NEW THERAPEUTIC STRATEGY TO CONTRAST INFLAMMATION AND LUNG REMODELLING PROCESSES IN A MURINE MODEL OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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SUMMARY

Chronic obstructive pulmonary disease (COPD) is characterized by chronic and abnormal lung inflammation leading to progressive and irreversible airflow obstruction. According to the latest World Health Organization (WHO) estimates, 64 million people currently have moderate to severe COPD, and WHO predicts that COPD will become the third leading cause of death worldwide by 2030.

Validation of a new murine model of COPD

Since COPD is a multifactorial disease, it is difficult to reproduce a valid animal model that is able to express all the phenotypical features clinically observed in patients. The main experimental protocols used so far require genetic approaches or the exposure of animals to noxious stimuli, such as tobacco smoke and irritant agents. In the literature to date no protocol has considered chronic treatment with multiple risk factors. The aim of this study was to develop a model of COPD, exposing animals to environmental conditions similar to those of patients with COPD in order to reproduce a chronic inflammatory state, emphysema and bronchial remodelling. The validation of this model was useful for therapy studies.

Mice were exposed for 1, 3 or 6 weeks to the main COPD risk factors: cigarette smoke; lipopolysaccharide to mimic bacterial exacerbations of the disease and particulate matter (PM-10) from urban pollution.

The first week of exposure is able to induce an increase in inflammatory cytokines, which progressively reduce in the following weeks. Real time PCR has shown, from the third week, an increase in gene expression of TGFβ and αSMA (key factors in the remodelling pathway) and proteolytic enzymes, with a peak in the sixth week of exposure, confirmed by immunohistochemistry and Western blot. Histological analysis showed a progressive thickening of the bronchial wall, destruction of lung tissue, enlargement of air spaces (emphysema) and the formation of lymphoid follicles, in particular in the sixth week of combined treatment.

Thus, the use of this new experimental protocol is able to trigger inflammatory processes which, in the later stages of exposure, induce destruction of the lung parenchyma and airway remodelling (bronchial wall thickening, goblet cell hyperplasia and B-cell follicles), which are irreversible after treatment cessation. This demonstrates that the pathogenesis observed in mice reflects the natural history of COPD.

Effect of anti-inflammatory and bronchodilator therapy administered in the early stage of COPD

The therapeutic goal of COPD treatment, in addition to clinical improvement, is the modification of the natural history of the disease, reducing mortality. Nevertheless, all available therapies are essentially symptomatic and contribute to improving the quality of life, without counteracting the progressive decline
of the lung function. In contrast to current GOLD guidelines, which provide the use of inhaled corticosteroids in advanced stages of COPD (when tissue remodelling has already been occurred), the aim of this study was to test the hypothesis that a combination of bronchodilators and anti-inflammatory treatment is able to influence the progression of COPD only in early stages of pathogenesis, when tissue damage is less relevant and bronchial structural changes are still reversible.

Mice with COPD-like phenotype were treated with a combination of antinflammatory (fluticasone) and bronchodilators (salmeterol and tiotropium) drugs, at different times:

- mice early treated (treatment started at the first week of exposure to risk factors, for 4 weeks);
- mice late treated (treatment started at the sixth week of exposure to risk factors, for 4 weeks).

Pharmacological treatment significantly decreases level of cytokines in a group early treated compared to untreated. No significant effects were observed in groups treated later, since levels of cytokines resulted similar between group untreated and those treated. Early therapy was able to attenuate the activation of remodelling processes (bronchial wall thickening, goblet cell hyperplasia, formation of B cell follicles). Moreover, therapy in the first stage of the disease was able to reduce the degree of lung tissue destruction (emphysema). No significant effects on bronchial remodelling were observed in groups treated later.

These data confirmed that a bronchodilator and antinflammatory therapy began too late is not able to counteract tissue changes, despite effective action on gene and tissue expression of remodelling factors. Instead, an early pharmacological treatment reduced the peak of inflammation contributing to attenuate bronchial alterations that are still reversible. Thus, the addition of an anti-inflammatory drug to bronchodilator therapy in the early stages could be a useful new therapeutic strategy to reduce the extent of bronchial obstruction and to effectively counteract the progression of the disease.
1. CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

1.1 DEFINITION

Chronic obstructive pulmonary disease (COPD) is an important cause of morbidity and mortality and represents a significant economic burden throughout the world. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defined COPD as a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases.¹

It is possible to prevent the disease increasing smoking cessation, treating patients with primary asthma or detecting early the disease in order to reduce exposure to risk factors. Extrapulmonary effects are frequently observed in patients with COPD, related to impairment of respiratory function: these are cardiovascular diseases, osteopenia, chronic infections, reduced fat mass, depression and anxiety.¹ Airways obstruction in COPD is linked to several lung mechanisms, such as parenchymal destruction and bronchial wall thickening that together contribute to the narrowing of bronchial lume. COPD is not fully reversible since the limitation does not revert, even after the use of bronchodilators and anti-inflammatory drugs.

1.2 EPIDEMIOLOGY AND RISK FACTORS

Globally, COPD is now the fourth leading cause of death, representing a worldwide social problem since as it is expected to become the third leading cause of death in the next 20 years. Currently, more than 219 million people have the disease in the world (more than 15 million in the United States).²,³ Traditionally, COPD is a disease that predominantly affects males; however, NHANES studies in United States showed that the prevalence of moderate COPD in woman increased during the last 20 years, in contrast to the prevalence in men that decreased during the same period.⁴,⁵ A similar trend has been observed in other developed countries, whereas in developing ones the prevalence of COPD is still higher in men compared to woman.⁶,⁷ COPD is a multifactorial disease induced by exposure to environmental factors (tobacco smoke, air pollution, work exposure, respiratory infections) and host factors (age, gender, genetic predisposition) acting singly or synergistically among themselves.⁸ Tobacco smoke is the main risk factor for the disease, but in industrialized and developing countries additional risk factors are important and preventable causes of COPD.
1.2.1 ENVIROMENTAL RISK FACTORS

**Tobacco smoke** Several epidemiologic studies have shown that cigarette smoking is the most important risk factor for COPD. Smokers have lung function abnormalities and a progressive decline in forced expiratory volume in the first second (FEV1); moreover, mortality of COPD in smokers compared to non-smokers is at least 7 times higher in light smokers (1-14 cigarettes/day), 10-fold increased in moderate smokers (15-24 cigarettes/day) and 21-fold in heavy smokers (≥ 25 cigarettes/day). Reducing the number of cigarettes smoked daily has a significant positive effect on health; indeed, an encouraging study demonstrated that who stopped smoking at an age between 35 and 44 years showed a survival curve that was similar to that of non-smokers. Po Delta study from Northern Italy confirms the relationship of smoking and COPD: a higher prevalence of chronic cough and mucus hyperproduction was found in smokers compared to non-smokers in both sexes. A population-based study by Hagstad showed that exposure to passive smoke is a risk factor among never-smokers to develop COPD. The relationship becomes stronger with increasing exposure. In multiple environmental tobacco smoke exposure settings the risk of developing COPD is even comparable to moderate smoking. Active and passive maternal smoking has a negative effects during human pregnancy: maternal smoking is associated with placental damage in all trimester of pregnancy, indeed tobacco toxins dysregulate trophoblastic and fetal cells biological functions; effects are reduction of fetal growth and weight, fat mass and most anthropometric parameters. Each puff of a cigarette contains than 2000 xenobiotic compounds, 10 free radicals, toxins including cyanide, sulphides, cadmium, carcinogenic hydrocarbons, nicotine and nitric oxide that injure lung tissue by different mechanisms, such as increased release of proteolytic enzymes by immune cells, reduced anti-proteases defences, lipid peroxidation of membrane, cleavage of proteins and increase in epithelial permeability. Tobacco smoke is known to cause increased sequestration of neutrophils in the pulmonary vasculature and airspaces in humans, with alterations in the oxidant/antioxidant balance. Oxidative stress is responsible for many of cigarette smoking effects, since oxidants, such as H₂O₂, have been shown to inhibit apoptosis and induce necrosis. Moreover, cigarette smoke condensate decreases epithelial-cell adherence and increases detachment and lysis in a human type II alveolar epithelial-cell line.

**Air pollution** While active cigarette smoking is the most important preventable risk factor globally, outdoor and indoor air pollutants can cause or exacerbate COPD. In developed countries there is a clear evidence that exposure to high levels of outdoor air pollutants is associated with increased mortality and morbidity due to COPD and related cardiorespiratory diseases. The degree of exposure to outdoor air pollutants however is variable over time primarily due to changes in pollutant emissions and weather conditions. Studies in the last 20 years continue to show increased risk associated mainly with particulate matters, even at much lower levels. Populations in low-income countries are largely exposed to indoor
air pollutants from the combustion of biomass fuels, such as coal, straw, animal dung, crop residues and wood which are used to heat and cook in poorly ventilated homes; this condition contributes significantly to the burden of COPD-related diseases, particularly in non-smoking women.\textsuperscript{16}

A recent multinational study performed in Latin America (ESCALA) has shown that levels of particulate matter are significantly associated with increased mortality from respiratory and cardiovascular causes, in particular COPD.\textsuperscript{17} Another recent cohort study demonstrated that long-term exposure to elevated traffic-related fine particulate air pollution (black carbon, particulate matter with aerodynamic diameter <2.5\,\mu m, nitrogen dioxide and nitric oxide) and wood smoke was related to an increased risk of COPD hospitalization (+15\%) and mortality (+7\%).\textsuperscript{18}

Several studies have shown that air pollutants, such as particulate matter, ozone (O\textsubscript{3}) and NO\textsubscript{2} can produce deleterious effects on lung tissue: increase in bronchial reactivity\textsuperscript{19}, airway oxidative stress\textsuperscript{20}, pulmonary and systemic inflammation\textsuperscript{21}, amplification of viral infections\textsuperscript{22} and reduction in airway ciliary activity.\textsuperscript{23}

**Occupational risk** Exposure to dusts, vapours, chemicals and fumes in the workplace is associated with an increased risk of developing COPD, in particular in countries of low and middle income, where occupational exposures to noxious compounds could be greater than high-income countries because of less severe laws. Exposure to high levels of organic particles such as bacterial/fungal toxins (farming activities), cotton dust (textile industry), mineral dusts or heavy metals (industrial work) is associated with an increased risk of obstructive lung disease.\textsuperscript{24} The risk is lesser than that of smoking and interactions between smoking and occupational exposure to various agents is relevant. A study performed in Northern Italy demonstrated the relationship between work exposure and COPD: among exposed workers there was an increase in symptoms of bronchitis and a decrease in lung function compared to not exposed.\textsuperscript{25,26}

**Respiratory infections** Infections have an important role in development and progression of COPD, as they could predispose to bronchiectasis or changes in airways responsiveness. Epidemiological studies have demonstrated the association between childhood respiratory infections and the presence of symptoms and reduced lung function in adulthood.\textsuperscript{27} In the general population study of *Po Delta*, a higher prevalence of respiratory symptoms and diagnosed COPD was noted in subjects with previous childhood respiratory infections compared to those without, particularly in smokers.\textsuperscript{28}

Furthermore, bacterial and viral infections commonly induce COPD exacerbations that are linked to worsening the quality of life\textsuperscript{29} and rapid decline in lung function with more frequent and longer lasting hospital admissions.\textsuperscript{30} The typical viruses known to cause exacerbations are rhinoviruses, which cause most common colds, followed by respiratory syncytial virus (RSV), influenza, coronaviruses and parainfluenza viruses (PIVs). Severe acute exacerbations are associated with a high risk of death, with
about 10-30% of the subjects dying during hospital admission and about 40-60% dying during the year following admission.\textsuperscript{31,32}

1.2.2 ENDOGENOUS RISK FACTORS

**Genetic factors** It is clear that there is a high variation in susceptibility to COPD between individuals due to genetic factors which have a key role in the development of the disease, regulating the lung responses to environmental exposures. The best known genetic factor linked to COPD is a deficiency of the serine protease $\alpha_1$-antitrypsin, which affects 1-3\% of patients with COPD: although $\alpha_1$-antitrypsin is mainly produced in the liver, its main function is to protect the lung against proteolytic damage from neutrophil elastase; therefore, lower concentrations of this enzyme increase the risk of alveolar destruction and emphysema, particularly in combination with smoking or other exposures.\textsuperscript{33} The $\alpha_1$-antitrypsin deficit is due to a hereditary defect of two alleles of the protein situated in the gene locus of the segment of chromosome 14q32.1. The homozygote phenotype PiZZ has been associated with a severe form of pulmonary emphysema. Not every homozygote individual develops the disease, as tobacco smoking is generally necessary. Moreover smoking also seems to significantly increase the risk of disease in the heterozygote forms.\textsuperscript{34}

Numerous other genes have been studied and implicated in COPD susceptibility, based on various pathogenic pathways, such as inflammation (interleukin-4, -6, -13, TNF$\alpha$, TGF$\beta$), protease/antiprotease balance (MMP-9, TIMP2, SERPINA3) and oxidative stress (glutathione transferase, superoxide dismutase).\textsuperscript{35}

**Ageing** COPD prevalence, morbidity and mortality increase with age. Lung function reaches its peak level in young adults and starts to decline in the third and fourth decades of life.\textsuperscript{36} The link between aging and the pathogenesis of COPD is strongly supported by numerous studies\textsuperscript{37-38}: senescence is a complex outcome of endogenous and environmental factors (ex. oxidative stress) and therefore the role of cigarette smoke, noxious gas and air pollutants is a key factor linking aging lung to COPD.

**Gender** Several studies suggested a gender difference in susceptibility to harmful effects of smoking in the lung: women may be biologically more susceptible to the adverse effects of cigarettes than men, due to sex differences in smoke metabolism.\textsuperscript{39} The airways in women are anatomically smaller and each cigarette may represent a proportionally greater exposure.\textsuperscript{40} Dimensional, immunological and hormonal determinants are other biological possibilities for a gender difference.\textsuperscript{41}
1.3 PATHOGENESIS OF COPD

The hallmark of COPD is the abnormal chronic inflammation in the lung in response to inhalation of noxious agents, mainly cigarette smoke. Host factors including genetic susceptibility, epigenetic changes and oxidative stress contribute by amplifying inflammation induced by cigarette smoke.\(^{42}\) The epithelial cells of the airways and alveoli are a major source of inflammatory mediators and proteases. Triggered by smoking, they produce several factors (TNF\(\alpha\), TGF\(\beta\), IL1\(\beta\), IL8, GM-CSF) which cause activation of fibroblast and small airways fibrosis. The epithelial cells protect the lung through the production of mucus which traps bacteria and compounds (defensins, cationic proteins) which have antimicrobial properties. Cigarette smoke or other toxic and irritants agents induce the release of pro-inflammatory cytokines and chemokines also by alveolar macrophages, eliciting the expression of adhesion molecules on endothelial cells and the recruitment of neutrophils and inflammatory monocytes to the lungs. Activated dendritic cells induce adaptive immune responses encompassing CD4+ T cells, CD8+ cytotoxicity and B cells responses, which lead to the development of lymphoid follicles. This condition causes lung destruction, through the release of oxygen radicals and proteolytic enzymes (elastase, metalloproteases) with consequent bronchial remodelling.\(^{43}\)

1.3.1 AIRWAYS INFLAMMATION

**Innate immune response** Airways have innate defence mechanisms encompassing the epithelial barrier, mucociliary clearance, humoral factors and cells (macrophages, dendritic cells, monocytes, neutrophils, natural killer cells and mast cells). Innate immunity is a rapid, aspecific response to infections and tissue injuries that stimulate the development of specific adaptive immune responses. Acute exposure to noxious factors leads to the release of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) molecules. The recognition of PAMPs and DAMPs by several families of pattern recognition receptors (PRRs) expressed in alveolar macrophages, dendritic and epithelial cells is crucial in mediation of inflammatory responses to infections and tissue damage. The PRRs include transmembrane Toll-like receptor (TLRs), cytosolic NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). Xenobiotic compounds and free radicals contained in cigarette smoke injure lung epithelial cells and extracellular matrix to a degree that is directly proportionate to their concentrations. Products derived from injury (hyaluronate, hyglican) are ligands for TLR4 and TLR2 leading to the activation of nuclear factor kB (NF-kB)\(^{44,45}\) that induce epithelial cells to produce inflammatory cytokines. These mediators are able to activate alveolar macrophages\(^{46,47}\) which secrete further cytokines (IL8, IL1\(\beta\), TNF\(\alpha\), GM-CSF, MCP-1, ICAM-1) eliciting the expression of adhesion molecules on endothelial cells and the recruitment of neutrophils and inflammatory monocytes to the lungs. Numbers of neutrophils and macrophages are increased in the lungs of smokers and patients with COPD. Activated neutrophils and macrophages cause lung destruction through the release of oxygen radicals and proteolytic enzymes (elastase, metalloproteases) with consequent bronchial remodelling.
radicals and proteolytic enzymes, such as neutrophil elastase and matrix metalloproteinases (MMPs). Elastase, cathepsin-G and proteinase-3, secreted by neutrophils, cause over-stimulation of submucosal mucous glands and goblet cells. This chain of events induces dendritic cells to mature and migrate to local lymphatic organs; stimulation by TLRs leads to the expression of CD80-CD86 and cytokines, creating a propitious environment for T-cell antigen presentation and proliferation into effector CD4+ type 1 helper (Th1) T cells and cytolytic CD8+ T cells. Interleukin-6, secreted by dendritic cells, favours the production of effector T cells by overcoming the signals from regulatory T (Treg) cells. Upon activation, effector T cells express tissue-specific chemokine receptors. CD8+ T cells and natural killers contribute to lung damage through the release of other proteases (granzyme B and perforin).

In this step, if innate inflammation is minimized or controlled, inflammation processes will not progress to adaptive immunity and the disease may be arrested.\textsuperscript{48}

\textbf{Adaptive immune response} Adaptive immune inflammation is characterized by the presence of CD4+ type 1 helper (Th1) T cells, cytolytic CD8+ T cells and IgG-producing B cells. CD8+ cytotoxic T cells are predominant in large airways, small airways and lung parenchyma.\textsuperscript{49,50} The number of CD8+ T cells in the lung correlates with the degree of airflow obstruction and emphysema, suggesting their role in tissue injury. In the lung of smokers with COPD, epithelial and endothelial cells undergo apoptosis and this process is correlates with the number of CD8+ T cells and proteases (perforin and granzymes) secreted in the lung. This condition contributes to lung destruction in COPD.\textsuperscript{48} CD4+ T cells are also found in large numbers in the airways and parenchyma of smokers with COPD. The effector functions of the CD4+ T cell are mainly mediated by Th1 cytokines, which promote transendothelial migration of inflammatory cells to site of injury.\textsuperscript{48}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{innate_response.png}
\caption{Innate immune response following smoke exposure.}
\textbf{Source:} Cosio et al. N Engl J Med 2009\textsuperscript{48}
\end{figure}
B cells are increased in large airways of patients with COPD; moreover, they organized into lymphoid follicles around small airways and in lung parenchyma of patients, especially in the severe stages. Lymphoid follicles are anatomically and functionally well organised and consisting of memory and naïve B cells, T cells, dendritic cells, essential for T-cell and B cell priming and clonal expansion. These lymphoid follicles are responsible for antigen retention, immunoglobulin class switching and affinity maturation; B cells are oligoclonal in nature, suggesting antigen-specific induction of the B cell follicles: antigens involved could derive from microorganisms, cigarette-smoke, breakdown products of extracellular matrix and autoantigens. Therefore, the pathogenic role of the follicular B-cell response is controversial: it might be beneficial if protective against microbial colonisation and infections in the airways; or it could be destructive if directed against lung tissue antigens, suggesting an autoimmune component in COPD.

The recruitment and activation of inflammatory cells, macrophages, neutrophils, eosinophils, CD4+ and CD8+ T cells, and B cells progress as COPD worsens.

![Fig.2 Adaptative immune response in COPD.](source)

1.3.2 EMPHYSEMA

Emphysema is defined as an abnormal permanent enlargement of peripheral airspaces of the lung, including respiratory bronchioles, alveolar ducts and alveoli, accompanied by destruction of alveolar walls. Human emphysema was originally described by Laennec\textsuperscript{54,55}, who noted \textit{marked variations in the size of the air vesicles, which might be smaller than a millet seed or as large as a cherry stone or haricot}. \textit{Vesicles of the latter size were produced by the coalescence of adjacent air spaces following rupture of the alveolar walls}. Emphysema generally develops between the ages of 45 and 60 as a component of COPD in smokers\textsuperscript{56}, but lung destruction has also described in other non-smoking-related disorders.

**Pathogenesis** The pathogenesis of emphysema is driven by three distinct processes: 1) chronic exposure to inhaled toxic agents (mainly cigarette smoke) leads to inflammatory cell recruitment in the terminal airspaces; 2) these inflammatory cells release proteases that destroy the extracellular matrix of the lung; 3) ineffective repair of elastin and other extracellular matrix components leads to development of emphysema.\textsuperscript{57}

The inflammatory response in emphysema is characterized by the activation of innate and adaptive processes. Indeed, the alveolar spaces are infiltrated by neutrophils, macrophages and lymphocytes, which cause the release of potentially destructive proteolytic enzymes and the damage of alveolar-capillary cells. Production of proteases is not restricted to inflammatory cells: structural cells such as epithelial and endothelial cells are also able to produce proteases.\textsuperscript{58,59} As described above, innate immunity is based on pattern recognition receptors that recognize structures, such as LPS and endogenous ligands; activation of TLR4 by LPS stimulate production of cytokines and ROS, involved in pathogenesis of emphysema, as demonstrated by several experimental studies: for example, overexpression of TNF\textsubscript{α} in mice lung is able to cause emphysema.\textsuperscript{60} In addition to inflammation, oxidative stress deriving from cigarette smoke inhalation plays an important role in pathogenesis of emphysema: the major consequence is the activation of the transcription factor NF-kB, which activates proinflammatory cytokine transcription.\textsuperscript{61,62} Thus, oxidant damages and lung inflammation are synergistically responsible for the increased alveolar destruction and tissue repair defects.

In the lung, elastin fibers organize into a dense network around capillaries; loss of the alveolar septal scaffold after enzymatic degradation of matrix proteins induces airspace enlargement. Moreover, elastin fragments are chemotactic and can attract additional inflammatory cells to sites of injury, generating a vicious cycle.\textsuperscript{63} Emphysema leads to a progressive decline in the alveolar surface area available for gas exchange. Furthermore, loss of alveoli causes airflow limitation because of a decrease in elastic recoil and airflow limitation. Moreover, loss of the alveolar supporting structure leads to airway narrowing, which further limits airflow.
**Protease-antiprotease balance** The protease-antiprotease hypothesis and the concept that an excess of proteolytic activity can lead to lung destruction are confirmed by clinical and experimental studies. In 1964, Laurell and Eriksson described, for the first time, the association between α1-protease inhibitor deficiency and pulmonary emphysema. Moreover, experimental studies conducted by Gross showed that instillation into the lungs of enzymes with elastolytic activity induced emphysema and that proteolytic enzymes lacking the ability to degrade elastin did not cause emphysema, confirming hypothesis deriving from clinical observations. Several enzymes with elastolytic activity contribute to the development of emphysema, as observed in the lung of COPD patients, neutrophil elastase, chymotrypsin, proteinase 3, metalloproteases, cysteine proteases. Inhibitors of these enzymes are also present in the normal lung, thus expanding the concept of protease-antiprotease balance. This balance could be modified by several mechanisms in addition to a congenital deficiency of an antiprotease. An inflammatory response could lead to the increased release of proteases, as reported in the lower respiratory tract of patients with COPD, and the amount of elastase in the lower respiratory tract has been correlated with the severity of emphysema. Similarly, levels of matrix metalloprotease (MMP9 and MMP12) are increased in COPD.

Antiproteases, including α1-protease inhibitor, are susceptible to oxidation. Oxidation of the methionyl residue at the active site of α1-protease inhibitor blocks protein activity. As a result, smokers with an inflammatory response may develop an acquired form of α1-protease inhibitor deficiency. Probably, tissue destruction results from the integrated activity of multiple proteases, indeed there are important interactions between the various classes of proteases. First, both cathepsins and metalloproteases are able to inactivate serine protease inhibitors by proteolysis. Similarly, serine proteinases can degrade tissue inhibitors of metalloprotease (TIMPs). Thus, these classes of proteases are capable of altering relative proteolytic-antiproteolytic balance by affecting inhibitors of other classes. In addition, metalloproteases are generally released as inactive forms and serine proteases are able to activate these proenzyme. Thus, serine proteases may lead to tissue destruction by activating metalloprotease cascades.

Proteases likely contribute to the pathogenesis of COPD by several mechanisms in addition to destruction of the extracellular matrix. In this context, the proteolytic cleavage of elastin by MMPs generates peptides that are potent chemoattractants for macrophages. MMPs, moreover, may play a role in the activation of other cytokines, including TGF-β and TNFα. Neutrophil elastase can directly stimulate fibroblast contraction which contribute to airways narrowing. Furthermore, neutrophil elastase can induce activation of the epidermal growth factor (EGF) receptor and leads to goblet cell metaplasia.

Cigarette smoke contains approximately 6,000 chemical compounds and many of these are highly reactive oxidant species. Moreover, the inflammatory response typically generates oxygen-free radicals that can lead to tissue damage. In defense against oxidant damage, several endogenous antioxidants are present in the lower respiratory tract. This has led to an oxidant-antioxidant hypothesis analogous to that of the protease-antiprotease hypothesis.
Effects of smoking on emphysema: cigarette smoke induces neutrophils and macrophages recruitment in the lung, with consequent increased release of proteolytic enzymes. This condition causes progressive destruction of the lung parenchyma, resulting in chronic activation of cellular repair processes and airway remodelling. Source http://www.helpmedicose.com/emphysema/

Classification There are three morphological types of emphysema:

- **Centriacinar (or centrilobular)** emphysema begins in the respiratory bronchioles and spreads peripherally. This form is associated with chronic cigarette smoking and predominantly involves the upper half of the lungs.

- **Panacinar** emphysema destroys the entire alveolus uniformly and is predominant in the lower half of the lungs. This phenotype is observed in patients with homozygous α1-antitrypsin deficiency.

- **Paraseptal (or distal acinar)** emphysema preferentially involves the distal airway structures, alveolar ducts and alveolar sacs. The process is localized around the septae of the lungs or pleura. Although airflow frequently is preserved, the apical bullae may lead to spontaneous pneumothorax; moreover, giant bullae occasionally cause severe compression of adjacent lung tissue.83
Airway remodelling is defined as an alteration in size, mass or number of tissue structural components, leading to thickening of bronchial wall, occurring in response to irritant agents and/or inflammation. In COPD, remodelling may occur in response to smoking-induced damage to the airways and it is characterized by large and small airway wall thickening, metaplasia of the epithelium, loss of epithelial cilia, increase in goblet cells size and number, submucosal gland and smooth muscle hypertrophy/hyperplasia.

**Epithelial changes and goblet cells hyperplasia** The epithelial response to inhaled noxious compounds represents attempts by the bronchial epithelium to protect itself and repair the injury caused by toxic factors, as cigarette smoke. The epithelium is a site of inflammation which shows altered permeability and is the source of a number of growth factors and cytokines which modulate inflammatory processes and remodelling pathways below the basement membrane. Cigarette smoke induces the release of IL-1β, IL-8 and granulocyte colony-stimulating factor (G-CSF) from bronchial epithelial cells through oxidative pathways, inducing a neutrophil and monocytes chemotactic activities. The normal pseudostratified ciliated epithelium is replaced by goblet cells and, in more severe disease, by squamous metaplasia, which is the reversible replacement of the columnar epithelium by squamous epithelium. In addition, there is associated hypertrophy of mucus glands. These anatomical changes are associated with alterations in the expression of mucin genes. Goblet cell hyperplasia is more pronounced in smokers with COPD than in those without COPD; these changes, therefore, lead to the production of mucus that is abnormal in quality and increased in quantity and, at the same time, mucociliary clearance is impaired, contributing to increase morbidity and mortality. The increase in mucus production and reduction in mucociliary clearance caused by cigarette smoking represent an innate host defence response to exogenous injuries. The biochemical mechanisms and cellular processes responsible for alteration in epithelial cell populations are incompletely defined. However, inflammatory mediators, including proteases and oxidants, are able to have effects on epithelial cells. Goblet cell hyperplasia may involve the activation of EGF-receptor, which may be upregulated by oxidants in cigarette smoke and release of cytokines, such as TNFα and IL-8. Activation of the EGF-receptor, which may take place either by direct ligand activation or by non-ligand-mediated activation through either oxidant or proteolytic pathways, can lead to altered mucin gene expression. EGF-receptor activation also appears to be a key process in goblet cell metaplasia in animal models.

**Smooth muscle** A significant increase in airway smooth muscle in small airways of patients with COPD has been reported in several studies and the amount of airway smooth muscle has been inversely correlated with lung function (FEV1% predicted). The amount of airway smooth muscle was increased by nearly 50% in patients with more severe COPD. Although the airway smooth muscle mass is
increased, it is unknown whether this is caused by an increased number of airway smooth muscle cells, an increase in airway smooth muscle cell size or both.

Airway smooth muscle cells have contractile properties, but also are able to produce and release cytokines, chemokines, growth factors and proteases, participating in the inflammatory and remodelling mechanisms.

**Fibrosis** It is likely that the process responsible for lung fibrosis resembles the mechanism responsible for fibrosis in other chronic disorders. In this context, TGFβ plays a central role, particularly in wound healing. It is a potent activator of fibroblasts, converting them to a myofibroblast-like phenotype, through the increase in αSMA expression and inducing the production of extracellular matrix. TGFβ also stimulates fibroblast recruitment and augments the ability of fibroblasts to contract and reduce their surrounding extracellular matrix. TGFβ activates a receptor that functions as a serine/threonine kinase and phosphorylates Smad proteins, particularly Smad-2 and Smad-3, although other signaling pathways may also play a role; in particular, Smad-3 signaling appears to be key in both wound healing and fibroblast activation. In addition to TGFβ, a variety of other cytokines likely present in the lung in COPD can also modulate fibroblast activity. Increased levels of the Th2 cytokines IL-4 and IL-13, which can stimulate fibroblasts, have been reported in tissues in COPD. Imbalance between tissue destruction and tissue repair can also contribute to the development of emphysema; cigarette smoke can inhibit fibroblast repair responses, suggesting that smoke may shift the balance toward the development of emphysema by impeding repair as well as by augmenting destruction. Modulation of the fibrotic response in the small airways could have an effect in modifying the natural history of COPD.

**Multiple roles of TGFβ in airway remodelling** As mentioned above, TGFβ has multiple effects in the same cell type and in different cells, depending on microenvironmental and cellular conditions. TGFβ induces apoptosis in airway epithelial cells and is involved in the regulation of adhesion properties of epithelial cells, leading to damage of the epithelial cell layer. Moreover, TGFβ has a role in hyperproliferation of goblet cells and hypersecretion of mucus. The most important effect of TGFβ is the inducing action on fibroblast proliferation, differentiation and extracellular matrix protein production. Furthermore, TGFβ contributes to enhance the proliferation of airway smooth muscle cells and is involved in the abnormal proliferation of blood vessels. Therefore, it is evident how all these properties of TGFβ confirm its key role in the exacerbation of airway remodelling.
Fig. 4 Multiple roles of TGFb in lung remodelling.

2. COPD THERAPY

2.1 CLINICAL FEATURES

Patients often ignore symptoms, modify their lifestyle to minimize dyspnea and ignore cough and sputum production. Typically they present with a combination of signs and symptoms of chronic bronchitis, emphysema and reactive airway disease, including:

☑ productive cough that worsens in the mornings and produces a small amount of colorless sputum;
☑ breathlessness which is the most significant symptom, but it usually does not occur until the sixth decade of life. By the time the FEV1 has fallen to 50% of predicted, the patient is usually breathless upon minimal exertion. In fact, the FEV1 is the most common variable used to grade the severity of COPD, although it is not the best predictor of mortality;
☑ wheezing which may occur in some patients, particularly during exertion and exacerbations.

According to the 2011 guidelines, a history of more than 40 pack-years of smoking was the best single predictor of airflow obstruction; however, the most helpful information was provided by a combination of the following 3 signs:\textsuperscript{120}:

☑ self-reported smoking history of more than 55 pack-years;
☑ wheezing on auscultation;
☑ self-reported wheezing.

If all 3 signs are absent, airflow obstruction can be nearly ruled out. With disease progression, intervals between acute exacerbations become shorter and each exacerbation may be more severe.

COPD is now known to be a disease with systemic manifestations and the quantification of these manifestations has proved to be a better predictor of mortality than lung function alone. Many patients with COPD may have decreased fat-free mass, impaired systemic muscle function, osteoporosis, anemia, progressive exercise intolerance, alteration in mental status, pulmonary hypertension and even left-sided heart failure. Depression is not uncommon in subjects with COPD.\textsuperscript{121}

2.2 STAGES OF COPD

The GOLD classifications are the main method used to describe the severity of COPD. GOLD classifies people with COPD based on their degree of airflow obstruction. The airflow limitation is measured during pulmonary function tests: when blowing out forcefully, people with normal lungs can exhale most of the air in their lungs in one second. Pulmonary function tests measure this and other values and are used to diagnose COPD and its severity. The volume in a one-second forced exhalation is called the \textit{forced expiratory volume in one second} (FEV1), measured in liters. The total exhaled breath is called the \textit{forced vital capacity} (FVC), also measured in liters. In people with normal lung function, FEV1 is at least
70% of FVC. Because of lung damage, people with COPD take longer to blow air out; therefore, an FEV1 less than 70% of FVC can make the diagnosis of COPD in someone with compatible symptoms and history. Classifications are then used to describe the severity of the obstruction or airflow limitation. The lower their FEV1, the worse a person's airflow limitation, thus as COPD progresses, FEV1 tends to decline. GOLD introduced four categories of severity for COPD, based on the value of FEV1:

<table>
<thead>
<tr>
<th>STAGE</th>
<th>FEV1/FVC</th>
<th>FEV1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Mild COPD</td>
<td>FEV1/FVC&lt;0.70</td>
</tr>
<tr>
<td>Stage II</td>
<td>Moderate COPD</td>
<td>FEV1/FVC&lt;0.70</td>
</tr>
<tr>
<td>Stage III</td>
<td>Severe COPD</td>
<td>FEV1/FVC&lt;0.70</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Very Severe COPD</td>
<td>FEV1/FVC&lt;0.70</td>
</tr>
</tbody>
</table>

Table 1. GOLD classification based on FEV1/FVC and FEV1 values.

2.3 COPD TREATMENT

The goal of COPD treatment is to improve a patient’s functional status and quality of life by preserving optimal lung function, improving symptoms and preventing the recurrence of exacerbations. Currently, no treatments aside from lung transplantation have been shown to significantly improve lung function or decrease mortality. Once the diagnosis of COPD is established, it is important to educate the patient about the disease and to encourage his active participation in therapy.

Approaches to management include recommendations such as those provided by GOLD:

- **Stage I** (mild obstruction): reduction of risk factors (influenza vaccine, smoking cessation); short-acting bronchodilator as needed.
- **Stage II** (moderate obstruction): reduction of risk factors (influenza vaccine, smoking cessation); short-acting bronchodilator as needed; long-acting bronchodilator; cardiopulmonary rehabilitation.
- **Stage III** (severe obstruction): reduction of risk factors (influenza vaccine, smoking cessation); short-acting bronchodilator as needed; long-acting bronchodilator; cardiopulmonary rehabilitation; inhaled glucocorticoids if repeated exacerbations.
- **Stage IV** (very severe obstruction or moderate obstruction with evidence of chronic respiratory failure): reduction of risk factors (influenza vaccine, smoking cessation); short-acting bronchodilator as needed; long-acting bronchodilator; cardiopulmonary rehabilitation; inhaled glucocorticoids if repeated exacerbation; long-term oxygen therapy; consider surgical options such as lung volume reduction surgery and lung transplantation.
Oral and inhaled medications are used for patients with stable disease to reduce dyspnea and improve exercise tolerance. Most of the medications used are directed at the following four potentially reversible causes of airflow limitation in a disease state that has largely fixed obstruction:

- Bronchial smooth muscle contraction
- Bronchial mucosal congestion and edema
- Airway inflammation
- Increased airway secretions

Inadequate nutritional status associated with low body weight in patients with COPD is associated with impaired pulmonary status, reduced diaphragmatic mass, lower exercise capacity and higher mortality rates. Nutritional support is an important part of comprehensive care in patients with COPD.122

### 2.3.1 BRONCHODILATOR TREATMENT

Bronchodilator drugs are the key aspect of COPD treatment regimen. They are able to dilate airways, increasing airflow and decreasing dynamic hyperinflation. Nevertheless, these drugs provide symptomatic relief but do not alter disease progression or decrease mortality.

β2-agonist bronchodilators activate specific β2-adrenergic receptors on the surface of smooth muscle cells, which increases intracellular cyclic adenosine monophosphate (cAMP) and smooth muscle relaxation. Even patients who have no measurable increase in post-bronchodilator expiratory airflow may benefit from treatment with β2-agonists. The inhaled route is preferred because it minimizes adverse systemic effects.

Anticholinergic drugs compete with acetylcholine for postganglionic muscarinic receptors, inhibiting bronchomotor tone and inducing bronchodilation. They block vagally mediated reflex arcs that cause bronchoconstriction. These agents are poorly absorbed systemically and are relatively safe.

Historically, β2-agonists were considered first line and anticholinergics were added as adjuncts. Studies have shown that combination therapy results in greater bronchodilator response and provides greater relief. Monotherapy with either agent and combination therapy with both are acceptable options. Generally, long-acting bronchodilators are more beneficial than short-acting ones.123-124

Guidelines recommend combination therapy involving two long-acting bronchodilators with differing modes of action in patients whose COPD is not sufficiently controlled with monotherapy1. As mentioned, airway smooth muscle relaxation can be achieved via two main routes: inhibition of acetylcholine signaling via muscarinic M3 receptors on airway smooth muscle with a muscarinic antagonist, or stimulation of β2-adrenoceptors with a β2-agonist.125,126 Targeting these two mechanisms of bronchoconstriction has the potential to optimize the bronchodilator response without increasing the dose of either component. The interaction between the two systems has yet to be fully elucidated; however, β2-agonists can amplify the bronchial smooth muscle relaxation directly induced by the muscarinic
antagonist by decreasing the release of acetylcholine via modulation of cholinergic neurotransmission. Additionally, muscarinic antagonists have been demonstrated to augment β2-agonist-stimulated bronchodilation by reducing the bronchoconstrictor effect of acetylcholine in preclinical models.125

**Short-acting muscarinic antagonist (SAMA) plus short-acting β2-agonist (SABA)** The concept of adding a muscarinic antagonist to a β2-agonist is not new. A fixed-dose combination of the short-acting agents ipratropium and albuterol and of fenoterol and ipratropium provides significant benefits over monotherapy with either component.127-128 Additionally, dual bronchodilation with ipratropium and albuterol resulted in a consistently longer response and more patients achieved a pre-specified response level (12-15%) in FEV1 with the combination compared to individual components127-129, with an equivalent or improved safety profile.

**Long-acting muscarinic antagonist (LAMA) plus long-acting β2-agonist (LABA)** Relatively few studies have examined the combination of LAMAs and LABAs. Several randomized controlled trials have reported improved lung function for tiotropium plus formoterol versus tiotropium alone.130,131 Some trials have also identified significant improvements in symptom scores.132-133 A recent meta-analysis confirmed the benefits of tiotropium plus formoterol on average FEV1 and Transition Dyspnea Index (TDI).134 Currently available data on tiotropium plus salmeterol are conflicting: initial investigations indicated the benefits of tiotropium plus salmeterol versus either monotherapy alone, while suggesting co-administration of once-daily salmeterol plus tiotropium was inadvisable, due to the shorter duration of bronchodilation provided by salmeterol.135 The Canadian Optimal Therapy of COPD trial investigated the impact of tiotropium plus placebo, tiotropium plus salmeterol, or tiotropium plus salmeterol/fluticasone on clinical outcomes in 449 patients with moderate to severe COPD.136 Tiotropium plus salmeterol/fluticasone (but not tiotropium plus salmeterol) statistically improved lung function and quality of life and reduced the number of hospitalizations for exacerbations compared to tiotropium plus placebo.136 A more recent study, however, demonstrated significant improvements in FEV1 with salmeterol once or twice daily plus tiotropium.137 Tiotropium plus indacaterol has been demonstrated to improve lung function and inspiratory capacity, as well as providing a further reduction in use of rescue medication.138 These data confirm that combination therapy has the potential to improve outcomes versus monotherapy.

The safety profiles of both LAMAs and LABAs are well understood; given that both muscarinic antagonists and β2-agonists can have a detrimental effect on the cardiovascular system139,140, these adverse events need to be monitored carefully in development programs for combination products. Initial results suggest that the cardiovascular safety profile of glycopyrronium plus indacaterol is similar to placebo, with no clinically significant differences observed versus placebo141 and, to date, no safety concerns have been identified with tiotropium plus olodaterol.142 Free combinations of LAMA/LABA also seem well tolerated. No differences in blood pressure and pulse rate were observed with tiotropium plus salmeterol versus single-agent therapies.137
2.3.2 MANAGEMENT OF INFLAMMATION

Inflammation plays a significant role in the pathogenesis of COPD. Systemic and inhaled corticosteroids attempt to temper this inflammation and positively alter the course of disease. The use of systemic steroids in the treatment of acute exacerbations is widely accepted and recommended, given their high efficacy. A meta-analysis concluded that oral and parenteral corticosteroids significantly reduced treatment failure and the need for additional medical treatment and that they increased the rate of improvement in lung function and dyspnea over the first 72 hours.\textsuperscript{143} On the other hand, the use of oral steroids in persons with chronic stable COPD is widely discouraged, given their adverse effects which include hypertension, glucose intolerance, osteoporosis, fractures and cataracts. A Cochrane review showed no benefit at low-dose therapy and short-lived benefit with higher doses (>30 mg of prednisolone).\textsuperscript{144}

Inhaled corticosteroids (ICS) provide a more direct route of administration to the airways and, similar to other inhaled agents, are only minimally absorbed. Consequently, the systemic adverse effects of these medications at standard doses are negligible. Despite the theoretical benefit, the current consensus is that inhaled corticosteroids do not decrease the decline in FEV1, although they have been shown to decrease the frequency of exacerbations and improve quality of life for symptomatic patients with a FEV1 of less than 50%.\textsuperscript{145} The 2013 ICSI (Institute for Clinical Systems Improvement) guidelines conclude that inhaled steroids are appropriate in patients with recurrent exacerbations of COPD.\textsuperscript{146} Inhaled corticosteroids are not recommended as monotherapy and should be added to a regimen that already includes a long-acting bronchodilator. The TORCH trial (\textit{TOwards a Revolution in COPD Health}) showed that a combination of an inhaled corticosteroid and a long-acting β\textsubscript{2}-agonist was more beneficial than inhaled corticosteroids alone.\textsuperscript{147} These data suggest that in patients with COPD, inhaled corticosteroids should be used only in conjunction with a long-acting β\textsubscript{2}-agonists. However, patients treated with inhaled corticosteroids were noted to have an increased rate of pneumonia. Despite the possible increased risk of pneumonia associated with inhaled corticosteroids, a retrospective cohort study showed that in patients with COPD hospitalized with pneumonia, prior use of inhaled corticosteroids was actually associated with decreased mortality and less mechanical ventilation.\textsuperscript{148}

Nonsteroidal anti-inflammatory medications have not been shown conclusively to have any benefit in COPD. No response has been shown to medications targeting IL-8 and TNFα. Leukotriene inhibitors commonly used in asthma have also not proven to be beneficial in COPD. However, macrolide antibiotics have been shown to have anti-inflammatory effects in the airways of COPD patients. More specifically, azithromycin has been shown to improve the phagocytic function of pulmonary macrophages and to be a potent anti-inflammatory.\textsuperscript{149}

\textbf{Combination of corticosteroids and bronchodilators} The combination of LABA and ICS is the most common in use for both COPD and asthma. The physiological and clinical benefits of LABAs have
been shown to be enhanced when administered in conjunction with ICS.\textsuperscript{150} ICS and LABAs combination have been shown to improve lung function, symptoms and health status, and they reduce exacerbations in patients with moderate to severe COPD.\textsuperscript{151-152} Results of a recent meta-analysis showed that ICS/LABA did not decrease the number of severe exacerbations, respiratory mortality and cardiovascular mortality compared with LABA monotherapy.\textsuperscript{153} This meta-analysis showed that the superior FEV1 achieved with the ICS/LABA compared with LABA monotherapy was able to increase the frequency of pneumonia. This is in conflict with an earlier analysis of the same database that found that ICS/LABA combination therapy was superior to LABA monotherapy for exacerbation frequency reduction.\textsuperscript{154}
3. ANIMAL MODELS OF COPD

COPD humans studies are limited to morphological evaluations of lung biopsies or *in vitro* research. Thus, the development of animal models is of great importance for analysing molecular mechanisms, histological and functional alterations, commonly observed in patients with COPD, and for evaluating new therapeutic approaches.

To date, many species have been used, including rodents, guinea-pigs, monkeys and sheep.\(^{155-156}\) Mice offer the greatest ability to explore pathogenesis of COPD, because of the low cost, the ability to produce animals with genetic modifications that are useful to investigate specific pathways, the big amount of antibody probes and the availability of numerous occurring mouse strains with different reactions to smoke exposure. In mice, features of human COPD can be modelled by exogenous administration of proteases, chemicals, particulates and cigarette smoke exposure\(^ {155,157-158}\). Regardless of the method of exposure, many of the hallmark features of human COPD, as chronic lung inflammation, impaired lung function, emphysema, mucus hypersecretion, small airway thickening, lymphoid aggregates can be mimicked in the smoking murine model. Nevertheless, there is no COPD animal model that replicates all the phenotype observed in humans.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Human features</th>
<th>Experimental approaches</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of exposure to risk factors</td>
<td>Tobacco smoke</td>
<td>Exposure-based experimental protocol</td>
</tr>
<tr>
<td></td>
<td>Occupational dusts and chemicals</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indoor/outdoor air pollution</td>
<td></td>
</tr>
<tr>
<td>Chronic lung inflammation</td>
<td>Elevated levels of macrophages, neutrophils and T-</td>
<td>Assessment of airway cytokine levels</td>
</tr>
<tr>
<td></td>
<td>cells and cytokines in lung</td>
<td></td>
</tr>
<tr>
<td>Airflow obstruction</td>
<td>Decrease in FEV(_1)</td>
<td>Lung function test</td>
</tr>
<tr>
<td>Hypersecretion</td>
<td>Chronic sputum production</td>
<td>Morphological assessment of hypersecretion</td>
</tr>
<tr>
<td>Emphysema</td>
<td>Progressive impairment of lung function</td>
<td>Morphological assessment of airspace enlargement and lung function test</td>
</tr>
<tr>
<td>Airway remodelling</td>
<td>Bronchial wall thickening</td>
<td>Morphological assessment of bronchial wall diameter</td>
</tr>
</tbody>
</table>

Table 2. Indicators for COPD observed in human that should ideally be present in animal models and available for analysis.
3.1 EXPOSURE TO TOXIC OR IRRITANT AGENTS

**Cigarette smoke** Most animal models require the exposure to cigarette smoke, the main risk factor. Exact comparisons of findings from groups are difficult because of different types of cigarettes, doses, instruments, exposure protocols and variety of mouse strains. A number of factors must be considered to the design of cigarette smoke-induced models of COPD. The method of smoke production and exposure is a critical factor. Mainstream smoke refers to the smoke produced and drawn back through a cigarette by the act of inhalation or puffing. This type of exposure occurs in active smokers. Sidestream smoke refers to the smoke produced passively from the burning end of a cigarette that escapes into the surrounding atmosphere before being inhaled. Exposure to sidestream and exhaled mainstream smoke occurs during passive smoke exposure. Although both types of smoke have the same chemical composition, there is a considerable difference in the relative concentration of components, but some studies suggested that the two types of smoke may not generate large differences in COPD phenotype. At present, two exposure systems are used: whole-body and nose-only. Both systems have been used widely to provide important insights into COPD pathogenesis. Nose-only system allows to reduce the potential confounding ingestion of nicotine, tar and other chemical agents by animals from their fur and to generate intermittent smoke exposures mimicking the puff profile of human smokers.

The inflammatory response induced by cigarette smoke occurs in two phases: an acute phase characterized by neutrophils and a chronic phase (after 2-4 weeks of exposure) with neutrophils, macrophages and lymphocytes permeation. Churg et al., in a murine model of acute exposure (4 cigarettes for 1 hour), observed that the inflammation is associated with NF-κB and TNFα activation, responsible for the up-regulation of E-selectin. Vlahos et al. demonstrated that mice fully exposed to cigarette smoke for 4 days showed significant high levels of macrophages, neutrophils and proteases in BAL, besides the increased gene expression of inflammatory cytokines. Chronic application of the same protocol (3-6 months) was able to induce a persistent airway inflammation accompanied by emphysema. First studies of chronic exposure to smoke (6 months) have shown an increase in goblet cell number, a thickening of bronchi wall, a reduction in alveolar attachments and the formation of lymphocyte aggregates around bronchial wall, as observed in humans. Lungs of mice and rats exposed to smoke for 6 hours/day, 5 days/week for 7 or 13 months, showed significant differences in two species: bronchial alterations observed in mice at the thirteenth month of treatment are greater than those observed in rats; in addition, inflammatory infiltrate in mice showed a higher level of neutrophils, indicating that exposure to tobacco smoke induces a COPD-like phenotype more significant in mice despite of rats. Further studies have shown that cigarette smoking promotes the release of proteolytic enzymes by inflammatory cells with imbalance between the levels of protease and antiprotease, the process underlying the development of pulmonary emphysema. Experimental models described so far include several disadvantages:
✓ exposure to cigarette smoke for several months, with high costs;
✓ phenotype obtained generally equivalent to early or moderate stages of COPD;
✓ difficulty to reproduce emphysema or significant remodelling, typical of severe stages of COPD;
✓ after smoking cessation, emphysema is not progressive and goblet cell metaplasia regresses, unlike what was observed in advanced stages of COPD, where structural alterations progress even after smoking cessation.155

**Bacterial lipopolysaccharide** Lipopolysaccharide (LPS) is a component of the outer wall of gram-negative bacteria, also present as a contaminant in the smoke of cigarette, air pollution and organic dusts. LPS administration induces an inflammatory response with increased levels of neutrophils and pro-inflammatory cytokines (TNFα, IL-1β) in BAL, enhanced production MMP9 and MMP12 and decreased lung function. Furthermore, it has been observed that exposure to LPS induces bronchial wall thickening, hyperplasia of goblet cells, collagen deposition.167 LPS administration may occur through direct instillation into nasal cavities or intratracheal or intravenous injection and inhalation. Exposure by inhalation is the most widely used, since it induces a greater inflammatory response compared to intratracheal instillation, because aerosolized LPS distributes uniformly throughout the lung, while by instillation, distribution is irregular and can cause a significant inflammatory response exclusively where LPS is deposited.168 Thus, LPS administered either alone or in combination with cigarette smoking is useful to induce a strong bronchial inflammation, in order to mimic events of exacerbations that commonly occur in concomitance with respiratory infections.155

**Sulfure dioxide, nitrogen dioxide, oxidants and particulate** Sulfur dioxide (SO₂) is an irritant gas used to induce, in rodent or guinea pigs, lung damages. Exposure to high levels (200 to 700 ppm for 4-8 weeks) is able to trigger pulmonary inflammation, with increased polymorphonuclear and mononuclear inflammatory cells in airways, mucus overproduction and goblet cell hyperplasia. Mechanisms underlying this effect have not yet been clarified; however, the interruption of exposure to SO₂ leads to total remission of inflammation within 1 week.166

A brief inhalation to nitrogen dioxide (NO₂) induces an initial response with extensive tissue damage, followed by a late phase in which repair processes are activated. The exposure of rats to 10 ppm of NO₂ for more than 24 hours induce ciliar damages and hypertrophy of bronchial epithelium. A concentration of 15 ppm for 7 days leads to an increased mitochondrial activity and oxygen consumption in the airways. Doses of 50-150 ppm (mg/m³ 94-282) can determine significant lung injury, edema, hemorrhage, pleural effusion and death. Exposure to ozone has a toxic effect on the respiratory tract because of the strong oxidative stress that induces increased production of cytokines and decreased lung function. Some experimental protocols require the use of silica, coal dust and ultrafine particles, commonly found in air pollution derived by combustion. Inhalation of these substances causes an intense oxidative stress that results in pulmonary emphysema. Exposure to diesel exhaust particles (DEP), known for its high
oxidizing power, is another type of approach used in some models of chronic inflammation of the airways. A long-term treatment can determine the onset of cancer.\textsuperscript{166}

### 3.2 TREATMENT WITH PROTEASES

Single intratracheal administration of proteolytic enzymes, such as human neutrophil elastase, porcine pancreatic elastase or papain is sufficient to induce morphological changes such as emphysema. Porcine pancreatic elastase is the most used because of the lower cost and greater availability compared to human neutrophil elastase.\textsuperscript{155} Proteases promote up-regulation of inflammatory cytokines (TNFα, IL-1β, IL-6 and IL-8) resulting in lesions of lung parenchyma, loss of collagen and elastin and consequent enlargement of the airspaces and emphysema. The advantage of these models is that a single intratracheal administration of protease leads to rapid and significant changes, although less significant than those obtained with exposure to cigarette smoke.\textsuperscript{155}

### 3.3 GENE-TARGETING APPROACHES

Genetically-altered monogenic and polygenic models to mimic COPD have been developed in recent years using modern techniques of molecular biology.\textsuperscript{156,169} Gene-depletion and gene-overexpression in mice provide a powerful technique to identify the function and role of distinct genes in the regulation of pulmonary homeostasis \textit{in vivo}. Mice lacking elastin were generated since destruction of alveolar elastic fibers is implicated in the pathogenic mechanism of emphysema and elastin is a major component of the extracellular matrix. It was shown that these animals have an arrest in the development of terminal airways accompanied by fewer distal air sacs that are dilated with attenuated tissue septae.\textsuperscript{170} Also, deficiency of the microfibrillar component fibulin-5 and platelet derived growth factor A (PDGF-A) leads to airspace enlargement.\textsuperscript{171,172} PDGF-A (-/-) mice lack lung alveolar smooth muscle cells, exhibit reduced deposition of elastin fibers in the lung parenchyma and develop lung emphysema due to a complete failure of alveogenesis.\textsuperscript{173} Fibroblast growth factors are known to be essential for lung development. Mice simultaneously lacking receptors for FGFR-3 and FGFR-4 have an impaired alveogenesis with increased collagen synthesis.\textsuperscript{174}

A further mechanism to induce emphysema-like lesions is to expose developmentally normal genetically-modify animals to exogenous noxious stimuli such as tobacco smoke. This also allows to identify potential molecular mechanisms involved in the pathogenesis of COPD. Using MMP12 gene depletion studies it was shown that in contrast to wild type mice, the lung structure of MM12 gene-depleted animals remains normal after long term exposure to cigarette smoke.\textsuperscript{175}

Gene-targeting techniques display very useful tools to examine potential molecular mechanisms underlying human COPD.
4. AIMS OF THE STUDY

Validation of a new murine model of COPD

*In vivo* models of airway inflammation and lung damage are the preferred preclinical system for evaluating mechanisms and mediators related to COPD. As mentioned above, the major risk factor, acute and/or chronic exposure to cigarette smoke (CS), is the model most used.\(^{161,176}\) Wagner et al.\(^ {177}\) identified a model of airway inflammation and hypersecretion exposing rats to different levels of SO\(_2\); other models use acute NO\(_2\) exposure\(^ {178}\) or administration of elastolytic enzymes, including pancreatic elastase, neutrophil elastase and proteases, which results in air-space enlargement.\(^ {179,180}\) Moreover, in recent years models to mimic COPD have been developed using modern techniques of molecular biology that induce lung emphysema secondary to the failure of alveolar septation.\(^ {172}\) However, these approaches are able to reproduce a limited amount of COPD features, such as emphysema and chronic inflammation. The focus should also be on crucial processes in the progression of the disease, such as bacterial exacerbations and remodelling mechanisms.

In a first part of this work, the aim was to reproduce an innovative murine model of COPD through the exposure to a combination of three main risk factors: cigarette smoke, LPS to mimic bacterial exacerbations, and particulate matters with a diameter less 10 μm from environment. A time course was performed by sacrificing animals at the first, third and sixth week of exposure to monitor progression of lung injury.

Anti-inflammatory and bronchodilator therapy in the early stage of COPD

As observed in previous studies on murine model, exposure to risk factors induces a peak of inflammation in the first stage of treatment, responsible for the following activation of remodelling pathways. The aim of this study was to evaluate effects of an early treatment with a combination of anti-inflammatory and bronchodilator drugs to attenuate the inflammatory processes and subsequent tissue changes. This is in contrast to current GOLD guidelines, which provide an anti-inflammatory treatment only in severe stages of COPD, when tissue remodelling has already been occurred. The key point of COPD therapy could be the attenuation of acute inflammation that occurred in the early stage of pathogenesis, when tissue damage is less relevant and bronchial structural changes are still reversible.
a) According to current GOLD guidelines, inhaled corticosteroids should be added to a regimen that already includes a long-acting bronchodilator in advanced stages of COPD, when tissue remodelling has already been occurred.

b) The hypothesis is that the addition of inhaled corticosteroids to a bronchodilator therapy in early stages of pathogenesis could be able to influence the progression of COPD, as tissue damage is less relevant and bronchial structural changes are still reversible.
5.1 ANIMALS

The study protocol was reviewed and approved by the Committee on Ethics in Animal Experiments of the Italian National Institutes of Health. Seventy BALB/c male mice (10 weeks of age; n=5 for group) were obtained from Charles River Laboratory (Calco, Italy), fed a normal mouse diet *ad libitum*, housed under biosafety level 2 conditions, and cared for according to the standard and specific procedures outlined by the Italian National Institutes of Health. Animals were divided into 14 groups:

- Control mice exposed to room air and aerosolized with saline
- Mice exposed to particulate matter (PM-10) for 1, 3 and 6 weeks
- Mice exposed to LPS for 1, 3 and 6 weeks
- Mice exposed to cigarette smoke for 1, 3 and 6 weeks
- Mice exposed to PM-10, LPS and cigarette smoke for 1, 3 and 6 weeks
- Mice exposed to PM-10, LPS and cigarette smoke for 6 weeks followed by suspension of treatment for 4 weeks.

At the end of the respective exposures mice were sacrificed under anesthesia with intraperitoneal urethane (1.6 g/kg, Sigma-Aldrich, St Louis, MO, USA), bronchoalveolar lavage (BAL) was performed and the lungs were removed. Lungs were immediately dissected and then separated into individual lobes: one was fixed in 4% buffered formalin, routinely processed and embedded in paraffin for histological analysis; the other was frozen in isopentane and stored at −80°C for molecular analysis.

5.1.2 PM-10 EXPOSURE

Mice were exposed to two different types of particulate matter (PM-10): ERM-CZ100 and ERM-CZ120 (Sigma-Aldrich, St Louis, MO), containing respectively organic and inorganic fractions. This product was certified by the Institute for Reference Materials and Measurements (IRMM).
<table>
<thead>
<tr>
<th>ERM-CZ100</th>
<th>ERM-CZ120</th>
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<tbody>
<tr>
<td>Benzo[a]anthracene</td>
<td>Arsenic</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>Cadmium</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>Lead</td>
</tr>
<tr>
<td>Benzo[j]fluoranthene</td>
<td>Nickel</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
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</tr>
<tr>
<td>Indeno[1,2,3-c,d]pyrene</td>
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</tr>
<tr>
<td>Sum of benzo[b]fluoranthene,</td>
<td></td>
</tr>
<tr>
<td>benzo[k]fluoranthene, benzo[j]</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Composition of organic (CZ100) and inorganic (CZ120) fraction of PM-10.

Mice were treated with 2 mg aerosolized PM-10 (1 mg of ERM-CZ100 + 1 mg of ERM-CZ120, diluted in 6 ml of saline) once a day for 5 days/week. The control group was aerosolized with saline only. The aerosol was generated with an ultrasonic nebulizer (Flaem, Universal plus) and introduced into a Plexiglas chamber box where the mice were placed.

5.1.3 LPS EXPOSURE

Aerosolization was conducted by nebulization of LPS (*Escherichia coli* serotype 026:B6; Sigma-Aldrich, St Louis, MO) diluted with saline solution, using an ultrasonic nebulizer (Flaem, Universal plus) and introduced to a Plexiglas chamber box where the mice were placed. Animals were treated twice weekly with 2.5 mg of LPS (diluted in 6 ml of saline) blown into the chamber box for approximately 20 minutes; controls were aerosolized with endotoxin-free saline.

5.1.4 CIGARETTE SMOKE EXPOSURE

Mice were exposed to the smoke of 21 cigarettes/day (3 cycles of 7 cigarettes/cycle), for 5 days/week, using commercial cigarettes (10 mg tar, 0.8 mg nicotine, 10 mg CO) in a special cage (Tecniplast, Buguggiate, Italy). Control mice were exposed to room air.

The smoke was produced by the burning of a cigarette and was introduced into the chamber with the airflow generated by a mechanical ventilator (7025 Rodent Ventilator, Ugo Basile, Biological Research Instruments, Comerio, Italy) at a frequency of 25 strokes/minute and a volume of 8 ml/stroke (flow 200 ml/min).
5.2 PHARMACOLOGICAL TREATMENT

Mice with COPD-like phenotype were treated with different drugs at different times, as described in Tables 4-5. Drugs and doses:

- β₂-agonist: 6 ml salmeterol/die (15 µg/ml)
- Antimuscarinic: 6 ml tiotropium bromide/die (7 µg/ml)
- Corticosteroid: 6 ml fluticasone furoate/die (43.2 µg/ml)

Drugs were aerosolized using an ultrasonic nebulizer (Flaem, Universal plus) and introduced to a plexiglas chamber box, once a day, for approximately 20 minutes. Control mice were aerosolized only with saline.

Table 4. Mice with COPD-like phenotype were treated with different combination of drugs, starting therapy in early stage for some groups and in late phase for others.
<table>
<thead>
<tr>
<th>Group</th>
<th>Exposure to smoke, PM10 and LPS</th>
<th>Pharmacological aerosol treatment (for 4 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control mice</td>
<td>No exposure to risk factors</td>
<td>No treatment (n=5)</td>
</tr>
<tr>
<td>Mice with COPD-like phenotype</td>
<td>Exposure for 5 weeks</td>
<td>No treatment (n=5)</td>
</tr>
<tr>
<td></td>
<td>Exposure for 9 weeks</td>
<td>No treatment (n=5)</td>
</tr>
<tr>
<td>Mice with COPD-like phenotype and early treatment (started at the 1st week of exposure to risk factors)</td>
<td>Exposure for 5 weeks</td>
<td>$\beta_2$ agonist (n=5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antimuscarinic (n=5)</td>
</tr>
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<td></td>
<td></td>
<td>Corticosteroid (n=5)</td>
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<tr>
<td></td>
<td></td>
<td>$\beta_2$ agonist + Corticosteroid (n=5)</td>
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<td></td>
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<td>Antimuscarinic + Corticosteroid (n=5)</td>
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<tr>
<td></td>
<td></td>
<td>$\beta_2$ agonist + Antimuscarinic + Corticosteroid (n=5)</td>
</tr>
<tr>
<td>Mice with COPD-like phenotype and late treatment (started at the 6th week of exposure to risk factors)</td>
<td>Exposure for 9 weeks</td>
<td>$\beta_2$ agonist (n=5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antimuscarinic (n=5)</td>
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<td>Corticosteroid (n=5)</td>
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<td>$\beta_2$ agonist + Corticosteroid (n=5)</td>
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<td></td>
<td>Antimuscarinic + Corticosteroid (n=5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\beta_2$ agonist + Antimuscarinic + Corticosteroid (n=5)</td>
</tr>
</tbody>
</table>

Table 5. Treatment schedule of mice with COPD-like phenotype.
5.3 GENE EXPRESSION

5.3.1 RNA ISOLATION AND REVERSE TRANSCRIPTION

Total RNA was purified from the lungs using TRIzol reagent (Life Technologies, Carlsbad, CA). After purification, RNA was treated with DNase (DNA-free-Ambion), and cDNA was synthesized with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions.

5.3.2 REAL-TIME PCR

PCR reactions were performed with cDNA and 100 nM of commercially available target-specific Taqman-FAM-labelled probes (Applied Biosystems, USA), using TaqMan Universal PCR Master Mix. TaqMan probe/primers specific for 18S (Hs99999901_s1), TGFβ (Mm01176413_m1), αSMA (Mm00725412_s1), SphK1, SphK2, S1P3 and CXCL15 were Taqman Assays-on-Demand Gene Expression Products. All reagents were from Applied Biosystems. Real-time PCR was carried out using ABI PRISM 7000 (Applied Biosystems, Forster City, CA). Gene expression was assessed relative to 18S and as a fold change to control using the 2^(-ΔΔCt) method. All PCR assays were performed in duplicate.

5.4 CYTOKINE LEVELS

5.4.1 BRONCHOALVEOLAR LAVAGE (BAL)

The trachea was exposed and cannulated with a needle (20 gauge x 32 mm); lungs were then washed three times with sterile saline (0.3 ml, 0.3 ml, 0.4 ml). Liquid samples were centrifuged at 1200 g for 15 minutes and supernatants were collected at –80°C for ELISA tests.

5.4.2 ELISA TEST

Cytokine levels were measured using a mouse ELISA Ready-SET-Go! Kit (TNFα, IL1β, GM-CSF, IL-10) purchased from eBioscience (San Diego, CA), according to the manufacturer’s recommended procedure. Briefly, samples were first placed into 96-well plates which had been coated with a mouse monoclonal antibody as a catcher. Following incubation for 2 hours at room temperature, the plate was washed five times with phosphate-buffered saline. Biotin-conjugated antibody and avidin–horseradish peroxidase were then used to detect the presence and concentration of cytokines; the absorbance at 450 nm was determined by a microplate reader. The concentration of cytokines in each sample was extrapolated from a standard curve, obtained with scalar concentrations of recombinant cytokine, and assayed with ELISA simultaneously with the samples.
5.5 HISTOLOGICAL ANALYSIS

Lung sections (4 μm) were paraffin-embedded and stained with haematoxylin-eosin (Bio Optica, Milan, Italy) to measure bronchial wall thickness and tissue destruction, and Alcian blue–periodic acid–Schiff (AB-PAS) (Bio Optica, Milan, Italy) to evaluate goblet cell hyperplasia. Images of lung tissue sections were captured using a digital camera (AxioCam ICc1 R4; Zeiss, United States).

5.5.1 BRONCHIAL WALL DIAMETER

The thickness of the bronchial wall was measured by tracing around the basement membrane and the luminal surface of epithelial cells and calculating the mean distance between the lines using Axio Vision Rel. 4.8 software. A minimum of 10 bronchi per mouse measuring 150–350 μm luminal diameter were analysed by two separate double-blinded operators using Axio Vision Rel. 4.8 software (Zeiss, United States) which was calibrated with a reference micrometre slide.

5.5.2 GOBLET CELL PERCENTAGE

For goblet cell determination, using ImageJ software, AB-PAS-stained sections were evaluated and a percentage of the goblet cell area of each bronchus was assessed. A minimum of 10 bronchi per mouse were analysed by two separate double-blinded operators.

5.5.3 EMPHYSEMA

Pulmonary emphysema was quantified based on the degree of alveolar destruction, as determined by measuring the mean linear intercept (L_m) in micrometers. To measure the intercepts, a sheet with 10 horizontal and 11 vertical lines was laid over the images. The L_m is obtained using the equation L_m = L_{tot}/L_i, where L_{tot} is the total length of the lines in the microscopic field, and L_i is the number of intercepts of alveolar structures with the lines of the reticulum.

5.5.4 IMMUNOHISTOCHEMICAL STAINING AND QUANTIFICATION

Applied antibodies were subjected to in-house validation by the manufacturer for immunohistochemical analysis on paraffin-embedded material. The antibodies used in the study were anti-elastase, anti-TGFβ and anti-αSMA (ThermoScientific, Waltham, MA). Sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the specimens in 0.25 mM EDTA solution (pH 8.0) at 95°C for 45 min. Slides were incubated in 3% H₂O₂ in methanol for 10 minutes to block endogenous peroxidases; primary antibodies were incubated overnight at 4°C and then with ImmPRESS reagents (VECTOR Laboratories, Burlingame, USA) for 30 minutes. Sections were incubated in DAB substrate kit for peroxidase (VECTOR Laboratories, Burlingame, USA) and counterstained. Using ImageJ software, immunostained sections were evaluated and a percentage of the
positive area of each bronchus was assessed. A minimum of 10 bronchi per mouse were analysed by two separate double-blinded operators.

5.6 WESTERN BLOT

Proteins were extracted from lung tissue using protein extraction buffer (150 mM sodium chloride; 1% Triton; 50 MM Tris pH 8.0; 1× protease inhibitor cocktail (ThermoScientific, Rockford, U.S.A.). Tissue was homogenized, maintained in constant agitation for 2 hours at 4°C and then centrifuged for 20 minutes at 13,000 g at 4°C. Protein concentrations were measured with QuantumMicro Protein assay (EuroClone, Pero, Italy). A total of 25 μg of protein lysates were subjected to SDS-PAGE on 12% acrylamide gel and transferred on to Amersham Hybond-ECL membrane (GE Healthcare, Orlando, FL). The blot was incubated with 5% non-fat dry milk at room temperature for 1 hour to block non-specific binding sites and then incubated for 2 hours at room temperature with specific antibodies against α-SMA, TGF-β and tubulin (Sigma-Aldrich, ST Louis, MO) as endogen control. The membrane was incubated in HRP-conjugated secondary antibody for 1 hour at room temperature and detected with an ECL kit (LiteAblot Plus, EuroClone, Pero, Italy).

5.7 AIRWAY RESPONSIVENESS

Mice were sacrificed and bronchial tissues was rapidly dissected and cleaned from fat and connective tissue. Rings of 1-2mm length were cut and placed in organ baths mounted to isometric force transducers (Type 7006, Ugo Basile, Comerio, Italy) and connected to a Powerlab 800 (AD Instruments, Ugo Basile, Comerio, Italy). Rings were initially stretched until a resting tension of 0.5g was reached and allowed to equilibrate for at least 30 min. In each experiment bronchial rings were challenged with carbachol (10^-6 mol/L) until the response was reproducible. Once a reproducible response was achieved bronchial reactivity was assessed performing a cumulative concentration-response curve to carbachol (1x10^-8 -3x10^-5 mol/L).

5.8 STATISTICAL ANALYSIS

Data were analysed with Prism GraphPad statistical software (GraphPad Software, Inc., San Diego, CA, USA) using one-way analysis of variance (ANOVA), followed by post hoc comparison using Bonferroni’s t-test. We considered P values <0.05 to be significant.
6. RESULTS

6.1 MURINE MODEL OF COPD

PM10, LPS and smoke have a synergistic action, inducing an early and stronger activation of inflammation

Evaluation of inflammatory cytokines in BAL collected from mice has shown that treatment with a combination of risk factors (PM-10, LPS and smoke) is able to trigger a strong and rapid inflammatory response in the first week of exposure. In the following weeks cytokine levels tended to reduce, particularly in the last week of exposure. This trend was not observed in groups exposed to single risk factors: indeed, they have shown a delayed inflammatory response (Fig. 1A,B,C). Moreover, gene expression of lungkine (CXCL15), which is involved in lung-specific neutrophil trafficking, was increased in all weeks of combined exposure, unlike what was observed in a single exposure that induced a significant increase of lungkine only in the last week of treatment with PM10 and LPS (Fig. 2). Interestingly, IL-10 levels were significantly reduced over control values in all groups from the third week of treatment. Therefore, it is evident that PM10, LPS and smoke have a synergistic action, anticipating the activation of inflammatory pathways and establishing a condition of chronic inflammation, highlighted by progressive lower levels of IL-10 (Fig. 1D).

The degree of tissue destruction is progressively increased in all groups

Gene expression of neutrophil elastase and MMP-9 is significantly increased in the sixth week of combined exposure (Fig. 3A); protein expression in bronchial tissue, evaluated by immunochemistry, is enhanced during all periods of combined exposure (Fig. 3B). Histological analysis has shown that both single and combined exposures were able to induce a progressive destruction of lung parenchyma, as particularly evidenced in the last week of combined treatment (Fig. 3C).

Remodelling processes are progressively activated and lung function reduced

Both single and combined exposures were able to progressively increase relative gene expression of key remodelling factors (TGFβ and αSMA); levels are particularly high in the sixth week of combined treatment (Fig. 4A). These data were confirmed by the evaluation of bronchial tissue expression of these key proteins (Fig. 4B-C). Moreover, single and combined exposures were able to increase relative gene expression of kinases that activate S1P (SphK1-2) and receptor S1P3. The trend is similar to that observed with other remodelling factors (Fig. 5).

Histological analysis has shown that all treatments are able to increase bronchial wall diameter and goblet cell hyperplasia, but this effect is particularly evident in the sixth week of combined exposure to risk factors (Fig. 6A-B). Moreover, we observed that B cells tend to organize into follicles only in groups exposed to a combination of factors, with more evidence in the last week of treatment (Fig. 6C).
Persistent lung injuries and consequent bronchial remodelling impair the physiological functions of the lung. This is confirmed by airway responsiveness measurements which has shown a progressive and significant decrease in bronchial reactivity in mice exposed to a combination of risk factors, particularly in those treated for 6 weeks (Fig. 7).

**Bronchial modifications are irreversible after treatment suspension**
After 6 weeks of exposure, discontinuation of treatment for another 4 weeks was unable to improve lung remodelling, as there were no significant changes in bronchial wall diameter, in tissue destruction and in goblet cell hyperplasia compared to mice treated and sacrificed at 1, 3 and 6 weeks of exposure (Fig. 8).

### 6.2 THERAPY STUDY

**Cytokine release is reduced only with an early therapeutic treatment**
Anti-inflammatory and bronchodilator treatment significantly decreases levels of TNFα, IL1-β and GM-CSF in a group early treated compared to untreated. No significant effects were observed in groups treated later, since levels of cytokines resulted similar between group untreated and those treated (Fig. 9A-B-C).

**Pharmacological treatment counteracts remodelling processes only if administered during early phase of lung injury**
Treatment with corticosteroids and bronchodilators, alone and/or in combination, was able to significantly decrease TGFβ and αSMA mRNA levels in lungs, both in groups treated early than in those treated later (Fig. 10A). This trend was confirmed with immunochemistry which revealed a significant reduction in bronchial expression of proteins (Fig. 10B). These data demonstrate that pharmacological treatment had a positive effect on molecular pathway involved in remodeling processes, whatever the period of administration.

Therapy with corticosteroid and bronchodilator drugs, particularly if combined, was able to significantly counteract lung tissue destruction, bronchial wall thickness and goblet cell hyperplasia when administered in the early stages. Indeed, bronchial alterations remained at control levels. Late treatment was able to contrast emphysema and goblet cell hyperplasia, but bronchial tissue resulted still significantly remodelled compared to controls (Fig. 11A-B-C). Similar trend was observed with lymphoid follicles positive for B cells, a marker of severe stage of COPD (Fig. 11D).
7. DISCUSSION

7.1 COMBINED EXPOSURE TO CIGARETTE SMOKING, ENVIRONMENTAL POLLUTIONS AND LIPOPOLYSACCHARIDE REPRODUCES AN EXPERIMENTAL MURINE MODEL OF COPD

In contrast to the large number of experimental studies of allergic airways inflammation, investigations into COPD are limited. The current challenge is to identify the role of different mediators and molecular mechanisms potentially involved in the pathophysiology of COPD, as no effective treatments able to reverse COPD progression have yet been discovered. Thus validation of animal models is necessary to understand pathological mechanisms and identify new therapeutic strategies.

In 1965, Gross et al.\textsuperscript{182} demonstrated for the first time that the intratracheal instillation of papain could cause emphysema-like lesions in rats; moreover, a clinical study by Laurell and Erickson\textsuperscript{64} showed that patients deficient in α1-antitrypsin had an increased risk of emphysema. These data were the scientific basis for the hypothesis of protease–antiprotease imbalance in the pathogenesis of emphysema. In subsequent years additional animal models were developed, leading to a greater understanding of lung biology in pathological conditions similar to those of COPD. Models are generally based on the induction of emphysema-like lesions through the instillation of proteases, or on the triggering of a chronic inflammation via the inhalation of noxious agents (smoke or SO\textsubscript{2})\textsuperscript{161,177,180}, depending on the duration and doses required by the experimental protocol. An important approach is the use of knockout animals, such as PDGF-A-deficient mice, which develop emphysema.\textsuperscript{172}

In contrast to the complexity of COPD in humans, currently available animal models are limited because animals do not develop the disease spontaneously; moreover, experimental protocols reproduce a limited number of phenotypical characteristics, neglecting crucial aspects, such as exacerbations due to respiratory infection, that worsen airways inflammation (already present in COPD patients) and accelerate the decline of lung function. In addition, it is important to carefully evaluate morphological and molecular modifications to determine how far experimental models overlap with clinical observations in patients.

The aim of this study was to validate an innovative COPD model based on combined exposure to the main risk factors: cigarette smoke, environmental pollution and bacterial exacerbations, using LPS that mimics injury by infections. Using molecular and morphological analysis, we compared single exposure to each factor as well as combined exposure, in order to highlight a possible synergistic effect between different treatments. In addition, a time course was performed by sacrificing animals at 1, 3 and 6 weeks of exposure, to monitor the progression of lung injury. The protocol lasted 6 weeks, as combined exposure for 9 weeks induces inflammation and remodelling processes that are no different from those in the group exposed for 6 weeks, thereby revealing a plateau of pathogenesis.
Assessment of proinflammatory cytokines in BAL showed that combined exposure to risk factors is able to induce a strong inflammatory response in the first week of treatment; in the following weeks levels of cytokines were reduced, but nevertheless remained significantly higher than in the control group, unlike what was found in the groups with a single exposure that showed a delayed inflammatory response. Interestingly, levels of IL-10 in BAL were reduced in all groups in the third and sixth weeks of exposure, with more evidence in groups exposed to a combination of factors; this is consistent with the low levels of IL-10 observed in patients with moderate/severe COPD and in smokers. Gene expression of CXCL15, a chemokine specifically expressed by lung bronchoepithelial cells and involved in lung-specific neutrophil trafficking, was increased during all periods of combined exposure. Therefore, these data demonstrate that a combination of risk factors can induce an acute inflammatory response in the first week of treatment; in the following weeks a chronic inflammatory state is established, confirmed by lower levels of IL-10.

A persistent inflammatory process leads to the release of several proteolytic enzymes, in particular by neutrophils and macrophages; an increase in lung parenchymal protease levels induces a progressive destruction of bronchial tissue, with consequent loss of alveolar attachments and emphysema. This condition activates repair processes through the release of several growth factors; over time, in pulmonary chronic diseases such as COPD, a continuous repair of tissue leads to a modification of the original architecture of the tissue, with consequent bronchial remodelling. In this study we observed that gene expression levels of MMP-9 and neutrophil elastase were significantly increased only in groups with combined exposures from the third week onwards, with an evident peak in the sixth week of exposure. These data were confirmed by histological analysis, which showed a significant increase in emphysema-like lesions, not only in groups exposed to a combination of factors, but also in groups exposed to smoke and to LPS. Further confirmation was derived by the detection of elastase by immunochemistry.

We also analysed the key remodelling factors TGFβ and αSMA: several studies have confirmed that TGFβ, a profibrotic cytokine, is increased in the remodelled airways of patients with COPD and/or asthma. The fibrogenesis process depends on differentiation of fibroblasts into myofibroblasts, mediated by αSMA. An increase of αSMA release is directly induced by TGFβ, through Smads proteins. Moreover, Bossè et al. showed that TGFβ is responsible for the increased proliferation of bronchial smooth muscle cells, synergistically with FGF2. In our study, gene expression levels of these remodelling factors were significantly increased in the combined exposure groups in the third week of exposure, and even more increased in the sixth week. In a group treated with a single factor the trend was similar, albeit smaller. The detection of TGFβ and αSMA proteins using immunochemistry and Western blot analysis in bronchial tissue confirmed the results obtained with real-time PCR.

We also analysed the expression of sphingosine-1-phosphate (S1P), as some studies have demonstrated its role in bronchial inflammation and remodelling; Kono et al. showed that activation of the SphK1/S1P pathway regulates the differentiation of fibroblasts into myofibroblasts, mediated by TGFβ
through the S1P3 receptor. The trend towards gene expression of kinases that activate S1P (SphK1-2) and receptor S1P3 is similar to that observed with other remodelling factors. Morphometric analysis showed that bronchial wall thickness is increased from the third week of exposure, and even more so in the sixth week, particularly in groups treated with a combination of risk factors. Similar data were observed with goblet cell hyperplasia, which is responsible for mucus hyperproduction and airflow obstruction in humans.190 Several clinical studies have demonstrated that the progression of COPD from mild to moderate/severe is characterized by the formation of lymphoid follicles positive for B cells, consistent with increased immune surveillance in the bronchial mucosa, as the close relationship between epithelium, APC cells and lymphoid follicles facilitates activation of the adaptive immune response.51 In this study we observed that B cells tend to organize into follicles in lung tissue in the sixth week of combined exposure, particularly around the bronchial walls. No significant evidence was observed in groups exposed to a single factor (data not shown).

We evaluated pulmonary function through airway responsiveness measurements, which showed a progressive reduction in bronchial reactivity, with significant evidence in the last week of treatment; this is consistent with the hypothesis that continuous differentiation of fibroblasts into myofibroblasts impairs the physiological functions of the lung.

Remodelling and loss of alveolar attachments are probably irreversible. This was an interesting result derived from the evaluation of bronchial structural changes 4 weeks after the cessation of treatment; we observed no significant variations from a group exposed for 6 weeks and immediately sacrificed, demonstrating that this protocol induces irreversible bronchial remodelling, even if the exposure to risk factors is suspended.

Validation of new murine models must be based on the possibility of making comparisons between the morphological and molecular analyses in mice and clinical observations in patients. As described by Hogg51, the primary host defences against noxious stimuli are the innate and adaptive inflammatory immune responses. The innate defence system of the lung includes the mucociliary clearance system and the epithelial barrier, supported by the acute inflammatory response that follows tissue injury; chronic inflammation is then accompanied by irreversible alterations to lung tissue and small airway obstruction. Moreover, cigarette smoking alters the composition of the lung in such a way that fibrosis becomes self-perpetuating, even after patients have stopped smoking.191 Indeed, the inflammatory changes in the airways caused by cigarette smoke exposure were only partially reversed after smoking cessation.

The application of this new experimental protocol is able to trigger inflammatory processes which, in the later stages of exposure, induce destruction of the lung parenchyma and airways remodelling (bronchial wall thickening, goblet cell hyperplasia and B-cell follicles) that are irreversible after treatment cessation. This demonstrates that the pathogenesis observed in mice reflects the natural history of COPD.
Graphical representation of the progressive pathophysiological changes observed in this innovative murine model during all period of combined exposure to risk factors.

Earlier stages of COPD are associated with more reversible changes, whereas later stages show more structural alterations and irreversibility. Thus a careful assessment of the structural phenotype of COPD patients is likely to lead to optimal categorization for trials, and earlier disease is more likely to respond to therapies.

7.2 AN EARLY ANTINFLAMMATORY AND BRONCHODILATOR THERAPY COUNTERACTS LUNG INFLAMMATION AND REMODELLING PROCESSES

Although COPD is a major global health problem with a rising incidence and morbidity, few pharmacotherapeutic advances have been made over the past decades. The disease causes approximately 2.75 million deaths annually, and the number is projected to increase. COPD is characterized by progressively deteriorating lung function, accompanied by breathlessness (particularly after physical exertion), cough, sputum production, respiratory failure, and eventually death. The aim of COPD treatment should be to increase lung function, prevent disease progression, decrease symptoms and exacerbations, and improve quality of life; however, the approach to therapy has generally focused on symptomatic relief. With the exception of smoking-cessation programs for patients with early disease, oxygen treatment for persistent hypoxemia, and lung-reduction surgery for selected patients with emphysema, no treatment has been shown to reduce mortality. However, newer therapeutic strategies could improve quality of life and reduce serious morbidity, particularly exacerbations of disease and hospital admissions.

The recently updated GOLD guidelines recognize that long-acting bronchodilator therapy is central to the symptomatic management of moderate COPD. LABAs, such as salmeterol, achieve this goal providing long-term sustained bronchodilation without tolerance, each dose improves airflow limitation for
more than 12 hours\textsuperscript{201,202}, reduces breathlessness and exacerbation rate, and is associated with a clinically significant improvement in health status.\textsuperscript{202} The potential anti-inflammatory and cytoprotective properties of LABAs and the documented effect on exacerbation rate could have a significant impact on patient survival.

Anti-inflammatory drugs, such as inhaled corticosteroids, have little or no effect on the rate of decline of lung function\textsuperscript{203,204}, but may reduce the frequency of exacerbations\textsuperscript{203,205}, especially when combined with an inhaled long-acting $\beta_2$-agonist.\textsuperscript{206} Nevertheless, a combination therapy with corticosteroid and $\beta_2$-agonist doesn’t reduce mortality rate, as demonstrated by the TOwards a Revolution in COPD Health (TORCH) study. This randomized, double-blind trial compared salmeterol at a dose of 50 μg plus fluticasone propionate at a dose of 500 μg twice daily (combination regimen), administered with a single inhaler, with placebo, salmeterol alone, or fluticasone propionate alone for a period of 3 years. Outcomes for the comparison between the combination regimen and placebo were death from any cause, frequency of exacerbations, health status, and spirometric values. Although during the 3 years of the study combination regimen resulted in significantly fewer exacerbations, improved health status and lung function, as compared with placebo, it did not significantly reduce mortality from any cause.\textsuperscript{147}

The 4-year randomized, double-blind trial Understanding Potential Long-Term Impacts on Function with Tiotropium (UPLIFT) compared therapy with either tiotropium or placebo in patients with COPD. This study showed that tiotropium was associated with improvements in lung function, quality of life, and exacerbations, but did not significantly reduce the rate of decline in FEV1.\textsuperscript{207}

Current GOLD guidelines indicate an advantage in the use of long acting $\beta_2$-agonists and/or anticholinergics combined with inhaled steroid in the moderate/severe stages, characterized by frequent exacerbations, even though these combination therapies administered in advanced stages of COPD seem to be unable to slow down the progression of the disease and reduce mortality. We hypothesize that a combined treatment could be useful to influence the progression of COPD only in the early stages of pathogenesis, when tissue damage is less relevant and bronchial structural changes are still reversible.

To verify this statement, we set up an experimental therapeutic protocol using a previously described murine model of COPD, which requires a combined exposure to cigarette smoke, PM10 and bacterial LPS. The combined exposure is able to trigger inflammatory processes that, in the later stages, induce destruction of the lung parenchyma and airway remodelling such as bronchial wall thickening, goblet cell hyperplasia and B-cell follicles. These modifications were irreversible after exposure cessation, reflecting the natural history of COPD. Animals were treated at different times with a long acting $\beta_2$-agonist (salmeterol), an anticholinergic (tiotropium) and a corticosteroid (fluticasone), either alone or in combination. One group was treated from the second week of exposure to harmful factors (early therapy), when remodelling is still insignificant; the other group began the therapy from the sixth week (late therapy), when remodelling processes are strongly activated. Drug treatments lasted 4 weeks in all groups.
Molecular and morphological evaluation was based on the comparison between the group with COPD-like phenotype and not pharmacologically treated, and groups treated with different drug combinations, in order to assess the effects of early and late treatment on inflammatory and bronchial remodelling processes.

In a first analysis, we evaluated BAL fluids cytokine levels through ELISA tests showing that early therapy was able to drastically reduce inflammation, while late therapy didn’t induce significant changes in the release of inflammatory cytokines, compared to untreated group. This result is consistent with those observed in preliminary studies on murine model where the inflammation reaches its peak in the early weeks of exposure to risk factors and tends to gradually decrease.

We also analysed gene and tissue expression of key factors involved in tissue remodelling. Several studies confirmed that TGFβ, a profibrotic cytokine, is elevated in thickened airways and in BAL fluid of patients with asthma and/or COPD, suggesting a prominent role in bronchial modifications.\textsuperscript{186,187} Indeed, a hallmark of pulmonary remodelling is the differentiation of fibroblasts into myofibroblasts, typically mediated by αSMA.\textsuperscript{106} TGFβ contributes to lung fibrosis increasing fibroblast survival and inducing an increase in αSMA levels, by through the activation of Smad proteins. Moreover, Bosse and colleagues\textsuperscript{188} showed that TGFβ is also responsible for increased proliferation and survival of bronchial smooth muscle cells, contributing to muscular hypertrophy and hyperplasia. We observed a significant reduction in gene and tissue expression of TGFβ and αSMA, both with early and late therapy, revealing a positive effect on molecular pathway, regardless of dosing time.

Instead, histological analysis revealed a different trend for tissue modifications where only early treatment significantly counteracted the thickening of the bronchial wall and the emphysema, compared to untreated group. Assessment of goblet cell hyperplasia, responsible for mucus overproduction in the bronchial lumen, revealed that all groups had high percentages of mucus cells but only early therapy was able to significantly reduce them, particularly the triple therapy. Indeed, late pharmacological treatment reduces hyperplasia, although with a lesser effect. Several clinical studies have demonstrated that the progression of COPD from mild to moderate/severe is characterized by the formation of lymphoid follicles positive for B cells.\textsuperscript{51} Our data showed that bronchial tissue positivity to B lymphocytes was significantly reduced only with the early therapeutic treatment.

In conclusion, these data confirmed that a late bronchodilator and anti-inflammatory therapy is not able to counteract tissue changes, despite effective action on gene and tissue expression of remodelling factors. Instead, an early pharmacological treatment reduces the peak of inflammation contributing to attenuate bronchial alterations that could be still reversible.

Thus, the addition of an anti-inflammatory drug to bronchodilator therapy in the early stages, besides decrease symptoms, reduce exacerbations and improve quality of life, could be useful to increase lung function, decrease the extent of bronchial obstruction and effectively counteract the progression of the disease.
8. FIGURES

FIGURE 1

A. TNFα

B. IL-1β
C. GM-CSF

D. IL-10

Legend:
- **Control**
- **PM10**
- **LPS**
- **Smoke**
- **PM10+LPS+Smoke**
**Figure 1. ELISA tests on BAL to assess inflammatory cytokine levels.**

**A.** Single exposure to risk factor (PM10, LPS, smoke) is able to increase TNFα levels from the third week of treatment, with particular evidence in the sixth one in groups exposed to LPS and smoke († p<0.001 vs PM10 1 wk; ‡ p<0.001 vs LPS 1 wk; § p<0.001 vs smoke 1 wk). Combined exposure enhances TNFα levels in the first week with a progressive and significant reduction in the following weeks (II p<0.001 vs P+L+S 1 wk).

**B.** IL-1β levels are significantly enhanced in the third and sixth week of single exposure († p<0.001 vs PM10 1 wk; ‡ p<0.001 vs LPS 1 wk; § p<0.001 vs smoke 1 wk). Treatment with a combination of risk factors significantly increases cytokine levels in the first and third week of treatment; in the sixth weeks levels return to basal values (II p<0.001 vs P+L+S 1 wks).

**C.** GM-CSF dosage has shown significant high levels in the first week of single treatment with PM10 and LPS († p<0.001 vs PM10 1 wk; ‡ p<0.001 vs LPS 1 wk). Combined exposure enhances levels in the first and third week of treatment (II p<0.001 vs P+L+S 6 wks).

**D.** In all groups of treatment levels of IL-10 are significantly reduced during the third and sixth week of exposure († p<0.001 vs PM10 1 wk; ‡ p<0.001 vs LPS 1 wk; § p<0.001 vs smoke 1 wk; II p<0.001 vs P+L+S 1 wk).

* p<0.001 vs CTRL. Data represent the mean ± SD.
Figure 2. CXCL15 (murine homologue of human IL-8) mRNA expression in lungs determined by real-time PCR analysis.

* p<0.001 vs CTRL. Data represent the mean ± SD.
Figure C.

Legend:
- **Control**
- **PM10**
- **LPS**
- **Smoke**
- **PM10+LPS+Smoke**

Graph showing **L_m (Mean Linear Intercept)** for different conditions:
- **CTRL**
- **PM 10 1 wk**
- **PM 10 3 wks**
- **LPS 1 wk**
- **LPS 3 wks**
- **LPS 6 wks**
- **Smoke 1 wk**
- **Smoke 3 wks**
- **Smoke 6 wks**
- **P+L+G 1 wk**
- **P+L+G 3 wks**
- **P+L+G 6 wks**

Significance marked with an asterisk (*) for certain conditions.
Figure 3. Evaluation of gene and tissue expression of proteolytic enzymes and assessment of the degree of lung destruction.

A. Real time PCR showed a significant increase of elastase relative gene expression in the sixth week of combined exposure (II p<0.001 vs P+L+S 1-3 wks). Relative gene expression of MMP9 increased in the sixth week of combined exposure (II p<0.001 vs P+L+S 1-3 wks).

B. Assessment of positive bronchial area to elastase immunoreaction has shown that protein expression is significantly increase in the sixth week of exposure to LPS (‡ p<0.001 vs LPS 1-3 wks), in all groups exposed to smoke (* p<0.001 vs CTRL) and to a combination of risk factors, with particular evidence in the sixth week (II p<0.001 vs P+L+S 1-3 wks).

C. Representatives images of haematoxylin-stained lung section (20X) in control mice (I), in mice exposed to a combination of smoke, PM10 and LPS for 1 week (II), 3 weeks (III) and 6 weeks (IV).

Evaluation of Mean Linear Intercept on lung tissue section has shown significant variations in group exposed for 6 weeks to LPS, to smoke and to a combination of three factors (II p<0.001 vs P+L+S 1-3 wks).

* p<0.001 vs CTRL. Data represent the mean ± SD.
FIGURE 4

A.
C.

![Image of Western Blot and Bar Graphs](image-url)
Figure 4. Gene and tissue expression of remodelling key factors, TGFβ and αSMA.

A. Relative gene expression of TGFβ and αSMA, analysed through real time PCR, is significantly increased in groups exposed to LPS, smoke and to a combination of toxic agents (‡ p<0,001 vs LPS 1wk; § p<0,001 vs smoke 1wk; II p<0,001 vs P+L+S 1 wk).

B. Representatives images of TGFβ-positive bronchial area (20X) in lung of control mice (I), mice exposed to a combination of smoke, PM10 and LPS for 1 week (II), 3 weeks (III) and 6 weeks (IV). Analysis of positive bronchial area to TGFβ immunoreaction has shown a significant increase in tissue expression in groups exposed to LPS for 6 weeks (‡ p<0,001 vs LPS 1 wk and 3 wks), in groups treated with smoke for 3 and 6 weeks (§ p<0,001 vs smoke 1 wk) and in all those exposed to a combination of risk factors (II p<0,001 vs P+L+S 1 wk).

Tissue expression of αSMA resulted significantly enhanced in groups exposed only to smoke (§ p<0,001 vs smoke 1 wk) and in groups treated with a combination of agents, particularly in the sixth week of exposure (II p<0,001 vs P+L+S 1 wk).

C. Western blot analysis has shown a significant increase in αSMA and TGFβ tissue expression in group exposed for 6 weeks to a combination of risk factors (II p<0,01 vs P+L+S 1-3 wks).

* p<0,01 vs CTRL. Data represent the mean ± SD.
Figure 5. Gene expression of kinases that activate S1P (SphK1-2) and receptor S1P3.

Relative gene expressions of SphK1, SphK2 and S1P3 receptor, analysed through real time PCR, are significantly increased in groups exposed to LPS, smoke and to a combination of toxic agents (‡ p<0.001 vs LPS 1wk; § p<0.001 vs smoke 1wk; † p<0.001 vs P+L+S 1wk; II p<0.001 vs P+L+S 1 wk).

* p<0.01 vs CTRL. Data represent the mean ± SD.
FIGURE 6

A.
c. Control

P+L+S 1 week  P+L+S 3 weeks

P+L+S 6 weeks
Figure 6. Evaluation of remodelling processes: bronchial wall thickness, goblet cells hyperplasia and B cell-positive lymphoid follicles.

A. Representatives images of haematoxylin-stained bronchial sections (20X) in control mice (I), in mice exposed to a combination of smoke, PM10 and LPS for 1 week (II), 3 weeks (III) and 6 weeks (IV).

Measurement of bronchial wall diameter (μm) has shown a significant thickening in all groups of exposure (* p<0.001 vs CTRL). In particular, combined treatment induces a progressive increase in wall diameters, with a peak in the sixth week of exposure (II p<0.001 vs P+L+S 1 wk and 3 wks).

B. Representatives images of P.A.S.-stained lung sections (20X) in control mice (I), in mice exposed to a combination of smoke, PM10 and LPS for 1 week (II), 3 weeks (III) and 6 weeks (IV).

Percentage of P.A.S. positive area resulted increased in all groups treated, particularly in those exposed to a combination of risk factors for 6 weeks (II p<0.001 vs P+L+S 1-3 wks).

C. Representatives images of B cell-positive bronchial area (anti CD45, 20X) in lung of mice untreated and those exposed to a combination of smoke, PM10 and LPS for 1 week, 3 weeks and 6 weeks. B cells localized around bronchial wall from the first week of combined exposure, and tend to organize into follicles in the third and sixth week of treatment. No significant positive immunoreaction was observed in groups exposed to a single toxic agent (data not shown).

* p<0.01 vs CTRL. Data represent the mean ± SD.
Figure 7. Evaluation of lung function.

Airway responsiveness measurements revealed a progressive reduction of lung contractility, significant at the sixth week of exposure to noxious agents.

* p<0.01 vs CTRL. Data represent the mean ± SD.
Figure 8. Evaluation of histological changes in a group treated for 6 weeks followed by a suspension of exposure for 4 weeks, compared to groups treated for 1, 3 and 6 weeks.

Measurements of bronchial wall diameter (μm), Mean Linear Intercept and percentage of P.A.S. positive area have shown no significant variations after a suspension of treatment for 4 weeks, compared to groups treated and sacrificed at first, third and sixth week of exposure.
FIGURE 9

A.

B.

C.
Figure 9. ELISA tests on BAL to measure inflammatory cytokine levels.

A. TNFα levels were significantly decreased in groups early treated with a combination fluticasone+salmeterol, fluticasone+tiotropium and fluticasone+salmeterol+tiotropium, compared to the group untreated (‡ p<0,001 vs P+L+S 5wks). No significant variations were observed in grouped treated later. No significant variations were observed in grouped treated later.

B. IL1β dosage showed that cytokine levels were reduced in all groups early treated (‡ p<0,001 vs P+L+S 5wks). No significant variations were observed in grouped treated later.

C. Levels of GM-CSF were significantly decreased in groups early treated with a combination fluticasone+salmeterol, fluticasone+tiotropium and fluticasone+salmeterol+tiotropium, compared to the group untreated (‡ p<0,001 vs P+L+S 5wks). No significant variations were observed in grouped treated later.

* p<0,01 vs CTRL. Data represent the mean ± SD.
FIGURE 10

A. 

[Bar graphs showing TGF-β and αSMA relative expression under different conditions: CTRL, P+S+S 5 weeks, FLU, SALM, TIO, FLU+SALM, FLU+TIO, P+S+S 9 weeks, FLU, SALM, TIO, FLU+SALM, FLU+TIO. Legend includes symbols for Control, Early therapy, and Late therapy.]
**Figure 10. Gene and tissue expression of TGFβ and αSMA.**

**A.** Relative gene expression of TGFβ and αSMA, evaluated through real time PCR, was significantly reduced by early and late treatment (‡ p<0.001 vs P+L+S 5wks; § p<0.001 P+L+S 9wks).

**B.** Representatives images of TGFβ-positive bronchial area (20X) in lung of mice exposed to risk factors for 5 weeks and not treated, mice exposed to risk factors for 5 weeks and treated with fluticasone+salmeterol+tiotropium, mice exposed to risk factors for 9 weeks and not treated, mice exposed to risk factors for 9 weeks and treated with fluticasone+salmeterol+tiotropium. Analysis of positive bronchial area to TGFβ and αSMA immunoreaction has shown a significant reduction in tissue expression in all groups early treated (‡ p<0.001 vs P+L+S 5wks) and in groups treated later with fluticasone, fluticasone+salmeterol, fluticasone+tiotropium and fluticasone+salmeterol+tiotropium (§ p<0.001 vs P+L+S 9wks).
FIGURE 11

A.
D.

**P+L+S 5wks**

**FLUT+SALM+TIOTR**

**P+L+S 9wks**

**FLUT+SALM+TIOTR**
Figure 11. Morphometric analysis of lung sections to evaluate remodelling processes: tissue destruction, bronchial wall thickness, goblet cells hyperplasia and B cell-positive lymphoid follicles.

A. Representatives images of haematoxylin-stained lung section (20X) in control mice (I), in mice exposed to a combination of smoke, PM10 and LPS for 1 week (II), 3 weeks (III) and 6 weeks (IV).
Evaluation of Mean Linear Intercept on lung tissue section has shown that early treatment with fluticasone+salmeterol, fluticasone+tiotropium and fluticasone+salmeterol+tiotropium and late therapy with fluticasone+tiotropium and fluticasone+salmeterol+tiotropium were able to contrast processes that induce emphysema (‡ p<0,001 vs P+L+S 5wks; § p<0,05 P+L+S 9wks).

B. Representatives images of haematoxylin-eosin stained lung section (20X) of mice exposed to risk factors for 5 weeks and not treated, mice exposed to risk factors for 5 weeks and treated with fluticasone+salmeterol+tiotropium, mice exposed to risk factors for 9 weeks and mice exposed to risk factors for 9 weeks and treated with fluticasone+salmeterol+tiotropium.
Measurement of bronchial wall diameters showed that only groups early treated with fluticasone+salmeterol, fluticasone+tiotropium and fluticasone+salmeterol+tiotropium had a diameter significantly reduced compared to untreated (‡ p<0,001 vs P+L+S 5wks).

C. Representatives images of P.A.S.-stained lung section (20X) of mice exposed to risk factors for 5 weeks and not treated, mice exposed to risk factors for 5 weeks and treated with fluticasone+salmeterol+tiotropium, mice exposed to risk factors for 9 weeks and mice exposed to risk factors for 9 weeks and treated with fluticasone+salmeterol+tiotropium.
Percentage of P.A.S. positive area resulted reduced in groups early treated with fluticasone, tiotropium, fluticasone+salmeterol, fluticasone+tiotropium († p<0,05 vs P+L+S 5wks) and particularly fluticasone+salmeterol+tiotropium (‡ p<0,001 vs P+L+S 5wks). Goblet cells were decreased in groups treated later with tiotropium, fluticasone+salmeterol, fluticasone+tiotropium and fluticasone+salmeterol+tiotropium (§ p<0,05 vs P+L+S 9wks).

D. Representatives images of B cell-positive bronchial area (anti CD45, 20X) in lung of mice exposed to risk factors for 5 weeks and not treated, mice exposed to risk factors for 5 weeks and treated with fluticasone+salmeterol+tiotropium, mice exposed to risk factors for 9 weeks and mice exposed to risk factors for 9 weeks and treated with fluticasone+salmeterol+tiotropium.
Early therapy counteracts the formation of B cells positive lymphoid follicles, compared to untreated. No significant differences were observed between groups treated later and group untreated.
* p<0,01 vs CTRL. Data represent the mean ± SD.
REFERENCES


