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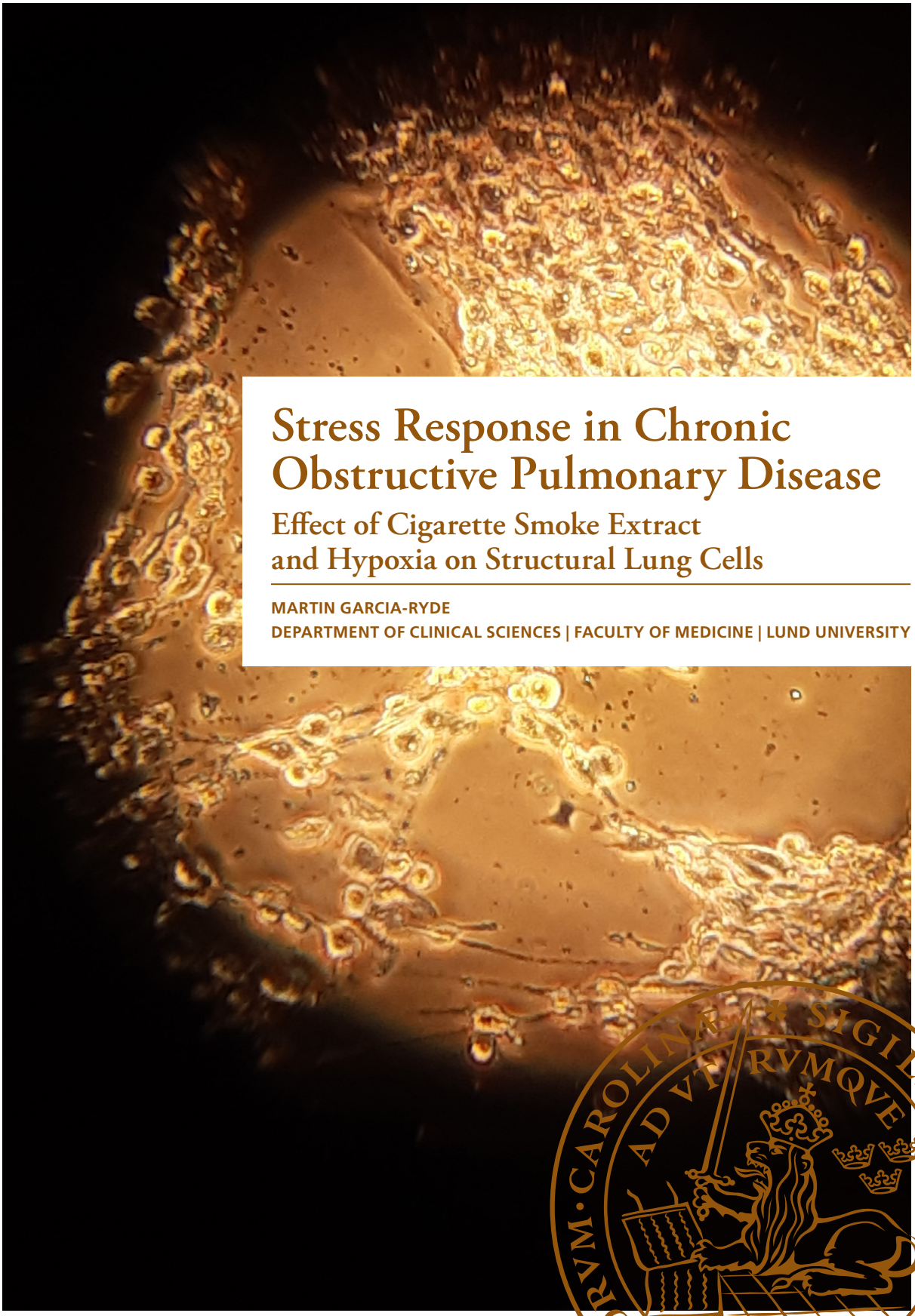

Stress Response in Chronic Obstructive Pulmonary Disease

Effect of Cigarette Smoke Extract and Hypoxia on Structural Lung Cells

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Martin Garcia-Ryde

LUND
UNIVERSITY

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Abstract:

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide according to the world health organization. It is a disease characterized by chronic inflammation and emphysema, and cigarette smoking is the main cause of COPD development. There are several forms of stress present in the lungs of COPD patients, such as smoking induced endoplasmic reticulum stress or hypoxic stress caused by pathological changes in the lung. Many of the mechanisms behind COPD are still unknown, such as why some people develop COPD while others do not despite similar smoking habits. We have investigated differences in how lung fibroblasts from healthy and COPD subjects react at the transcriptional level to cigarette smoke extract or hypoxic exposure. We have also stained the cells to visualize and measure stress related proteins. Additionally, two epithelial cell lines of alveolar or bronchial origin were investigated in a similar way. From these investigations, we have found that there is a difference in how COPD subjects respond to stress, compared to healthy subjects. The healthy subjects go through several changes in expression to try to solve the stress, while this response is lacking in subjects with COPD. This difference is especially noticeable in pathways relating to apoptosis and cell proliferation, but also in pathways relating to hypoxic and endoplasmic reticulum stress. Lung fibroblasts from healthy subjects go into senescence in response to the stress and if the cell fails to resolve the stress, it undergoes apoptosis. Lung fibroblasts from COPD subjects on the other hand regulate different pathways and go straight into apoptosis. This atypical and deficient response in COPD subjects could be a contributing factor to disease progression and to why some people develop the disease.

Key words:

COPD, Cigarette smoke, hypoxia, gene expression, lung, fibroblast, epithel

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Abstract

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide according to the world health organization. It is a disease characterized by chronic inflammation and emphysema, and cigarette smoking is the main cause of COPD development. There are several forms of stress present in the lungs of COPD patients, such as smoking induced endoplasmic reticulum stress or hypoxic stress caused by pathological changes in the lung. Many of the mechanisms behind COPD are still unknown, such as why some people develop COPD while others do not despite similar smoking habits. We have investigated differences in how lung fibroblasts from healthy and COPD subjects react at the transcriptional level to cigarette smoke extract or hypoxic exposure. We have also stained the cells to visualize and measure stress related proteins. Additionally, two epithelial cell lines of alveolar or bronchial origin were investigated in a similar way. From these investigations, we have found that there is a difference in how COPD subjects respond to stress, compared to healthy subjects. The healthy subjects go through several changes in expression to try to solve the stress, while this response is lacking in subjects with COPD. This difference is especially noticeable in pathways relating to apoptosis and cell proliferation, but also in pathways relating to hypoxic and endoplasmic reticulum stress. Lung fibroblasts from healthy subjects go into senescence in response to the stress and if the cell fails to resolve the stress, it undergoes apoptosis. Lung fibroblasts from COPD subjects on the other hand regulate different pathways and go straight into apoptosis. This atypical and deficient response in COPD subjects could be a contributing factor to disease progression and to why some people develop the disease.

Popular Summary

Chronic obstructive pulmonary disease (COPD) is a common lung disease among smokers but also affects non-smokers. It is a complex inflammatory disease that leads to impaired airflow in the airways and the breakdown of the outermost parts of the lungs. This leads to difficulty breathing with typical symptoms such as paleness, weakness, coughing, excess phlegm, shortness of breath and wheezing. The disease develops slowly but becomes more serious with time. In the beginning, patients may just have difficulty carrying out physically demanding activities, but eventually, they may have difficulty breathing even at rest. The disease often alternates between better and worse periods, which can be exacerbated by infections, exertion or air pollution. COPD is usually diagnosed by a breathing test.

In Sweden, COPD is strongly linked to smoking with some studies showing that up to half of all smokers suffer from COPD later in life. Still, it often takes at least 30 years to develop clear symptoms, and passive smokers are also affected by the disease. Approximately 6% of Sweden's population has been diagnosed with COPD, and the disease accounts for approximately 3% of all deaths. Globally, the cause of COPD is more commonly air pollution, both outdoors and in the home (burning with wood, coal or dung with poor ventilation). Certain occupations also carry an increased risk of COPD such as those working in dusty environments and those working with harmful chemicals and particles. The disease can also have genetic causes, and some studies indicate that women are more susceptible than men. Alarmingly, COPD is now the third leading cause of death according to the World Health Organization, and the number of deaths due to COPD is increasing.

There is no cure for COPD, but symptoms can be reduced. The most important intervention is removing the cause of the disease, such as by quitting smoking or avoiding polluted air. There are also several medications that can widen the airways or reduce inflammation, making it easier to breathe. Still, these can only reduce the symptoms and slow down the progression of the disease but not cure it.

There is much we still do not know about COPD. For example, we don't know why some people can smoke their entire adult life without developing COPD, while others who do not smoke at all still get COPD. In our research, we have investigated how a certain cell type from the lung (fibroblasts) reacts to cigarette smoke extract, as well as how lung fibroblasts and another type of lung cells (epithelial cells) react to low oxygen levels (hypoxia) to try and mimic the harmful conditions that promote COPD. Epithelial cells are found in the outermost layer of the inside of the airways, and this means that they are in direct contact with the air, making them sensitive to harmful substances that are inhaled. Fibroblasts are a common cell type in the connective tissue and produce substances that build up what is between cells, the so-called connective tissue. This is altered in COPD, which contributes to the

disease. Therefore, we have studied lung fibroblasts to try to understand how this has changed in COPD.

We have seen that lung fibroblasts from people with COPD have a deficient response to the biological stress caused by cigarette smoke and hypoxia. This is most clearly seen in factors linked to cell death, growth of cells and new formation of blood vessels. Lung fibroblasts from healthy individuals stop dividing and if they cannot resolve what caused the stress, the cells enter programmed cell death. In COPD, on the other hand, other genes are affected, and the cells undergo programmed cell death without first stopping their growth. These differences in defense against stress may contribute to the development of the disease and be one of the reasons why some individuals develop COPD. Ultimately, more research along this vein can help us develop a better way of finding those at risk of developing COPD.

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Populärvetenskaplig sammanfattning

Kroniskt obstruktiv lungsjukdom (KOL) är en vanlig lungsjukdom bland rökare som även drabbar icke-rökare. Det är en komplex inflammatorisk sjukdom som leder till försämrat luftflöde i luftvägarna och att lungblåsorna som sitter längst ut i luftrören går sönder. Detta leder till svårigheter att andas och syresätta bloded med typiska symptom så som blekhet, avmagring, slemmig hosta, andfåddhet och väsande eller pipande andning. Sjukdomen kommer först smygande men blir succesivt allvarligare. Först blir det svårt att utföra krävande aktiviteter men så småningom kan patienten ha svårt att andas även vid vila, vilket är svårt funktionsnedsättande. Sjukdomen går ofta omväxlande i bättre och sämre perioder (skov), vilka kan orsakas av infektioner, ansträngning eller luftföroreningar. KOL diagnostiseras vanligen med andningsmätningar (spirometri).

I Sverige är KOL starkt kopplat till rökning och studier visar till och med att upp till hälften av alla som röker drabbas av KOL senare i livet, men det tar ofta minst 30 år för att utveckla tydliga symtom och även passiva rökare drabbas. Ungefär 6% av Sveriges befolkning har diagnostiserats med KOL, och sjukdomen står för ca 3% av alla dödsfall. I ett globalt perspektiv är andra orsaker mer vanliga, speciellt förorenad luft, både i hemmet (eldning med trä, kol eller dynga med dålig ventilation) eller utomhus (luftföroreningar). Vissa yrkesverksamheter ger också förhöjd risk för KOL, t.ex. miljöer med mycket damm (t.ex. spannmål eller mjöl) eller skadliga kemikalier och partiklar (t.ex. kadmium eller svetsning). Sjukdomen kan också ha genetiska orsaker och en del studier indikerar att kvinnor är mer känsliga än män. KOL är nu den tredje vanligaste dödsorsaken i världen enligt världshälsoorganisationen.

Det finns inget botemedel mot KOL, däremot kan man minska symptomen. Det viktigaste är att ta bort det som orsakat sjukdomen, d.v.s. sluta röka eller försöka undvika förorenad luft. Det finns också mediciner som används för att vidga luftvägarna eller minska inflammationen. Dessa kan i allmänhet bara minska symptomen och sakta ner sjukdomsförloppet, men inte bota sjukdomen.

Det är mycket som vi fortfarande inte vet om KOL. Till exempel vet vi inte varför vissa kan röka hela sitt vuxna liv utan att utveckla KOL medan andra som inte alls röker ändå får KOL. I vår forskning har vi undersökt hur en viss celltyp från lungan (fibroblaster) reagerar på extrakt från cigarettrök, samt hur lungfibroblaster och en annan typ av lungceller, epitelceller, regerar på låga syrenivåer (hypoxi) för att försöka efterlikna den skadliga miljön som lungan kan vara utsatt för vid KOL. Epitelceller finns ytterst på luftvägarnas insida och utgör en skyddsbarriär där de är i direkt kontakt med luften, vilket gör dem känsliga mot skadliga substanser som andas in. Fibroblaster är en vanlig celltyp i bindväven och producerar faktorer som bygger upp bindväven, det vill säga lungans struktur som finns mellan celler. Denna

är förändrad i KOL vilket bidrar till sjukdomen. Därför har vi studerat lungfibroblaster för att försöka förstå hur detta är förändrat vid KOL.

Våra studier visar att epitelceller är känsliga för hypoxi. Vi har också sett att lungfibroblaster från personer med KOL har ett bristfälligt svar mot den biologiska stress som orsakats av cigarettrök och hypoxi. Detta märks tydligast i faktorer kopplade till celldöd, tillväxt av celler och nybildning av blodkärl. Lungfibroblaster från friska individer slutar att dela sig och om de inte kan lösa det som orsakat stressen går cellerna in i programmerad celldöd. I KOL däremot så är andra gener påverkade och cellerna genomgår programmerad celldöd utan att först sluta sin tillväxt. Dessa skillnader i försvar mot stress kan bidra till sjukdomens utveckling och vara en av anledningarna till varför vissa individer utvecklar KOL.

List of Papers

Paper I

Ryde M, van der Burg NMD, Berlin F, Westergren-Thorsson G, Bjermer L, Ankerst J, Larsson-Callerfelt AK, Andersson C, Tufvesson E, Expression of stress-induced genes in BAL cells and lung fibroblasts from healthy and COPD subjects.

Manuscript in preparation.

Paper II

Garcia-Ryde M, van der Burg NMD, Larsson CE, Larsson-Callerfelt AK, Westergren-Thorsson G, Bjermer L, Tufvesson E. Lung Fibroblasts from Chronic Obstructive Pulmonary Disease Subjects Have a Deficient Gene Expression Response to Cigarette Smoke Extract Compared to Healthy. Int J Chron Obstruct Pulmon Dis. 2023 Dec 18;18:2999-3014. doi: 10.2147/COPD.S422508. PMID: 38143920; PMCID: PMC10742772.

Paper III

Ryde M, Marek N, Löfdahl A, Pekny O, Bjermer L, Westergren-Thorsson G, Tufvesson E, Larsson-Callerfelt AK, Altered hypoxia-induced cellular responses and inflammatory profile in lung fibroblasts from COPD patients compared to control subjects.

Manuscript in preparation.

Paper IV

Berggren-Nylund R, Ryde M, Löfdahl A, Ibáñez-Fonseca A, Kåredal M, Westergren-Thorsson G, Tufvesson E, Larsson-Callerfelt AK. Effects of hypoxia on bronchial and alveolar epithelial cells linked to pathogenesis in chronic lung disorders. Front Physiol. 2023 Mar 13; 14:1094245. doi: 10.3389/fphys.2023.1094245. PMID: 36994416; PMCID: PMC10040785

Author's contribution to the papers

Paper I

Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original draft, Writing – review & editing, Visualization, Project administration.

Paper II

Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original draft, Writing – review & editing, Visualization, Project administration.

Paper III

Methodology, Formal analysis, Investigation, Writing – Original draft, Writing – review & editing, Visualization.

Paper IV

Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original draft, Writing – review & editing, Visualization, Project administration.

Abbreviations

ATF6	Activating transcription factor-6
AEC	Alveolar epithelial cell
BAL	Bronchoalveolar lavage
Bcl-2	B-cell lymphoma 2
COPD	Chronic obstructive pulmonary disease
CSE	Cigarette smoke extract
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
FEV1	Forced expiratory volume in one second
FGF-2	Fibroblast growth factor 2
FVC	Forced vital capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HIF	Hypoxia inducible factor
IL	Interleukin
IRE1	Inositol-requiring enzyme 1
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PG	Prostaglandin
PRKN	Parkin RBR E3 ubiquitin protein ligase
RT-qPCR	Reverse-transcription quantitative polymerase chain reaction
SMAD	Mothers against decapentaplegic homolog
TNF-α	Tumor necrosis factor alpha
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Introduction

The lung

The lung is the organ where gas exchange takes place in the human body. The lung is divided into lobes, with the right lung having three lobes, the upper, middle, and lower right lobe, whereas the left lung has two lobes, the upper and lower left lobe. The airways can be divided into large and small airways or conducting and respiratory zones. The conducting zone transports the air to the respiratory zone, where gas exchange takes place (1). The large airways are comprised of the trachea, bronchi, and ends in the bronchioles. All of these airways have cartilage to support the structure. The small airways consist of the terminal bronchioles (bronchioles smaller than 2 mm in diameter), which divide into alveolar ducts, and alveoli, all of which have no cartilage to support the structure. It is in the alveoli that gas exchange takes place (2). Oxygen is brought into the lungs with each breath and carbon dioxide is expelled with every expiration. To make gas exchange as efficient as possible, the alveoli have a very large surface that is covered by an extensive system of capillary blood vessels and surfactant producing cells. Connective tissue and smooth muscle surround the structures in the lung. The connective tissue contains a mixture of cells and extracellular matrix (ECM). It plays an important role for the elasticity of the lung, necessary for breathing, but it also gives structural support, and facilitates signaling and interactions between cells. Among the cells present in the lung we find the mesenchymal cell type fibroblasts and epithelial cells (3, 4) which are the focus of this thesis.

Since the lungs are in contact with the outside world through the air, they need proper protection to prevent infection. The first line of defense is ciliated cells and mucus that catch and transport bacteria and particles out of the lungs. Coughing also helps to clear out the mucus. In addition to mucus, antimicrobial compounds and immunoglobulin A are also secreted as part of the defense (5). Immune cells such as macrophages, dendritic cells, T cells, and B cells can also be found in the epithelial lining of the lung (6).

Fibroblasts

Fibroblasts are a type of mesenchymal-derived cell that is part of the connective tissue. They can be found embedded within the ECM and have an elongated spindle

shape, usually with some kind of protrusion, but the shape varies tremendously. Fibroblasts are able to migrate to places where they are needed. This ability to migrate, increased proliferation and contractility make them effective for wound healing (7, 8). The fibroblasts produce and organize ECM to shape the structure in all tissues and organs (9). The ECM creates a unique microenvironment with different proteins depending on the tissue. The ECM is also involved in signaling of different kinds, including effects such as migration, proliferation, and differentiation (10, 11). Additionally, fibroblasts regulate the immune system, produce chemokines, growth factors, and cytokines, and facilitate cell–cell interactions (9, 12, 13).

Fibroblasts is a heterologous group of cells and different types can be found even in the central and distal parts of the lung (14, 15), as is evident by a difference in proliferative ability, gene expression, and protein production.

Fibroblasts play an important role in certain lung diseases such as chronic obstructive pulmonary disease (COPD), which is the focus of this thesis. In COPD, fibroblasts are associated with a pathological process called fibrosis. In general, fibrosis is pathological scarring during wound healing after injury or chronic inflammation (16). This process leads to excessive production of ECM and tissue remodeling. In the lung, this affects the elasticity, lessens alveolar space, and thickens the cell wall, leading to difficulties in breathing, reduced diffusion capacity, and decline of the lung function. In COPD, it has also been shown that the components of the ECM are altered (17).

Epithelial cells

Epithelial cells are one of the first lines of defense against harmful particles that have been inhaled. Epithelial cells can change into mesenchymal cells through a process known as epithelial-to-mesenchymal transition (EMT), whereby they gain increased motility. However, this contributes to fibrosis in COPD (18, 19), as fibrosis is associated with airway remodeling. This indicates that EMT plays an important role in COPD, especially in smokers (20).

In the alveoli, there are alveolar epithelial cells of two different types. The squamous alveolar epithelial cells type 1 (AECI) form a monolayer of cells through which the gas exchange occurs, whereas the cuboidal alveolar epithelial cells type 2 (AECII) produce surfactants which reduce surface tension to make breathing more effective (2). AECII also replenishes the AECI cells when the epithelium is damaged. These cells are critical for gas exchange and destruction of them can impair lung function.

COPD

Definition

Chronic obstructive pulmonary disease (COPD) is a lung disease that leads to obstruction of the airways and breathing problems. COPD is characterized by breakdown of the alveoli (emphysema) and inflammation in the lungs (bronchitis). Recently, a new definition of COPD was made by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (21). The new definition is based on a publication by Celli B. et al 2022 (22) and reads: "Chronic Obstructive Pulmonary Disease (COPD) is a heterogenous lung condition characterized by chronic respiratory symptoms (dyspnea, cough, sputum production, and/or exacerbations) due to abnormalities of the airways (bronchitis, bronchiolitis) and/or alveoli (emphysema) that cause persistent, often progressive, airflow obstruction". The progressive part means that it is a disease that worsens with time and there is currently no way to treat the disease, except lung transplantation.

COPD is classified based on forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) measurements using spirometry. In healthy individuals, the FEV1/FVC ratio is above 0.7 and if it is 0.7 or below the individual is spirometrically diagnosed with COPD. This diagnosis is then used to further divide COPD into groups depending on severity of the disease, so called GOLD stages. This division is based on the measured FEV1, given as a percentage of the expected healthy FEV1. The mildest stage is called GOLD I, for which FEV1 is equal to or above 80% of the expected value. The moderate stage GODL II has a FEV1 between 80% and 50% of the expected level. GOLD III is a severe stage with an expected FEV1 of 50% to 30%. The very severe stage is GOLD IV with less than 30% of the predicted FEV1. This classification is used in this thesis.

Symptom assessment and exacerbation history could also be considered in the definition of COPD, using stages A to D. Here, subjects with stage A have less symptoms and low risk of exacerbations, B have more symptoms but still low risk of exacerbations, C have less symptoms and higher risk of exacerbations, and D have more symptoms and high risk of exacerbations. An even newer system that came out with the GOLD 2023 report, fuses group C and D into group E (which stands for exacerbation). This ABE system has mostly implications for the medical treatment and has yet to be validated by clinical research.

Symptoms

One of the first symptoms of COPD is chronic cough, although it is often disregarded because it is often considered to be normal or expected when smoking or living in polluted environments. Dyspnea is another main symptom and is the major reason for the disability and the anxiety that comes with COPD (23). Patients

typically describe dyspnea as an increased effort to breath, heaviness of the chest, hunger for air, and gasping (24). One of the most common and distress-causing symptoms of COPD is fatigue (25). This is often described as feeling "drained of energy" or a "general tiredness" and it has an impact on everyday activities and the quality of life (26, 27). Wheezing when breathing is another symptom of COPD and chest tightness can also occur. Though treatable, these symptoms are related to increased exacerbation risk, worse health status, and emergency hospital admission (28).

Pathology

Several changes occur in the lungs of COPD patients, such as inflammation, emphysema, and remodeling. These changes are major players in COPD disease progression.

Inflammation

Chronic inflammation is one of the hallmarks of COPD and the inflammatory response seems to be different compared to in healthy subjects. The abnormal inflammation causes severe damage to the lung. Lymphocytes, activated neutrophils, and alveolar airway macrophages are generally increased in the lung in COPD. Multiple inflammatory mediators and growth factors are released by inflammatory cells, epithelial cells, and other structural cells (29). These inflammatory mediators and growth factors function by attracting inflammatory cells, inducing structural changes, and enhancing inflammatory processes (30). Tumor necrosis factor alfa (TNF-α), interleukin 6 (IL-6), and interleukin 8 (IL-8) are mediators for inflammation and have been shown to be increased in COPD (31, 32). Additionally, monocyte chemotactic protein 1 (MCP-1) is a cytokine that recruits immune cells to sites of inflammation and has been shown to increase in COPD subjects with emphysema (33).

Remodeling

Remodeling caused by inflammation, cigarette smoke, or repeated injury to the airway wall can lead to impaired reparation of the lung, hyperplasia of smooth muscle cells and fibrosis (34). This can be a contributing factor to the development of obstruction in the airways (35). There is evidence for an imbalance in COPD patients between proteases that break down connective tissue, such as matrix metalloproteinases and neutrophil elastase, produced by epithelial and inflammatory cells, and antiproteases, that prevent this breakdown, such as alpha-1 antitrypsin (36). Proteases mediate the destruction of elastin, a component of connective tissue, which is a feature of emphysema (37). In patients with COPD and asymptomatic smokers, peribronchiolar fibrosis and interstitial opacities have been reported (38-40). In smokers and COPD patients, an excessive production of growth factors, such as

profibrotic transforming growth factor beta (TGF-β) and platelet-derived growth factor A and B, may be found in the airway wall (41). TGF- β is important for ECM production and is a primary driver of fibrosis (42). The vasculature in the lung can also be altered in COPD patients e.g. by thickening of the epithelial cell layer leading to increased resistance (43). This alteration of vasculature can impact both the bronchial circulation, which supply nutrients and oxygen to the cells of the lung, and the pulmonary circulation, which brings de-oxygenated blood to the lung. Additionally, a less diverse microbiome has been found in the lung of severe COPD patients and there is an immune response to the microorganisms in the lung, which in turn seem to contribute to COPD pathogenesis (44).

Lung function

Lung function trajectories

In recent years, much focus has been put on what have been referred to as lung function trajectories. The lungs are not fully developed at birth and the lung function tend to peak at 20–25 years (45). This is then followed by a slight decrease in lung function over time. This normal decline can be influenced by many different factors during the lifetime of a person and may decrease the maximum lung function, shorten the plateau of constant lung function, or accelerate the decline of lung function (46). Finding individuals with reduced lung function may help to identify people at risk of developing COPD (47, 48). Various factors during early childhood can affect lung function later in life, such as birth weight (49-52). The lung function declines over time as an individual ages, but it is not clear if natural aging cause COPD or if it is the cumulative exposures to smoke or pollutants throughout life that cause COPD. Structural changes during aging of the airway and parenchyma are very similar to those in COPD (53) and conversely, COPD patients have shown evidence of increased aging of the lung (54). Increased risks of exacerbations and mortality in COPD patients have been associated with age-related epigenetic changes in immune cells (55, 56). The finding that COPD can result from decreased peak lung function or accelerated lung function decline opens up for new investigations on opportunities for prevention, early diagnosis, and treatment of the disease (46, 57).

Airflow obstruction and air trapping

Increased airway resistance in COPD is caused by small airway obstruction and emphysema, involving destruction of the parenchyma, leading to reduced elastic recoil. Relative contributions, occurrence, and time of development vary from person to person. The lung parenchymal destruction, luminal liquid in the small airways, narrowing small airways, and structural changes in the lung are caused by the chronic inflammation in COPD. These changes make it more difficult for the airways to stay open during breathing. The loss of small airways may also cause

further airflow obstruction and problems with the clearance of mucus (58). COPD patients also have a reduced number of small airways (57, 58). Taken together, all these things lead to difficulties in emptying the lungs of air during expiration, decreased FEV1, decreased FEV1/FVC ratio, air trapping, and lung hyperinflation (59). When there is still air left in the lung after exhalation, it will cause lung hyperinflation. This has the effect that with every breath, more and more air stays in the lung. This makes breathing more difficult, especially during work, leading to exercise intolerance (60-62).

Abnormal gas exchange

As COPD progresses, the gas exchange gets more limited. Emphysema indicates destruction of the parenchyma, which leads to a low oxygen uptake, measured as a decrease in lung diffusion capacity. Hypoxic vasoconstriction occurs in regions with poor ventilation. COPD patients have an altered ventilation–perfusion ratio due to abnormalities in the structure of the airway, alveoli, and pulmonary circulation (63). The structural abnormalities are the main cause of gas exchange problems, leading to arterial hypoxemia (64).

Exacerbations

Exacerbations are deterioration of the symptoms of COPD and can last for days or weeks, requiring drug intervention and possibly hospitalization. There is also often an increase in both systemic and local inflammation (65, 66). Exacerbations get more frequent as COPD progresses and may lead to permanent lung damage, increased decline of lung function, and poor prognosis (67). There are several triggers of exacerbations, such as viral or bacterial infections or environmental factors, but sometimes the cause is unknown.

Risk factors for COPD development

There are several risk factors for developing COPD, such as smoking, occupational exposure, environment, and genetics. This makes COPD a very heterogenous disease. It has been estimated that roughly 50% of COPD cases are caused by exposure to other things than smoking, such as environmental pollution (68, 69). Gender and social pressure can influence the disease development such as pressure to start smoking. A longer life expectancy also means more risk exposure. COPD is the result of a complex combination of accumulated interactions during the lifetime of a person that damage the lung and alter normal development or aging. (70).

Smoking

There are several adverse effects of cigarette smoking that relate to COPD, such as greater decline of FEV1, higher mortality rate, and a higher prevalence of abnormal lung functions and respiratory symptoms (45). However, even with all these adverse

effects of smoking there are other factors that also need to be considered. In fact, not all heavy smokers develop COPD and half of all COPD cases worldwide are caused by other factors than smoking (69). It should also be noted that passive exposure to cigarette smoke may cause COPD or other respiratory symptoms (71).

Cigarette smoke contains a lot of different chemical compounds. Even if some of them are caught in the filters of cigarettes, thousands of chemical compounds remain that can affect the smoker. They include nicotine, heavy metals, polyaromatic hydrocarbons, phenols, nitrosamines, carbonyls, aromatic amines, alcohols, heterocyclic compounds, carbon dioxide and monoxide, nitrogen gases, and tar (72-77). The concentrations of the toxic chemicals are not fully known.

Several factors impact which compounds that are found e.g. smoking behavior, blend of tobacco, cultivation practices, and ventilation. The resulting end-products also depend on the temperature of combustion. This introduces an element of uncertainty in what chemical compounds a person is exposed to when smoking. It is further complicated by the fact that there are volatile compounds that may evaporate before consumption.

Several adverse effects of cigarette smoke have been reported. In lung fibroblasts, cigarette smoke has been shown to induce inflammation (78). Proliferation and migration is inhibited in lung fibroblasts after exposure to cigarette smoke extract (CSE) (79) and CSE has been shown to induce senescence in lung fibroblasts (80). Type 1 collagen and α-smooth muscle actin are induced by CSE (81). Cell death in the form of apoptosis has also been found after CSE exposure and at CSE concentrations above 40%, necrosis is more prevalent than apoptosis (82-84). Additionally, CSE has been shown to cause oxidative stress, contributing to disease progression (85, 86). Oxidative stress is when there is an imbalance between reactive oxygen species and antioxidant defenses, which can lead to damage to cellular targets such as DNA or proteins which in turn leads to endoplasmic reticulum (ER) stress.

Occupational exposure

Several occupations have been identified that give an increased risk of COPD (87). These include sculptors, gardeners, and warehouse workers. Exposure to organic or inorganic dust, chemical agents, and fumes are risk factors for COPD (88, 89). Inhaled pesticides have been shown to induce a higher incidence of COPD and respiratory problems (90, 91). Household air pollution, such as burning of wood and coal, animal dung, crop residues, and cooking without good ventilation are also risk factors for COPD (92, 93). It is not known how COPD caused by occupational factors differs from COPD caused by cigarette smoking. This needs to be further studied as these COPD patients might have different clinical features and lung trajectories, implying that they may need a different treatment.

Environmental exposure

Air pollution has been shown to be a risk factor for COPD development (94). In low- and middle-income countries, air pollution is responsible for a large part of COPD cases. The main components of air pollution are particulate matter of different sizes (such as metals, nitrogen, and sulfur oxides), ozone, and other greenhouse gases. The risk caused by air pollution is dose dependent and there is no "safe" threshold. Particulate matter of 2.5 μm size and nitrogen dioxide has been shown to be harmful and the increases risk of COPD even in places with low general air pollution (95). Air pollution increases the risk of exacerbations, hospitalizations, and mortality in COPD patients (96). Therefore, it is very important to reduce indoor and outdoor air pollution to prevent and reduce COPD.

Genetics

It has been shown that smoking siblings of COPD patients have an increased risk of airway obstruction, indicating that there is a genetic factor for susceptibility to COPD (97). SERPIN1A is the best documented gene with mutations that lead to an increased risk of COPD. These mutations lead to a hereditary deficiency of α-1 antitrypsin (AATD) (98). AATD may lead to liver disease and emphysema (and thereby COPD). However, only a small part of COPD cases is caused by AATD deficiency. Another genetic factor that has been shown to have an effect in COPD is Sulfatase Modifying Factor 1 (*SUMF1*). Some single nucleotide polymorphisms of *SUMF1* are associated with lower lung function and COPD (99, 100). There are many other genes that are associated with reduced lung function and a risk of COPD, but the individual effect of each gene is small (101) .

Multimorbidity

It is common for COPD patients to suffer from comorbidities that have the same risk factors as COPD. These risk factors include smoking, diet, aging, alcohol, and inactivity, and they can have a major impact on survival and health status. Cardiovascular disease, depression, metabolic syndrome, anxiety, and osteoporosis are comorbidities that are frequently found in patients with COPD (102, 103). Conversely, COPD can increase the risk of other diseases, such as lung cancer and emphysema-related diseases (104, 105). High blood pressure is a common problem in smokers and patients with COPD. Abnormalities in the pulmonary circulation, including increased cell and smooth muscle growth, have been found in smokers with normal spirometry and patients with COPD with mild airflow obstruction (106, 107). Hypoxic vasoconstriction of small pulmonary arteries or loss of pulmonary capillary beds can lead to development of pulmonary hypertension in later stages of COPD. Airway hyper–responsiveness has been found to be a leading risk factor of COPD, second only to cigarette smoking, according to the European Community Respiratory Health Survey (108). Chronic mucus hypersecretion has also been shown to worsen COPD (109).

Sex differences

Some studies suggest that women are more sensitive than men to the adverse effects of smoking (110, 111), but this is controversial. It has also been shown that the same level of smoking leads to more severe disease in women (112).

Socioeconomic status

Poverty has been shown to be associated with obstructed airflow (113). Additionally, having a lower socioeconomic status has been shown to be associated with an increase of the risk of developing COPD (114, 115). The causes of these associations remain to be elucidated. Possible factors are air pollutants (both household and outdoor), crowdedness, diseases, or insufficient nutrition. More investigations of these factors are needed.

Treatment

Although there is currently no way to cure COPD, except for lung transplantation, there are ways to slow down the progression and treat the symptoms of the disease.

Smoking cessation

Stopping smoking is one of the treatments with the greatest effect on COPD progression. Despite this, roughly 40% of people with COPD continue to smoke and this leads to a poor prognosis and progression of the disease (116). Other sources of nicotine can be used to reduce the use of tobacco products (117). Electronic cigarettes can be used for smoking cessation (118), but has become a way into nicotine dependence for young never-smokers. E-cigarette use has been linked to different forms of lung injury, bronchiolitis, and other lung related health problems, including death (119, 120), but long-term health effects have so far not been investigated.

Pharmacotherapy

There are several classes of drugs used to treat COPD symptoms. Bronchodilators widen the airways and make it easier to breathe. This family of drugs also reduce air trapping, improve breathlessness, and lead to better exercise capacity (121). Bronchodilators are primarily used to reduce or prevent symptoms. Short-acting (SABA) or long-acting (LABA) $beta_2$ adrenergic agonists are used to improve FEV1, lung volume, exacerbation rate, dyspnea, and health status (122, 123).

Antimuscarinic drugs target muscarinic receptors on the airways smooth muscle cells to prevent parasympathetic effects, thereby reducing the bronchoconstriction in the lungs, making airways wider and breathing easier. There are both short-acting (SAMA) and long-acting (LAMA) antimuscarinics, which are used to improve lung function, avoid exacerbations, and reduce mucus hypersecretion (124, 125).

Using a combination of the above-mentioned bronchodilators of different mechanisms can give more bronchodilation and less side effects than increasing dosage of just one of them (126, 127). Inhaled corticosteroids (ICS) can be added to further improve patient outcomes, avoid exacerbation, reduce mortality, and amend lung function (128-131).

There are also additional drugs that are less used. These include methylxanthines (bronchodilators), oral glucocorticoids (exacerbation management), phosphodiesterase-4 inhibitors (anti-inflammatory), antibiotics (exacerbation management), mucolytic drugs (exacerbation management), vaccination, and biologics against diverse targets (132).

Biomarkers

Finding biomarkers for COPD is of major interest since it would give an objective measure of the disease and could be used to find signs of the disease at an early stage. Blood eosinophil levels can be used as a biomarker for exacerbations and indicate when it is beneficial to use inhaled corticosteroids (133). One aim of this thesis was to identify potential biomarkers for COPD.

Cellular stress

Hypoxia

The air we breathe contains 21% oxygen and the level decreases to roughly 14% as the air makes its way through the lungs down to the alveoli. This is due to a higher concentration of water vapor and carbon dioxide in the alveoli. This is considered to be the normal oxygen levels (normoxia) in the lung. Different factors, such as bronchoconstriction and remodeling, can reduce the concentration of oxygen in the lungs further. This leads to constriction of blood vessels to try to redirect blood to areas with more oxygen. The reduced airflow, remodeling, and death of alveolar epithelial cells in COPD makes it difficult for oxygen to be transported efficiently, which creates hypoxic environments in the lungs (134). The hypoxia in turn leads to upregulation of angiogenic factors, such as vascular endothelial growth factor (VEGF) (135) and matrix metalloproteases, causing increased growth of blood vessels and vascular remodeling. Two of the VEGF variants are VEGF-A, which binds to the VEGF receptors VEGFR1 and VEGFR2, thereby promoting angiogenesis, and VEGF-C, which binds to VEGFR2 and VEGFR3, promoting lymphangiogenesis (136).

Increased vascularization and remodeling of lung tissue has been observed in COPD (137, 138). Vascular remodeling is common in COPD and cardiovascular comorbidities impact the disease negatively (139). Hypoxia inducible factor (HIF) is a marker for hypoxia and works as a transcription factor to regulate oxygen

homeostasis (Figure 1). HIF regulates genes related to angiogenesis, fibrosis, apoptosis, inflammation, and migration (140). Two forms of HIF are active at different timepoints during hypoxia. HIF1 is active in the acute phase of hypoxia, while HIF2 is active during chronic hypoxia (141). HIF1 and HIF2 induce similar genes, but there are differences in the pathways induced (142). VEGF can be induced by HIF1 (143), HIF2 (144), prostaglandin E_2 (145), TGF-β1 (146), and activating transcription factor 4 (ATF4) (1).

ER stress

The endoplasmic reticulum (ER) is an organelle that has functions relating to synthesis, folding, and transport of proteins. Incorrectly folded proteins are broken down within the proteasome. Under some conditions, unfolded or misfolded protein can accumulate, which leads to ER stress. This activates the unfolded protein response (UPR) in an attempt to clear unfolded and misfolded proteins (see Figure 1). The UPR consists of three pathways associated with activating transcription factor-6 (ATF6), inositol-requiring enzyme 1 (IRE1), and protein kinase RNA-like endoplasmic reticulum kinase (PERK), respectively. These pathways try to get rid of the un/misfolded protein by regulating other genes. If the problem cannot be solved, apoptosis is initiated. ATF6 is cleaved in the Golgi apparatus and then functions as a transcription factor for other genes. IRE1 activates TNF receptor associated factor (TRAF) and x-box binding protein 1 (XBP1), which then active other pathways or function as a transcription factor for other genes. PERK activates eucaryotic translation initiation factor 2 alpha (eIF2α), which inhibits transcription and lead to activation of ATF4. This leads to induction of VEGF (angiogenesis), nuclear erythroid 2-related factor 2 (Nrf2, antioxidant response), and DNA damage-inducible transcript 3 (CHOP, apoptotic). B cell lymphoma 2 (Bcl2) is inhibited by CHOP and this inhibition together with other genes activated by CHOP lead to apoptosis.

Previously it has been shown that the ER is more disorganized in individuals with COPD than in healthy individuals and a difference was found in the expression of ER stress response genes in lung in response to chemical stressors (147). It has also been shown that ER stress is increased in smokers and that COPD subjects have an impaired oxidant/antioxidant balance (148, 149). All this makes ER stress and UPR an interesting research topic, that has been investigated in this thesis.

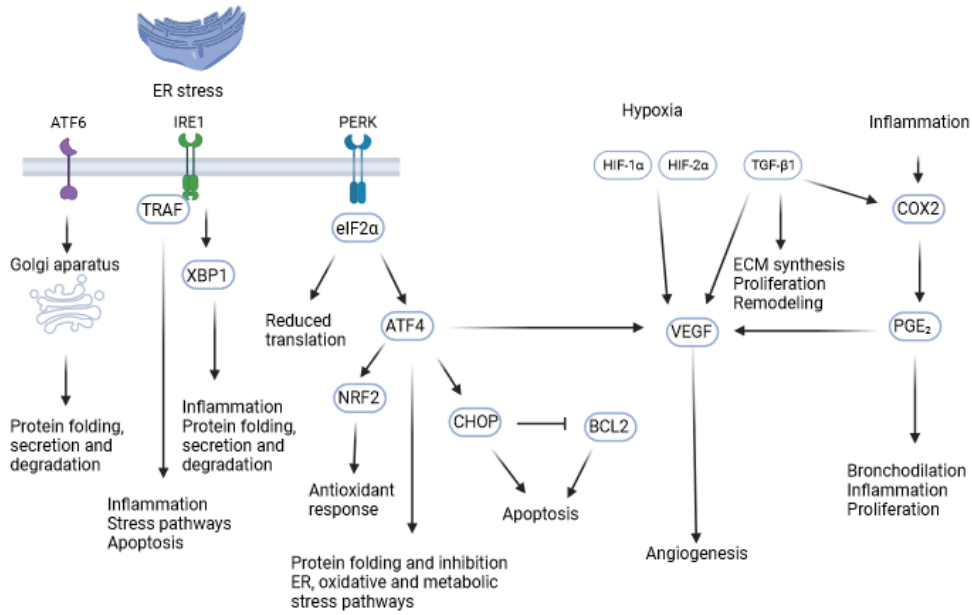

Figure 1
Pathways activated by ER stress, hypoxia, and inflammation and how these interact with each other to solve the stress. The UPR is activated in response to ER stress and there are three, ATF6, IRE1, and PERK. ATF6 is processed in the Golgi apparatus and then works as a transcription factor. IRE1 activates TRAF and XPB1, which in turn works as a transcription factor or activates other genes. PERK leads to reduced translation by activating eIF2α, which also activates ATF4. This in turn leads to activation of stress pathways, VEGF (angiogenesis), Nrf2 (antioxidant response), and CHOP (apoptosis). VEGF is also activated in response to HIF-1α, HIF-2α, TGF-β1, and PGE_2. TGF-β1 also leads to remodeling and activation of the inflammatory induced COX2, which in turn activates PGE_2 (inflammation and bronchodilation). Created with BioRender.com

Apoptosis

Apoptosis is a form of cell death that occurs under controlled conditions. Cell stress of different kinds, both from outside and inside the cell, can cause apoptosis. During apoptosis, caspases are activated and drive the apoptotic process. Apoptosis is a tightly regulated process with several ways to both induce and inhibit it. Examples of such regulators are the B-cell lymphoma (Bcl-2) family, mitogen-activated protein kinases (MAPK), and transcription factor Jun. They were all investigated in this thesis.

Cell senescence

Cellular senescence is when a cell stops dividing but remains metabolically active. It is a part of normal development but can also be caused by damage to cells, aging, and oxidative stress (150, 151). This process prevents unfit cells from multiplying and as such, these cells will not become a danger for the rest of the organism. Parkin RBR E3 ubiquitin protein ligase (PRKN) has been shown to be active during senescence in COPD and is affected by cigarette smoke (152). Other genes, such as Jun, MAPK, and Mothers against decapentaplegic homolog (SMAD), also have a role in cell senescence (153).

Materials and methods

Study design

Paper I

Bronchoalveolar lavage (BAL) cells were collected from 42 subjects (35 healthy and 7 COPD subjects) and lung fibroblasts were collected from 43 subjects (24 healthy and 19 COPD subjects. Lung fibroblasts from 16 subjects (9 healthy and 7 COPD subjects) were stimulated with cigarette smoke extract (CSE). The mRNA was collected from all the cells and the expression of ER stress genes was investigated using RT-qPCR. Fluorescent stainings were also done on both BAL cells and lung fibroblasts. Protein levels of ER stress mediators were measured with western blots. The effect of CSE on lung fibroblast cell proliferation was investigated from 6 subjects (3 healthy and 3 COPD subjects) using the HoloMonitor live cell analysis system.

Paper II

Lung fibroblasts from 6 healthy subjects and 6 subjects with COPD were stimulated with CSE after which mRNA was extracted. The mRNA was analysed using NanoString nCounter© Human Fibrosis V2 Panel, investigating 760 genes. Pathway enrichment using Maayan Lab Enrichr was used to investigate the biological process gene ontology (GO) terms.

Paper III

Distally derived lung fibroblasts from 14 subjects (7 healthy, 4 GOLD II and 3 GOLD IV COPD subjects) were exposed to hypoxic conditions with and without profibrotic stimuli. Genes and proteins related to oxidative stress response and endoplasmic reticulum stress, remodeling, and inflammation were investigated using RT-qPCR, multiplex, and immunostaining.

Paper IV

The bronchial cell line BEAS2B and the alveolar type 1 cell line hAELVi were exposed to hypoxic conditions with or without profibrotic stimuli. Genes and proteins related to disease pathways were analysed. Additionally, alterations in cell viability and metabolic activity were investigated.

Bronchoalveolar lavage

During bronchoscopy, phosphate buffered saline (PBS) was infused into the middle lobe of the lung and was then drawn back. This liquid is the bronchoalveolar lavage (BAL) and contains a mixture of different cell types from the lung.

Primary lung fibroblasts

Primary lung fibroblasts were derived from peripheral lung tissue after either lobectomies, explants or biopsies. During the lobectomies, the fibroblasts were obtained from lung parenchyma at least 1 cm or more away from the tumor that demanded the removal of an entire lung lobe. Lung fibroblasts from lung transplant explants were obtained from the lung parenchyma without bronchioles and vessels. Both these ways of collecting cells resulting in distal lung fibroblasts (154).

Additional primary lung fibroblasts were derived from central airway biopsies collected during bronchoscopies.

Cells that come directly from human tissue are less resilient than cell lines and often take longer time to culture. They also have to be used at a lower passage to avoid losing their phenotype. On the other hand, these primary cells are obtained from subjects with different degrees of lung disease, and will as such be much more indicative of real-life reactions and therefore these cells will be much more physiologically relevant.

Cell lines

Two human cell lines were used: human alveolar type I epithelial lentivirus immortalized cell line CI-hAELVi (hAELVi) (155) and human bronchial epithelial cell line (BEAS2B) (156). hAELVi is an immortalized human alveolar cell line with which can model the air–blood barrier of the peripheral lung. These cells have alveolar type 1 like properties. BEAS2B, on the other hand, is derived from human bronchial epithelium. This cell line is often used when studying respiratory diseases. It has also been shown that BEAS2B shares features associated with mesenchymal stem cells, which are similar to the fibroblast cells (156).

Cigarette smoke extract

Preparation of cigarette smoke extract

Smoke from research cigarettes from University of Kentucky were bubbled into cell media with the help of a vacuum (See Figure 2). One cigarette was smoked for 3 min for each 5 mL of cell media. A total of 100 mL was made per batch and two batches were made in total. The extract was filtered to remove particles and then aliquoted and stored. The raw extract was considered 100% CSE.

Figure 2
Setup used to create the cigarette smoke extract in a fume hood. Research cigarettes from University of Kentucky were put in a mouthpiece attached to a tube that went into a flask with media. The cigarette was then lit and bubbled into the media with the help of a vacuum. The media was stirred using a magnetic stirrer.

Cigarette smoke extract stimulation

In *Paper I,* lung fibroblasts were stimulated with cigarette smoke extract at concentrations of 0%, 5%, 10%, 20%, or 30% for 4 hours, after which the RNA was harvested. A range of CSE concentrations were investigated to see at which concentration the CSE has the largest effect without inducing cell death. For the HoloMonitor study in *Paper I,* concentrations of 0%, and 5% for 48 hours were

used. These concentrations were selected to investigate the effect of CSE both at lower concentrations of CSE and at the concentration where we saw the largest effect on ER stress. In *Paper II*, the lung fibroblasts were stimulated with 0% and 30% CSE, after which the RNA was harvested.

Hypoxia stimulation

Cells in cell culture wells or slides were incubated for 4 hours or 24 hours at hypoxic conditions (1 % O_2) in a hypoxic chamber in which temperature, humidity, O_2, N_2, and CO_2 were monitored (see Figure 3). To some cells, TGF-β1 (10 ng/mL) was added as a profibrotic stimuli. After stimulation, supernatants were collected, and the cell layer was harvested for RNA and protein analyses, or the cells were fixed using paraformaldehyde. There are other ways to create hypoxic conditions, such as having an abundance of liquid on top of the cells, but a hypoxic chamber gives an accurate control of the oxygen level.

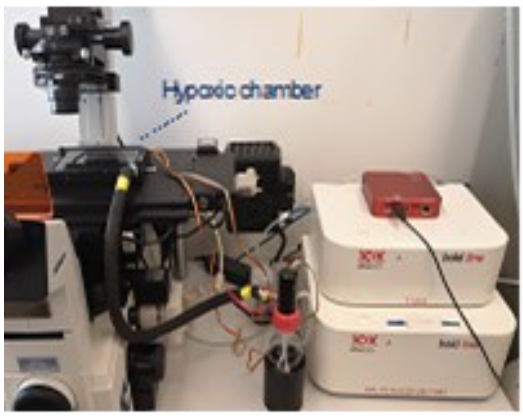

Figure 3
Setup of the hypoxic chamber used to conduct the hypoxia stimulation. The concentrations of O_2, N_2, and CO_2 were controlled and monitored to achieve hypoxic conditions. Figure by Anna Löfdahl.

RT-qPCR

The collected RNA was purified and converted into cDNA according to instructions supplied with the kits used. The cDNA and primers for relevant genes were then used to run reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analyses to measure gene expression. The genes investigated were involved in the unfolded protein response (UPR), oxidative stress, mitochondrial stress responses, endoplasmic reticulum stress, angiogenesis, remodeling, and inflammation.

NanoString analysis

NanoString is a technique in which a reporter probe with an attached barcode binds to a capture probe that binds to the RNA of interest. The number of barcodes is then counted automatically in a fluorescence microscope to measure the number of RNA molecules in the sample. This gives an absolute number instead of a relative number that you get from RT-qPCR. Additionally, the data are more reproducible using this approach. There is a complementary software, nSolver, that is used to analyze the data, which further increases reproducibility.

In *Paper II* the NanoString nCounter© Human Fibrosis V2 Panel containing 760 genes was used to investigate gene expression levels in lung fibroblasts after CSE exposure in healthy and COPD subjects. Despite suboptimal optical density, the samples passed the quality control. Genes with counts lower than the negative control in 50% or more of the samples were excluded. Remaining genes were analysed using nSolver software. From this analysis we get genes that have a difference in expression. Each gene is also annotated by NanoString based on KEGG (Kyoto encyclopedia of genes and genomes), Reactome, and literary sources, which give an indication of the function of the genes.

GO enrichment analysis

Gene ontology (GO) is an attempt at providing structure and creating a controlled vocabulary for annotating genes, sequences, and gene products (157). In this approach, genes are given a term based on their function. The terms are divided in three groups: biological process (BP), molecular function (MF), and cellular component (CC). The GO terms can be analysed to find if there are any classes of genes that are overrepresented among the genes that show statistically significant differences.

Enrichr

All genes with a significant change in expression after CSE stimulation were analysed based on their GO terms by running a pathway enrichment analysis using the Maayan Lab Enrichr software (158-160). Enrichr employs several sources for the enrichment analysis, and you can easily move between the information from different sources. Genes that showed significant changes in both healthy and COPD subjects were analysed to investigate overlap of GO terms.

DAVID

DAVID is a bioinformatics tool that gives a set of annotations of selected genes. It uses multiple sources for its functional annotations (161, 162). It can be used to get a better understanding of the function and grouping of genes. The software was used to identify the most common GO terms for genes with a significant change in expression after CSE stimulation. Additionally, a cluster analysis was performed to group GO terms with a similar function. This allows for an efficient interpretation of results involving many genes.

Enzyme linked immunosorbent assay and multiplex

The supernatant collected after hypoxia exposure was studied with enzyme linked immunosorbent assay (ELISA). This technique utilizes antibodies to detect the presence of a target protein. We used ELISA to investigate VEGF and prostaglandins. Additionally, cytokines and growth factors were investigated using multiplexed Luminex. Multiplex is a variant of ELISA in which the antibodies are bound to fluorescent beads. This approach allows for screening of thousands of analytes at the same time. Both ELISA and multiplex are good and efficient techniques for high-throughput screening of many samples, which makes it ideal for the analysis of the supernatant collected from the cell cultures.

Immunocytochemistry staining

Slides with lung fibroblasts, BEAS2B, or hAELVi cells were stimulated with CSE, or exposed to hypoxia. The cells in the slides were fixed with formaldehyde. For some of the stainings the cells were permeabilized to allow the primary antibodies to reach intracellular targets and a fluorophore conjugated secondary antibody together with DAPI for nuclei staining was added before taking pictures of the cells. These images were then quantified to compare the level of fluorescence corresponding to a greater presence of the target of the antibody. This lets us both visualize and compare proteins of interest present in the cells. It is a comparative method that does not give exact concentrations of the proteins of interest. Additionally, only a few proteins can be tested at a time, which is not optimal for patient samples which are fairly limited in supply. On the other hand, the staining provides pictures that are easier to interpret and discuss than numbers and they also show the localization of the proteins within the cell.

Cell viability and metabolic activity

Cell viability

Measurements of cell viability and cytotoxicity were done using a lactate dehydrogenase (LDH) assay. LDH is an enzyme that is important for cellular energy production. The presence of LDH's substrate (lactate) in the cell supernatant is an indication of damage (or rupture) of the cell membrane. By measuring the lactate concentration, we get an indication of how viable the cells are before and after stimulation with hypoxia or CSE. This assay only investigates the presence of lactate and not the kind of damage that has caused its presence. Neither does it give a count of how many cells are viable or not. Other assays, such as WST, Trypan blue, or TUNEL, can be used to elucidate this. However, the LDH assay has the advantage that it can be done on supernatant after the experiments and was therefore used for the CSE stimulated lung fibroblasts.

Metabolic activity

To complement the LDH cell viability test, the metabolic activity of cells exposed to hypoxic conditions was measured using a water-soluble tetrazolium salt test. In this assay, a dye is reduced by the presence of an enzyme. This yields a color of different intensity depending on the concentration of the enzyme. In *Paper IV* we used the tetrazolium salt WST-1 to measure the metabolic activity of BEAS2B and hAELVi cells. WST-1 has three important advantages over other tetrazolium salts, such as MTT. First, WST-1 does not need any solubilization step and can therefore be read immediately. Second, the signal from WST-1 is stronger. Third, it is less cytotoxic, partly due to the fact that WST-1 is not taken up into the cells.

Statistical Analysis

In *Paper I,* the data are presented as the median and the interquartile range. When comparing BAL from healthy and COPD subjects, the Mann–Whitney U test was used. When comparing smoke status of the BAL and lung fibroblasts, the Kruskal–Wallis test was used. For the paired samples, mixed-effects analysis was used. The CSE stimulated fibroblasts were analysed using mixed-effects analysis with uncorrected Dunn test or the uncorrected Fisher LSD test as a post-hoc test. For the NanoString expression analysis, the Mann–Whitney U test was used when comparing healthy and COPD subjects. Wilcoxon signed rank test was used to compare experiments with 0% and 30% CSE.

In *Paper II,* heatmaps were generated using NanoString nSolver 4.0. Thresholding and normalization of the transcript counts were produced by nSolver 4.0, further analyzed using IBM® SPSS® Statistics v 27.0.0.0, and finally Excel was used to manually validate the results. The comparison between healthy and COPD subjects was done using the Mann–Whitney U test. When comparing the difference between before and after CSE exposure for healthy and COPD subjects separately, Wilcoxon signed rank test was used.

In *Paper III,* values are presented as individual medians with interquartile range. GraphPad Prism 9.1.2 was used to analyse the data. The matched and grouped data were analysed using repeated measurements two-way ANOVA with post-hoc analysis using Fisher's LSD.

In *Paper IV,* the statistical analysis was done using GraphPad Prism 9.3.1. The data were presented as the mean with one standard deviation. When comparing two groups, Student's t-test was used. For the VEGF, PGE_2 and multiplex analysis, one-way ANOVA with post-hoc analysis using Fisher's LSD was used. One sample t-test was used to analyse the qPCR data.

In all papers, a *p*-value below 0.05 was considered significant. The only exception was the Enrichr GO terms, which were considered significant if the *p*-value was below 0.01.

Ethical approval

The collection of human lung fibroblasts and BAL in *Paper I* and *II* was approved by the Regional Ethical Review board in Lund (Dnr 2015/891 and 2008/413). The human material used in *Paper III* was approved by the Regional Ethical Review board in Lund (Dnr 2015/891) and the Regional Ethical Review board in Gothenburg (Dnr 675-12/2012). All subjects or closest relative signed written informed consent.

Results

Paper I

Stress-related gene expression in BAL cells

The expression of stress-related genes in the BAL cells was investigated. Stainings of ATF6, IRE1, PERK, and CHOP showed that these stress genes co-stain with the ER. However, when comparing healthy and COPD subjects, we could not find any statistically significant differences in the expression of any of the ten stress-related genes investigated. This was likely due to the sample, since BAL is a mixture of structural cells from the epithelium and innate immune cells, such as macrophages, lymphocytes, neutrophils, eosinophils, and mast cells (163). Therefore, it is difficult to tell which cell type would produce the investigated targets.

Stress-related gene expression in lung fibroblasts

To avoid the confounding factors of mixed cell types, we focused on investigating the expression of stress-related genes in lung fibroblasts instead. As in the BAL cells, stainings of ATF6, IRE1, PERK, and CHOP showed that these stress genes co-stain with the ER and western blot protein analysis even found higher levels of ATF6 in COPD lung fibroblasts. Expression levels of CHOP were lower in COPD subjects (and remained lower at increasing levels of CSE exposure) but protein quantification levels of CHOP were higher, indicating patients with both COPD and a smoking history tended to have higher but arrested apoptosis signaling than healthy subjects. In a post-hoc analysis of the NanoString results in Paper II, we found that 30% CSE exposure resulted in a significant upregulation of CHOP and downregulation of BCL2L1. These two genes together support the suggestion that CSE exposure increased apoptosis signaling in both healthy and COPD subjects. Additionally, healthy subjects also had a downregulation of ATF4, PSMB1, and PSMC3. ATF4 is part of the PERK pathway and induces CHOP expression, so healthy subjects may respond to CSE with a stronger apoptosis signaling than COPD subjects. This was simulated by assessing proliferation using the HoloMonitor live cell imaging system to monitor the cell growth. However, 5% CSE induced a similar level of cell death in both healthy and COPD lung fibroblasts. Smoking history is also related to differential expression of several stress related

genes (IRE1, Nrf2, PSMA1 and OXR1) at baseline. Though the higher expression of IRE1 in current smokers actually paired with lower IRE1 protein levels at increasing CSE exposure. The post-hoc NanoString analysis also found that after 30% CSE exposure, COPD downregulated two proteosome subunits while healthy downregulated four, indicating a stronger signaling for protein breakdown in lung fibroblasts from healthy than from COPD subjects. During increasing levels of CSE exposure, healthy lung fibroblasts responded by significantly downregulated ATF6 (at 5% CSE), implying that COPD subjects have an impaired ER stress response compared to healthy subjects, but this finding needs to be further investigated.

Summary

We have found that lung fibroblasts from COPD subjects have a deficient ER stress response to CSE, likely leading to premature apoptosis. Healthy subjects changed the expression of several genes, while COPD subjects had fewer genes with a change in expression. This deficiency may be one reason why COPD develops. Even at baseline, there was a difference in apoptosis-related gene expression. No difference in ER stress related gene expression could be found in BAL cells.

Paper II

As there were only a few differences between healthy and COPD lung fibroblasts when focusing on ER stress alone, we endeavored to broaden the scope by utilizing a whole fibrosis-related panel of gene expressions. In this study we investigated lung fibroblasts from healthy and COPD subjects before and after 30% CSE exposure using the NanoString fibrosis panel, targeting over 700 gene transcripts. This approach allowed us to assess two types of differential expression, the general difference (at either baseline or after 30% CSE exposure) between healthy and COPD subjects, and the response difference to 30% CSE (i.e. the change from baseline to after CSE exposure) between healthy and COPD subjects. KEGG pathway maps and Enrichr gene ontology were ultimately used to help define relevant pathways involved. However, DAVID analysis and the functional annotations provided by NanoString were also processed and consulted and are included here as additional findings to the paper.

Differences in gene expression

At baseline, we found a lower expression of 16 genes and higher expression in one gene in COPD subjects compared to healthy subjects. Those with the strongest difference (>30% difference in the geomean) indicated that lung fibroblasts from

COPD subjects are more predisposed to lower proliferation and more apoptosis, particularly related to the Notch and MAPK signaling pathways, than lung fibroblasts from healthy subjects. After CSE exposure, COPD subjects had a lower expression of four genes and higher expression of one gene compared to healthy subjects. The strongest difference again indicated lower proliferation predisposition in COPD but this time, it was specifically related to the hedgehog signaling pathway (although it is important to note that the three pathways are closely linked).

The major response difference to CSE between healthy and COPD lung fibroblasts was that the healthy fibroblasts responded by altering significantly more genes (207) than COPD lung fibroblasts (70, with an overlap of 51 genes between healthy and COPD). Interestingly, healthy lung fibroblasts responded to CSE by altering 11 of the 17 genes that were differentially expressed at baseline, showing that many of these genes already expressed by healthy fibroblasts are actively used to respond to CSE. Out of the strongest gene changes in response to CSE (>50%), healthy fibroblasts most consistently (<25% coefficient of variation) downregulate four genes and COPD most consistently upregulated just one gene, and all these five genes are strongly related to the MAPK signaling pathway. An in depth Enrichr analysis supported the strongest gene changes, identifying two unique GO terms for the healthy subjects' response to CSE (MAPK cascade and regulation of cell population proliferation) and one unique GO term for the COPD subjects' response to CSE (Notch signaling pathway). Assessing the genes involved in these GO terms in response to CSE stimulation, all healthy and COPD unique GO terms were significantly downregulated.

Additional functional analysis

There are several ways to investigate the function of a large number of genes. To complement the results gained from the Enrichr analysis we also consulted the functional Annotations supplied by NanoString and DAVID to create automated clusters of GO terms with a similar or related function (169, 170). The results gained from the Enrichr analysis (that focused on biological processes) were easier to interpret, which is why it was used in the publication. The additional data gained from the NanoString annotations and the DAVID cluster analysis (that included biological processes, cellular compartment, and molecular function) has a lot of supporting additional information, albeit mostly only for the healthy subjects and they are therefore presented here in the thesis.

As there were significantly more genes that responded differentially to CSE in the healthy than the COPD lung fibroblasts, the NanoString annotations (Figures 4 and 5) and DAVID clusters (Tables 1) were much stronger (more than four genes or $p<0.01$, respectively) in healthy subjects. We found in the healthy subjects an upregulation of energy producing pathways and fatty acid metabolism in the NanoString annotations and mitochondrial energy production in the DAVID

analysis, which indicates metabolic activity. We could also see a downregulation of the annotation MAPK cell stress and three clusters each of cell proliferation and transcription in the DAVID analysis. Both these findings are in line with what we found with Enrichr and indicates cell senescence. In COPD subjects, we found a downregulation of transcription related genes in the DAVID analysis and an upregulation of apoptosis related genes in the NanoString annotations. This too is in line with the Enrichr results, where we found Notch pathway to be changed, also indicating apoptosis.

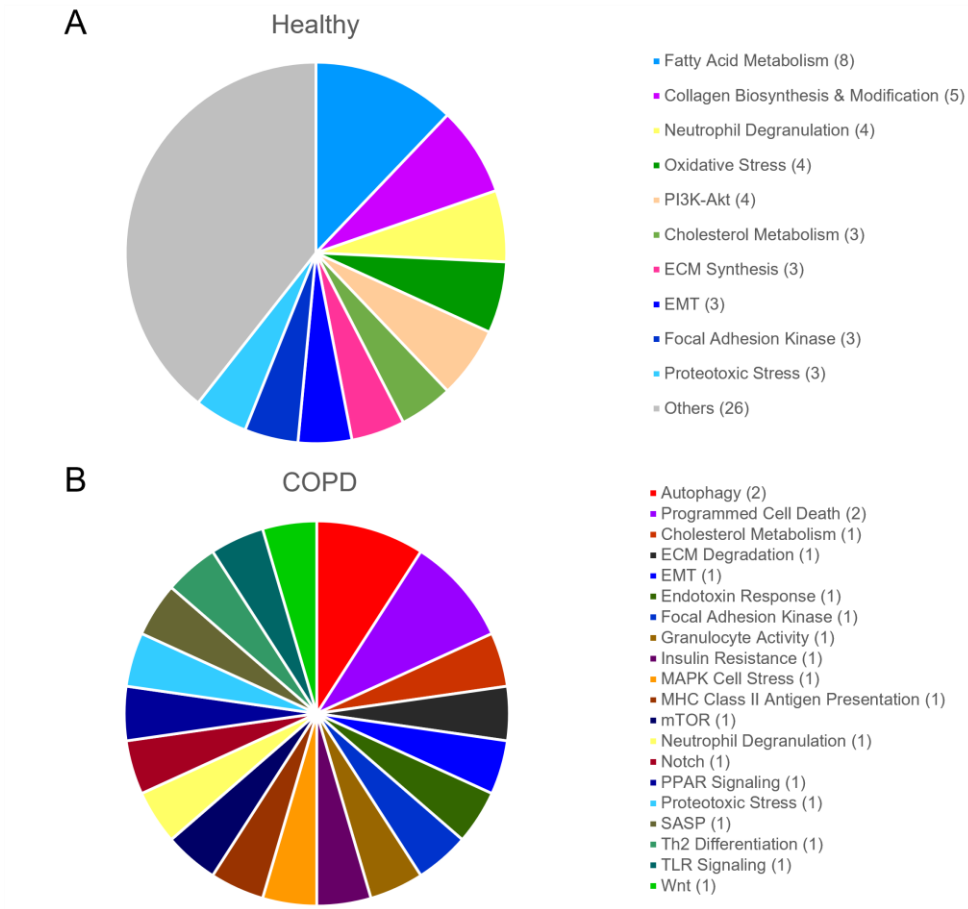

Figure 4
Pie charts of the top ten NanoString functional annotations of genes with a significantly upregulated expression in response to cigarette smoke extract. The number in brackets is the number of significantly upregulated genes with that annotation. A) Chart of gene annotations that were upregulated in healthy subjects only. B) Chart of annotations of genes that were upregulated in COPD subjects only.

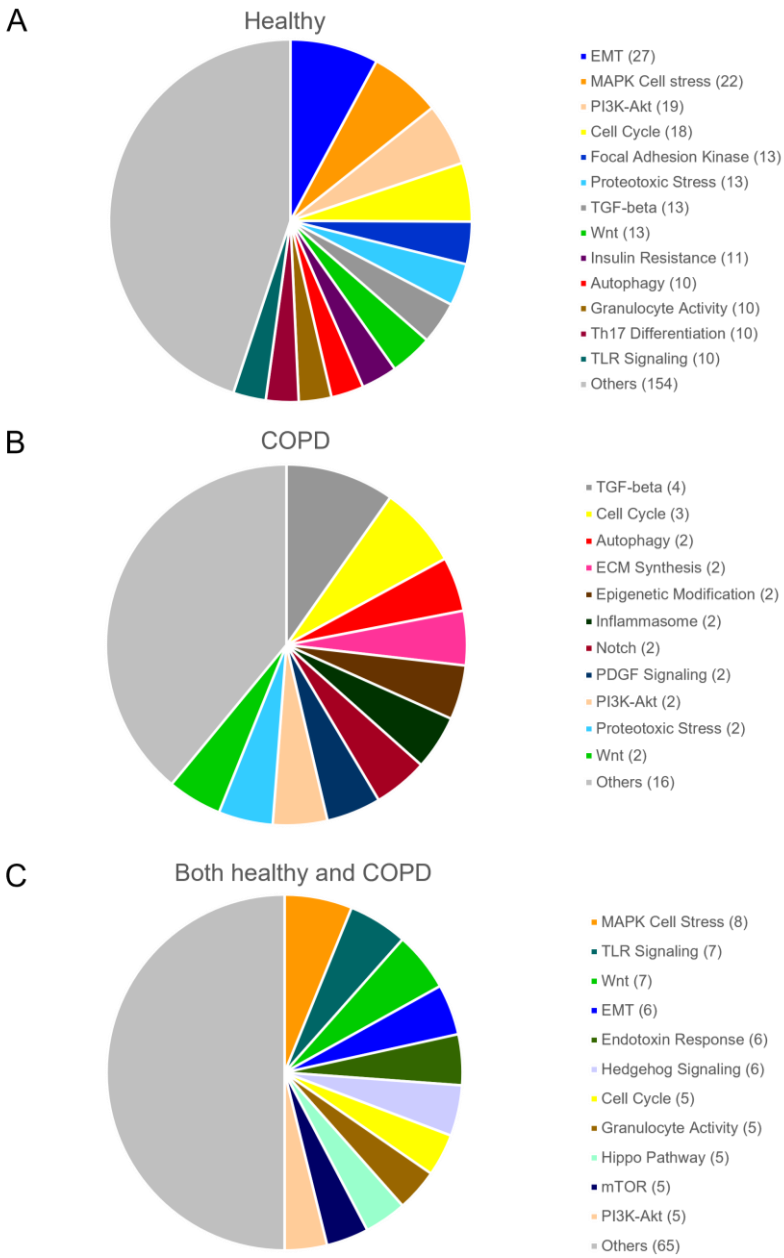

Figure 5
Pie charts of the top ten NanoString functional annotations of genes with a significantly downregulated expression in response to cigarette smoke extract. The number in brackets is the number of significantly downregulated genes with that annotation. A) Chart of annotations of genes that were downregulated in healthy subjects only. There are 154 additional gene annotations with a lower frequency. B) Chart of annotations of genes that were downregulated in COPD subjects only. There are 16 gene annotations present at lower frequency. C) Chart of annotations of genes that were downregulated in both healthy and COPD subjects. There are 65 gene annotations present at lower frequency.

Table 1. Cluster analysis of significantly modified genes.
Results of the cluster analysis with DAVID. The results have simplified by combining the GO terms into more general functions.

Cluster function	Count	Genes
Significantly upregulated genes in healthy subjects		
Chemical stimuli response	5	CAT, COL1A1, EGR1, HADH, TXN2
Extracellular localization	16	CAT, COL1A1, COL1A2, COL6A3, FABP5, GNG2, LGALS3, LOXL1, LOXL4, MASP1, PGM1, RPLP0, S100A4, SERPINF1, SIL1, SSR4
Mitochondrial energy generation	11	CASP8, CAT, COX6B1, COX7C, HADH, LGALS3, MMUT, NDUFA1, NDUFB5, NDUFB8, TXN2
Extracellular matrix	4	COL1A1, COL1A2, COL6A3, SIL1
Transcription	5	EGR1, SREBF1, STAT5A, TCF7L2, TXN2
Significantly downregulated genes in healthy subjects		
Phosphate kinase activity	34	ACSL4, ATG101, BRAF, CREBBP, CSNK1E, CSNK2A1, CSNK2B, FNIP2, GSK3B, JAK1, JAK2, KRAS, LATS1, LATS2, MAPK1, MAP2K1, MAPK8, MAPK9, MET, MKI67, OGT, PIK3CA, PIK3CB, PIK3C3, PPARA, PRKAB2, PRKDC, PTK2, ROCK2, SRC, TGFB1, TGFBR1, TGFBR2, UBE2D2
RNA polymerase II promoter regulation	36	ATF4, CD36, CREB3, CREBBP, CTBP2, CTNNB1, CUL3, E2F4, FNIP2, FGF2, GREM1, HDAC4, IL11, JAK2, KAT6A, LRP6, MECP2, MEF2A, MET, NCK2, OGT, PPARA, PPM1A, PRKDC, PSMC3, RELA, RELB, RORA, SIRT1, SKI, SMAD4, SNAI2, STAT3, TGFB1, TRAF6, XBP1
Proliferation and cell survival	7	IL6ST, JAK1, JAK2, PTPN1, SMAD4, SRC, STAT3
Cell development and growth	26	ADAM9, CDKN1A, CFLAR, CSNK2B, CTNNB1, FGF2, GSK3B, HTRA2, JAK2, LOX, MEF2A, PPARA, PTK2, RNF111, ROCK2, SIRT1, SKI, SMAD4, SMURF1, SNAI2, SRC, STAT3, TGFB1, TGFBR1, TGFBR2, TRAF6
Signaling	8	CBL, CRK, CRKL, NCK2, MAPK1, PTPN1, STAT3, YWHAG
Cell proliferation	8	KRAS, MAPK1 MAP2K1, PIK3CA, PPP2CA, SOS1, SRC, TGFB1
Energy/fat homeostasis	5	CD36, LEPR, PIK3CA, SIRT1, STAT3
Cell proliferation	9	BRAF, CRKL, CTNNB1, FGF2, MAPK1, MAP2K1, PRKDC, SMAD4, TGFBR1
Tyrosine modification/cell division	14	ATG101, BRAF, CBL, CRK, FGF2, GSK3B, JAK1, JAK2, MAP2K1, MET, PTK2, SIRT1, SRC, SRCJAK2
Transcription	13	ATF4, CREB3, CREBBP, CTNNB1, E2F4, KAT6A, MEF2A, PPARA, RELA, PRKDC, SKI, SMAD4, STAT3
Cell barrier function	4	RAP1B, RAPGEF1, RAPGEF2, ROCK2
Cell proliferation	8	ADAM9, CSNK1E, FGF2, KRAS, PPM1A, PTK2, SRC, TGFB1
Inflammation	10	CD36, CFLAR, CTNNB1, GREM1, KRAS, PPM1A, RELA, STAT3, TGFB1, TRAF6
Membrane traffic	4	OGT, PIK3C3, PIK3CA, PIK3CB
Cell adhesion	11	ADAM9, ADAM17, CD36, CD44, CTNNB1, FAP, FGF2, ITGA1, PTK2, RAC1, SRC
Apoptosis	4	DAXX, MAPK1, MAPK8, MAPK9
Protein breakdown	11	CBL, CUL3, CTNNB1, PSMD13, RNF111, SIRT1, SMURF1, TRAF6, UBE2D2, UBE4B, XBP1

Signal transduction	9	CDKN1A, CRKL, FGF2, GSK3B, KRAS, RAC1, RAPGEF1, RAPGEF2, SOS1
Transcription	9	CREBBP, CTBP2, CTNNB1, DAXX, GSK3B, HDAC4, KAT6A, MECP2, SIRT1
Transcription	34	APLP2, ATF4, CREB3, CREBBP, CRK, CSNK2B, CTBP2, CXCL8, DAXX, E2F4, ERO1A, FAP, GREM1, HDAC4, HTRA2, KAT6A, LRP6, MAPK1, MECP2, MEF2A, MKI67, OGT, PPARA, RELA, RELB, RORA, SMAD4, STAT3, SIRT1, SNAI2, SOS1, SKI, XBP1
Protein breakdown	5	CSNK2B, MAPK1, PSMC3, PSMD13, PSMB1
Genome maintenence	9	CREBBP, CTBP2, CTNNB1, DAXX, JAK2, KAT6A, RNF111, SKI, SIRT1
Significantly downregulated genes in COPD subjects		
Transcription	3	ATF7IP, EP300, SMAD3
Significantly downregulated genes in both healthy and COPD subjects		
Phosphorylation	12	ATP7A, CHUK, CREB1, CSNK1A1, CSNK1G3, MAP3K1, MAML1, PIK3R4, PRKACA, PRKACB, SMAD2, STK4
Transcription	15	ARHGAP35, BRPF3, CHUK, CREB1, HCFC1, KMT2D, MAML1, PPARD, SMAD2, SP1, SP3, STK4, TBL1XR1, YWHAB

Summary

Among the fibrosis related genes, we found that healthy lung fibroblasts regulate significantly more genes than COPD fibroblasts in response to CSE exposure. The healthy response relied heavily upon downregulating the MAPK cascade and cell proliferation, while the COPD response slightly reduced the Notch signaling pathway. This was supported by changes in the fatty acid metabolism, programmed cell death, MAPK cell stress, Notch annotations, the mitochondrial energy generation, cell proliferation, and transcription clusters, as seen in the additional analysis. Overall, we theorize that COPD subjects have a significantly deficient response to CSE while healthy show compelling signs of cell senescence, which could be a possible reason why COPD is maintained.

Paper III

In Paper III, we hypothesized that lung fibroblasts from COPD patients would respond differently to hypoxic conditions and profibrotic stimuli compared to healthy controls, since cigarette smoking has been shown to reduce tissue oxygen (164). With this aim, we exposed distally derived lung fibroblasts from COPD and healthy subjects to hypoxic conditions for either 4 or 24 hours, and assessed 16 gene expressions related to angiogenesis, hypoxia, remodeling, UPR, oxidative stress, and apoptosis.

Hypoxia-induced response in lung fibroblasts

After 4 hours of exposure, healthy subjects upregulated expression of 5-HTR2B (remodeling/fibrosis) (165), Collagen 7 (basal membrane remodeling in airways) (166), IRE1 (UPR), SOD3 (oxidative stress), and c-Jun (apoptosis), while HIF-1α (hypoxic response) was downregulated. Lung fibroblasts from COPD subjects, on the other hand, increased expression of VEGFR2 (angiogenesis) and Nrf2 (antioxidant response). We have also found a lower expression of 5-HTR2B in COPD subjects compared to healthy subjects.

After 24 hours of exposure, we found an increase of Bcl2 (apoptosis) and a decrease of PARK (UPR) in healthy subjects. We also found a higher expression of ATF6 (UPR) and lower expression of SOD3 (oxidative stress) and c-Jun (apoptosis) in COPD than in healthy subjects. No changes in gene expression were found in lung fibroblasts from COPD subjects after hypoxia exposure.

Taken together, these results indicate a difference in how lung fibroblasts from COPD subjects react to hypoxia compared to lung fibroblasts from healthy individuals. The lung fibroblasts from healthy subjects changed expression of six genes after four hours of hypoxia exposure and two genes after 24 hours of hypoxia exposure. In contrast, the gene expression in COPD fibroblasts was only changed in two genes at four hours of hypoxia and none after 24 hours. The genes with a changed expression in COPD subjects are also of different pathways than those that changed in healthy subjects. This indicates a deficient response to hypoxic exposure in lung fibroblasts from COPD subjects.

In COPD subjects, we found higher levels of IL-6 at hypoxic conditions and IL-8 at normoxic conditions. There were also higher levels of MCP1 and lower levels of RANTES in COPD subjects without any profibrotic stimuli. Hypoxia increased VEGF-C levels in both COPD and healthy subjects. We also investigated how a profibrotic stimuli, TGF-β1, modulates the inflammatory response and the response to hypoxia. The addition of profibrotic stimuli led to changes in both healthy and COPD subjects, by increasing VEGF-A and IL-6, and decreasing MCP-1, VEGF-C and HGF in lung fibroblasts from both COPD and healthy subjects. The TNF-α level also decreased but only in healthy subjects. In COPD subjects, we found higher levels of IL-6 and IL-8. There were also higher levels of MCP1 in COPD subjects without any profibrotic stimuli compared to healthy subjects.

Summary

In this study we investigated how lung fibroblasts respond to hypoxia. We found differences in gene expression of remodeling, oxidative stress, apoptosis, and stress related genes after hypoxia exposure, but also differences in gene expression between healthy and COPD subjects. Hypoxia showed less effects on expression of inflammatory markers and growth factors, but TGF-β had a profound effect on

inflammatory markers and suppressed some of the hypoxia-induced responses. There were also differences in inflammatory markers between healthy and COPD subjects. Once again, we found deficiencies in the response of COPD subjects to stress stimuli. This gives further insight into the role of lung fibroblasts in COPD.

Paper IV

Since paper III showed that lung fibroblasts from COPD subjects respond differently to hypoxia compared to lung fibroblasts from healthy subjects, we investigated in Paper IV whether hypoxia could induce mechanistic alterations in lung epithelial cells that could contribute to the development of pulmonary disease. To study this, we utilized bronchial (BEAS2B) and alveolar (hAELVi) cell lines to see whether the response to hypoxia would differ in different lung epithelial cell populations.

Hypoxia-induced response in epithelial cells

We found an overall downregulation in the expression of genes, involving hypoxic markers (HIF1α and HIF2α), oxidative stress (Nrf2), remodeling (VEGFR3 and 5-HTR2B), inflammation (PTGS2), ER-stress (PSMA1, PSMB6, and PSMD11), and mitochondrial dysfunction (PINK1) in both cell lines after exposure to hypoxia compared to normoxic cell culture conditions.

Using immunocytochemistry, we further investigated the expression of HIF2α and VEGFR to see whether the protein expression follows the gene expression. Indeed, we found a significant increase in HIF2α levels in hAELVi but not in BEAS2B cells, indicating a response to the hypoxic exposure as HIF2α is a marker of hypoxia and typically increases under hypoxic conditions. In contrast, VEGFR2 was undetectable, while VEGFR3 was detectable in both cell lines but there was no significant change after hypoxic exposure in either cell line, meaning both cell lines have a similar capacity for angiogenesis through this receptor.

We then investigated whether the cell viability was affected by hypoxia. Using an LDH assay, we did not find any differences between normoxic and hypoxic conditions in either cell line. We then used a WST-1 assay to measure the metabolic rate. Both BEAS2B and hAELVi cells had increased metabolic activity after hypoxia exposure. Finally, we detected no significant difference in cell density in BEAS2B nor hAELVi cells exposed to normoxic and hypoxic conditions.

Addition of profibrotic stimuli

We also studied whether the addition of TGF-β1, a profibrotic stimulus, may modulate the effects of hypoxia. VEGF-A and VEGF-C were increased at both normoxic and hypoxic conditions in BEAS2B cells when TGF-β1 was added, while in hAELVi neither VEGF-A or VEGF-C levels were affected by hypoxia nor TGF-β. In BEAS2B cells, hypoxia induced an increase in tenascin levels compared to normoxia but not when TGF-β1 was present. While in hAELVi cells, FGF-2 levels decreased after exposure to hypoxia without TGF-β1, there was no difference in endothelial growth factor (EGF) in hAELVi cells exposed to normoxic and hypoxic conditions, but the EGF levels were significantly higher with hypoxia and TGF-β1 than with hypoxia alone.

Finally, when investigating inflammatory mediators, stimulation with TGF-β alone was associated with an increase in most of the investigated inflammatory mediators. In BEAS2B cells, we found higher levels of IL-6 after hypoxic exposure compared to normoxic conditions, while in hAELVi the IL-6 level decreased with hypoxia compared to normoxia but not when TGF-β was present. The levels of MCP-1 were increased in BEAS2B cells in response to hypoxia. In BEAS2B cells, the levels of IL-8 decreased by hypoxic exposure without TGF-β, while in hAELVi cells, the IL-8 levels were lower after hypoxia both with and without TGF-β. Moreover, PGE_2 levels were also lower in hAELVi cells after hypoxia exposure both with and without TGF-β.

Summary

Hypoxia induces pathological changes related to peribronchial fibrosis, which can be seen in early stages of COPD. Investigating the response to hypoxia in bronchial and alveolar epithelial cells, we have found alterations in mechanisms that are important in pulmonary diseases. This included downregulation of mitochondrial and oxidative stress markers, alterations in remodeling and inflammatory response. The response to profibrotic stimuli together with hypoxia was also altered in both cell types, but more so in bronchial epithelial cells than in alveolar cells (see Figure 6). This would mean that bronchial epithelial cells are more sensitive to changes in oxygen concentrations and processes that lead to remodeling than are alveolar epithelial cells.

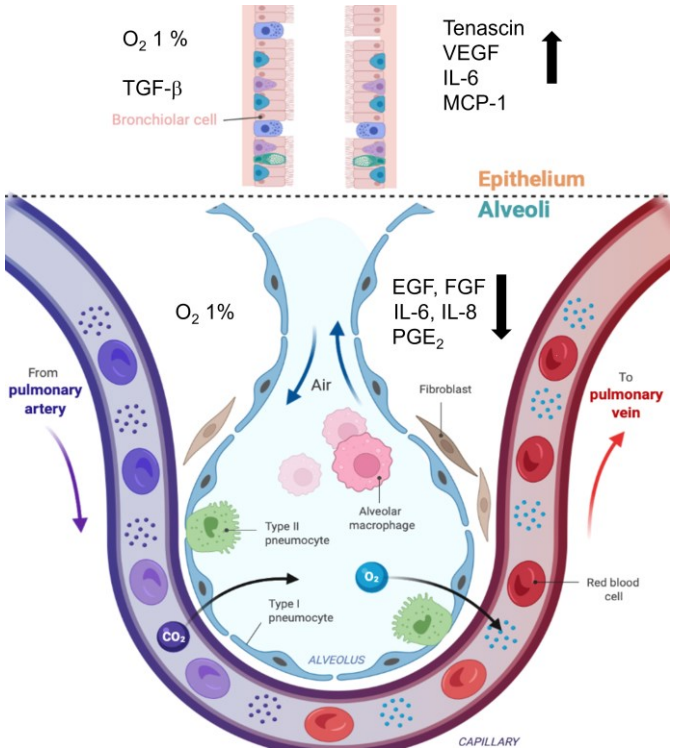

Figure 6
Scematic overview of the differences in response induced by hypoxia (1% O_2) in bronchial and alveolar epithelial cells. The bronchial epithelial cells upregulate Tenascin, VEGF, IL-6, and MCP1, while the alveolar epithelial cells downregulate EGF, FGF, IL-6, IL-8, and PGE_2. Figure by Anna-Karin Larsson-Callerfelt and Rebecca Berggren-Nylund.

Discussion

In this thesis we have investigated the stress response of lung fibroblasts from healthy and COPD subjects. Both cigarette smoke and hypoxia are sources of stress, but it is poorly understood how lung fibroblasts respond to these kinds of stresses. We have tried to bridge this gap by investigating lung fibroblast response to CSE and hypoxia.

Cigarette smoke exposure induced stress response

In *Papers I* and *II* we have investigated genes related to fibrosis and stress in BAL cells and human lung fibroblasts. For the BAL cells, we found some differences between the expression in never, ex, and current smokers, but no difference when comparing healthy and COPD subjects. Most of the differences in gene expression was found in ex-smokers. These findings indicate that the smoke status has an effect on the expression of stress related genes. The difficulty of obtaining statistically significant results could be due to the fact that BAL is a mixture of immune cells and it has not yet been investigated which fraction of the BAL cells are responsible for the expression of the stress related genes.

We also found a similar difference depending on the smoke status in human lung fibroblasts. There were fewer genes with a significant difference. Additionally, when lung fibroblasts were stimulated with CSE, we found differences between healthy and COPD subjects in stress gene expression. Healthy subjects showed the most prominent change in expression of stress related genes. For them, there were both up- and down-regulated genes that are part of the IRE1 and PERK pathways of the UPR, more specifically of the apoptotic UPR response, indicating that lung fibroblasts from healthy subjects are gearing up for apoptosis due to the toxic CSE. The healthy subjects had a changed expression in many more genes than the COPD subjects and the change was larger in healthy than in COPD subjects. The function of the genes with a changed expression also varied. Downregulation of cell proliferation and upregulation of energy generation in the mitochondria was of particularly interest. This might indicate a desperate last attempt of the cell to survive before going into apoptosis.

Taken together, the results in *Papers I* and *II* indicate a difference in how lung fibroblasts from healthy and COPD subjects react to CSE. This difference takes the form of a reduced response to the CSE in COPD subjects. It is not yet clear if this difference is the cause of COPD or if it is an effect of COPD or smoke status. Still, the results suggest that there are different mechanisms and pathways active in COPD subjects that are likely to contribute to the disease. The lung fibroblasts from healthy subjects have a proper response to CSE: They either try to survive by using the stress gene response or they try to mitigate the damage by going into apoptosis. Lung fibroblasts from COPD subjects, on the other hand, are unable to cope with the stress and go straight to apoptosis.

Stress response to hypoxia

We have shown significant changes in gene expression and metabolic activity in alveolar epithelial (hAELVi) and bronchial epithelial (BEAS2B) cells after hypoxia exposure. The HIF1α, HIF2α, Nrf2, PINK1, VEGFR3, 5-HTR2B, PTGS2, PSMA1, PSMB6, and PSMD11 genes are related to important mechanisms in lung diseases, such as oxidative stress, remodeling, and ER stress. The bronchial epithelial cells had a more marked response to profibrotic stimuli during hypoxia than the alveolar cells. This suggests that the bronchial epithelium is more responsive to remodeling and decreased oxygen during disease. A possible explanation for this is that the alveolar epithelium might be protecting itself with prostaglandins and is more adaptive to changes in O_2 concentration. Alternatively, it could be because alveolar epithelial cells are found deeper in the lung, where the oxygen concentration is naturally lower.

Hypoxia exposure induced less alterations in lung fibroblasts. We found a difference in VEGF-C release after hypoxic exposure, but we could not detect any statistically significant alterations of VEGFR3 at the mRNA level for the different treatments of the lung fibroblasts. This could be due to the fact that fibroblasts in general are less responsive to hypoxia since they are located in regions deeper in the lung where the oxygen concentration is lower and they may therefore be more adapted to a hypoxic environment. Hypoxia induced more differences in VEGF-C and PGE_2 levels in fibroblasts obtained from COPD patients diagnosed with GOLD II stage. This could be an indication of early remodeling and could be a potential biomarker for early disease. It makes sense that cells exposed to hypoxic conditions try to induce production of more blood vessels. The primary way of transporting oxygen is through the blood, so creating blood vessels would (from the perspective of the cells) increase the availability of oxygen to the hypoxic areas. In disease states, this contributes to remodeling by swelling, stiffening, and enlargement of the blood vessels (134) and might not necessarily solve the lack of oxygen. In other words,

the upregulation of vascular growth factors is an attempt at solving a problem that may have detrimental effects on the individual.

The findings in lung fibroblasts indicate a difference in gene expression and in inflammatory pathways in COPD subjects in response to hypoxia. This gives us additional insight into the pathology and heterogeneity of COPD. Interpretation of the results are complicated by interpatient differences and there is also the possibility that the oxygen level in the fibroblast is naturally fairly hypoxic. There are currently no studies that measure oxygen concentration at the fibroblasts *in vivo* (because it is difficult to measure). We know that the oxygen concentration in the lung is not 21% O_2 but the exact concentration that the studied cells are exposed to naturally is not known.

Concluding remarks

To sum up, COPD is a heterogeneous disease and there are still a lot of remaining questions. Through the work in this thesis, we have shed light on some of the mechanisms underlying COPD. We have found that lung fibroblasts from subjects with COPD have a deficient response to stress induced by CSE or hypoxia, both of which are commonly encountered for COPD subjects. In healthy subjects, the lung fibroblasts do their best to survive the stress. The lung fibroblasts from COPD subjects, on the other hand, barely react to the exposure of stress at all. This deficiency may be an adaptation to the environment in the COPD lung. Since both hypoxia and inflammation are common in the disease (29, 134), this constant environment of stress could desensitize the lung fibroblasts to external stimuli. In doing so, the deficient cells respond in an insufficient way to stress, which would not allow the lung to handle external stress properly. This could lead to progression of the disease instead of healing. If this is the case, changing the environment (167), or helping the lung fibroblasts to respond in a proper way could be a future way of treating COPD.

Future perspectives

The findings in this thesis have shed light on the deficient response in lung fibroblasts from COPD subjects after exposure to CSE or hypoxia. Though a lot of new knowledge has been gained, there are still many questions that needs to be answered in future projects.

One is whether the differences found in this thesis are consistent even when sub-dividing the subjects depending on their smoke status and GOLD stage. We found some indication in *Paper I* that most differences originate in the ex-smokers sub-group and in *Paper III* there also appear to be differences between GOLD II and GOLD IV. Therefore, investigating more subjects with more varied smoke status and GOLD stage could be an important step in trying to elucidate what impact these variables have during COPD progression. In particular, it would be interesting to study never-smokers both with and without COPD. Investigating never-smokers would give a more accurate baseline for the other results and would give insight into what a smoke-naïve response would look like.

It would also be interesting to investigate COPD caused by other reasons than smoking, such as environmental effects, genetics, aging, or abnormal lung development, to explore if the cause of the disease gives rise to a different response to CSE or hypoxia, or if the response is the same regardless of cause. If there is a difference, it would indicate a difference in mechanisms, and therefore different ways to handle and treat the various forms of COPD would be needed.

Only a few samples were investigated in our NanoString analysis. Additional transcriptomic analysis would add value to our data. It would also be beneficial to use more kinds of omics technologies, including more different kinds, such as proteomics, genomics, metabolomics, and epigenomics, to get a more comprehensive understanding of COPD. It has been shown that more kinds of omics increase the accuracy in diagnosing COPD (168). Several kinds of omics also allow the scientist to get accurate results with a comparatively low number of subjects or give more comprehensive information about the subjects in a study. Making omics more accessible would dramatically simplify data collection and would speed up the investigative process.

Continuing on the findings in *Paper IV*, it would be interesting to investigate primary bronchial epithelial cells because we found that the bronchial epithelial cell line had a more pronounced effect than the alveoli cell line. This would give us more

biologically relevant results, allowing more accurate conclusions. Additionally, it would also be of interest to further investigate the difference between central and distal fibroblasts. In this thesis we only briefly investigated this matter and we managed to find a difference, but a more thorough investigation would give information regarding the roles different types of fibroblasts play in the lung.

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Stress Response in Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of death worldwide. It is a chronic disease that gets progressively worse with time and there is currently no cure. Cigarette smoking is one of the main risk factors, but there is still a lot we do not know about how COPD develops. In this thesis we have investigated the effect of cigarette smoke extract and hypoxia on structural lung cells and we have found the cells from COPD subjects have a deficient stress response to these stimuli. This helps us develop a better understanding of COPD and could help to develop better ways of finding those at risk of developing the disease.

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