047
ATG4D	1 (0-3)	1.6 (0-6)	3.2 (1-6) #*	0.018
ATG5	1.2 (0-3)	1.1 (0-4)	3.1 (1-6) #*	0.019
Alveolar septa (score 0-6)				
ATG4A	1.4 (0-3)	1.7 (0-6)	4.5 (2-6) #*	0.0036
ATG4B	1.9 (0-4)	1.1 (0-6)	4 (3-6) #*	0.0054
ATG4C	1.1 (0-3)	1 (0-4)	3.5 (2-6) #*	0.0064
ATG4D	1.4 (0-3)	1.7 (0-6)	4 (2-6) #*	0.0084
ATG5	1.4 (0-4)	1.1 (0-4)	3.7 (1-6) #*	0.0088
ATG7	2 (0-4)	1.5 (0-6)	4.2 (1-6) #*	0.018

Scored (0-6) data expressed as mean value and range; Mann Whitney U test: * significantly different from control non smokers (CNS); # significantly different from control smokers (CS); NE=not evaluable.

Table 8.

Gene ID	Database object name	CS vs CNS		CS vs COPD		COPD vs CNS		CNS_mean	COPD_mean	CS_mean
		Log FC	P-value	Log FC	P-value	Log FC	P-value			
CASP1	Caspase-1	0,09	0,74	0,13	0,61	-0,04	0,86	42,58	41,22	46,58
CASP10	Caspase-10	0,12	0,63	0,01	0,97	0,11	0,63	18,55	20,06	20,96
CASP4	Caspase-4	-0,02	0,94	-0,22	0,35	0,2	0,36	75,4	88,35	78,78
CASP8	Caspase-8	0,27	0,33	0,18	0,5	0,09	0,73	25,31	26,88	31,21
CEBPA	CCAAT/enhancer-binding protein alpha	-0,23	0,57	0,3	0,43	-0,53	0,15	34,23	23,41	29,13
CELA1	Chymotrypsin-like elastase family member 1	1,29	0,37	-1,26	0,29	2,55	0,04	0,03	0,25	0,11
CELA2A	Chymotrypsin-like elastase family member 2A	1,08	0,45	-0,54	0,67	1,62	0,21	0,06	0,21	0,14
CELA2B	Chymotrypsin-like elastase family member 2B	-0,57	0,63	-1,7	0,14	1,13	0,28	0,44	0,87	0,27
CMA1	Chymase	-1,14	0,3	-1,13	0,3	-6,37E-03	0,99	0,55	0,49	0,23
CTRC	Chymotrypsin-C	-3,34	0,03	-0,96	0,56	-2,38	0,04	0,41	0,06	0,03
CTRL	Chymotrypsin-like protease CTRL-1	0,56	0,49	0,48	0,54	0,08	0,91	1,08	1,23	1,7
CTSG	Cathepsin G	-0,26	0,68	-1	0,1	0,74	0,2	3,3	5,61	2,94
CTSK	Cathepsin K	-0,47	0,31	-0,84	0,06	0,37	0,39	22,89	28,86	17,14
CTSL	Cathepsin L1	0,07	0,83	0,26	0,4	-0,19	0,52	110,18	96,18	118,21
CTSS	Cathepsin S	0,14	0,64	-0,15	0,61	0,3	0,3	277,8	341,1	308,45
ELANE	Neutrophil elastase	-0,84	0,37	-0,64	0,48	-0,19	0,82	0,63	0,53	0,37
F2R	Proteinase-activated receptor 1	-0,36	0,26	-0,52	0,09	0,17	0,57	44,98	49,3	35,67
F2RL1	Proteinase-activated receptor 2	-0,05	0,94	0,1	0,88	-0,14	0,8	1,68	1,48	1,65
GZMB	Granzyme B	0,02	0,97	-0,49	0,25	0,51	0,21	19,56	27,8	20,26

KLK2	Kallikrein-2	2,57	0,1	1,95	0,17	0,62	0,71	0,02	0,04	0,26
KLK3	Prostate-specific antigen	-0,04	0,99	-2,55	0,23	2,5	0,16	0	0,05	0
NPM2	Nucleoplasmin-2	1,7	0,03	-0,08	0,92	1,78	0,01	1,1	4,14	3,95
PLAT	Tissue-type plasminogen activator	0,01	0,97	-0,1	0,77	0,11	0,72	40,65	44,96	42,86
PLAU	Urokinase-type plasminogen activator	-0,38	0,28	0,23	0,5	-0,61	0,06	13,63	9,09	10,69
PLAUR	Urokinase plasminogen activator surface receptor	-0,2	0,62	0,2	0,61	-0,39	0,28	108,44	85,95	100,61
SERPINA1	Alpha-1-antitrypsin	0,51	0,14	0,3	0,36	0,21	0,5	126,05	145,51	185,77
SERPINA10	Protein Z-dependent protease inhibitor	NaN	NaN	NaN	NaN	NaN	NaN	0	0	0
SERPINA11	Serpin A11	2,19	0,22	2,18	0,23	0,01	1	0	0	0,05
SERPINA3	Alpha-1-antichymotrypsin	**-2,32**	**6,02E-04**	-0,29	0,66	**-2,03**	**1,07E-03**	221,56	60,56	51,53
SERPINA5	Plasma serine protease inhibitor	0,9	0,37	0,53	0,59	0,37	0,69	0,5	0,68	0,99
SERPINA6	Corticosteroid-binding globulin	1,38	0,27	1,37	0,27	7,14E-03	1	0,06	0,05	0,15
SERPINA9	Serpin A9	-0,02	0,99	-0,39	0,76	0,37	0,76	0,16	0,19	0,14
SERPINB1	Leukocyte elastase inhibitor	-0,18	0,52	-0,09	0,74	-0,09	0,73	177,4	171,37	164,32
SERPINB10	Serpin B10	3,06E-03	1	-0,36	0,77	0,37	0,75	0,11	0,13	0,1
SERPINB11	Serpin B11	**5,28**	**8,22E-03**	0,25	0,84	5,03	9,42E-03	0	0,49	0,61
SERPINB12	Serpin B12	NaN	NaN	NaN	NaN	NaN	NaN	0	0	0
SERPINB13	Serpin B13	4	5,28E-03	2,43	0,06	1,57	0,26	0,08	0,26	1,51

SERPINB2	Plasminogen activator inhibitor 2	1,87	0,13	2,4	0,05	-0,54	0,65	0,5	0,38	2,08
SERPINB3	Serpin B3	1,25	0,27	**2,65**	**0,02**	-1,4	0,19	1,95	0,8	5,32
SERPINB4	Serpin B4	2,17	0,28	-0,32	0,82	2,49	0,19	0	0,07	0,05
SERPINB5	Serpin B5	-0,42	0,74	**3,4**	**0,01**	**-3,82**	**2,67E-03**	2,29	0,17	1,98
SERPINB6	Serpin B6	0,09	0,69	0,11	0,6	-0,02	0,9	76,07	75,2	84,48
SERPINB7	Serpin B7	2,99	0,12	0,82	0,53	2,17	0,25	0	0,05	0,1
SERPINB8	Serpin B8	0,02	0,96	-0,06	0,9	0,08	0,85	5,06	5,68	5,36
SERPINB9	Serpin B9	-0,22	0,48	-0,37	0,23	0,15	0,61	25	28,48	22,89
SERPINC1	Antithrombin-III	-0,02	0,99	-0,06	0,96	0,04	0,98	0,17	0,17	0,16
SERPIND1	Heparin cofactor 2	**2,19**	**8,56E-03**	-0,9	0,25	**3,09**	**5,31E-05**	0,71	5,88	3,28
SERPINE1	Plasminogen activator inhibitor 1	-0,12	0,84	-0,57	0,3	0,45	0,39	160,32	234,96	163,87
SERPINE2	Glia-derived nexin	-0,37	0,37	-0,55	0,16	0,18	0,62	13,12	15,08	10,6
SERPINF1	Pigment epithelium-derived factor	-0,35	0,36	-0,58	0,12	0,23	0,51	68,07	79,94	55,28
SERPINF2	Alpha-2-antiplasmin	0,91	0,01	0,66	0,06	0,25	0,46	17,27	20,47	32,86
SERPING1	Plasma protease C1 inhibitor	-0,4	0,13	-0,47	0,07	0,07	0,78	447,95	470,1	350,15
SERPINH1	Serpin H1	-0,11	0,63	-0,08	0,74	-0,04	0,87	91,84	90,99	88,75
SERPINI1	Neuroserpin	-0,83	0,11	-0,48	0,35	-0,35	0,45	4,38	3,45	2,42
SERPINI2	Serpin I2	-0,63	0,49	-1,07	0,23	0,44	0,59	1,12	1,51	0,69
SPINK1	Serine protease inhibitor Kazal-type 1	-0,33	0,74	-1,25	0,19	0,92	0,31	3,15	5,92	2,63
SPINK5	Serine protease inhibitor Kazal-type 5	0,26	0,48	0,18	0,63	0,09	0,8	36,72	41,23	48,1

Table 9 Immunohistochemical quantification of Serpins signaling proteins in the lung parenchyma

Localization	Control non Smokers	Control Smokers	COPD Patients	Kruskal Wallis p-Value
Bronchiolar Epithelium (score 0-3)				
Serpin A3	1.0(0.25-1.5)	0.75(0.5-1.5)	0.75(0.25-1.5)	0.673
Serpin A6	1.58(0.75-2.0)	0.81(0.25-1.36) *	1.25(0.41-2.0)	**0.025**
Serpin B2 (PAI-2)	0.25(0.0-0.625)	0.125(0.0-0.625)	0.25(0.0-0.5)	0.968
Serpin B3	2.56(2.0-3.0)	2.4(1.0-2.5)	2.56(2.0-2.95)	0.116
Serpin B5	2.45(1.25-3.0)	3.0(1.75-3.0) *	2.75(1.75-3.0) &	**0.016**
Serpin B11	2.75(2.33-3.0)	2.5(1.37-2.87)	2.87(2.33-3.0)	0.163
Serpin B13	0.44(0.0-0.62)	0.77(0.0-1.5)	0.67(0.0-2.0)	0.130
Serpin C1	3.0(1.8-3.0)	2.83(1.5-3.0)	3.0(2.5-3.0)	0.244
Serpin D1	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.1)	0.621
SPINK1	2.96(1.5-3.0)	2.75(1.83-3.0)	2.95(2.5-3.0)	0.205
PLAUR (UPAR)	2.75(1.25-3.0)	2.95(1.75-3.0)	2.75(1.5-3.0)	0.199
tPA	1.75(0.75-2.87)	2.75(0.75-3.0) *	2.75(0.5-3.0) *	**0.050**
Cathepsin K	0.55(0.0-1.75)	0.75(0.0-1.12)	0.5(0.15-1.16)	0.772
Cathepsin L	2.25(0.75-3.0)	2.75(1.5-3.0)	2.87(1.75-3.0)	0.158
Caspase1	2.92(2.75-3.0)	2.5(1.5-3.0)	2.85(1.5-3.0)	0.122
Bronchiolar Lamina Propria (score 0-3)				
Serpin A3	1.0(0.25-1.5)	0.75(0.5-1.5)	0.75(0.25-1.5)	0.789
Serpin A6	1.0(0.5-1.5)	0.5(0.0-1.0) *	0.5(0.25-1.5) *	**0.029**
Serpin B2 (PAI-2)	0.0(0.0-0.5)	0.5(0.0-1.0)	0.5(0.0-0.5)	0.590
Serpin B3	1.0(0.75-2.5)	1.0(0.5-1.0)	1.0(1.0-2.5)	0.128

Serpin B5	2.41(1.25-3.0)	2.87(1.75-3.0) *	2.75(1.75-3.0)	**0.065**
Serpin B11	2.5(2.0-2.92)	2.25(1.25-2.75)	2.5(2.0-2.5)	0.252
Serpin B13	0.12(0.0-0.5)	0.5(0.0-1.0) *	0.5(0.0-2.0) *	**0.027**
Serpin C1	2.5(1.5-2.87)	2.5(1.0-3.0)	2.75(2.0-3.0)	0.099
Serpin D1	0.0(0.0-0.12)	0.0(0.0-0.0)	0.0(0.0-0.05)	0.582
SPINK1	2.5(1.5-2.5)	2.5(1.5-2.75)	2.5(2.0-2.5)	0.580
PLAUR (UPAR)	2.5(1.25-2.75)	2.5(1.75-3.0)	2.5(1.5-2.75)	0.700
tPA	1.0(0.5-2.5)	2.0(0.5-2.5)	2.25(0.5-2.5)	0.160
Cathepsin K	0.25(0.0-0.5)	0.25(0.0-0.5)	0.25(0.0-0.5)	0.917
Cathepsin L	2.0(0.5-2.5)	2.5(1.5-3.0)	2.5(1.5-2.5)	0.201
Caspase1	2.75(2.5-3.0)	2.0(1.0-3.0) *	2.5(1.5-3.0)	**0.021**
Alveolar Macrophages (score 0-3)				
Serpin A3	0.75(0.25-1.36)	1.0(0.5-1.5)	0.75(0.5-1.75)	0.319
Serpin A6	1.75(0.5-2.0)	1.25(0.5-2.0)	1.0(0.41-2.0)	0.266
Serpin B2 (PAI-2)	0.5(0.0-2.25)	0.89(0.1-2.0)	0.5(0.25-2.0)	0.322
Serpin B3	1.5(0.75-2.5)	1.25(0.75-2.25)	1.0(0.5-2.25)	0.482
Serpin B5	2.0(1.5-2.5)	2.5(1.75-3.0) *	2.25(1.83-3.0)	**0.017**
Serpin B11	2.25(1.5-2.87)	2.0(1.5-2.5)	2.25(1.25-2.75)	0.093
Serpin B13	1.0(0.12-2.0)	2.0(0.17-2.5) *	2.0(0.17-2.5) *	**0.012**
Serpin C1	3.0(2.5-3.0)	2.75(2.0-3.0)	2.87(2.25-3.0)	0.140
Serpin D1	0.04(0.0-0.57)	0.06(0.0-1.0)	0.07(0.0-0.25)	0.708
SPINK1	2.5(2.0-2.92)	2.5(2.0-3.0)	2.5(1.0-3.0)	0.778
PLAUR (UPAR)	2.5(2.0-3.0)	3.0(2.0-3.0) *	2.75(1.83-3.0) &	**0.026**
tPA	1.75(0.75-2.5)	2.0(1.0-2.75)	2.0(0.75-2.75)	0.215
Cathepsin K	1.5(0.25-2.25)	1.75(0.5-2.5)	1.37(0.2-2.25)	0.617

Cathepsin L	2.25(1.25-2.75)	2.75(1.75-3.0) *	2.75(1.5-3.0) *	**0.063**
Caspase1	2.75(2.25-3.0)	2.5(1.5-3.0)	2.5(2.25-3.0)	0.111
Alveolar Septa (score 0-3)				
Serpin A3	1.0(0.5-1.7)	0.83(0.5-1.33)	0.79(0.5-125)	0.385
Serpin A6	0.75(0.0-1.5)	0.375(0.0-1.5)	0.25(0.125-1.5)	**0.080**
Serpin B2 (PAI-2)	1.0(0.125-1.5)	1.0(0.08-1.5)	1.25(0.25-2.0)	0.604
Serpin B3	1.5(1.0-2.5)	1.25(0.75-1.5) *	1.25(0.75-2.25)	**0.058**
Serpin B5	1.5(1.0-2.5)	2.25(1.5-3.0) *	2.0(1.5-2.75) *	**0.029**
Serpin B11	1.87(1.5-2.5)	1.5(1.0-2.0) *	1.9(1.25-2.5) &	**0.015**
Serpin B13	0.25(0.08-0.83)	1.0(0.16-1.5) *	0.75(0.0-2.0) *	**0.022**
Serpin C1	1.91(1.5-2.5)	1.5(1.0-2.5)	1.75(1.42-2.75)	0.533
Serpin D1	0.0(0.0-0.29)	0.0(0.0-0.25)	0.0(0.0-0.25)	0.576
SPINK1	1.5(1.0-2.0)	1.5(1.0-2.0)	1.75(1.0-2.5)	0.466
PLAUR (UPAR)	2.0(1.0-2.0)	1.5(1.0-2.0)	1.5(1.0-2.5)	0.628
tPA	0.5(0.25-1.5)	1.0(0.25-2.0)	1.25(0.5-1.75)	0.112
Cathepsin K	0.5(0.0-1.0)	0.5(0.25-1.0)	0.37(0.25-0.75)	0.155
Cathepsin L	1.4(0.5-2.25)	2.0(1.0-2.5)	2.0(1.25-2.5) *	**0.043**
Caspase1	2.5(2.0-3.0)	2.0(1.0-3.0) *	2.5(2.0-2.75)	**0.046**
Lung Vessels (score 0-3)				
Serpin A3	1.12(0.5-1.5)	1.0(0.5-1.5)	1.0(0.5-1.75)	0.256
Serpin A6 [e]	0.5(0.0-1.0)	0.0(0.0-1.0) *	0.375(0.0-1.0)	**0.063**
Serpin B2 [e] (PAI-2)	0.5(0.0-1.0)	0.5(0.0-1.5)	0.5(0.0-1.0)	0.801
Serpin B3 [e]	0.75(0.5-1.5)	0.75(0.5-1.0)	0.75(0.5-1.0)	0.973
Serpin B5 [e]	1.5(1.0-2.0)	1.5(1.0-2.5)	1.75(1.25-2.5)	0.156
Serpin B11 [e]	1.5(1.5-2.5)	1.5(0.75-2.0) *	2.0(1.0-2.5) &	**0.045**

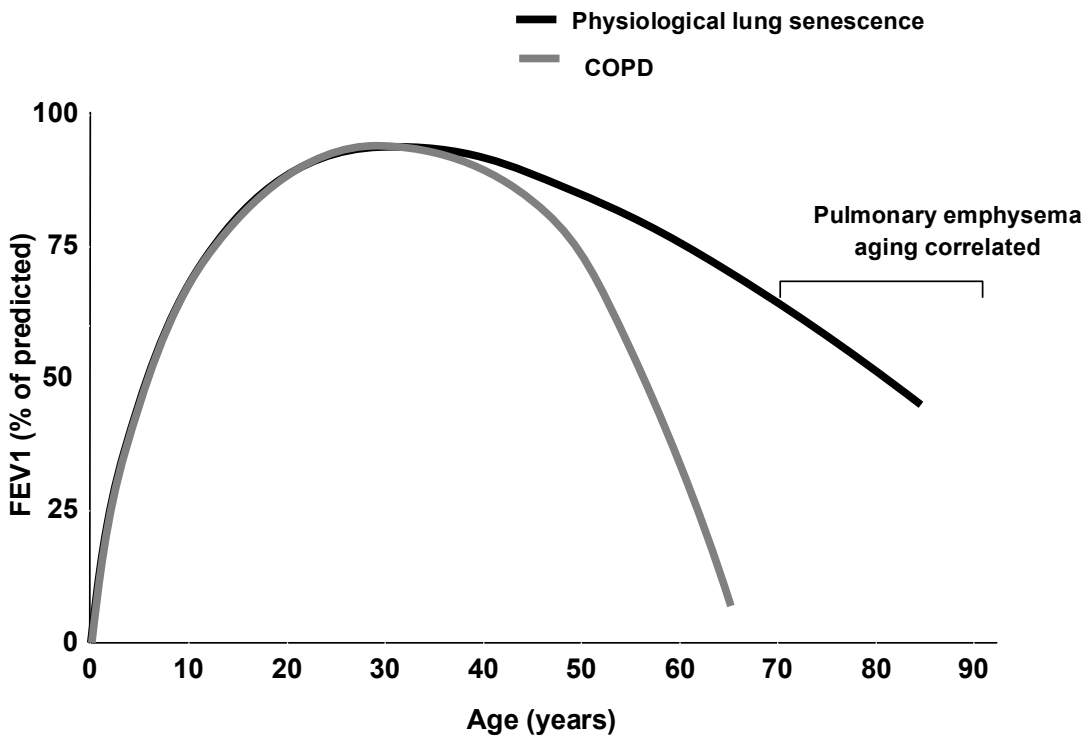
Serpin B13 [e]	0.5(0.0-0.75)	1.0(0.0-1.25) *	0.75(0.0-1.5) *	**0.013**
Serpin C1	2.0(2.0-2.5)	2.0(1.0-2.5)	2.0(1.0-2.5)	0.139
Serpin D1	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	NV
SPINK1 [e]	1.5(1.0-2.0)	1.5(1.0-1.5)	1.5(1.0-2.0)	0.611
PLAUR (UPAR)	1.62(1.0-2.0)	1.5(1.25-2.0)	1.5(1.0-2.5)	0.676
tPA	0.87(0.0-1.5)	1.0(0.25-1.75) *	1.25(0.5-2.0) *	**0.055**
Cathepsin K	0.5(0.0-1.5)	0.75(0.0-1.0)	0.5(0.0-1.0)	0.374
Cathepsin L [e]	1.12(0.25-2.0)	1.25(1.0-2.5)	1.5(0.5-2.0)	0.399
Caspase1 [e]	2.25(1.25-2.5)	1.5(1.0-2.5) *	1.75(1.5-2.5) *	**0.010**

Abbrevations: COPD, chronic obstructive pulmonary disease. Data expressed as median(range).

Startistics: the Kruskal-Wallis test was used for mulpiple comparison followed by Mann-Whitney U test for comparison between groups: *, $p<0.05$, significantly different from control non smokers; &, $p<0.05$, significantly different from control smokers whit normal lung function. Lung vessels-e, endothelial immunopositivity in the lung vessels. The exact "p" values for comparison between groups are given in the results section and/or figures.

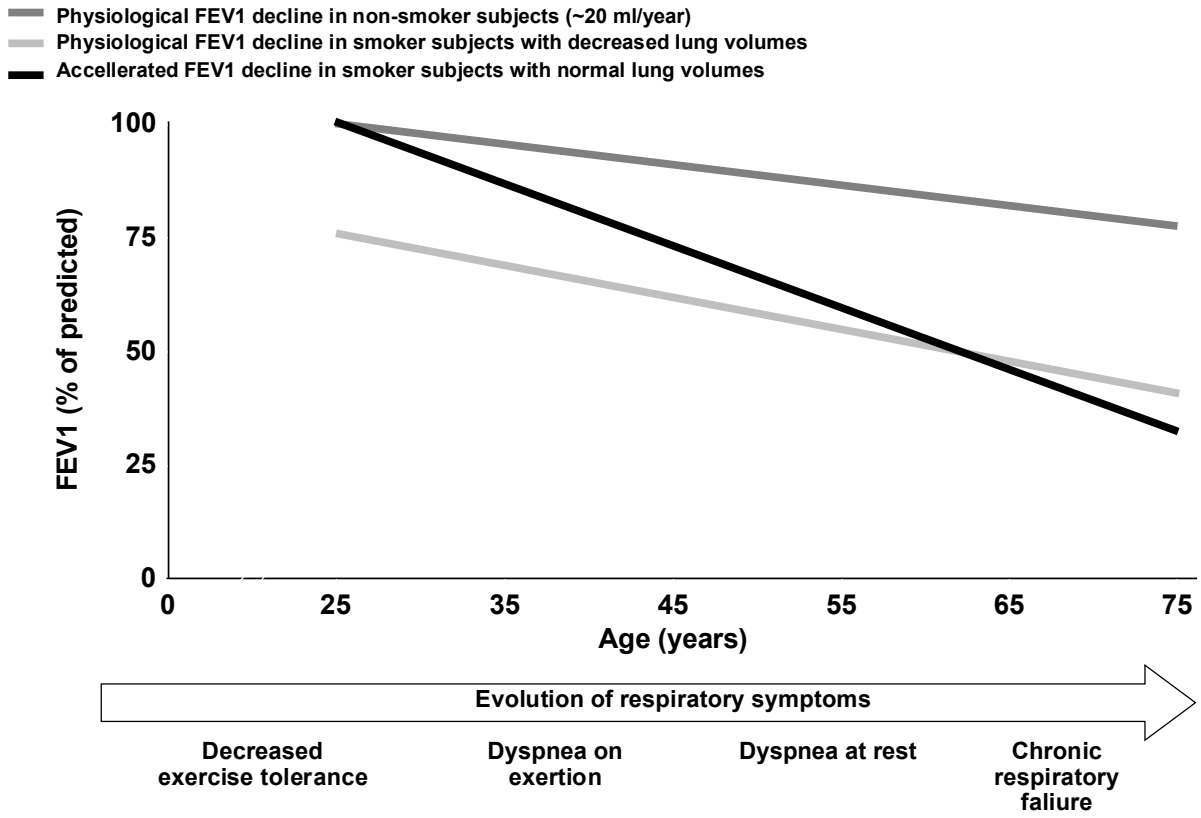
Figure legends

Figure 1 Physiological pulmonary senescence and during COPD Physiological pulmonary senescence and during COPD



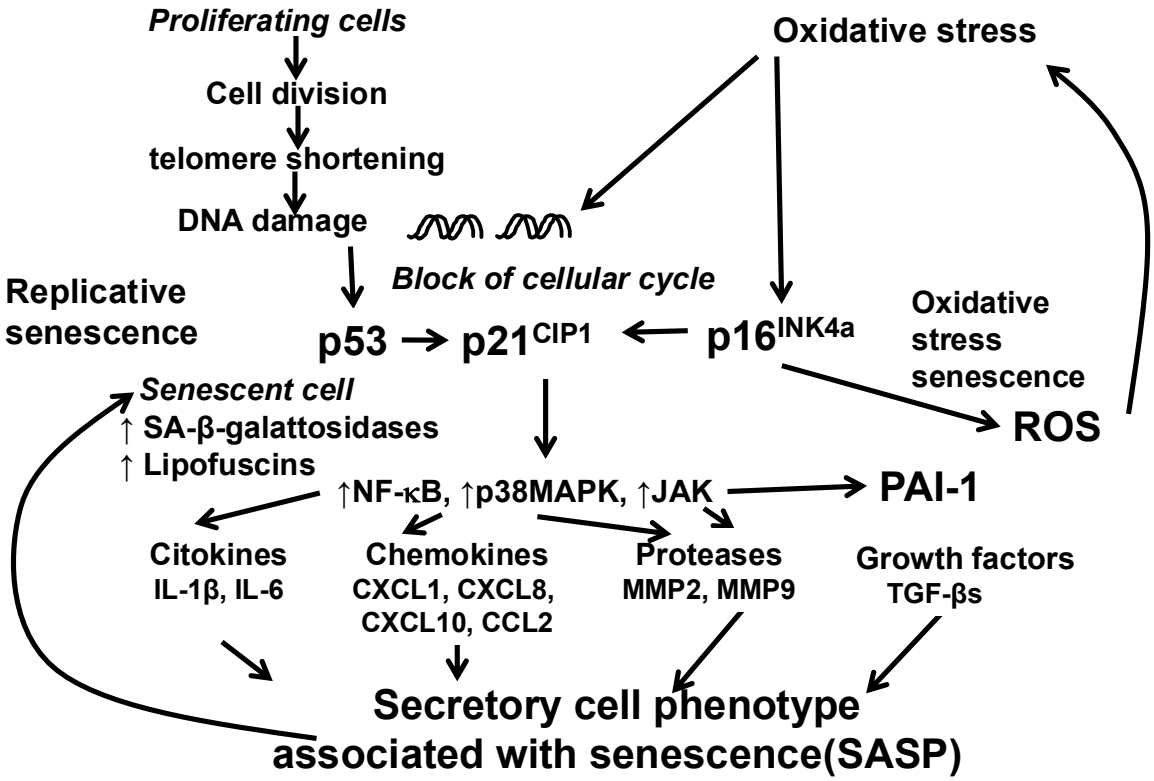
Abbreviations: COPD: Chronic Obstructive Pulmonary Disease. FEV1: forced expiratory volume in 1 second

Figure 2 Comparison of decline in physiological FEV1 secondary to aging and induced by chronic exposure to cigarette smoke



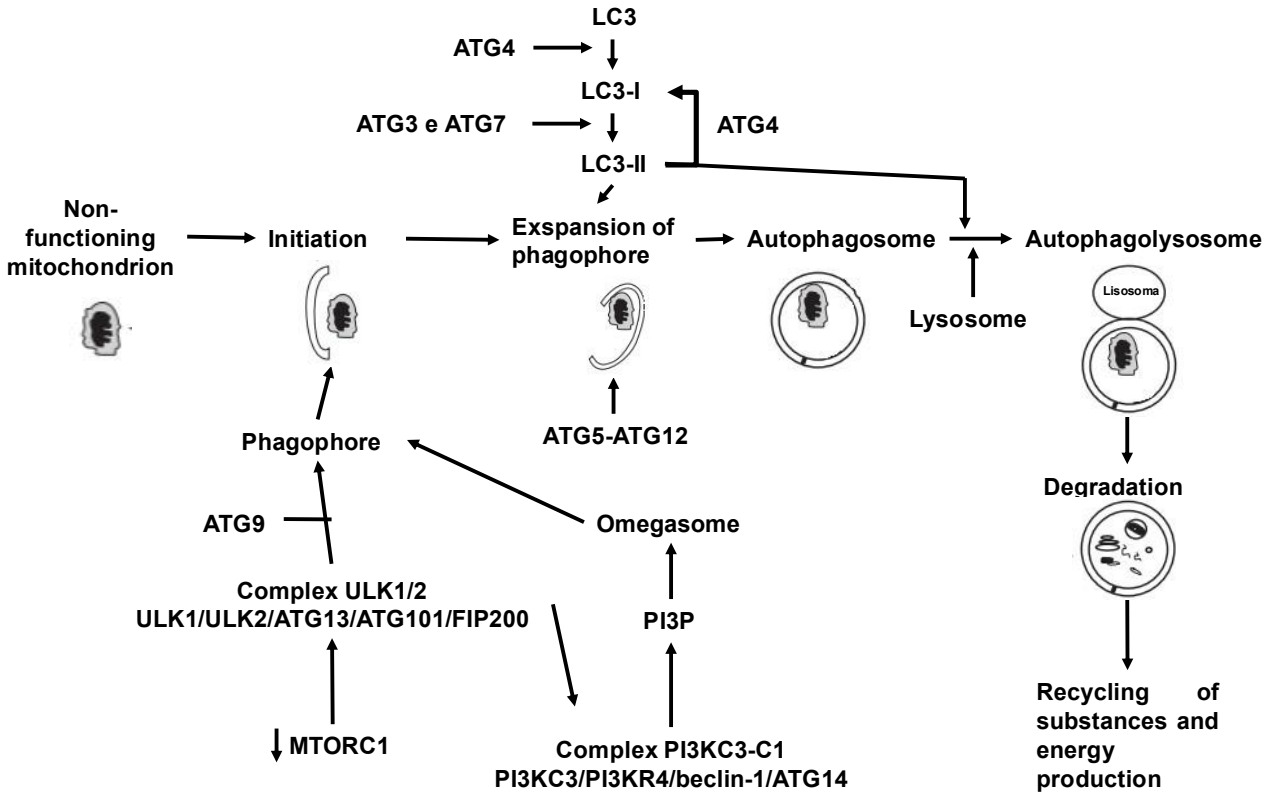
Abbreviations: FEV1: forced expiratory volume in 1 second

Figure 3 Pathways oxidative-stress mediated involved in aging and SASP production



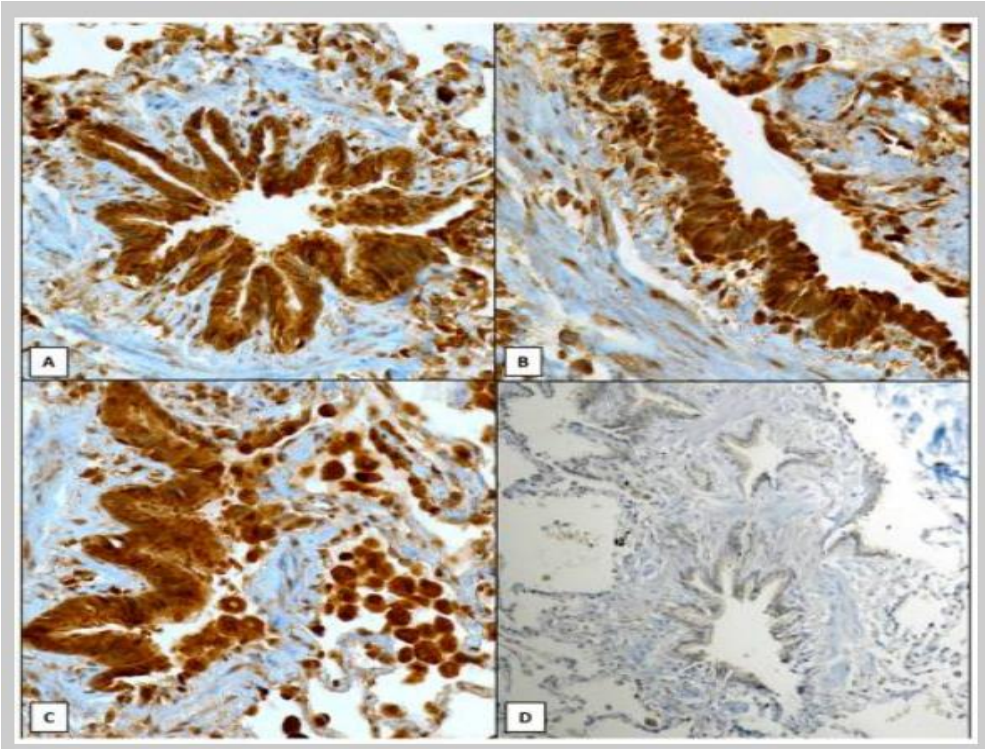
Abbreviations: CXCL: C-X-C chemokine; DNA: deoxyribonucleic acid; IL: interleukin; JAK: Janus kinase; MAPK: MAP kinase; MMP: metalloprotease; NF-κB: Nuclear factor kappa light chain enhancer of activated B lymphocytes; PAI-1: plasminogen activator type 1; ROS: oxidizing agents deriving from oxygen; SASP: senescence-associated secretory phenotype; TGF-β: transforming growth factor-β.

Figure 4 Intracellular pathway of macroautophagy



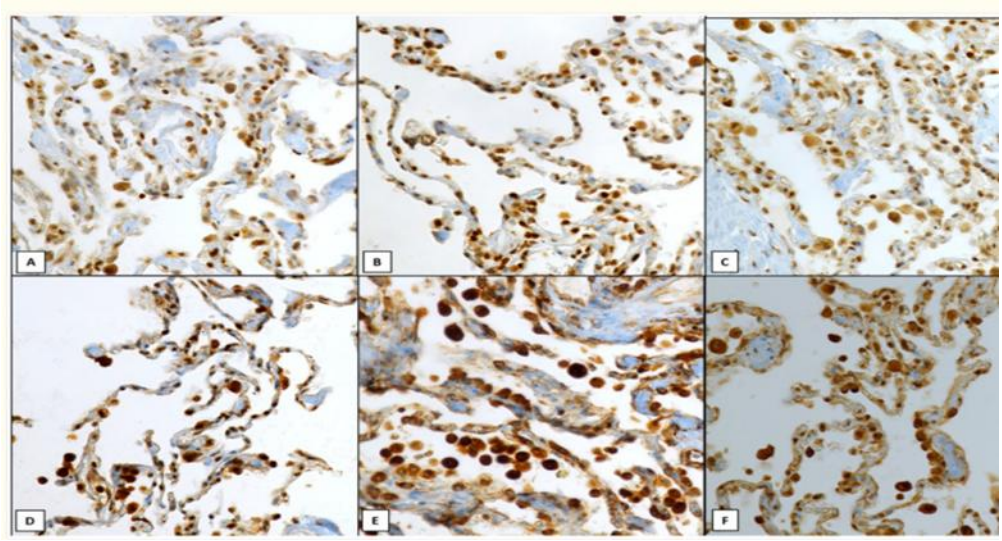
Abbreviations: ATG: autophagy-related proteins; LC3: microtubule-associated light chain protein; MTORC1: Mammalian target of rapamycin C1; PI3P: phosphatyl-inositol-3-phosphate; ULK1: Unc-51-like kinase activating autophagy.

Figure 5 Immunohistochemical quantification of autophagic molecules in the bronchiolar epithelium of patients with COPD, in control smokers and non-smoking subjects.



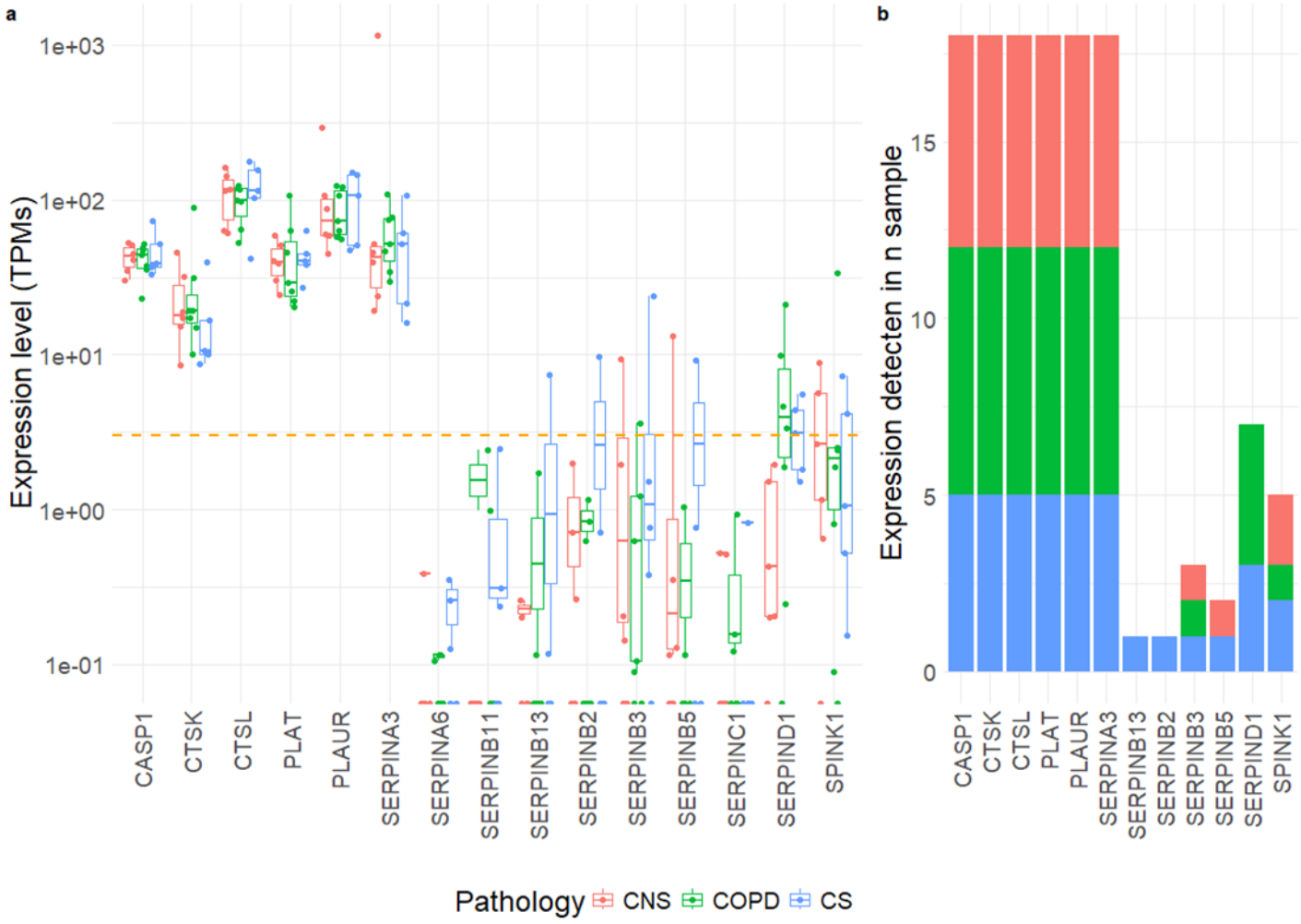
Immunoreactivity of ATGs in bronchial epithelium: a diffuse strong nuclear and cytoplasmic (score 6) staining was encountered with ATG4A (**A**, 400×), ATG4D (**B**, 400×), ATG5 (**C**, 400×) in patients with stable COPD; note the absence of immunoreaction in CNS/CS patients (**D**, 200×). Nuclear hemalum counterstain was made in all cases.

Figure 6 Immunohistochemical quantification of autophagic molecules in the peripheral lung of patients with COPD, in control smokers and non-smoking subjects.



Immunoreactivity of ATGs in alveolar lining: a diffuse strong nuclear and cytoplasmic (score 5–6) staining was encountered with ATG4A (A, 400×), ATG4B (B, 400×), ATG4C (C, 400×) ATG4D (D, 400×), ATG5 (E, 400×) and ATG7 (F, 400×) in patients with stable COPD, Note also the immunopositivity in alveolar macrophages. Nuclear hemalum counterstain was made in all cases.

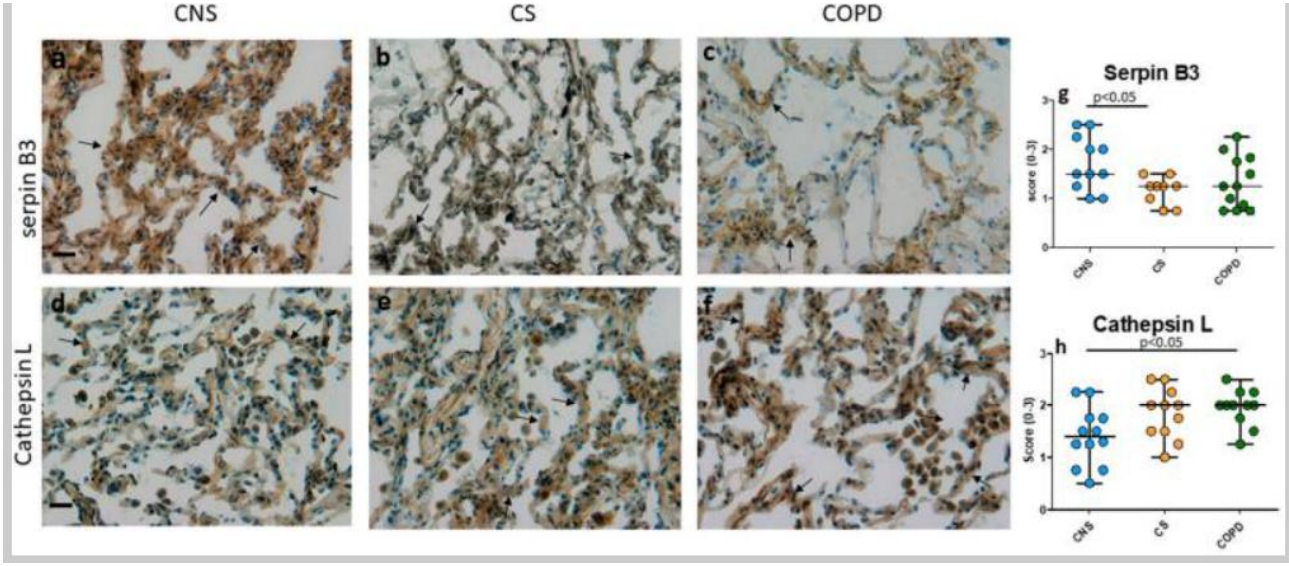
Figure 7 Gene Expression Level of SERPIN-Signaling Molecules in Lung Parenchyma



Expression levels of 15 selected genes obtained from lung parenchyma of control nonsmokers (CNS, n = 6), control smokers (CS, n = 5) and patients with chronic obstructive pulmonary disease (COPD, n = 7). The box plot shows the median and the distribution of expression values per gene reported as transcript per millions (TPMs): (a) the orange dotted

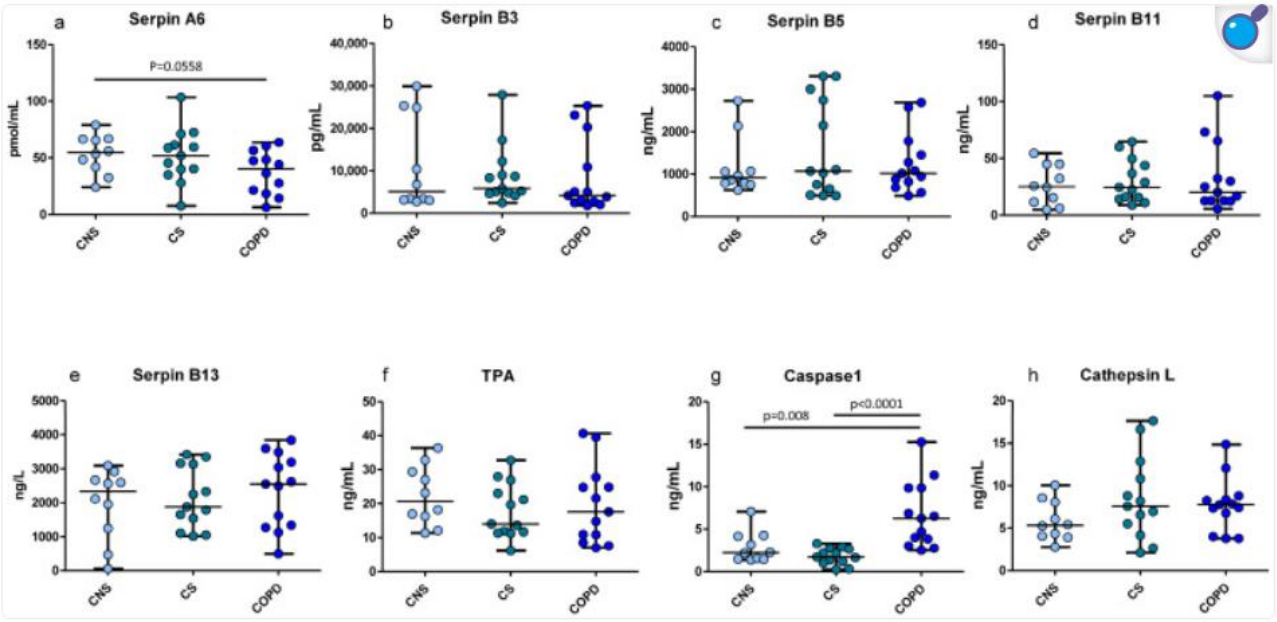
line shows the lower detection limit arbitrary set to 3 TPMs; and (b) the number of samples with detectable expression levels for the same genes, divided by conditions. The color codes are as follows: red for CNS; green for COPD; and blue for CS samples.

Figure 8 Immunohistochemical quantification of SERPINB3 in the peripheral lung of patients with COPD, in control smokers and non-smoking subjects.



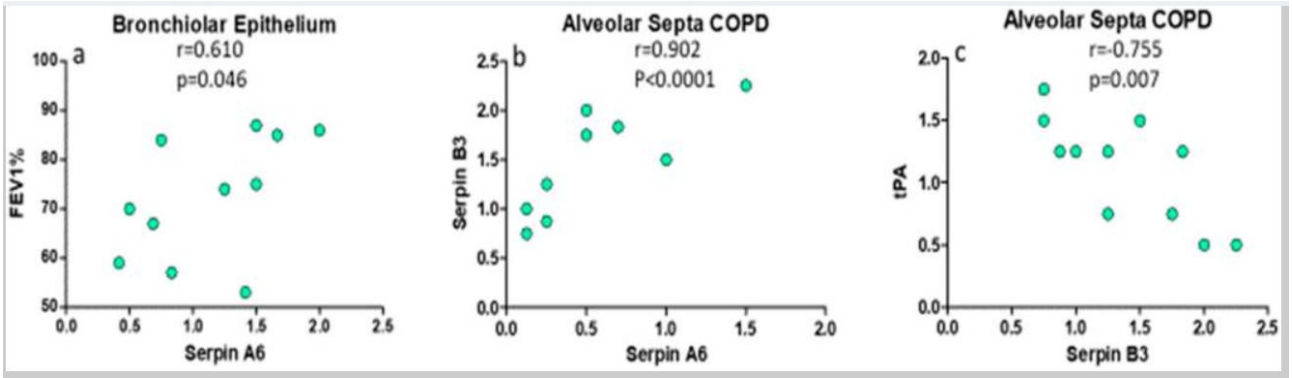
Photomicrographs showing alveolar septa from: (a,d) control nonsmokers; (b,e) control smokers, and (c,f) mild-moderate stable COPD patients immunostained for the identification of SERPINB3+ cells (a–c) and cathepsin L+ cells (d–f). Results are representative of those from 12 nonsmokers, 14 smokers with normal lung function and 13 with mild-moderate stable COPD. SERPINB3 is tendentially decreased in the alveolar septa of COPD patients compared to CNS. Cathepsin L is increased in the alveolar septa of COPD patients compared to CNS. Arrows indicate some immunopositive alveoli. Panels (g,h) show the quantitative analyses performed in lamina propria for immune expression of SERPINB3 (g) and cathepsin L (h). CNS, control nonsmokers; CS, control smokers with normal lung function, COPD, chronic obstructive pulmonary disease. Bar = 20 micron.

Figure 9 Tests for SERPIN-Signalling Proteins in Homogenized Peripheral Lung Tissue



ELISA tests performed on frozen lung tissue homogenates of patients with COPD (n = 13), CS (n = 14) and CNS (n = 12) for quantitation of SERPINs A6, B3, B5, B11, B13, tPA, caspase1 and cathepsin L: (a) SERPINA6 tendentially decreased in COPD compared to CNS; (g) caspase 1 increased in COPD compared to CNS and CS; and (b–f,h) no other significant differences were observed. The Mann–Whitney test was applied for comparison between groups. CNS, control nonsmokers; CS, control smokers with normal lung function, COPD, chronic obstructive pulmonary disease.

Figure 10 Correlations Between Clinical Parameters, SERPINs, Proteases and ATGs in Lung Parenchyma



Regression analysis in COPD patients alone: (a) in these patients, there was a significant positive correlation between SERPINA6 in bronchiolar epithelium and the predicted post-bronchodilator FEV1%; (b) in alveolar septa, SERPINA6 was positively correlated to SERPINB3; and (c) SERPINB3 was inversely correlated to tPA. Correlation coefficients were calculated by using the Spearman rank method.