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The role of accelerated ageing in aberrant lung tissue repair and remodelling in COPD

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, ***Roy Woldhuis*** declare that this thesis, is submitted in fulfilment of the requirements for the award of ***Doctor of Philosophy*** in the ***School of life sciences*** at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of the requirements for a degree at any other academic institution except as fully acknowledged within the text. This thesis is the result of a Collaborative Doctoral Research Degree program with ***The University of Groningen, Groningen, The Netherlands.***

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide, while the prevalence is still increasing. Since the exact COPD pathogenesis is still unknown and no effective therapeutics are available to stop the progression of the disease, novel insights into the pathogenesis of COPD are urgently needed. Accelerated ageing has been postulated to play a role in COPD with characteristic of ageing demonstrated in lungs from COPD patients compared to age-matched smokers without COPD. Recently, extracellular matrix (ECM) dysregulation has been described as an additional ageing hallmark of the lungs. ECM dysregulation can cause aberrant lung tissue repair and remodelling. Lung fibroblasts and airway smooth muscle cells (ASMCs) are the major producers and regulators of the ECM and therefore play an important role in lung tissue repair and remodelling. The aim of this thesis was to elucidate the role of accelerated ageing in aberrant tissue repair and remodelling in COPD.

We analysed ageing markers and ECM changes in lung fibroblasts and ASMCs from COPD patients compared to ex-smoker controls without COPD matched for age, gender, and smoking history. In addition, we assessed the functional effects of induction of cellular senescence, which is a major ageing hallmark.

We found characteristics of accelerated ageing in COPD-derived fibroblasts compared to lung fibroblasts from matched controls, including higher levels of cellular senescence. The increase in cellular senescence in COPD-derived fibroblasts was associated with lower levels of the ECM protein decorin and higher levels of pro-inflammatory protein secretion. Induction of cellular senescence in lung fibroblasts also resulted in ECM changes, secretion of pro-inflammatory proteins and impaired tissue repair functions of the fibroblasts. Furthermore, ASMCs had higher levels of cellular senescence compared to lung fibroblasts from the same patients, but in ASMCs no differences were found between COPD and control. Finally, we showed that E-cigarette extract induces cellular senescence in lung fibroblasts, and the induction in cellular senescence resulted in impaired tissue repair functions. Therefore, we concluded that E-cigarettes, commonly used as smoking alternative or as smoke cessation aid, are not a safe alternative for tobacco smoking.

These studies indicate that accelerated ageing plays a role in aberrant tissue repair and remodelling in COPD and thereby contributes to the pathogenesis of COPD. Future studies should unravel the exact mechanisms that lead to accelerated ageing in COPD to discover therapeutics targets to develop therapies that target accelerated ageing in COPD patients.

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CHAPTER 1

General Introduction & Scope of this Thesis

Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory lung disease that causes severe respiratory symptoms and a poor quality of life. COPD is characterized physiologically by airway obstruction and histologically by chronic inflammatory processes in the lungs that drive disturbed lung tissue remodelling, including emphysema and chronic bronchitis (1-3). Emphysema includes the loss of alveoli and collapse of small airways, functionally this results in impaired gas exchange and breathlessness. Chronic bronchitis causes airflow limitation as a result of inflammation-induced excessive mucus production and airway wall thickening (2-4). Excessive mucus production is also a major contributor to the development of chronic cough, which together with breathlessness affects daily activities. COPD develops slowly and symptoms are apparent later in life. Thereby, COPD is mainly prevalent in the elderly with an age of approximately 65 years or older (5). COPD is diagnosed through clinical assessment in combination with spirometry to measure lung function, whereas the latter also determines the Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages, which classifies the severity of disease (Table 1) (6, 7). Recently, symptoms and risk of exacerbations have been included in grouping the disease severity of patients, called the ABCD assessment (Figure 1) (7).

The major risk factor for the development of COPD is cigarette smoking. Exposure to other noxious gases, including air pollution and occupational exposures can also result in COPD. These exposures cause inflammation that is thought to lead to lung tissue destruction and the thickening of the airway walls. However, not all smokers develop COPD and 25-45% of COPD patients have never smoked (7, 8). Hence, genetic susceptibility plays an essential role in disease pathogenesis. Till now, alpha-1 antitrypsin deficiency is the best known genetic risk factor for emphysema (9), which causes disease in smokers, and only occasionally in non-smokers. Furthermore, genome-wide association studies have found multiple COPD susceptibility genes that were associated with lower lung function and COPD (10-13). However, the exact role of these genes in the pathogenesis of COPD is not fully understood yet.

The WHO estimated a global COPD prevalence of 251 million cases in 2016 and expects an increase in prevalence in the coming years (14). This expected increase in prevalence is caused by higher smoking prevalence in low and middle-income countries and ageing of the population globally. COPD mortality in 2015 has been estimated at 3 million, which is 5% of global deaths and thereby the third leading cause of death (14). Moreover, COPD is the 6th and 5th leading cause of death in 2018 in The Netherlands and Australia respectively. The burden of disease, expressed in Disability Adjusted Life Years, of COPD was ranked 4th and 6th in The Netherlands and Australia respectively. The total healthcare costs of COPD in the EU has been estimated at €141.4 billion, which is an average annual cost of €6,147 per patient (15). In The Netherlands, the number of hospital admissions was 33,735 with on average 7.6 days of admission, and the total healthcare

costs were €912 million in 2017 (Dutch National Institute for Public Health and the Environment; RIVM). In Australia, the number of hospital admissions was 77,660 and the total healthcare costs were estimated at $977 million in 2016 (Australian Institute of Health and Welfare; AIHW). Patients with severe COPD with more symptoms represent a significant proportion of hospital admissions and healthcare costs.

Since the pathogenesis of COPD is largely unknown, current treatment strategies are limited and mainly aimed at improving symptoms, without reducing disease progression (7). At the moment the most effective measure to slow down the progression of the disease is preventing exposure to the noxious gases, including cessation of cigarette smoking. E-cigarettes are used as an alternative or cessation device for cigarette smoking, but the effectiveness and safety as cessation aid are largely unknown and controversial (16-18). In addition, an increasing number of studies are being published showing that E-cigarette use is not harmless, including more reports on cases of E-cigarette vaping associated lung injury (EVALI) (19-23). To relieve symptoms, bronchodilators are used that reduce the airflow limitation mainly by relaxation of the airway smooth muscle. Short-acting bronchodilators are used to treat acute bronchoconstriction and long-acting bronchodilators are used to control and prevent symptoms (7). Anti-inflammatory agents are used to treat COPD exacerbations and reduce future risk of exacerbation, which is an acute worsening of the symptoms that are often caused by an infection (7). In patients with severe COPD, oxygen supplementation therapies are used after acute respiratory failure, including mechanical ventilation treatments. Novel strategies targeting severe emphysema are various bronchoscopic lung volume reduction methods that reduce breathlessness and improve lung function and quality of life (24, 25). Ultimately, the only therapeutic option left for very severe COPD patients is lung transplantation. So, since no effective treatment options are available to reduce disease progression, new therapeutic targets need to be found. Therefore, novel insights into the pathogenesis of COPD are urgently needed.

Table 1: Classification of COPD GOLD stages based on airflow limitation

GOLD stage	Severity	FEV_1 % predicted
I	Mild	> 80 %
II	Moderate	50 – 80 %
III	Severe	30 – 50 %
IV	Very severe	< 30 %

COPD is being diagnosed when FEV_1/FVC <70%.

FEV_1: Forced expiratory volume in 1 second. Determined by spirometry measurements.

FVC: forced vital capacity. Determined by spirometry measurements.

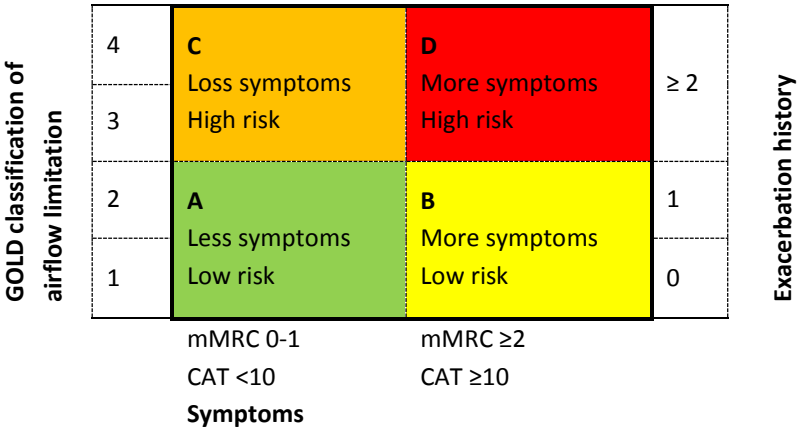

Figure 1: Assessment of COPD severity by use of ABCD disease groups. *Exacerbation risk is estimated by GOLD stages of airflow limitation (left) and exacerbation history (right). Symptoms (bottom) are assessed by modified Medical Research Council dyspnoea score (mMRC) and COPD Assessment Test score (CAT). Figure adapted from Agusti et al. (26) and Vogelmeier et al. (7).*

Normal and accelerated lung ageing

Since life expectancy is increasing worldwide, the interest in the role of ageing in health and disease has increased. As the general population is ageing, the prevalence of chronic and age-related diseases will rise as well. Ageing is described as the progressive decline in normal homeostasis, which leads to an increased risk of diseases and death (27). Recently, nine hallmarks of ageing have been described (Figure 2), which roughly can be divided into three categories; causes of damage, responses to damage and the phenotypic changes (27). During normal lung ageing, lung function declines over time and the alveolar spaces enlarge, called senile emphysema (28-30). Senile emphysema is mainly caused by loss of lung structure and elasticity. The characteristics of senile emphysema are to some extent comparable to pathologic emphysema seen in COPD. However, in COPD, chronic inflammation and tissue damage cause emphysema, including the destruction of alveolar septa and alveolar structure, which is in general maintained in senile emphysema (29). The rate of lung function decline is influenced by genetic factors and environmental exposures, including cigarette smoking (31-35). It should be noted that 4-13% of individuals never reach the maximum average lung function (FEV_1 of 100% predicted), which can increase their risk of COPD development, because of less spare lung capacity (36). Previous studies found multiple similarities between aged lungs and COPD lungs (37, 38). Features of ageing demonstrated in COPD include more inflammation, DNA damage, oxidative stress and cellular senescence, and reduced ability to repair DNA and protein damages (see chapter 2 for a complete review on this topic). Therefore, COPD has been postulated as a disease of accelerated ageing.

Severe, early-onset COPD

With respect to accelerated ageing, a group of patients that is of particular interest are severe, early-onset (SEO-) COPD patients. These patients develop very severe disease (GOLD stage IV, see Table 1) at a relatively young age (age <53, as defined by Silverman *et al.* (39)) with relatively low numbers of pack-years of cigarette smoking compared to the majority of COPD patients that develop symptoms from around 65 years of age (40). Therefore, SEO-COPD patients appear to have a high susceptibility to develop COPD. A large study, called COPDGene, found that 9% of severe (GOLD III-IV) COPD patients were SEO-COPD patients (41). Although the SEO-COPD patients represent a small subgroup of patients, these patients account for a significant proportion of hospital admissions and healthcare costs (42-44). Since SEO-COPD patients develop severe symptoms at a young age, we hypothesize that accelerated ageing may especially play a role in these patients.

Figure 2: *The Hallmarks of ageing. Figure reused with permission from Elsevier (27).*

ECM dysregulation in ageing and COPD

Recently, extracellular matrix (ECM) dysregulation has been proposed as an additional hallmark for lung ageing (38). The ECM is essential for the structure of the lung and tissue repair and remodelling processes. Major components of lung ECM are collagen, elastin, fibronectin and proteoglycans. Upon ageing the ECM changes, with in general an increase in fibrosis and loss of elasticity (45, 46). General COPD-associated ECM changes include increased fibrosis of the airway walls and ECM breakdown and lack of ECM repair in the alveoli, resulting in emphysema (47-50). Recently, a study in our group demonstrated differences in ECM gene expression with ageing in human lung tissue (51). Moreover, pathway analysis of the interaction analysis between age and COPD suggested that age-related changes in ECM, including several collagen genes, were larger in COPD patients as compared to non-COPD controls. Lung fibroblasts are the major producers of ECM and regulate ECM homeostasis and therefore play an important role in lung repair and remodelling processes. In COPD, the repair functions are impaired and remodelling processes are altered leading to the loss of alveoli and fibrosis around the airways. Airway smooth muscle cells are another cell type that has a role in tissue repair and remodelling in COPD. In COPD the ASM mass is increased, accompanied with enhanced ECM deposition (52). Alterations in ECM regulation have been demonstrated in COPD lungs previously, including higher collagen and versican production, and lower elastin, decorin and perlecan production. (48, 53-56). In addition, *in vitro* treatment with cigarette smoke extract (CSE) and TGF-β resulted in altered ECM production, including increased ECM protein production (53, 57, 58), where TGF-β levels are higher in COPD lungs and therefore often used to mimic *in vivo* COPD conditions. The role of accelerated ageing in aberrant lung tissue repair and remodelling in COPD and age-related changes in lung fibroblasts remains to be elucidated.

SCOPE OF THIS THESIS

We hypothesize that accelerated ageing is involved in the pathogenesis of COPD by affecting lung tissue repair and remodelling processes (Figure 3). Therefore, the overall aim of this thesis is to elucidate the role of accelerated ageing in aberrant tissue repair and remodelling in COPD.

Firstly, **chapter 2** gives an overview of the evidence available at the start of this project on the role of ageing in lung tissue repair and remodelling in COPD. It describes the similarities between lung ageing and COPD in more detail and gives a comprehensive overview of all data from published studies that demonstrated ageing hallmarks in lung tissue and structural cells from COPD patients or *in vitro* cultured primary structural lung cells treated with cigarette smoke extract (CSE). Finally, this review describes the gap in the scientific knowledge regarding the role of *accelerated* ageing in tissue repair and remodelling in COPD, which formed the basis for the experiments described in this thesis.

In the first experimental chapter (**chapter 3**) of this thesis we measured differential gene and miRNA expression with increasing age in biopsies from healthy individuals to assess key genes and regulators (miRNA's) involved in normal lung ageing. In **chapter 4**, we assessed whether primary parenchymal lung fibroblasts from COPD and SEO-COPD patients have features of accelerated ageing compared to fibroblasts from non-COPD controls by analysing multiple ageing hallmarks in these cells. In addition, we assessed whether accelerated ageing has functional consequences on ECM regulation of the fibroblasts. Following on our results in chapter 4, we aimed to define the senescence-associated secretory phenotype (SASP) of primary parenchymal lung fibroblasts in **chapter 5** and assessed whether these SASP proteins were secreted in higher levels by COPD-derived fibroblasts compared to non-COPD control-derived fibroblasts. Since we found a link between cellular senescence and ECM regulation in COPD-derived fibroblasts, and we know that ASMCs play a role in ECM regulation as well, we assessed in **chapter 6** whether COPD-derived ASMCs also have higher levels of cellular senescence compared to ASMCs from non-COPD controls and whether this is linked with ECM regulation. In the last experimental chapter (**chapter 7**), we assessed whether E-cigarette vapour exposure, similar to CSE, induces cellular senescence in primary parenchymal lung fibroblasts and whether this affects the repair function of these fibroblasts. Finally, in **chapter 8** we summarize all findings from this thesis, discuss the relevance and implications of these findings, and describe some of the future perspectives in the field of accelerated ageing and lung tissue repair and remodelling.

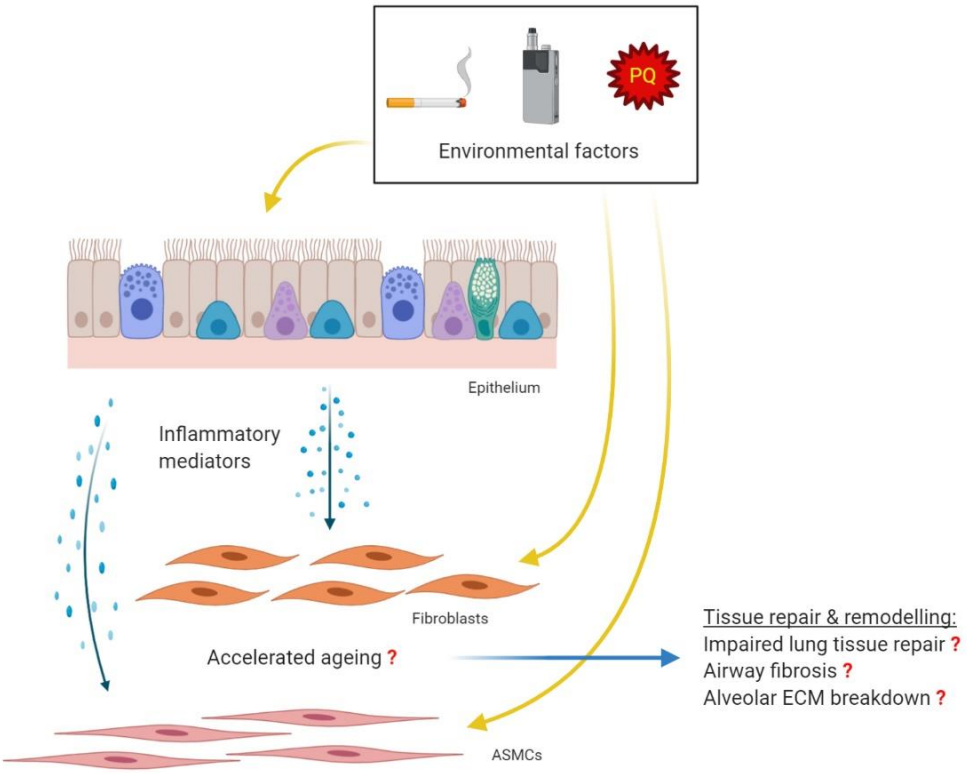

Figure 3: Hypothesized role of accelerated ageing in tissue repair and remodelling in COPD. *We hypothesize that lung fibroblasts and airway smooth muscle cells (ASMCs) from COPD patients have an accelerated ageing phenotype. We propose that this phenotype can be caused by environmental factors, including cigarette smoke, E-cigarette vapour, and Paraquat (PQ), which is another COPD risk factor by occupational exposure, directly or via the effect on the epithelium in combination with an impairment in age-related repair and maintenance mechanisms. In addition, we hypothesize that this ageing phenotype affects tissue repair and remodelling with impaired lung tissue repair, induced airway fibrosis and alveolar ECM breakdown. Created with BioRender.com.*

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CHAPTER 2

Lung ageing and COPD: is there a role for ageing in abnormal tissue repair?

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ABSTRACT

COPD is the fourth leading cause of death worldwide with increasing prevalence, in particular in the elderly. COPD is characterized by abnormal tissue repair resulting in (small) airways disease and emphysema. There is accumulating evidence that ageing hallmarks are prominent features of COPD. These ageing hallmarks have been described in different subsets of COPD patients, in different lung compartments, and also in a variety of cell types, and thus might contribute to different COPD phenotypes. A better understanding of the main differences and similarities between normal lung ageing and the pathology of COPD may improve our understanding of the mechanisms driving COPD pathology, in particular in those patients that develop the most severe form of COPD at a relatively young age, i.e. severe early onset COPD patients. In this review, after introducing the main concepts of lung ageing and COPD pathology, we focus on the role of (abnormal) ageing in lung remodelling and repair in COPD. We discuss the current evidence for the involvement of ageing hallmarks in these pathologic features of COPD. In the last part, we highlight potential novel treatment strategies and opportunities for future research based on our current knowledge of abnormal lung ageing in COPD.

What is ageing?

As the world indicates, ageing is a process that mainly affects elderly people. With the quickly growing elderly population, the negative aspects of ageing are becoming increasingly apparent. Ageing is defined as the progressive decline in homeostasis after the reproductive phase is complete, which results in increased risk of disease or death (1). As such, ageing is one of the main driving forces of the development and increasing burden of non-communicable diseases (NCDs), i.e. chronic diseases. Worldwide, NCDs are the leading cause of mortality and responsible for 38 million deaths each year. Of these deaths, 4 million can be attributed to respiratory diseases (2). In many of the NCDs including ischaemic heart disease, diabetes, Alzheimer's disease and chronic obstructive pulmonary disease (COPD), it is proposed that acceleration of the normal ageing process is involved in the disease pathogenesis (3).

In this review we will first describe the processes involved in the normal ageing lung and the disease pathology of COPD and then summarize the similarities and the differences. We will specifically focus on the role of abnormal ageing in lung remodelling and repair in COPD and discuss the current evidence for ageing hallmarks in the pathologic features of COPD. Finally, we will discuss potential novel treatment strategies based on the current evidence for lung ageing in COPD.

The ageing lung

On average, the human lung is growing until 10-12 years of age and further matures until it reaches its maximum function at approximately 20 years of age for females and 25 years of age for males (4). From then on lung function progressively declines with increasing age as a consequence of structural and physiological changes of the lung (4).

To start with the structural changes of the ageing lung, we can broadly divide these structural changes in three categories: changes in lung structure, changes in the chest wall and changes in respiratory muscles (5). The changes in the structure of the lung are mainly attributed to an increase in the size of the alveolar space without any inflammation or alveolar wall destruction, so called 'senile emphysema'. This microscopic emphysema increases in a linear fashion with age in non-smokers whereas when smoking a progressive increase in alveolar space size can be observed in specific (susceptible) individuals only (6-8). Senile emphysema might be a consequence of loss of the supporting structure of the lung parenchyma (4,5). Additionally, it has been observed that the elastic recoil of the lung reduces with increasing age. It has been postulated that this phenomenon is rather caused by reduced surface tension forces from the alveoli due to increased individual diameter size than by changes in elastin and collagen in the lung parenchyma (5). Upon increasing age, the compliance of the chest wall decreases progressively, which can be explained by several, synergistically acting, age-related processes. Firstly, the shape of the thorax may change with age due to reduced thickness of intervertebral discs, leading to reduced intra thoracic volume. Secondly, age-associated osteoporosis may cause vertebral fractures resulting in changes in the shape of the thorax. Thirdly, the stiffness of the ribs increases with age, thereby enhancing the forces needed

for movement of the chest (5,9). In general, muscle strength diminishes with age. This loss in muscle strength is also reflected in the diaphragm, the most important respiratory muscle, and thus affects the breathing pattern (5). These structural changes of the ageing lung have a clear effect on the overall lung function and several physiologically parameters are altered upon ageing. Both the forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) are decreasing with age and the rate of decline has shown to be higher for males than for females. As a consequence of the reduced elastic recoil and compliance of the chest wall, the residual volume (RV) increases, while the vital capacity (VC) decreases (9). Of interest, the total lung capacity (TLC) does not change with increasing age, since the reduction in elastic recoil observed upon ageing is counterbalanced by the decrease in chest wall compliance and muscle strength of the chest (5,9). Although the distribution of alveolar ventilation and perfusion across the lungs is very heterogeneous as a consequence of the decline in alveolar surface area, density of lung capillaries and pulmonary capillary blood volume, the overall transfer capacity of the lung for carbon monoxide (TLCO) is reduced with increasing age. Clinically, this might influence the physical activity and the development of sleep-discorded breathing (5,9).

Next to changes in lung function with increasing age, the natural defence mechanisms of the lungs are also gradually less functional, leading to increased infection risk (4). For example, the antioxidant response to prohibit the accumulation of reactive oxygen species (ROS) is deteriorated in the ageing lung, consistent with an increase in ROS levels upon ageing (10). Furthermore, intercellular communications become less effective with ageing (11) contributing to two phenomena known as immunosenescence and inflammageing. The first relates to dampened immune responses following an infection or injury, and the second term relates to the chronic activation of immune responses in aged subjects in the absence of a real immunologic challenge (12). As a result of immunosenescence, innate and adaptive immune responses decrease with age, which is characterized by an increase in memory and effector cells at the expense of naïve T cells and the overall T cell repertoire (13,14). Of interest, several of the pro-inflammatory mediators associated with inflammageing, like tumour necrosis factor (TN)-α, interleukin (IL)-1β and IL-6, are present as pro-inflammatory mediators in the senescence-associated secretory phenotype (SASP). Another factor contributing to increased inflammation in aged lungs is poor airway clearance of particles. Over time, muscles become atrophic, resulting in less strength for effective cough (15). Also, mucociliary clearance is known to be compromised with age (16), which might in particular contribute to viral and bacterial inflammation and thus acute exacerbations of lung diseases like COPD.

Pathology of COPD

COPD is a heterogeneous disease involving both the alveolar and airway compartment resulting in (small) airways disease and emphysema (Figure 1). The extent of pathologic changes in these different lung compartments is however variable in individual patients (17). The aetiology involves in general exposure to external noxious particles or gases. In the Western world this is in particular by (cigarette) smoking, and in the non-Western

world mainly by indoor cooking. COPD pathology is driven by chronic inflammation (18-20), which is still observed after stopping smoking (21) even after one or more years (22,23). The combination of the exposure and inflammation leads to lung tissue damage resulting in remodelling of the lung. This remodelling shows remarkable features: a common main aetiology, smoke exposure, leads to fibrosis (extracellular matrix increase) with thickening of (large and in a particular small) airway walls with lumen reduction and concurrently to emphysema with ECM destruction in the lung parenchyma. (19,24). Another main histopathologic feature of COPD is seen in the vasculature with in particular increased thickness of the arterioles, resulting in pulmonary hypertension as an important complication of COPD (25).

The chronic inflammation is mainly characterized by macrophages and ($CD8^+$) T cells and can also show increased plasma cells, neutrophilic granulocytes and sometimes eosinophilic granulocytes (26-28). B-cells are also found, often in aggregates or small primary or secondary follicles (29-31). These have been described in association with airways as tertiary follicles, or reactive bronchus associated lymphoid tissue (BALT), but also have been observed scattered in the parenchyma (31,32). The presence of such follicles is variable, most pronounced in patients with severe COPD (30,32), and not only seen in COPD patients but also, to a lesser extent, in heavy smokers without COPD (31). As oligoclonality of these follicles has been shown (31). It is most likely that these are induced by local antigen stimulation (33-35). Cigarette smoke components, micro-organisms and matrix components (36) have been considered as etiologic factors for this antigenic stimulation, but none have been convincingly shown yet (34,37).

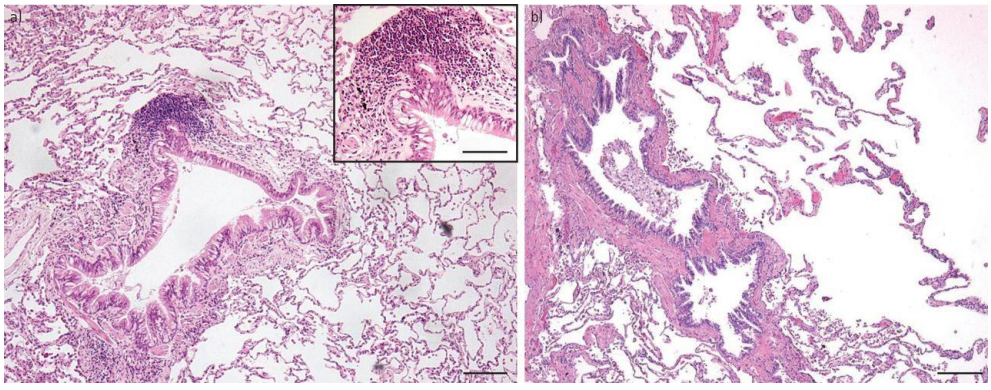

Figure 1; Pathologic changes in COPD. A) *Characteristic picture of airways changes, with increase of goblet cells, a thickened airway wall with some adventitial inflammation and a small lymphoid follicle at the left upper side. Emphysema is hardly present here (haematoxylin and eosin, x200).Insert shows the magnification of part of the airway with a lymphoid follicle.* ***B)*** *At the left almost longitudinal cross-section of a small airway, while at the right side severe parenchymal destruction by emphysema (haematoxylin and eosin, x200).*

In the large and small airways, epithelial changes are observed with increase of goblet cells, basal cell hyperplasia and squamous metaplasia, sometimes with dysplasia (20,38-40). Overall, these changes are in general more pronounced in the large airways, where the primary and most intensive exposure takes place. In the airway lumen increased and more sticky mucus can be present, which is produced by the increased number of goblet cells in combination with the enlarged mucus glands. The airway walls are thickened, caused by increased inflammation, increase of smooth muscle mass, increase in size of mucus glands, and, in due time, further changes with increase of extracellular matrix (ECM).

The matrix changes in the airways are in general of a fibrotic nature with increase of collagens in the submucosa but also in the adventitia of smaller airways (41-44). Similar to asthma, a thickening of the basement membrane is seen, although more irregular and of different composition (43). An interesting finding was that in the peribronchial area of small airways in severe COPD an impressive reduction was found for proteoglycans, most prominent for decorin and to a lesser extent biglycan (45,46). A main characteristic of decorin is that it can bind TGF-β, one of the main cytokines regulating matrix production, which is consistently upregulated in COPD (47,48). Another important feature is that decorin is the main proteoglycan connecting collagen fibrils, in this way regulating rigidness of the collagen (48,49). As decorin is reduced in COPD, this will result in a very loose type of fibrosis, contributing to increased airway collapsibility and reduced peribronchial tensile strength of the parenchymal attachments (19,24). An interesting observation by Hogg *et al.* in a limited number of patients was a reduction in the number of small airways with increasing emphysema severity within COPD lungs suggesting that part of the airflow limitation within COPD is primarily caused by destruction of small airways (50,51).

Emphysema is the characteristic pathology of COPD that occurs in the lung parenchyma. This is characterized by net destruction of alveolar walls, as a result from increased destruction in combination with failing tissue repair (19). The destructive part is caused by an unbalance between exposure to oxygen radicals from cigarette smoke and neutrophils and proteases from macrophages and neutrophils, and their counterparts, oxygen scavengers and anti-proteases (52). Also in the parenchyma inflammation is present, but far less research has been published with respect to this compartment (53,54). Similar to the airways, cytotoxic (CD8+) T cells are important infiltrating cells in alveolar septa and arterioles in COPD when compared to non-COPD controls (53). Whereas neutrophils were and are considered as a main inflammatory cell contributing to emphysema, already in early studies in smokers no association was found between parenchymal neutrophils and the severity of the destructive index (55). In addition to possible direct effects of both smoke components and proteases released from inflammatory cells, indirect effects have been shown to destroy alveolar walls in COPD by inducing apoptosis of endothelial and alveolar type 2 epithelial cells, likely contributing to emphysema (56,57).

The important vascular changes in COPD are mainly seen in the arterioles. Here, intimal thickening with smooth muscle proliferation and increase of collagen and elastin, together with hyperplastic increase of the media have been observed (25,58-61). Initially this was thought to be the result of hypoxia, but these events are also present in mild and early cases (60). More recently, the vascular changes have been attributed to "endothelial dysfunction" i.e. pathophysiological changes in the normal biochemical function of the endothelium (25,61,62). The end result are arterioles with a thickened wall, increased contraction and reduced lumen, but also with the reduced ability to vasodilate. Functionally, this leads to pulmonary hypertension which is a major cause of morbidity in COPD and a predictor of mortality (25,61).

Similarities and differences between lung ageing and COPD

One of the first reports on senile lungs compared with normal and emphysematous lungs by Verbeken *et al.* (63,64) demonstrated that the airspace enlargement in ageing, although comparable with smoking induced emphysema, differed in the fact that it was more regular in distribution without clear-cut destruction. Furthermore, increased thickening of alveolar septa was observed without inflammation or fibrosis with reduced density of the membranous bronchioles. They proposed the term senile lung for this condition. So, although similar in enlargement of airspaces at least part of the pathogenesis appears to be different. For loss of elasticity it is less clear whether this is a destructive effect in COPD or whether in both conditions there is an underlying defect in elastin fibrillogenesis. The functional effects on small airways in milder forms of emphysema are however comparable with the senile lung with loss of elastic recoil (64).

In the ageing process, Ito and Barnes (1) proposed that with increasing age the lung is less able to maintain organ integrity and protect itself against oxidative injury. Also, Kirkwood (65,66) indicated that cellular defects often cause inflammatory reactions contributing to damage, thereby causing a vicious circle of ongoing microscopic damage in due time with ageing. As yet, it is not readily clear to what extent these events are present during the total life course and when effects on tissue homeostasis become effective. In addition, it is not clear what the variation in the natural course of these events is with regard to their contribution to deterioration of the normal ageing lung. Taking the above mechanisms into account, several components observed in COPD, like the ongoing inflammation, unbalanced oxidative stress, and changes in the ECM are quite comparable as observed in the normal ageing lung. However in COPD, these changes will occur in general at an earlier age and to a larger extent compared to normal lung ageing. In the paragraphs below we will discuss in more detail whether premature or abnormal (lung) ageing aspects may or may not play a role in pathogenesis and natural course of COPD.

Lung ageing and COPD phenotypes

As described above, COPD is a very heterogeneous lung disease presented by different (mixed) phenotypes. Well-known phenotypes in COPD are chronic bronchitis with predominant airway related changes (inflammation and airway wall thickening) and

increased mucus production, and emphysema with (severe) alveolar wall destruction, hyperinflation and impaired gas exchange. Other phenotypes of COPD are related to the number of exacerbations (i.e. the frequent exacerbator) (67,68) or the age of onset of the disease, i.e. severe early onset COPD (SEO-COPD) (69). Given the difference in underlying pathology of these phenotypes, it can be envisaged that lung ageing is more or differently involved in some of these phenotypes than others. As discussed above, senile emphysema is an important hallmark of lung ageing and together with the structural changes, the ageing lung is in particular inclined to develop an emphysema-like phenotype. This is different from the bronchitis phenotype, where, apart from increased inflammation, there are very little similarities with the ageing lung and there is no indication of increased mucus production or airway wall thickening in the ageing lung, although decreased ciliary function with ageing likely contributes to increased coughing and decreased mucociliary clearance (15). The frequent exacerbator is an interesting phenotype as exacerbations are in general linked to infections and the susceptibility for infections increases with age (70,71). Moreover, age is a risk factor for COPD exacerbations (72) and hospital admissions for acute exacerbations of COPD (73).

Severe early-onset COPD is an interesting COPD phenotype with respect to ageing. Patients with this phenotype develop very severe COPD at a relatively young age, i.e. <53 according to Silverman *et al.* (69) and often with a relatively low number of pack years of smoking. This severe early-onset COPD (SEO-COPD) leads to a high personal burden and huge societal costs due to loss in working days and frequent hospitalizations. As these patients progress so quickly, we propose that, if accelerated ageing is an important contributor to COPD pathology, it should be most clear in these SEO-COPD patients. With respect to the pathology these patients are characterized by severe emphysema (69).

Ageing hallmarks in COPD

The main hallmarks of ageing were recently summarized in a review by Lopez-Orin (11) and this was followed by an overview of these hallmarks in lung ageing and lung disease (74). Broadly, the ageing hallmarks can be divided in processes affecting transcription (genomic instability, telomere attrition and epigenetic alterations), processes affecting the metabolism (loss of proteostasis, deregulated nutrient sensing and mitochondrial dysfunction) and cellular processes (cellular senescence, stem cell exhaustion and altered intracellular communication). We will now discuss the current knowledge about the possible role for these ageing hallmarks in COPD and mainly focus on findings in structural cells (alveolar and bronchial epithelial cells, smooth muscle cells and fibroblasts) and lung tissue. Subsequently, we will summarize the main evidence regarding the role of ageing hallmarks in disturbed repair and remodelling in COPD. All findings discussed in the paragraphs below are summarized in Table 1.

Transcription

Genomic instability

Ageing leads to increased DNA damage and to impaired ability to prevent and repair DNA damage. Several markers related to these features have also been demonstrated in COPD lungs and may contribute to the pathologic processes.

The DNA damage marker gamma- H2A histone family member X (ɣ-H2A.X) was increased in alveolar walls, including type I and type II epithelial cells and endothelial cells (75), as well as in small airways of COPD patients compared to controls (75,76). Another study, however, showed no differences in small airways in COPD versus control (77).

Smoke exposure increases ɣ-H2A.X levels in experimental animal models and cigarette smoke extract (CSE) treatment increases ɣ-H2A.X levels in bronchial epithelial cells and fibroblasts *in vitro* (76,78,79), suggesting an important role for oxidative stress. In addition, the anti-ageing protein sirtuin 6 (SIRT6) is considered to be protective against DNA damage and senescence. SIRT6 levels are decreased in lung tissue homogenates from COPD patients and overexpression and knockdown of SIRT6 in bronchial epithelial cells resulted in a decrease and an increase in ɣH2A.X levels, respectively (79).

The DNA repair marker Ku86 was decreased in parenchymal lung tissue of COPD patients, including small airways (77,80), while no differences were observed in Ku70 expression in these samples. Ku70 is another DNA repair marker which was decreased in leukocytes derived from COPD patients and its expression was negatively correlated with age (80).

Table 1: Evidence for ageing hallmarks in COPD

Hallmark	Marker	COPD vs non-COPD	COPD cell origin	CSE treated cells	References
Genomic instability	γ-H2A.X	↑	Lung tissue sections, AT1, AT2, HBEC & PV-EC	HBEC, HFL1 & MRC-5	(75,76,78,79)
	Ku70	↓	Peripheral leukocytes		(80)
	Ku80	↓	Lung homogenates		(77)
Telomere shortening	length	↓	Lung homogenates AT2, PA-SMC, PV-EC & peripheral leukocytes	SAEC (COPD) & HFL1	(78,81-86)
	telomerase	↓	PV-EC		(84)
	TPP1	↓	Lung homogenates	SAEC & HFL1	(78)
Epigenetic changes	HDAC activity	↓	Lung homogenates and bronchial biopsies		(92)
	SIRT-1 & -6	↓	Lung homogenates		(94,95)
Loss of proteostasis	Autophagy	↑	HBEC	SAEC	(100)
	Autophagy	↓		HBEC (COPD)	(100)
	Autophagosomes	↑	Lung homogenates	HBEC & BEAS-2B	(79,99,100)
	Ubiquitin	↑	Lung homogenates		(98,100)
	p62	↑	Lung homogenates		(79,100)
Deregulated nutrient sensing	S6K (mTOR)	↑*	Lung homogenates & peripheral leukocytes	HBEC	(79,103)
	IGF1	↑	SAEC		(104)
Mitochondrial dysfunction	ROS	↑		HBEC, BEAS-2B & MRC-5	(76,108,109)
	Ox-DNA	↑	Lung homogenates		(77)
	lipid peroxidation	↑	Lung homogenates		(105,106)
	NO	↑	Lung homogenates		(106)
	mitophagy	↑	Lung homogenates	HBEC & BEAS-2B	(108,109)
	Antioxidant	↓	Lung homogenates & HBEC		(107)
	Mitochondrial membrane potential	↓		BEAS-2B	(108)
Immune dysregulation	Klotho	↓	Lung homogenates	HBEC	(112)
	NF-κB	↑	Lung homogenates		(113)
	pro-inflammatory cytokines	↑	Lung homogenates		(113)
Senescence	SA-β-gal	↑	SAEC, PA-SMC, PV-EC & fibroblasts	SAEC (COPD), HBEC, A549, HFL1 & MRC-5	(76,78,79,84-86,115-117)
	p16	↑	Lung tissue sections, AT1, AT2, PA-SMC, PV-EC & fibroblasts	HFL1	(75,76,78,84-86,116,117)
	p21	↑	Lung homogenates, AT2, PA-SMC, PV-EC & peripheral leukocytes	HBEC, A549 & HFL1	(78-80,84-86,117)
	IL-6 & IL-8	↑	Lung tissue sections, AT1, AT2, PA-SMC & PV-EC	MRC-5	(75,76,84,85)
ECM dysregulation	ECM proteins	↑	Lung homogenates		(42)
	Elastogenesis genes	↑	Lung homogenates		(125)
	MMP/TIMP dysregulation	↑	Lung homogenates		(123)
Stem cell exhaustion	Circulating progenitor cells	↓*	Endothelial and haemopoietic progenitor cells		(137,138)
	Regenerative capacity	↓	Basal progenitor cells		(135)
	Stem cell function	↓	HBEC		(142)
	WNT signalling	↓*	Lung homogenates, AT2 & SAEC		(144,147)
	Notch pathway	↓*	SAEC		(143)

CSE: cigarette smoke extract; AT1: type I alveolar cells; AT2: type II alveolar cells; HBEC: human bronchial epithelial cells; PV-EC: pulmonary vascular endothelial cells; HFL1: foetal lung fibroblasts; MRC-5: foetal lung fibroblasts; TPP1: telomere protection protein 1; PA-SMC: pulmonary artery smooth muscle cells; SAEC: small airway epithelial cells; HDAC: histone deacetylase; SIRT: sirtuin; BEAS-2B: bronchial epithelial cell line (virus); mTOR: mechanistic target of rapamycin; IGF1: insulin-like growth factor 1; ROS: reactive oxygen species; SA-β-gal: senescence-associated-β-galactosidase; IL: interleukin; A549: alveolar basal epithelial cell line (carcinoma); ECM: extracellular matrix; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinase. #: mean age was significantly different between COPD and control group; for the other studies, information was not available or mean age was not different between groups.

Telomere shortening

Telomere shortening is an important inducer of senescence and a well-known phenomenon in ageing. Reduced telomere length in circulating leukocytes in COPD has been demonstrated in several studies (80-83), while data regarding telomere shortening in structural cells is still scarce.

Reduced telomere length was demonstrated in pulmonary vascular endothelial cells and pulmonary artery smooth muscle cells that were derived from COPD patients when compared to cells derived from smoking controls (84,85). Tsjui *et al.* used fluorescent in situ hybridization (FISH) to assess telomere length in alveolar type II and endothelial cells and demonstrated decreased telomere length in COPD patients when compared to non-smoking controls, but not compared to smoking controls (86). A recent study from Ahmad *et al.* assessed telomere length in lung tissue and reported an association with levels of telomere protection protein 1 (TPP1) (78). Both telomere length and TTP1 levels were reduced in lung homogenates from COPD patients compared to non-smoking controls, but not compared to smoking controls. This was further supported by decreased TTP1 levels and telomere length in CSE treated airway epithelial cells and lung fibroblasts (78).

The above findings of Tsuji *et al.* and Ahmad *et al.* (78,86) suggest an association with smoking rather than being COPD specific, although given that most COPD-patients are (ex-) smokers, this might be a contributing factor to disease risk and development.

Epigenetic changes

Epigenetic alterations caused by DNA methylation, histone modifications and noncoding RNA's are highly dynamic and influenced by ageing (87). It has even been postulated that the DNA methylation status of particular CpG-sites, also known as the 'epigenetic clock', can be used in an algorithm to predict the biological age (88,89). However, as far as we know, this algorithm has not been applied yet to COPD patients to test if the biological age of COPD patients determined by their methylation status is indeed increased compared to controls as would be expected. The majority of epigenome-wide methylation studies have been performed in whole blood and not much data is available on DNA methylation in whole lung tissue and lung tissue-specific cell types. While it has been widely established that cigarette smoke affects DNA methylation (90) and that COPD is highly associated with cigarette smoke exposure, in a recent systematic review by *Machin et al.*, no consistent differences were found in DNA methylation in peripheral blood in association with COPD or lung function (91). Therefore, the role of DNA methylation in COPD and thereby the role of age-associated differences in DNA methylation in COPD remains unclear.

Histone deacetylase (HDAC) enzymes can reduce the acetylation of histones, leading to enhanced expression of inflammatory genes involved in the disease pathogenesis of COPD. It has been shown that the HDAC activity is reduced in peripheral lung tissue, alveolar macrophages and bronchial biopsies of COPD patients compared to controls and this activity is further associated with the disease severity of COPD in peripheral lung tissue (92). The NAD^+-dependent Class III protein deacetylases known as

the Sirtuin family are frequently described as anti-ageing enzymes (93). The fact that SIRT1 and 6 have been shown to be decreased in peripheral lung tissue, and SIRT1 also in serum, of COPD patients compared to controls and, suggests age-associated acetylation differences in COPD (94,95). *Baker et al.* postulate that the reduced expression of both of the Sirtuins is regulated by the micro-RNA MiR-34a, a small endogenous non-coding RNA, which appears to be increased in COPD patients compared to controls. While the role of micro-RNAs in COPD has been extensively reviewed (96) the role of micro-RNAs in accelerated lung ageing is not extensively investigated and remains rather elusive (97).

Metabolism

Loss of proteostasis

Ageing cells are less able to maintain the homeostasis of proteins and contain more damaged proteins. In COPD lungs proteostasis of cells is decreased as well, which results in accumulation of damaged proteins. Accumulation of ubiquitinated proteins and the de-ubiquitinating enzyme and aggregation marker, ubiquitin C-terminal hydrolase L1 (UCH-L1), is increased in lung tissue of patients with severe COPD, and these levels negatively correlate with FEV_1 % predicted (98). Furthermore, several autophagy markers are increased in COPD lung tissue including p62, microtubule-associated proteins 1A/1B light chain 3B (LC3-II), autophagy related 4 (Atg4), Atg5-Atg12 and Atg7 (79,99).

Functional studies showed that autophagy activity (LC3-II flux) is increased in bronchial epithelial cells of COPD patients, without further increase upon CSE treatment (100). However, CSE treatment does increase the amount of autophagosomes in airway and bronchial epithelial cells (79,99). Inhibition of autophagy in bronchial epithelial cells results in accumulation of ubiquitinated protein and p62 (100). Again, the anti-ageing molecule SIRT6 may also regulate autophagy, as SIRT6 overexpression and knockdown resulted in an increase and a decrease of autophagosomes respectively (79).

Deregulated nutrient sensing

Nutrient sensing is a cell's ability to recognize and respond to fuel substrates such as glucose and recent findings suggest that nutrient sensing is increased in COPD lungs. Caloric restriction is strongly associated with longevity, and this is possibly mediated via two main pathways involved in nutrient sensing: mechanistic target of rapamycin (mTOR) and insulin like growth factor (IGF1)-signalling (101,102). The activity of mTOR, an important protein kinase in cell metabolism and nutrient sensing, is increased in total lung tissue and leukocytes of COPD patients (103) as well as in CSE-treated bronchial epithelial cells (79). In addition, SIRT6 is considered to attenuate the IGF1-mTOR pathway. The IGF1 pathway is important in cell growth and interacts with mTOR in the regulation of energy metabolism. Here, SIRT6 may play a role, as overexpression and knockdown of *SIRT6* resulted in a decrease in mTOR activity and increase in IGF1 signalling, respectively (79). Of interest, IGF1 protein levels were found to be increased in airway epithelial cells of patients with chronic bronchitis (104).

Mitochondrial dysfunction

With ageing, the function of the mitochondria decreases, which can lead to oxidative stress. Of interest, increased levels of oxidative stress are observed in COPD lungs as well. In whole lung tissue of COPD patients increased oxidative stress was found, as determined by reactive oxygen species (ROS) levels, oxidized-DNA, lipid peroxidation and nitric oxide (NO) levels (77,105,106). Moreover, lipid peroxidation correlated negatively with FEV_1 % predicted (105). In addition, gene expression and protein levels of the anti-oxidant nuclear factor, erythroid 2 like 2 (NRF2) were decreased in total lung tissue and bronchial epithelial cells of COPD patients (107) and *NRF2* expression was positively correlated with airway obstruction (FEV_1/FVC). Mitophagy, the degradation of mitochondria by autophagy, was increased in total lung tissue of COPD patients (108).

Furthermore, CSE treatment of bronchial epithelial cells resulted in increased ROS levels and mitophagy and decreased mitochondrial potential (108,109). Also in fibroblasts CSE treatment resulted in higher ROS levels (76).

Cellular processes

Immunosenescence and inflammageing

As described above, ageing is associated with immunosenescence and inflammageing. These two definitions underlie most of age-associated diseases and are important during COPD development in aged individuals. Several recent studies have investigated how ageing might affect immune dysregulation in COPD. In a study from 2016, John-Schuster *et al.* (110) demonstrated that aged mice exposed to cigarette-smoke are more susceptible to develop emphysema than younger mice. Aged animals had increased lung inflammation, with higher levels of inflammatory cells and mediators associated with lower repair. Two studies, also from 2015, observed that Klotho, an anti-ageing protein with anti-inflammatory properties, is reduced in alveolar macrophages (111) and airway epithelial cells of patients with COPD (112). The reduction was associated with high levels of oxidative stress, inflammation and apoptosis (111,112). Furthermore, decreased expression of miR-125a and b levels in COPD have been linked to inflammation and an impaired immune response. MiR-125a reduction resulted in NF-κB activation with a classical induction of pro-inflammatory cytokines, while in parallel, low levels of miR-125a and b suppress viral clearance (113). These data underscore the potential of targeting inflammation and at the same time increasing resistance to infections in the aged individual with COPD.

Cellular senescence

Cellular senescence is a cell state in which normal cells stop to divide as a mechanism to prevent tumorigenesis and tissue damage. Senescent cells can be cleared by the immune system, however upon ageing the number of senescent cells is accumulating in tissues. In here, these cells can have detrimental effects as they secrete several inflammatory factors

and may disturb normal tissue homeostasis and repair due to the loss of their proliferative capacity and normal physiologic function (114). Evidence is accumulating for increased cellular senescence in COPD lungs.

The percentage of senescent-associated β-galactosidase positive cells was increased in multiple cell types in COPD patients, including airway epithelial cells, smooth muscle cells, endothelial cells and fibroblasts as well as in CSE treated alveolar and bronchial epithelial cells (76,78,79,84,85,115,116). Another senescence marker, the cell cycle inhibitor p16, was found to be increased in total lung tissue, alveolar cells, airway epithelial cells, smooth muscle cells, endothelial cells and fibroblasts of COPD patients (75,76,84-86,116). Similarly, the presence of p21, another cell cycle inhibitor, was increased in total lung tissue, alveolar cells, smooth muscle cells, endothelial cells and leukocytes of COPD patients (79,80,84-86). P21 was also increased in CSE treated bronchial epithelial cells and fibroblasts (78,79,117). Moreover, the percentages of p16 and p21 positive cells were negatively correlated with FEV_1 % predicted in alveolar type II and endothelial cells (86). The levels of IL-6 and IL-8, two important cytokines that are secreted by senescent cells as part of the senescence-associated secretory phenotype, were increased in total lung tissue, alveolar cells, smooth muscle cells and endothelial cells of COPD patients as well as in CSE treated fibroblasts (75,76,84,85). Though these cytokines can also be the result of ongoing inflammation in COPD, these observations cannot directly be related to an increase in cellular senescence.

ECM dysregulation

Age-related changes in the lung can also be observed at the extracellular levels. Comparable to the ageing lung (118,119) the extracellular matrix (ECM) is altered in COPD (120). The main alterations in COPD include increases of several ECM proteins such as collagens, fibronectin and laminin (42), changes in the structural organization of collagen with more disorganized collagen fibres (121), and also a reduction in elastic fibres (122). An important contributing factor to these ECM changes is the imbalance between proteases, such as matrix metalloproteinase 12 (MMP12) and neutrophil elastase, and anti-proteases, like α-1 antitrypsin and tissue inhibitor of metalloproteinase (TIMP) 1-4, as reviewed by Navratilova *et al.* (123).

Elastin degradation plays an important role in the pathogenesis of COPD. Elastin fragments alone are known to induce inflammation, leading to destruction of lung tissue (124). It was previously thought that in COPD the lung loses its ability to repair, however, it has become increasingly more evident that there may be aberrant attempts at repair. A number of genes encoding for elastogenesis components, such as fibulin-5 (*FBLN5*), microfibril associated protein 4 (*MFAP4*), latent transforming growth factor binding protein 2 (*LTBP2*) and elastin (*ELN*) itself were identified in a large COPD patient cohort to be higher expressed (125). Whether these components are beneficial or further drive disease pathogenesis remains unclear, as extracellular proteins have the potential to interfere with different cellular pathways (126,127).

Another interesting observation in COPD is the change in lung fibroblast responses *in vitro*. Lung fibroblasts are the main cells involved in ECM homeostasis and repair in the lung and several studies have shown differences in terms of ECM production when comparing COPD fibroblasts to those derived from non-COPD controls (128-131), suggesting a disturbed or abnormal repair capacity of these cells.

Stem cell exhaustion

Adult lung tissue is thought to reside in a quiescent state. Upon injury, (stem) cells can get activated and are able to proliferate and (trans) differentiate into other cell types, according to their plasticity (132). Indeed, the lung harbours different cell populations including stem cells, responsible for its unique homeostatic capacity to ensure gas exchange (133,134). Airway basal cells represent a well-characterized stem cell population located in the trachea and bronchi. These cells have the ability to self-renew and give rise to secretory, ciliated and neuroendocrine cells (135). In the distal lung, alveolar type II cells (ATII) (136) have been shown to be able to replenish lost ATII and trans differentiate to alveolar type I cells (ATI), thus ensuring proper gas exchange (132,133). It is most likely that other progenitor or stem cell subpopulations exist, which is indicated by several studies in mouse tissue over the past years, however, the existence of these cell in the human tissue and the relevance for tissue injury and potentially impaired repair, remains elusive.

Moreover, COPD has been associated with reduced numbers and dysfunction of circulating progenitor cells (137,138). Cigarette-smoke, a major risk factor for COPD, was shown to reduce the repair potential of endothelial progenitor cells (139), and bone marrow mesenchymal stem cells by interfering with cell homing and proliferation capacities (140). Thus, stem cell exhaustion might contribute to COPD pathogenesis by reducing the endogenous renewal and repair capacity of the lung by local as well as recruited cells. Stem cell niches fail to respond effectively to additional demands for cell-turnover, moreover, deranged metabolic signalling and premature senescence might occur (141). In line with this, reduced regenerative capacity of basal progenitor cells has been reported in COPD (135). In addition, in a different study, an abnormal population of $TRP63^{+}$ $KRT5^{+}$ $KRT14^{+}$ basal cells was identified in regions of hyperplasia from sections of COPD human airways (142), suggesting abnormal stem cell function. Developmental pathways, such as WNT, Sonic Hedgehog and Notch, are important susceptibility factors for COPD (143-145) and are associated with the regulation of different stem cell functions (146). Canonical WNT signalling, which relies on stabilization of β-catenin for transcriptional activation, is decreased in COPD (144,147,148). Notably, pharmaceutical activation of the pathway led to an increase in surfactant protein C production and secretion along with increased alveolar type I cell marker expression in COPD lung tissue *ex vivo*, thus suggesting that the initiation of stem cell mediated repair in the COPD lung is possible (149).

<u>Evidence for ageing hallmarks and abnormal tissue repair in COPD</u>

Increased levels of DNA damage and decreased levels of DNA repair markers have been demonstrated in COPD, in particular in the alveolar compartment. Although the data is derived from a limited number of studies, it does indicate a role for these ageing markers in COPD, in particular in relation to emphysema development with increased alveolar wall destruction and lack of repair.

Another key hallmark in ageing is telomere shortening. Since information on telomere length in structural cells is mostly lacking it is difficult to speculate on a role of telomere shorting in relation to tissue repair and remodelling in COPD. Reduced TPP1 levels in relation to smoking in lung tissue and structural cells however does suggest an effect of smoking. Whether this also relates to smoking-induced COPD remains to be elucidated.

Regarding epigenetic changes in COPD, solid data on DNA methylation is lacking, and thus it is yet not possible to infer a role for DNA methylation in abnormal tissue repair in COPD. Several studies however have indicated involvement of histone modification (HDAC and Sirtuins) and miRNAs in COPD and it is of great interest to further evaluate if and how these changes contribute to accelerated lung ageing and abnormal tissue repair in COPD.

With respect to the metabolic changes in COPD, increased autophagy and accumulation of damaged proteins reflects ongoing tissue damage and high protein turnover in COPD. Whether this is cause or consequence (or both) of the abnormal repair response is currently unclear. Similarly, disturbed nutrient sensing (IGF-1-mTOR) and the oxidant anti-oxidant imbalance, indicate that, as with normal ageing, cell homeostasis is disturbed, which makes the cells vulnerable to disease. However, whether and how this contributes to abnormal repair should be evaluated by further studies.

Of all ageing hallmarks in COPD, the changes in cellular processes are probably the best studied. Multiple studies have shown increased cellular senescence and changes in ECM regulation in COPD, in particular in structural cells, including epithelial cells, smooth muscle cells and fibroblasts. The latter are of particular interest, as fibroblasts are the main regulators of tissue repair in the lung and changes in these cells possibly underlie the abnormal tissue repair responses in COPD. Together with the reduced numbers, dysfunction and regenerative capacity of progenitor cells in COPD these age-related cellular changes may very well explain the disturbed repair and remodelling capacity of COPD lungs, both in the alveolar and airway compartment.

Implications for treatment

COPD exacerbations are of major concerns in elderly, as they are highly susceptible to infections. Due to a dampened immune system, vaccination is not considered a successful preventive measure (150). Having this in mind, strategies which boost the immune system have been proposed for lung disease treatment. One of the strategies is the interference with gut microbiota (151). Local microbiota influences immunity at distal sites and organs. *Bifidobacterium breve* and *Lactobacillus rhamnosus* have been shown to reduce

inflammatory responses in macrophages that were exposed to cigarette smoke extract *in vitro* (152). Another potential future therapy is based on the application and usage of stem cells. In 2013, bone marrow-derived mesenchymal stem cells (MSCs) were first transplanted to patients with no adverse effects observed in older patients (153). More recently, an immunomodulatory mechanism has been associated with MSCs treatment which decreased lung inflammation and improved lung function (154). Furthermore, as mentioned above, pharmaceutical activation of the WNT pathway showed promising effects *ex vivo* and indicates the opportunities to induce endogenous stem cell mediated repair in COPD (149).

Although stem cell therapies hold promise as future therapeutic options, more regulations and clinical trials on the matter are needed to optimize therapeutic schemes, dosages, infusion rates and further identify possible risk groups and specific adverse effects (155). Ultimately, understanding the molecular biology of ageing in the lung is crucial for finding new ways of manageing COPD in older but also younger (SEO-COPD) patients.

Summary and conclusions

As summarized in this review, main ageing hallmarks are present in COPD and this supports the hypothesis that (abnormal) ageing contributes to COPD development. With respect to the role of abnormal ageing in tissue repair in COPD, the strongest indications come from cellular changes, i.e. increased cellular senescence, ECM dysregulation and stem cell exhaustion. Yet, to be able to answer our question whether accelerated or abnormal ageing is causally contributing to COPD pathogenesis and in particular impaired tissue repair, we need to integrate all findings and assess how age-related changes affect ECM homeostasis and tissue repair in the lung. Ideally this should not be restricted to single-cell culture models with primary lung cells, but also involve more complex co-culture and organoid models, lung tissue slices and/or lab-on-a-chip approaches. An important aspect that needs to be taken into account is the age-matching between the control and COPD groups. Indeed for some studies discussed in this review, the mean age was significantly different between the control and COPD group (indicated in Table 1). This may come as a challenge to distinguish the effects which are related to age and which are related to COPD. Finally, translation and comparison to *in vivo* models and to what happens in the lungs of the actual COPD patients is important for the identification of potential new therapeutic approaches. Evaluation in well-defined clinical samples is crucial to understand the clinical implications and potential benefit for COPD patients. This information may also guide us towards novel approaches aiming to stop or at least slow down accelerated lung ageing in COPD. Providing a future perspective for the most vulnerable group of COPD patients that suffers from the highest disease burden and lacks adequate treatment; severe early onset COPD.

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CHAPTER 3

Age-Related Gene and MiRNA Expression Changes in Airways of Healthy Individuals

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ABSTRACT

Knowledge on age-related miRNA changes in healthy individuals and their interaction with mRNAs is lacking. We studied age-related mRNA and miRNA expression changes and their interactions in normal airways.

RNA and small RNA sequencing was performed on bronchial biopsies of 86 healthy individuals (age: 18-73) to determine age-related expression changes. Per age-related miRNA we determined the enrichment of age-related predicted targets and their correlation.

We identified 285 age-related genes and 27 age-related miRNAs. Pathway enrichment showed that genes higher expressed with age were involved in synapse-related processes and genes lower expressed with age were involved in cell cycle regulation, the immune system and DNA damage/repair. MiR-146a-5p, miR-146b-5p and miR-142-5p were lower expressed with increasing age and we found a significant enrichment for predicted targets of these miRNAs among genes that were higher expressed with age. The expression levels of the enriched predicted targets *RIMS2* and *IGSF1* were negatively correlated with both miR-146a-5p and miR-146b-5p. *RIMS2* was present in the enriched process, i.e. positive regulation of synaptic transmission.

In conclusion, genes decreased with ageing are involved in several of the ageing hallmarks. Genes higher expressed with ageing were involved in synapse-related processes, of which *RIMS2* is potentially regulated by two age-related miRNAs.

INTRODUCTION

Worldwide, the proportion of individuals over 60 years old is predicted to increase from 12% in 2015 to 22% in 2050[1]. This rise in the number of elderly individuals in the population will lead to an increase in ageing-associated diseases. Ageing is a process in which the body homeostasis progressively declines, resulting in increased risk of disease or death[2]. Nine hallmarks have been defined for ageing: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication[3]. In the ageing lung, dysregulation of the extracellular matrix has been proposed as an additional hallmark[4].

During normal ageing lung function declines over time due to a variety of mechanisms and anatomic changes including smaller thoracic cavity, reduced respiratory muscle function, senile emphysema and reduced mucus clearance[5]. Knowledge about changes in the airways due to ageing is scarce. Previously it was shown that airway wall thickness was decreased with higher age[6] and a murine study showed that senescence of airway progenitor cells impairs airway regeneration[7].

It is likely that changes in gene and microRNA (miRNA) expression play a role in ageing-associated processes in the lung. To gain insight in these processes, several gene and miRNA expression studies have been performed. Previously, we identified 3,509 age-related genes in lung tissue that were involved in lung development, cell-cell contact, calcium signalling and immune response[8]. Dugo *et al.* found enrichment of genes involved in extracellular matrix production and function, pro-inflammatory responses and wound healing among the 217 age-related genes in lung tissue[9]. Multiple miRNAs have been proposed to be involved in the process of ageing[10-13] and especially in cellular senescence, for example by targeting genes that play a role in the p53/p21 and p16/Rb senescence pathways[14]. In addition, expression levels of miR-210 and miR-494 were induced by DNA damage and oxidative stress in human foetal lung fibroblasts and vice versa. These miRNAs induced DNA damage and oxidative stress via a positive feedback loop[15].

Although several genes and miRNAs have been suggested to be involved in ageing of the lungs, limited information is available about the underlying age-related mRNA-miRNA interactions. Moreover, so far no studies have been performed in healthy subjects with a normal pulmonary function and without respiratory complaints. In the present study we aimed to identify age-related mRNA and miRNA changes and their interactions in bronchial biopsies of 86 healthy individuals with an age range of 18-73 years.

RESULTS

Subject characteristics

After quality control, RNA sequencing data of 77 and small RNA sequencing data of 82 bronchial biopsies were available for further analyses, resulting in a total of 86 biopsies originating from subjects with an age range of 18-73 years (Table 1). Of these 86 subjects, 73 had both mRNA and small RNA sequencing data available.

Table 1. Subject characteristics

Characteristics	Subjects
N	86
Male / Female, n	48 / 38
Age range, years	18 – 73
Never-smoker / smoker	44 / 42
FEV_1, % predicted [a]	98.7 (93.6 – 107.7)
FEV_1/FVC, % [b]	78.8 (74.5 – 84.1)
Pack-years, n	15.4 (4.4 – 29.3)

[a] *FEV_1, % predicted = percentage of Forced Expiratory Volume in one second of the predicted normal value for an individual of the same sex, age and height.*
[b] *FEV_1/FVC, % = Forced Expiratory Volume in one second/Forced Vital Capacity ratio expressed in percentage.*
Medians (interquartile ranges) are shown unless otherwise stated.

Age-related genes in human bronchial biopsies

Significant age-related changes in expression were observed for 285 genes (FDR adjusted p-value <0.05, Figure 1, Supplementary Table 1A-B). Of these genes, 149 were higher expressed and 136 were lower expressed with increasing age. The association with age of the top-3 higher expressed genes (*TMTC1*, *CPS1* and *RP11-550F7.1*) and top-3 lower expressed genes (*TIMELESS*, *KNTC1* and *BRIP1*) with lowest FDR adjusted p-values are shown in Supplementary Figure 1A.

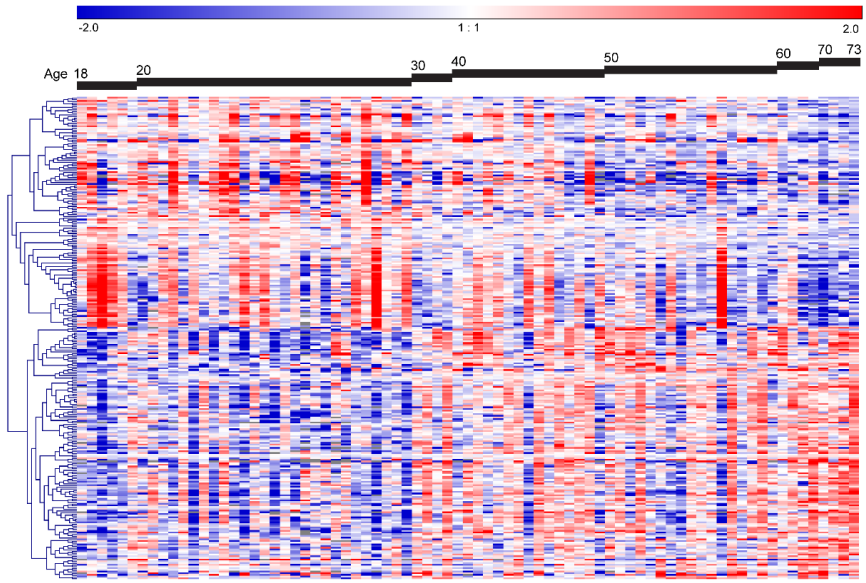

Figure 1. Age-related genes in human bronchial biopsies. *Heatmap of the supervised hierarchical clustering of the 285 age-related genes. The 77 subjects were ordered by age. The heatmap shows the median-centred expression of the 285 genes of which 136 genes were lower expressed and 149 were higher expressed with increasing age (FDR adjusted p-value<0.05).*

Replication of age-related genes in lung tissue

Of the 149 higher expressed and the 136 lower expressed genes with increasing age, 117 and 118 genes, respectively, were also detected in our previously published lung tissue dataset[8]. In this dataset we replicated the association with age for 58 out of 117 higher expressed genes (49.6%) and 43 out of 118 lower expressed genes (36.4%) (P-value<0.05, Supplementary Table 1A-B).

Biological processes and pathway enrichment of age-related genes

The biological processes and pathway enrichment analyses in Enrichr revealed amongst the age-related genes 155 and 116 significantly enriched processes and pathways respectively (FDR adjusted p-value<0.05, Supplementary Table 2). The genes higher expressed with age were enriched for synapse-related processes (n=19 genes) and pathway related to muscle contraction (n=8 genes). The genes lower expressed with age were enriched for processes and pathways related to cell cycle (n=54 genes), the immune system (n=44 genes) and DNA damage and repair (n=25 genes). Similar results were obtained using g:Profiler[16] (Supplementary Table 3).

Age-related miRNAs in human bronchial biopsies

Significant age-related expression changes were observed for 27 miRNAs (FDR adjusted p-value<0.05, Figure 2, Supplementary Table 1C-D). This included 13 miRNAs with lower expression levels and 14 with higher expression levels with increasing age. The association with age for the top-3 higher expressed miRNAs (miR-3195, miR-1247-5p, and miR-1-3p) and top-3 lower expressed miRNAs (miR-146b-5p, miR-155-5p and miR-20a-5p) based on FDR adjusted p-values are shown in Supplementary Figure 1B.

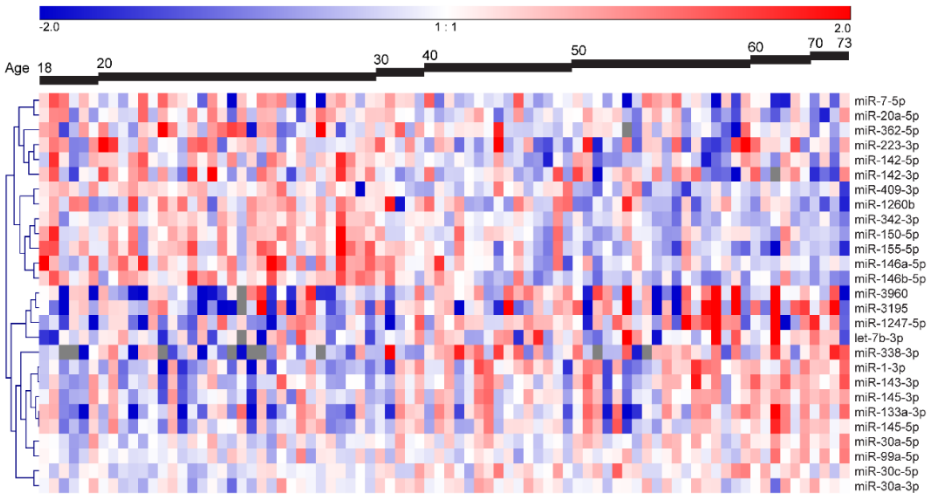

Figure 2. Age-related miRNAs in human bronchial biopsies. *Heatmap of the supervised hierarchical clustering of the 27 age-related miRNAs. The 82 subjects were ordered by age. The heatmap shows the median-centred expression of the 27 miRNAs of which 14 miRNAs were higher expressed and 13 miRNAs were lower expressed with increasing age (FDR adjusted p-value<0.05).*

Enrichment of age-related predicted miRNA target genes

To identify potential interactions between age-related mRNA and miRNA expression changes, we determined whether the predicted target genes of age-related miRNAs were enriched among the genes that were either higher or lower expressed with age as compared to all expressed genes. Of the 13 miRNAs that were lower expressed with increasing age, miR-146b-5p, miR-142-5p and miR-146a-5p showed a significant enrichment of their predicted target genes among genes higher expressed with increasing age (p-value<0.05), and a similar trend was observed for miR-409-3p (p-value=0.098, Figure 3). MiR-146b-5p, miR-142-5p and miR-146a-5p had five, 16 and five predicted

targets that were higher expressed with increasing age, respectively (Supplementary Table 4). Of the 14 miRNAs that were higher expressed with increasing age no significant enrichment of predicted target genes was found among genes lower expressed with increasing age.

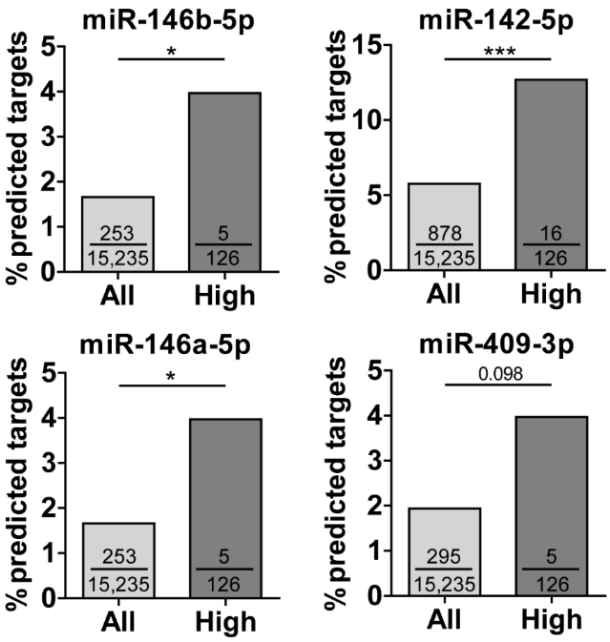

Figure 3. Enrichment of predicted miRNA target genes within the age-related genes. *MiRNA target gene enrichment analysis for miRNAs that were lower expressed with increasing age. All = all expressed genes (n total = 15,235 genes); High = genes that were higher expressed with age (n = 126 genes); The numbers in the bars indicate the number of predicted target genes divided by all expressed genes (15,235 genes, light grey bars) or by the 126 genes that were higher expressed with age (dark grey bars); * p-value<0.05, *** p-value<0.001.*

Correlation of miR-146b-5p, miR-142-5p and miR-146a-5p with their predicted targets

To further substantiate the connection between age-related gene and miRNA expression changes, we assessed the correlation between miR-146b-5p, miR-142-5p and miR-146a-5p and their enriched predicted targets that were higher expressed with increasing age (Supplementary Table 4). We identified nine significant negative correlations between the three miRNAs and their age-related predicted target genes (Figure 4). For miR-146b-5p, we found a significant negative correlation for four of the five enriched predicted target genes (Figure 4A). For miR-142-5p, we found a negative correlation for three out of sixteen (Figure 4B) and for miR-146a-5p for two out of five enriched predicted target genes (Figure 4C). The nine significant negative correlations involved seven different target genes. Of these, *RIMS2* and *IGSF1* were negatively correlated with both miR-146b-5p and miR-146a-5p. *RIMS2* was the 4th and *IGSF1* was the 6th most significant genes higher expressed with age (Supplementary Table 1A).

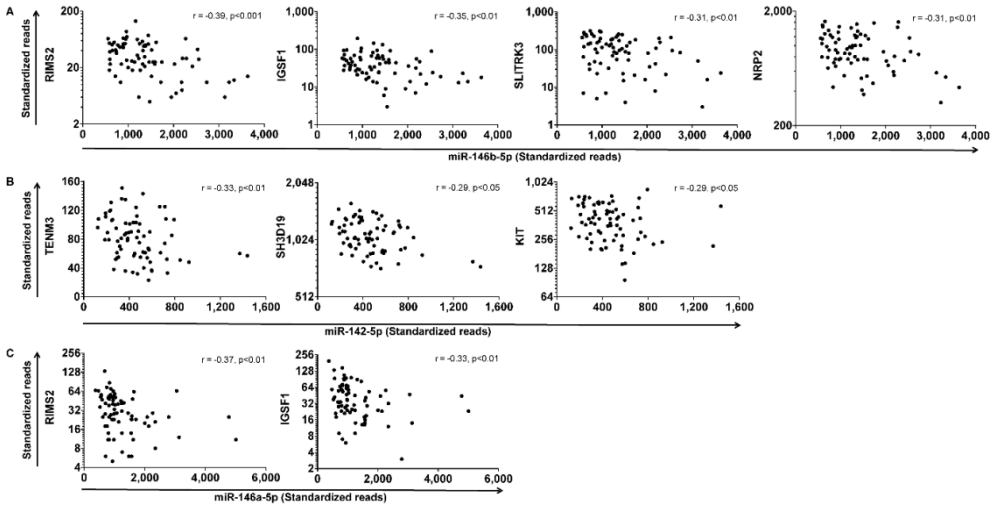

Figure 4. Correlation between miRNA expression and expression of their age-related predicted targets. *Lower expressed miRNAs with increasing age, (A) miR-146b-5p, (B) miR-142-5p and (C) miR-146a-5p correlated with their predicted target genes that are higher expressed with age. Spearman's correlation coefficient r and p-values are shown in the graphs.*

DISCUSSION

In this study, we investigated the potential role of miRNAs in the ageing process in healthy airways by combining age-related miRNA and gene expression changes. We identified 285 genes and 27 miRNAs of which the expression levels were changed with increasing age in bronchial biopsies. The genes with higher expression levels with increasing age were mainly involved in synapse-related processes. The genes with lower expression levels with increasing age were mainly involved in DNA damage and repair, cell cycle regulation and the immune system. MiR-146b-5p, miR-142-5p and miR-146a-5p expression levels were lower with increasing age and a significant enrichment of their predicted target genes was found among the genes higher expressed with increasing age. *RIMS2* and *IGSF1* were negatively correlated with miR-146b-5p and miR-146a-5p. Of these predicted target genes, *RIMS2* was involved in positive regulation of synaptic transmission, one of the significantly enriched biological processes amongst the age-related genes. To our knowledge, this is the first study in which age-related genes were connected to age-related miRNAs in airway biopsies from respiratory healthy subjects.

Interestingly, the above-mentioned miRNAs have been associated with age in previous studies. In accordance with our study, the levels of miR-142-5p in human serum were lower with increasing age[17]. Different to our findings, the expression levels of miR-146a-5p were shown to be higher with increasing age in human mesenchymal stem cells[18] and both miR-146a-5p and miR-146b-5p levels were increased in senescent compared to quiescent as well as proliferating human foreskin fibroblasts[19]. These disparate findings might be related to differences in cell type and/or tissue specific expression changes of these miRNAs with age. Previously, *MIR3142HG* was reported as a host gene of miR-146a[20], which was also significantly lower expressed with age in our study (p-value<0.05).

Our study showed that genes with higher expression levels with increasing age in bronchial biopsies were involved in synapse-related processes. The nervous system of the respiratory tract regulates the calibre of the bronchi and pulmonary vessels, but how the nervous system of the respiratory tract changes during ageing is still poorly understood[21]. *RIMS2,* known to be involved in the synapse-related processes, is one of the genes with higher mRNA expression levels with age. More specifically, RIMS2 is a presynaptic protein in the synaptic vesicle release site, so called the active zone, and interacts with several synaptic proteins including Rab3 to regulate Ca^{2+}-dependent neurotransmitter release from synaptic vesicles[22,23]. Previous studies suggested that ageing may decrease the active zone density of neuromuscular junctions in mice and rats which may affect neurotransmission[24]. However, *RIMS2* is lowly expressed in our study and so far, no studies have shown an association between *RIMS2* and ageing human airways. We demonstrated that *RIMS2* expression was negatively correlated with two miRNAs that

were lower expressed with age, i.e. miR-146b-5p and miR-146a-5p, suggesting that these miRNAs may regulate synapse-related changes during ageing.

In our correlation analyses, we found a significant negative correlation of *IGSF1* with expression levels of miR-146b-5p and miR-146a-5p, which are from the same miRNA seed family. This suggests that higher *IGSF1* expression is regulated by these miRNAs with increasing age. The *IGSF1* gene encodes an immunoglobulin superfamily glycoprotein of which the function and molecular mechanisms are not well defined yet[25]. Previous studies suggested that the IGSF1 protein may inhibit the TGF-β and activin signalling pathways by interacting with type I receptors[25,26]. As *IGSF1* is higher expressed with increasing age, the transcription of TGF-β- and activin-specific target genes might be negatively affected during ageing. The TGF-β pathway plays an important role in tissue repair and remodelling, and therefore the inhibition of this pathway may negatively affect the extracellular matrix protein production. Thus, our *IGSF1* findings would be in line with the previous observation of reduced airway wall thickness with ageing[6]. So far, the proposed correlations have not been experimentally validated[27].

The genes with lower expression levels with increasing age were mainly involved in DNA damage and repair, cell cycle regulation and the immune system. These processes and pathways are linked to three of the hallmarks of ageing, i.e. genomic instability, cellular senescence and altered intercellular communication[4]. Furthermore, the top-3 genes with the most significantly decreased expression levels with increasing age, i.e. *TIMELESS*, *BRIP1* and *KNTC1*, were enriched in the processes/pathways DNA damage and repair, and/or cell cycle regulation. TIMELESS mediates DNA repair by binding to PARP1 that is involved in various DNA repair pathways and in maintaining genomic instability[28,29]. BRIP1 is a DNA helicase and has been reported to be involved in double-strand break repair by interacting with BRCA1[30]. In addition to DNA repair, the interaction between BRIP1 and BRCA1 is involved in DNA damage–induced checkpoint control during the G2 to M phase transition[31]. KNTC1 is a mitotic checkpoint regulator that checks whether the chromosomes are properly aligned during cell division[32]. The decreased expression levels of *TIMELESS*, *BRIP1* and *KNTC1* may thus contribute to the decline in DNA repair capacity during ageing.

For the replication of the age-related gene expression changes we used our previously published lung tissue dataset[8]. Despite differences in tissue origin and subject inclusion, we were able to replicate our findings for a substantial proportion of the genes, showing the robustness of our results. Several aspects might explain why not all genes could be replicated in the lung tissue dataset. For example, the cell composition and partly also the function of cells differ between bronchial biopsies derived from central airways and lung tissues derived from peripheral lung. Moreover, we only included bronchial biopsies from respiratory healthy subjects, while in the lung tissue dataset both patients with lung diseases and control subjects with normal lung function were included. One of

the unique features of our study is that we used bronchial biopsies from respiratory healthy individuals (volunteers). This also brings us to the most important limitations, i.e. cross-sectional data, and availability of similar publicly available datasets for replication. As an alternative way of replication, we assessed the expression of six age-related miRNAs in lung tissue samples from 35 subjects with normal lung function with an age range of 42-82 years. We could not replicate the association with age for these miRNAs, as expression levels were quite variable in these samples and therefore power was limited (data not shown). Although our study showed negative correlations between the predicted and age-related target genes and the miRNAs within the same biopsies, additional experiments are required to show that these interactions can occur in relevant cell types present in the airways.

In conclusion, we identified changes in the expression of several genes and miRNAs and several potential mRNA-miRNA interactions in the airways during normal ageing. The genes that were lower expressed with increasing age are part of processes and pathways involved in three main hallmarks of ageing, i.e. genomic instability, cellular senescence and altered intercellular communication. The genes that were higher expressed with increasing age are involved in synapse-related processes, thus possibly in innervation of the airways of which *RIMS2* is potentially regulated by two age-related miRNAs miR-146b-5p and miR-146a-5p.

MATERIALS AND METHODS

Bronchial biopsies from respiratory healthy subjects

We included bronchial biopsies (airway tissue samples) obtained from 94 respiratory healthy subjects participating in the study to obtain normal values of inflammatory variables from healthy smoking and never-smoking subjects (NORM study) (ClinicalTrials Identifier = NCT00848406[33]). Subjects did not have a history of lung disease, had no respiratory symptoms and had a normal lung function, which was defined by absence of bronchial hyper responsiveness to methacholine (provocative concentration inducing a 20% fall in FEV_1 (PC_{20}) >16 mg/ml), forced expiratory volume in one second/forced vital capacity (FEV_1/FVC) higher than lower limit of normal and reversibility of the FEV_1 to salbutamol <10%. The study was approved by the Medical Ethical Committee of the University Medical Center Groningen (METc 2009/007) and conducted according to the Good Clinical Practice guidelines. All subjects gave their written informed consent[33].

RNA and small RNA sequencing

RNA and small RNA sequencing was performed to assess changes in mRNA and miRNA expression, respectively. Total RNA was isolated from the bronchial biopsies using AllPrep

DNA/RNA/miRNA Mini kit (Qiagen, Venlo, The Netherlands). RNA yield and quality was determined using the LabChip GX (Perkin Elmer, Waltham, MA, USA). The Ribo-Zero Gold kit (Illumina) was used to remove ribosomal RNA. RNA sequencing libraries were prepared using the TruSeq Stranded Total RNA Sample Preparation kit (Illumina) on the Caliper Sciclone NGS Workstation (PerkinElmer, Waltham, MA). Libraries were paired-end sequenced (2x100bp) on the HiSeq 2500 (Illumina), which resulted in read counts ranging from 44,780,324 to 113,186,108 that passed the Illumina's filter. FastQC (version 0.11.3) was used for the quality control of raw RNA sequencing data. HISAT (version 0.1.5[34]) and SAMtools (version 1.2[35]) were used for alignment to build b37 of the human reference genome and for sorting the aligned reads, respectively. Ensembl (release 75[36]) was used as the gene annotation database and the aligned reads were quantified with HTSeq (version 0.6.1p1). Quality control of the aligned reads was performed using Picard-tools (version 1.130). After filtering against genes with less than half of the samples achieving ≥5 standardized reads, 22,744 expressed genes were available for analyses.
Small RNA libraries were generated with the NEXTflex Small RNA-seq kit V3 (Bioo Scientific, Uden, The Netherlands) without gel-based purification. The libraries were single-end sequenced (1x50bp) on the Illumina HiSeq 2500 (Illumina, San Diego, CA, USA). Quality control was performed using FastQC (version 0.11.5). Trimming of the adapter sequences of the raw reads was performed using TrimGAlore 0.3.7. The miRDeep2 V2.0.0.8 software package[37] was used to allocate the reads to the known human miRNAs (miRBase Release 21, http://www.mirbase.org/) allowing one mismatch. After filtering out miRNAs with a median expression <1 FPM, 518 miRNAs were available for analyses.
Of the available 94 bronchial biopsies, some samples were excluded from the analysis due to absence of RNA, poor alignment metrics, low total read counts (<100,000 reads) or technical errors during library preparation. In the small RNA sequencing data, principal component analysis showed an effect of library preparation batch on expression levels of the samples. Therefore these data were corrected for this factor in the subsequent analysis.

Statistical analyses of (small) RNA sequencing data

The RNA and small RNA sequencing data analyses were performed using R version 3.3.2 with the Bioconductor-DESeq2 package (version 1.14.1). Associations between mRNA and miRNA expression and age were analysed using a generalized linear model. For these analyses, the following R-codes were run;

1) Design.mRNA = ~ gender + current smoking + age
2) Design.miRNA = ~ gender + batch + current smoking + age

Results were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR). Genes and miRNAs with an FDR adjusted p-value<0.05 were considered statistically significant associated with age.

Genesis software version 1.7.6 (Graz University of Technology, Graz, Austria)[38] was used for supervised hierarchical clustering of the age-related mRNAs and miRNAs using Pearson's correlation and to generate heatmaps. The subjects were ordered from young to old.

Replication of age-related genes in lung tissues

To replicate the age-related gene expression changes, we compared the 285 age-related genes of our present study with our previously published lung tissue dataset [8]. In our previously published study, lung tissues obtained from 1,197 subjects (age range: 4-85 years) were used for the gene-expression signature for lung ageing. In total, 3,509 significantly differentially expressed genes with age were identified in lung tissue (FDR<0.0001). In our present study, we compared the 285 age-related genes identified in bronchial biopsies with the 3,509 age-related genes identified in lung tissues. An age-related gene in our study was considered as replicated when the gene was also significantly related to age in the same direction in lung tissue at a nominal p-value<0.05.

Gene ontology and pathway analyses

Genes with higher and lower expression levels with age were separately subjected to Enrichr[39,40] and g:Profiler[16] to identify enriched biological processes (gene-set library: GO_Biological_Process_2018) and Reactome pathways (gene-set library: Reactome_2016 in Enrichr and Reactome_2018 in g:Profiler).

Predicted target gene enrichment

The potential role of the miRNAs with age-related expression changes were explored by identifying enrichment of their predicted targets in the set of genes with higher and lower expression levels with age. For the analyses of enrichment of the age-related genes with predicted binding sites for the age-related miRNAs, we included the genes in RNA sequencing data that are also present in the gene list of TargetScan (version 7.1[41]). Of the 149 genes that are higher expressed with age and the 136 lower expressed genes, 126 genes and 127 genes, respectively, were present in TargetScan. Of all expressed genes in RNA sequencing data, 15,235 genes were present in TargetScan. Significant enrichment was assessed as percentages of predicted target genes in age-related genes with either higher (n=126 genes) or lower (n=127 genes) expression levels, respectively, as compared to percentages of predicted target genes among all expressed genes (n=15,235). Differences were determined by Chi-square, considering a p-value smaller than 0.05 as significant. Correlation between gene and miRNA expression levels was assessed using the Spearman's correlation (two-tailed). A correlation with p<0.05 was considered statistically significant.

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AUTHOR CONTRIBUTIONS

AvdB, WT, JK, MvdB and CAB designed the research. KK, MMT, VG, CJV generated and/or pre-processed the (small) RNA sequencing datasets. IMB analysed the (small) RNA sequencing data. MdV performed the replication in the lung tissue dataset. JO and RRW performed the gene ontology and pathway analyses and the predicted target gene enrichment analyses. JO and RRW integrated the data and prepared the figures and tables. JO, RRW and CAB drafted the manuscript. All authors reviewed, edited and approved the manuscript.

ADDITIONAL INFORMATION

COMPETING INTERESTS

IMB reports consultancy fees paid to the university by GSK. CJV was supported by a grant from GSK. JO, RRW, AvdB, JK, KK, MMT, VG, MdV, WT, MvdB and CAB have no competing interests.

SUPPLEMENTARY DATA

The online supplement is available at:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6403379/

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CHAPTER 4

Link between Increased Cellular Senescence and Extracellular Matrix Changes in COPD

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ABSTRACT

COPD is associated with features of accelerated aging, including cellular senescence, DNA damage, oxidative stress, and extracellular matrix (ECM) changes. We propose that these features are particularly apparent in patients with severe, early-onset (SEO-)COPD. Whether fibroblasts from COPD patients display features of accelerated aging and whether this is also present in, relatively young, SEO-COPD patients is unknown. Therefore, we aimed to determine markers of aging in (SEO-)COPD-derived lung fibroblasts and investigate the impact on ECM.

Aging hallmarks and ECM markers were analyzed in lung fibroblasts from SEO-COPD and older COPD patients and compared with fibroblasts from matched non-COPD groups (n=9-11 per group), both at normal culture conditions and upon Paraquat-induced senescence. COPD-related differences in senescence and ECM expression were validated in lung tissue.

Higher levels of cellular senescence, including SA-β-gal positive cells (19% for COPD vs 13% for control) and p16 expression, DNA damage (γ-H2A.X positive nuclei), and oxidative stress (*MGST1*) were detected in COPD compared to control-derived fibroblasts. Most effects were also different in SEO-COPD, with SA-β-gal positive cells only being significant in SEO-COPD vs matched controls. Lower decorin expression in COPD-derived fibroblasts correlated with higher p16 expression and this association was confirmed in lung tissue. Paraquat treatment induced cellular senescence along with clear changes in ECM expression, including decorin.

Fibroblasts from COPD patients, including SEO-COPD, display higher levels of cellular senescence, DNA damage and oxidative stress. The association between cellular senescence and ECM expression changes may suggest a link between accelerated aging and ECM dysregulation in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory lung disease that causes severe respiratory symptoms and a poor quality of life. COPD is characterized by airway obstruction and chronic inflammatory processes in the lungs that drives disturbed lung tissue remodeling, including emphysema and chronic bronchitis (23). The pathogenesis of COPD is largely unknown and as a consequence current treatment strategies mainly act at improving symptoms, without reducing disease progression and mortality. Therefore, novel insights into the pathogenesis of COPD are needed.

Several studies demonstrated features of lung aging in the lungs of COPD patients (29, 30). Hence, COPD has been postulated as a disease of accelerated lung aging (25, 31). Aging is defined as the progressive decline of homeostasis, resulting in increased risk of disease or death (29). Features of lung aging including lung function decline, airspace enlargement, loss of elasticity, increased cellular senescence, genomic instability, and mitochondrial dysfunction are observed in COPD compared to matched healthy controls. All previous studies on lung aging in COPD were mainly focused on lung tissue changes in older COPD patients with mild-moderate COPD (8). However, with respect to accelerated lung aging, COPD patients who develop very severe COPD at an early age (previously defined as age <53 years (42)) are of particular interest. These severe, early-onset (SEO)-COPD patients often have progressive disease at an early age despite normal alpha-1-anti trypsin levels and relatively few pack-years of smoking (42). Until now, only telomerase mutations and shorter telomeres were linked to SEO-COPD (43), but no further studies have been done to investigate the role of accelerated aging in SEO-COPD patients.

Lung extracellular matrix (ECM) dysregulation has been described as one of the features of lung aging. ECM is important for the function and structure of the lung and plays a major role in tissue repair and remodeling (23, 39). Recently, we showed clear differences in gene expression in lung tissue associated with aging (16). Pathway analyses suggested that age-related differences in ECM composition were more pronounced in COPD patients compared to subjects without COPD. Therefore, we propose that accelerated lung aging contributes to the pathology of COPD by deregulating lung tissue repair and remodeling. Fibroblasts are important structural cells in the lung controlling extracellular matrix homeostasis, and as such have an essential function in lung repair and remodeling (23, 39). Previous studies have demonstrated alterations in lung fibroblast function and ECM production in patients with COPD (3, 20, 26, 46, 50). In addition, higher levels of markers of cellular senescence were detected in lung tissue and structural lung cells, including lung fibroblasts from patients with COPD (8, 13, 21, 33). However, it is unknown if lung

fibroblasts from SEO-COPD patients display an accelerated aging phenotype and if this has functional consequences.

Therefore, in this study we aimed to assess markers of aging in primary lung fibroblasts from SEO-COPD and older, mild-moderate, COPD patients. We focused on SEO-COPD, because accelerated aging may especially play a role in these patients. Moreover, the functional consequences of aging in fibroblasts on ECM regulation were studied and validated in lung tissue using the same patient groups.

METHODS

Subjects

Primary lung fibroblasts and peripheral lung tissue from subjects undergoing lung transplantation or tumor resection surgery were used. Resected lung tissue was isolated distal from the tumor and was macroscopically and histologically normal. Primary parenchymal lung fibroblasts were isolated as described before (35). The following inclusion criteria were used:

1) SEO-COPD patients; FEV_1/FVC <70% and FEV_1 <30% pred measured at an age <53 (according to (42)) and with age <56 at time of lung transplant surgery

2) non-COPD control subjects (SEO-COPD-matched); FEV_1/FVC >70%, age <60 at time of surgery

3) Older, mild-moderate, COPD patients; FEV_1/FVC <70% and FEV_1 30-80% pred, age >65 at time of surgery

4) non-COPD control subjects (Older COPD-matched); FEV_1/FVC >70%, age >65 at time of surgery

None of the COPD patients were alpha-1 antitrypsin deficient. To get sufficient SEO-COPD-matched non-COPD control subjects, subjects at an age <60 at the time of surgery were included, taken into account the age-matching with the SEO-COPD group.

The study protocol was consistent with the Research Code of the University Medical Centre Groningen and national ethical and professional guidelines ("Code of conduct; Dutch federation of biomedical scientific societies", htttp://www.federa.org). Lung fibroblasts and lung tissues used in this study are derived from left-over lung material after lung surgery and transplant procedures. This material was not subject to the act on medical research involving human subjects in the Netherlands and therefore an ethics waiver was provided by the Medical Ethical Committee of the University Medical Center Groningen (METc UMCG). All samples and clinical information were de-identified before experiments were performed.

Primary parenchymal lung fibroblast culture

The fibroblasts were cultured as described before (35). At passage 5, 25000 fibroblasts were seeded in 12-well plates and after 2 days treated with or without 250 µM Paraquat dichloride hydrate (PQ) (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 24 hours to induce cellular senescence (11). After 24 hours, PQ was removed and cells were either harvested immediately for flow cytometry or kept in culture for another 24 hours (gene expression analyses, γ-H2A.X staining) or 4 days (gene expression analyses, senescence-associated β-Galactosidase (SA-β-gal) staining and secreted proteins) (Figure S1). These time-points were carefully chosen based on pilot study results.

Senescence-associated β-Galactosidase staining

Cellular senescence was assessed with standard SA-β-gal staining as described before (18). Fibroblasts were fixed with 2% formaldehyde + 0.2% glutaraldehyde in PBS for 5 minutes. After fixation, cells were incubated with the described staining solution for 16 hours (in a dry incubator) at 37°C. After incubation, the staining solution was washed away and cells were covered with 70% glycerol in PBS for storage. Four random images of every well with cells were taken using a Nikon camera on a Leica light microscope at a total magnification of 200. SA-β-gal positive cells and total cells were scored blindly to calculate the percentage of SA-β-gal positive cells.

Immunofluorescence γ-H2A.X staining

DNA damage was assessed using immunofluorescence staining for the DNA damage marker γ-H2A.X. Fibroblasts were cultured on a 16 mm circle glass coverslip (Fisher Scientific, Landsmeer, the Netherlands) and as positive control for the staining, fibroblasts were treated with 500 µM H_2O_2 for 4 hours. 24 hours after PQ removal, fibroblasts were fixed with ice-cold 80% acetone in PBS for ten minutes at 4°C. After fixation, non-specific binding was blocked with 5% BSA in PBS. Fibroblasts were incubated with 2.5 µg/ml γ-H2A.X conjugated with Alexa Fluor® 555 antibody (EMD Millipore, Amsterdam, the Netherlands) in the dark for one hour at room temperature (RT). After incubation, fibroblasts were counterstained with DAPI for 5 minutes at RT and mounted on a slide with VECTASHIELD Antifade Mounting Medium (Vector Laboratories, Peterborough, United Kingdom). Four random images of every coverslip with cells were taken using a Leica LMD6000 fluorescence microscope at a total magnification of 400. Positive nuclei and total cells were scored blindly to calculate the percentage of γ-H2A.X positive cells. Representative examples of the staining are shown in Figure S2.

Analysis of reactive oxygen species

Levels of reactive oxygen species (ROS) were determined using flow cytometry. Directly after 24 hours of PQ treatment or untreated, fibroblasts were stained with 2.5 µg/ml chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; DCF) (Invitrogen, Landsmeer, the Netherlands) in PBS for one hour in a 5% CO_2 incubator at 37°C. After incubation, fibroblasts were trypsinized and collected in tubes for flow cytometry analyses on a BD LSR-II cytometer (BD Biosciences, Vianen, the Netherlands). The geometric mean fluorescence intensity (gMFI) of DCF in the live cell population was used.

Gene expression analyses

For multiple aging markers and ECM genes (Table S1) mRNA expression was measured using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). RNA was

harvested 24 hours and 4 days after PQ removal in TRIzol (Invitrogen) and total RNA was isolated according to manufacturer's protocol. RNA of lung tissue was isolated using an RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). RNA concentrations were measured using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). 400 ng of RNA was used for cDNA synthesis with random primers and Superscript II (Invitrogen) according to manufacturer's protocol. For gene expression analysis, 5 ng of cDNA was used for qRT-PCR with PowerUp SYBR Green Master Mix (Applied Biosystems, Bleiswijk, the Netherlands) using a LightCycler 480 PCR instrument (Roche, Woerden, the Netherlands). For ECM gene expression analyses, TaqMan gene expression assays (Applied Biosystems) were used. *18S rRNA (18S)* and *RNA polymerase II (RP2)* were used as reference genes. Sequences of used primers are listed in Table S2 and TaqMan assay IDs are listed in Table S3. Samples including a no template control as negative control were run in triplicate and $2^{(-\Delta Cp)}$ was calculated for relative mRNA expression levels.

Secreted protein analyses

Cell-free supernatants were harvested 4 days after PQ removal and stored in -80°C prior to ELISA analysis. Secreted IL-6, IL-8 and decorin levels were measured using Human DuoSet ELISA (R&D Systems, Abingdon, United Kingdom). As the numbers of cells were different at the end of culture between COPD and control-derived fibroblasts, and between untreated and PQ-treated (Figure 1C & 4C), we corrected the secreted protein levels for cell numbers counted at the end of culture.

Statistical analyses

SPSS software was used for the statistical analyses. Mann-Whitney U-tests were used to test differences between fibroblasts from COPD patients compared to controls. Upon significant difference between COPD and control, subgroup analyses were performed to test differences between SEO-COPD and older COPD compared to their matched control groups, using Mann-Whitney U-tests. The effect of PQ treatment was analyzed using paired analysis with Wilcoxon signed-rank tests. $P<0.05$ was considered statistically significant.

RESULTS

Patient characteristics

The characteristics of the 40 lung fibroblast donors are shown in Table 1. The SEO-COPD and older, mild-moderate, COPD groups were similar compared to their matched control groups in terms of age, sex, pack-years and months of smoking cessation. The male/female ratio was significantly different between the younger groups and the older groups. All SEO-COPD patients suffered from severe emphysema and had a FEV_1 % pred <30% before the age of 53.

Table 1: Subject characteristics of fibroblasts

Variable	Control (SEO-COPD-matched)	SEO-COPD	P-value	Control (Older COPD-matched)	Older, mild-moderate, COPD	P-value
Number	9	10		10	11	
Age, mean years (range)	52 (42-59)	50 (44-55)	0.349	70 (65-81)	73 (66-81)	0.176
Men/women, N	1/8	2/8	0.556	8/2	10/1	0.500
Pack-years	32 (28-35)	26 (14-30)	0.673	43 (28-51)	49 (19-53)	0.823
Stop-months	84 (18-168)	78 (63-93)	0.677	186 (81-252)	66 (27-96)	0.421
non-COPD, N	9	-		10	-	
COPD, N	-	10		-	11	
GOLD 1	-	-		-	-	
GOLD 2	-	-		-	7	
GOLD 3	-	-		-	4	
GOLD 4	-	10		-	-	
FEV_1 (% pred.)	87.0 (83.5-92.0)	16.5 (14.3-22.7)	**0.000**	90.7 (82.2-104.0)	66.7 (43.4-70.5)	**0.000**
FVC (% pred.)	92.8 (84.6-101.0)	42.6 (37.9-68.1)	**0.000**	89.5 (76.7-107.5)	83.5 (79.7-98.8)	0.647
FEV_1/FVC	75.9 (73.3-79.0)	27.6 (26.0-38.5)	**0.000**	72.1 (70.3-75.1)	50.0 (41.7-59.0)	**0.000**

- *Data are presented as medians with interquartile ranges (IQRs), unless otherwise stated.*
- *Significant differences between groups were tested using Mann–Whitney U-tests or unpaired T-tests. P-values are stated and boldfaced when significant different.*
- *Global Initiative for Chronic Obstructive Lung Disease (*GOLD*) stage based on FEV_1 % pred.*
- *FEV_1: forced expiratory volume in one second, FVC: forced vital capacity.*

Higher levels of cellular senescence in COPD-derived fibroblasts, including in SEO-COPD-derived fibroblasts

After seven days of basal cell culture, the percentage of SA-β-gal positive cells in lung fibroblasts from COPD patients was significantly higher compared to control subjects (Figure 1A+B). Subgroup analyses showed a significant difference between SEO-COPD and their matched controls and a trend between older COPD and their matched controls. In line with higher senescence, the total cell numbers at the end of culture were lower in COPD-derived fibroblasts compared to control subjects, which remained only significant between SEO-COPD and their matched controls in the subgroup analyses (Figure 1C). Gene expression of the senescence marker p16 (*CDKN2A*) was significantly higher in COPD-derived fibroblasts (Figure 1D) and a similar trend (p= 0.05) was observed for the senescence marker p21 (*CDKN1A*) (Figure 1E). The higher p16 expression was only significant in fibroblasts from older COPD patients compared to their matched control-derived fibroblasts. No differences were observed in the secretion of IL-6 between the groups (Figure 1F), while lower secretion of IL-8 was observed in fibroblast from COPD patients compared to controls (Figure 1G). Levels of secreted cytokines were normalized to cell numbers, but this did not have a big impact on the results (uncorrected data are depicted in Figure S3).

Higher levels of DNA damage and oxidative stress in COPD-derived fibroblasts, including in SEO-COPD-derived fibroblasts

The percentage of γ-H2A.X positive cells (DNA damage) was higher in lung fibroblasts from COPD patients compared to control subjects (examples of staining in Figure S2), which was only significant between SEO-COPD and their matched controls in the subgroup analyses (Figure 2A).

Expression of the oxidative stress response gene Microsomal glutathione S-transferase 1 (*MGST1*) was higher in fibroblasts from COPD patients compared to control subjects, which was only significant comparing SEO-COPD to matched controls (Figure 2B). We observed no significant differences in ROS levels between the groups (Figure 2C). However, a positive correlation was observed between ROS levels and γ-H2A.X positive cells, and *MGST1* gene expression (Figure 2D).

No significant differences were observed between the groups in genes involved in DNA repair (Ku70 and Ku80), nutrient sensing (*EIF4B* and *SHC1* gene expression), in mTOR activity (p-S6K1 protein levels) nor in genes or proteins involved in loss of proteostasis (*FOXO3, SIRT1* and *NRF2* gene expression, and autophagy markers LC3-II and p62); see Figure S4.

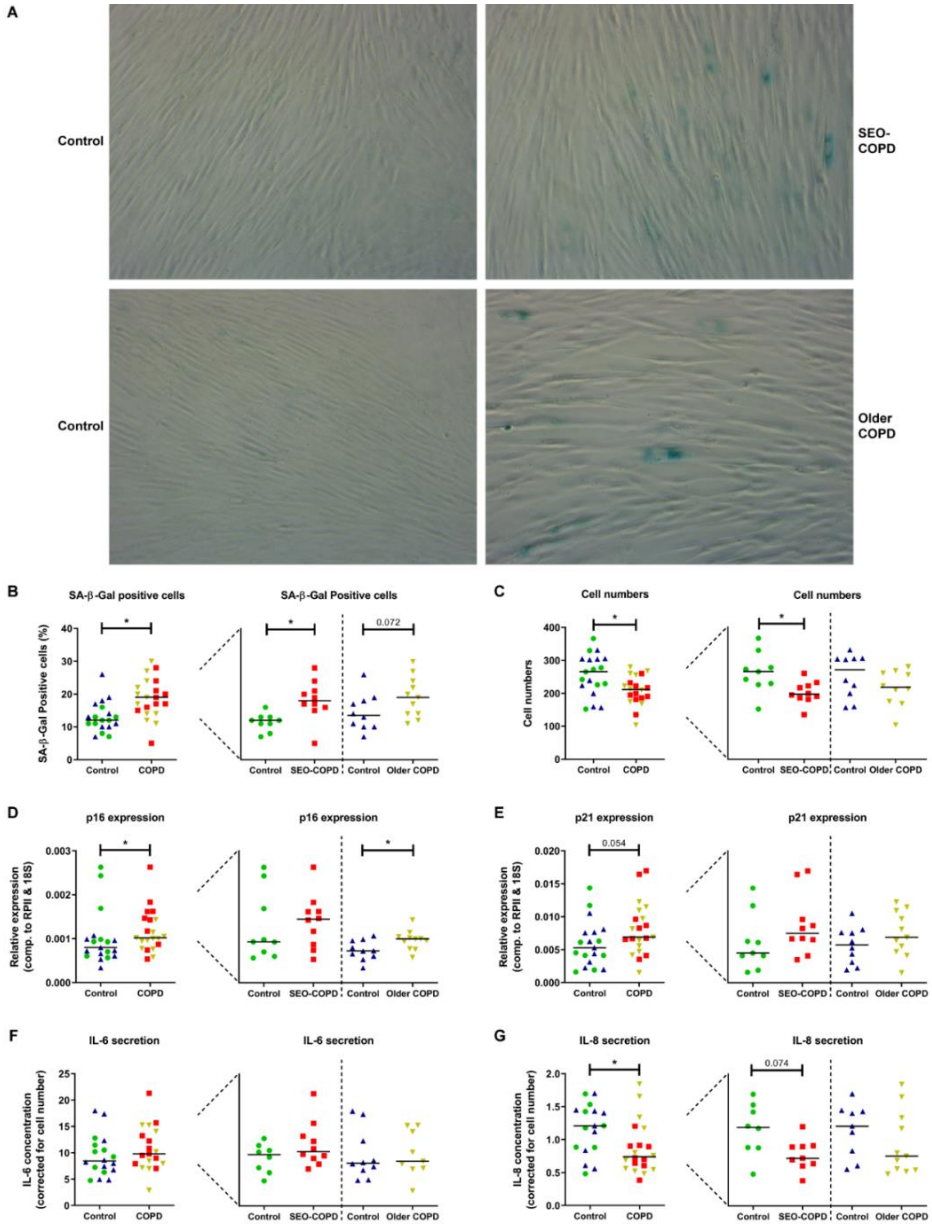

Figure 1: Higher levels of cellular senescence in COPD-derived fibroblasts.

*Examples of SA-β-gal staining of all 4 patient groups (A) and quantification of SA-β-gal positive cells (B) and total cell numbers (C). Dot plots show mRNA expression (24h) of p16 (D) and p21 (E) and secretion of IL-6 (F) and IL-8 (G) in cell culture medium (corrected for cell number) of all 4 patient groups. Green = SEO-COPD-matched control, red = SEO-COPD, blue = older COPD-matched control, yellow = older, mild-moderate, COPD. Lines represent medians. Significant differences tested with Mann-Whitney U tests. * P-value < 0.05 or p-value is indicated.*

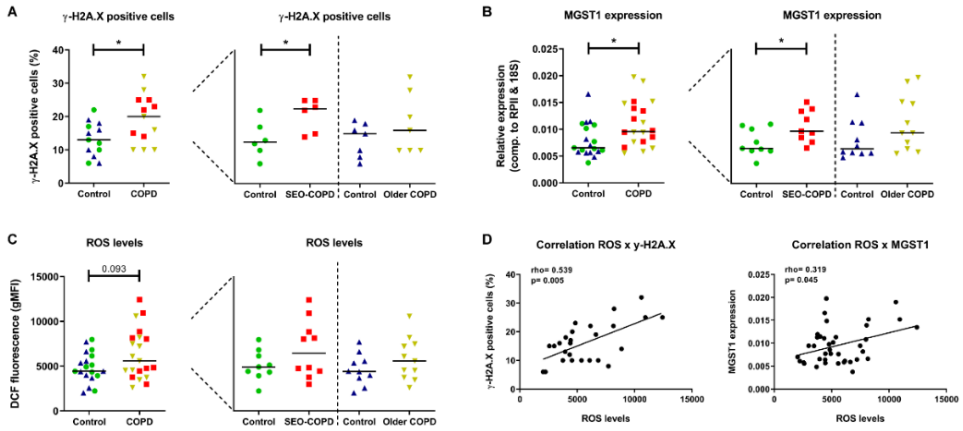

Figure 2: Higher levels of DNA damage and oxidative stress in COPD-derived fibroblasts.
*Dot plots show percentage of γ-H2A.X positive cells (A) MGST1 mRNA expression (24h) (B) and ROS levels as DCF gMFI (C) of all 4 patient groups. Green = SEO-COPD-matched control, red = SEO-COPD, blue = older COPD-matched control, yellow = older, mild-moderate, COPD. Lines represent medians. Significant differences tested with Mann-Whitney U tests. * P-value < 0.05 or p-value is indicated. Dot plots show correlation between ROS levels and γ-H2A.X positive cells and MGST1 mRNA expression (24h) (D). Significant differences tested with Spearman's rank tests. In the plots the Spearman rho and p-value are indicated and bold when significant.*

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Lower *DCN* gene expression in COPD-derived fibroblasts is correlated with higher markers of cellular senescence and lower lung function

To assess the impact of the accelerated aging phenotype on lung fibroblast function, gene expression of ECM proteins and alpha smooth muscle actin (*ACTA2*) was measured. Decorin (*DCN*) expression was lower in fibroblasts from COPD patients compared to control subjects, both in SEO-COPD and older COPD (Figure 3A). No differences in gene expression were observed for the other ECM genes nor *ACTA2* (Figure S5 & S6). *DCN* expression was positively correlated with lung function parameters FEV_1 and FEV_1/FVC (Figure 3B), and negatively correlated with the cellular senescence marker p16 (Figure 3C). Similar trends for negative correlation were observed for *DCN* and p21 (p= 0.064) and SA-β-gal (p= 0.074) (Figure 3C). To validate differences in *DCN* gene expression, we measured the levels of secreted decorin in the cell culture supernatants. A strong significant positive correlation between *DCN* gene expression and decorin protein secretion was found (Figure 3E). While the pattern was similar as observed for gene expression, no significant differences were observed for decorin protein secretion between the groups (Figure 3D).

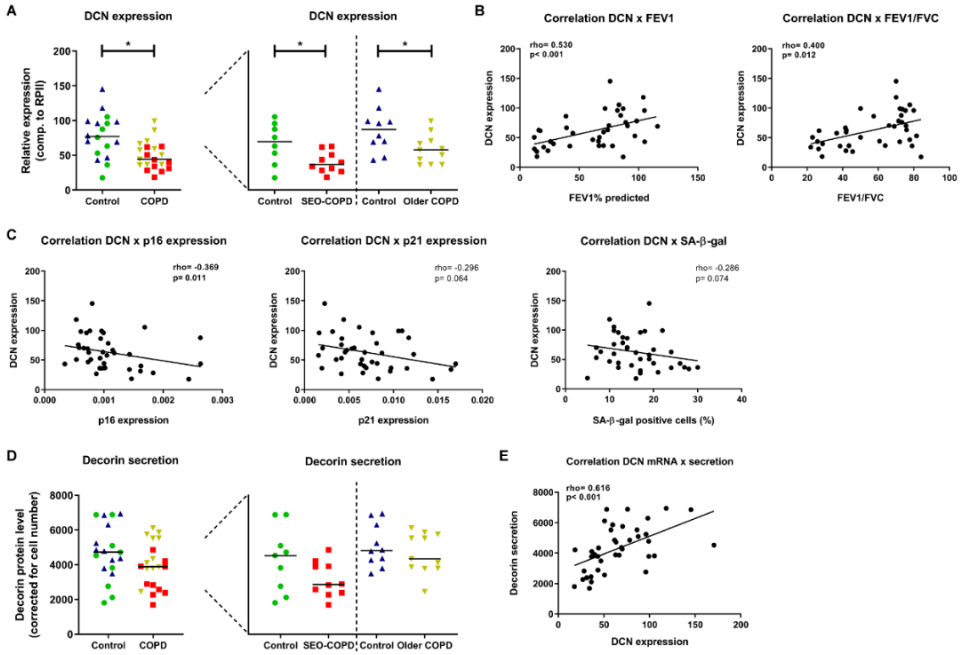

Figure 3: Altered DCN expression in COPD fibroblasts is associated with senescence.
*Dot plot shows mRNA expression (4d) of DCN of all 4 patient groups (A). Green = SEO-COPD-matched control, red = SEO-COPD, blue = older COPD-matched control, yellow = older, mild-moderate, COPD. Lines represent medians. Significant differences tested with Mann-Whitney U tests. * P-value < 0.05. Dot plots show correlation between DCN mRNA expression (4d) and FEV_1 % predicted and FEV_1/FVC (B), mRNA expression (24h) of p16 and p21, and SA-β-gal positive cells (C). Significant differences tested with Spearman's rank tests. In the plots the Spearman rho and p-value are indicated and bold when significant. Dot plot shows decorin protein secretion levels (corrected for cell number) of all 4 patient groups (D). Green = SEO-COPD-matched control, red = SEO-COPD, blue = older COPD-matched control, yellow = older, mild-moderate, COPD. Lines represent medians. Significant differences tested with Mann-Whitney U tests. Dot plot shows correlation between DCN mRNA expression (4d) and decorin protein secretion (E). Significant difference tested with Spearman's rank test. In the plot the Spearman rho and p-value are indicated and bold when significant.*

Paraquat treatment induces cellular senescence in primary lung fibroblasts

To induce cellular senescence in primary lung fibroblasts (see Table 1), we used the herbicide Paraquat (PQ), which causes oxidative stress (11). In occupational exposure, PQ has been documented as risk factor for COPD (10, 45). PQ treatment did not affect cell viability (data not shown). PQ treatment resulted in a significant increase in cellular senescence markers in primary lung fibroblasts, including increased SA-β-gal positive cells, lower cell numbers, increased p21 gene expression (24h & 4d after PQ removal) and IL-6 &

IL-8 secretion in fibroblasts from all subject groups (Figure 4A-G). Whereas a small, but significant decrease was observed for p16 after 24 hours of PQ removal (Figure 4D). After 4 days, p16 expression was not different between PQ treated and untreated, while p21 expression remained significantly increased upon PQ treatment (Figure S7). In addition to senescence induction, PQ treatment induced DNA damage (γ-H2A.X positive cells, see Figure S2 for example of staining) and ROS levels (Figure 4H+I) significantly, with only a trend towards significance for SEO-COPD in γ-H2A.X. None of the effects of PQ treatment were significantly different between COPD and control-derived fibroblasts.

Decreased *DCN* expression and protein secretion upon senescence induction in lung fibroblasts.

After PQ induced senescence, *DCN* gene expression and protein secretion were decreased 4 days after PQ removal (Figure 5A+B), which is the time-point SA-β-gal positivity is increased. At the earlier time-point, 24 hours after PQ removal, only a small decrease in *DCN* expression was observed (Figure S8A). Again, we found a clear correlation between *DCN* gene expression and protein secretion in untreated cells and in PQ treated cells as well (Figure 5C).

PQ-induced senescence results in altered ECM gene expression in lung fibroblasts

PQ-induced senescence resulted in striking changes in ECM gene expression and *ACTA2* gene expression 4 days after PQ removal. PQ-induced senescence resulted in decreased expression of collagen, type I, alpha 1 (*COL1A1*), fibulin-5 (*FBLN5*), elastin (*ELN*), fibronectin (*FN1*), *ACTA2*, and biglycan (*BGN*) and increased expression of versican (*VCAN*) (Figure 6). The decrease in *COL1A1*, *FN1*, and *BGN* was only significant in control-derived fibroblasts, but not in COPD-derived fibroblasts. *VCAN* expression upon PQ-induced senescence was significantly higher in COPD-derived fibroblasts compared to control-derived fibroblasts. After 24 hours of PQ removal, no or only small changes were found in ECM gene expression (Figure S8). *ELN* expression was increased after 24 hours, opposite to decreased expression after 4 days.

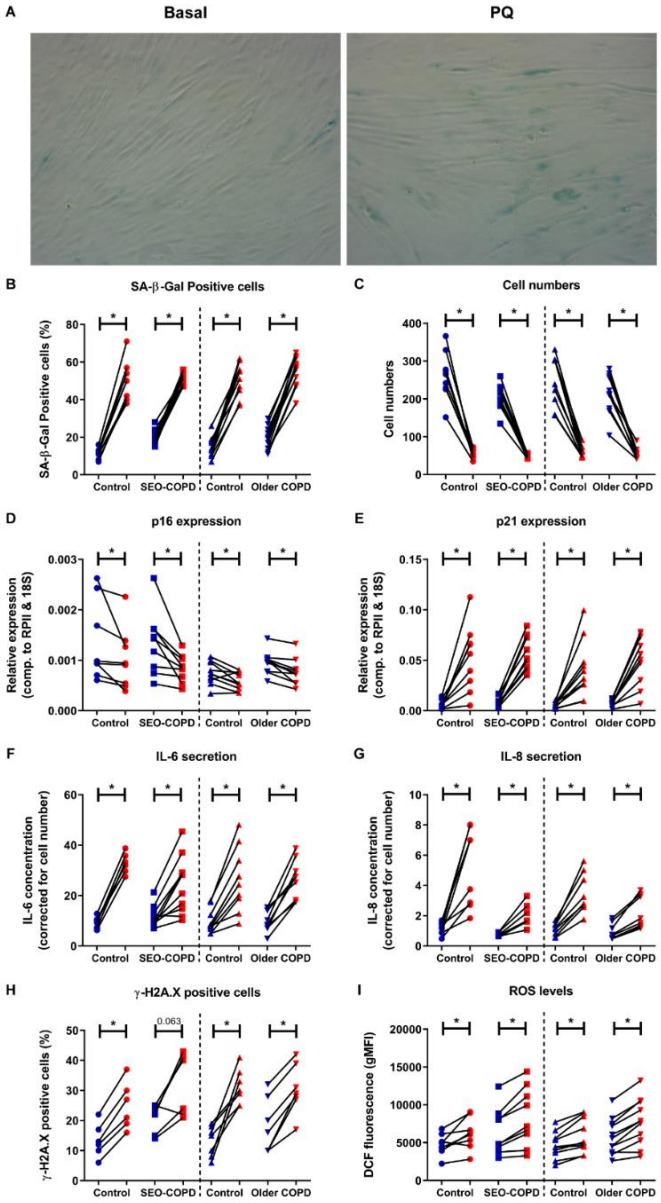

Figure 4: Induction of cellular senescence in primary lung fibroblasts.

*Examples of SA-ß-gal staining of untreated (basal) and PQ treated (PQ) fibroblasts (A) and quantification of SA-ß-gal positive cells (B) and total cell numbers (C). Dot plots show p16 (D) and p21 (E) mRNA expression (24h), secretion of IL-6 (F) and IL-8 (G) in cell culture medium (corrected for cell number), γ-H2A.X positive cells (H), and ROS levels (I) of untreated (basal) and PQ treated (PQ) fibroblasts per subgroup. Blue = basal and red = PQ. Significant differences between untreated and PQ treated are tested with Wilcoxon signed-rank tests and are indicated on top. * P-value < 0.05 or p-value is indicated.*

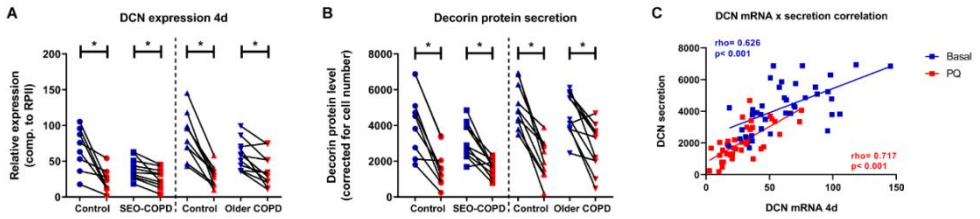

Figure 5: Decreased DCN expression and protein secretion upon senescence induction in primary lung fibroblasts.

*Dot plots show mRNA expression of DCN (A) and decorin protein secretion (B) after 4 days of untreated (basal) and PQ treated fibroblasts per subgroup. Blue = basal and red = PQ. Significant differences between untreated and PQ treated are tested with Wilcoxon signed-rank tests and are indicated on top. * P-value < 0.05. Dot plot shows correlation between DCN mRNA expression (4d) and decorin protein secretion (C) for untreated (Basal) and PQ fibroblasts. Blue = basal and red = PQ. Significant differences tested with Spearman's rank tests. In the plots the Spearman rho and p-value are indicated and bold when significant.*

COPD-derived fibroblasts respond differently to the induction of cellular senescence than control-derived fibroblasts

Next, the response to senescence induction upon PQ treatment was compared between COPD and control-derived fibroblasts. PQ-induced senescence was associated with reduced gene expression of Ku70 (*XRCC6*) in fibroblasts from SEO-COPD patients and Ku80 (*XRCC5*) in fibroblasts from SEO-COPD and older COPD patients, and SEO-COPD-matched control subjects (Figure 7A+B). For Ku70, the response on PQ-induced senescence was not different between the groups, whereas Ku80 gene expression was more decreased in older COPD-derived fibroblasts compared to their matched control subjects. Additionally, PQ-induced senescence caused lower induction of oxidative stress response genes *MGST1* and *FOXO3* in fibroblasts from COPD patients compared to control subjects, including in fibroblasts from SEO-COPD compared to their matched control subjects (Figure 7C+D). Relative expression levels are shown in the online supplement (Figure S9).

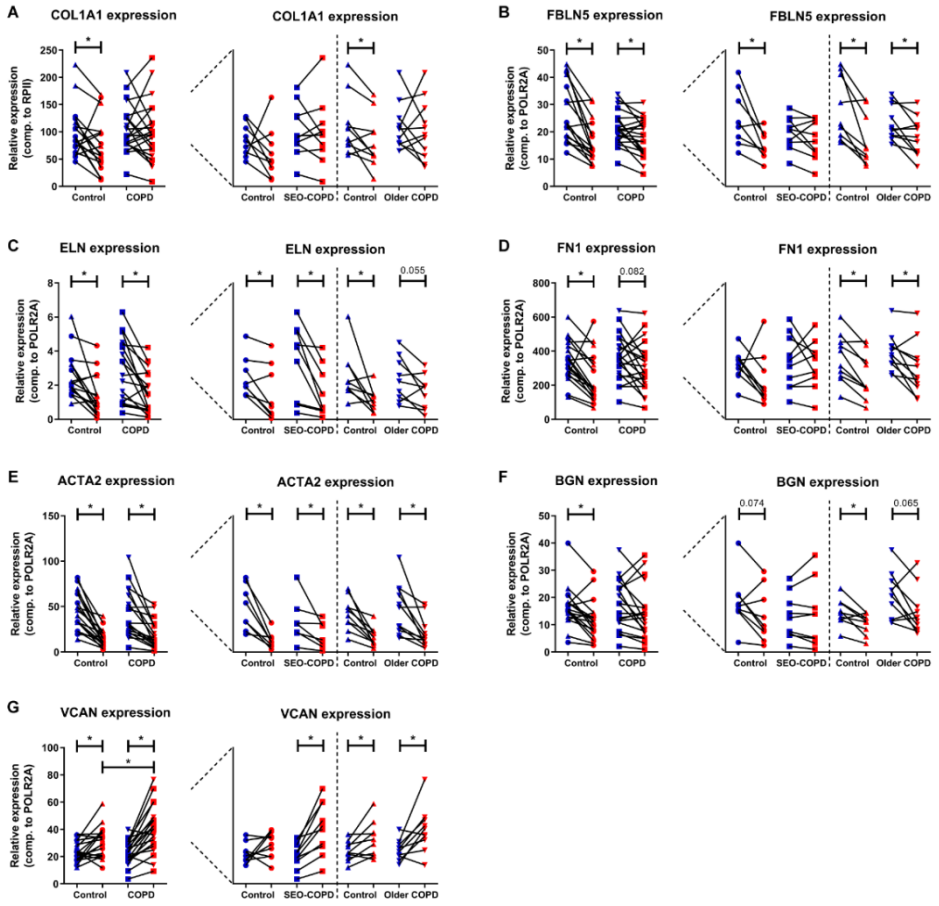

Figure 6: Altered ECM gene expression upon senescence induction in primary lung fibroblasts.
*Dot plots show mRNA expression of COL1A1 (A), FBLN5 (B), ELN (C), FN1 (D), and ACTA2 (E), BGN (F) and VCAN (G) after 4 days of untreated (basal) and PQ treated fibroblasts per groups and per subgroup. Blue = basal and red = PQ. Significant differences between untreated and PQ treated are tested with Wilcoxon signed-rank tests and are indicated on top. * P-value < 0.05 or p-value is indicated.*

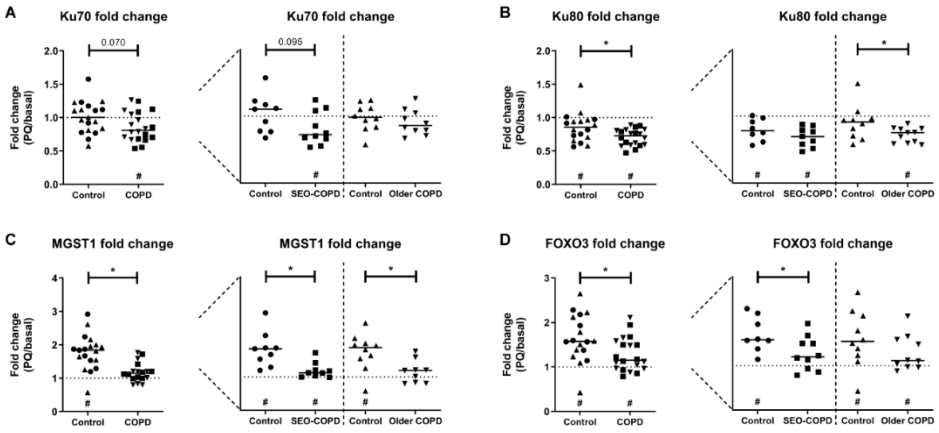

Figure 7: COPD-derived fibroblasts respond differently to induction of cellular senescence than control-derived fibroblasts.

*Dot plots show fold changes of mRNA expression after PQ treatment (24h) compared to untreated (basal) fibroblast expression of Ku70 (A), Ku80 (B), MGST1 (C) and FOXO3 (D) of all 4 patient groups. Lines represent medians. Significant differences between untreated and PQ treated per group are tested with Wilcoxon signed-rank tests and if significant are indicated above the x-axis with #. Significant differences in fold changes are tested with Mann-Whitney U tests and indicated at the right graph. * P-value < 0.05 or p-value is indicated.*

Patient characteristics of human lung tissue

To validate the association between senescence and ECM gene expression observed in fibroblasts, we used lung tissue from 59 donors using the same group definitions. The characteristics of the 59 lung tissue donors are depicted in Table 2. The SEO-COPD and their matched control group, and the older mild-moderate, COPD and their matched control group were similar in terms of age, sex, pack-years and months smoking cessation. The male/female ratio was significantly different between younger groups and older groups. All SEO-COPD patients suffered from severe emphysema and had a FEV_1 % pred <30% before the age of 53.

Table 2: Subject characteristics of lung tissue

Variable	Control (SEO-COPD-matched)	SEO-COPD	P-value	Control (Older COPD-matched)	Older, mild-moderate, COPD	P-value
Number	14	14		15	16	
Age, mean years (range)	52 (42-60)	52 (47-55)	0.763	71 (65-82)	71 (65-79)	0.971
Men/women, N	4/10	4/10	1.000	12/3	12/4	0.749
Pack-years	28 (20-35)	30 (22-40)	0.476	43 (25-52)	44 (21-50)	0.657
Stop-months	72 (15-252)	66 (51-93)	0.412	186 (54-288)	54 (15-123)	0.222
non-COPD, N	14	-		15	-	
COPD, N	-	14		-	16	
GOLD 1	-	-		-	-	
GOLD 2	-	-		-	11	
GOLD 3	-	-		-	5	
GOLD 4	-	14		-	-	
FEV_1 (% pred.)	90.1 (86.6-99.78)	17.6 (15.2-23.9)	**0.000**	87.0 (79.1-101.2)	64.5 (45.8-67.6)	**0.000**
FVC (% pred.)	97.3 (92.8-112.5)	46.7 (41.8-63.7)	**0.000**	86.5 (74.6-103.7)	83.9 (69.9-90.5)	0.426
FEV_1/FVC	75.2 (73.0-78.9)	27.3 (25.9-39.8)	**0.000**	72.7 (70.7-76.7)	54.2 (43.1-62.3)	**0.000**

- *Data are presented as medians with interquartile ranges (IQRs), unless otherwise stated.*
- *Significant differences between groups were tested using Mann–Whitney U-tests or unpaired T-tests. P-values are stated and boldfaced when significant different.*
- *Global Initiative for Chronic Obstructive Lung Disease (*GOLD*) stage based on FEV_1 % pred.*
- *FEV_1: forced expiratory volume in one second, FVC: forced vital capacity*

Confirmation of the association between cellular senescence and *DCN* gene expression in human lung tissue

Gene expression of the senescence marker p21, but not p16, was higher in lung tissue from COPD patients (Figure 8A+B). In the subgroup analyses this p21 difference was only significant in lung tissue from SEO-COPD patients compared to their matched control subjects (Figure 8B). No significant differences were observed in *DCN* expression (Figure 8C). In line with our findings in fibroblasts, a negative correlation was observed between *DCN* and p16 gene expression in lung tissue (Figure 8D).

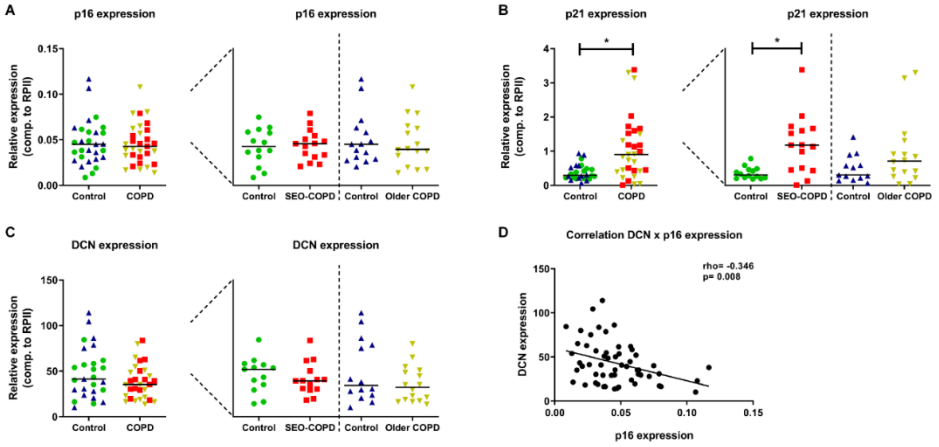

Figure 8: Association between senescence and DCN gene expression confirmed in human lung tissue.

*Dot plots show mRNA expression of p16 (A), p21 (B) and DCN (C) of all 4 patient groups. Green = SEO-COPD-matched control, red = SEO-COPD, blue = older COPD-matched control, yellow = older, mild-moderate, COPD. One of the dots of the SEO-COPD group contains the average of 2 lung samples from the same patient. Lines represent medians. Significant differences tested with Mann-Whitney U tests. * P-value < 0.05. Dot plot shows correlation between p16 mRNA expression and DCN mRNA expression (D). Significant difference tested with Spearman's rank test. In the plot the Spearman rho and p-value are indicated and bold when significant.*

DISCUSSION

In this study, we assessed the aging phenotype of lung fibroblasts from COPD patients and the consequences on ECM regulation, with a special focus on SEO-COPD. We observed higher levels of cellular senescence, DNA damage and markers of oxidative stress in lung fibroblasts from COPD patients compared to control subjects under normal cell culture conditions. Interestingly, part of these effects were more pronounced in SEO-COPD. With respect to ECM regulation, *DCN* gene expression was lower in COPD compared to control-derived fibroblasts and lower *DCN* expression was correlated with higher markers of cellular senescence. In addition, PQ-induced senescence resulted in clear alterations in ECM gene expression, including reduced *DCN* expression. Finally, we validated the higher levels of cellular senescence and the correlation between lower *DCN* expression and higher expression of the cellular senescence marker p16, in human lung tissue, despite the presence of different cell types in lung tissue.

With the increasing life expectancy worldwide, the interest in the role of aging in health and disease has massively increased (29). Especially for chronic degenerative diseases, including COPD, it has been postulated that acceleration of the normal aging process is involved in disease pathogenesis (25). Up to now, several studies have investigated the role of aging in COPD and most of these studies found indications for an accelerated aging process to be involved in COPD (8, 25, 30, 31). Our findings in lung fibroblasts on cellular senescence, oxidative stress and DNA damage are in line with previous studies. However, the uniqueness of our study is that we specifically focused on SEO-COPD patients. These COPD patients develop the most severe form of COPD at an earlier age with relatively low number of smoking pack-years compared to the majority of patients with mild-moderate COPD (42). Why these patients are particularly susceptible to develop this progressive and severe form of COPD is a major unsolved question. In particular the fact that they develop COPD at relatively young age is interesting for studies on the role of accelerated aging. We observed that the higher levels of cellular senescence in fibroblasts and lung tissue, and DNA damage in fibroblasts were most pronounced in SEO-COPD. As these relatively young patients already display this aging phenotype our data support a role for accelerated lung aging in SEO-COPD.

We found more DNA damage and oxidative stress in COPD and SEO-COPD-derived fibroblasts. These results are in line with previous findings in lung tissue and lung epithelial cells from COPD patients (2, 7, 41). DNA damage and oxidative stress can both induce cellular senescence, while oxidative stress can enhance DNA damage (12, 22, 34). The observed correlation between ROS and DNA damage (γ-H2A.X) suggests that ROS may have contributed to DNA damage in our study. In addition, we observed higher *MSGT1*

expression in SEO-COPD-derived fibroblasts. *MGST1* has been linked to aging in several studies (27, 40), including in lung aging (16). The positive correlation between *MGST1* and ROS levels in fibroblasts suggests that higher *MGST1* gene expression might be the result of higher ROS levels. In COPD patients, lung fibroblasts are chronically exposed to high levels of oxidative stress resulting from chronic inflammation, tissue damage, and also directly from oxidative stress exposure. These exposures may explain the higher levels of cellular senescence, DNA damage and oxidative stress in COPD-derived fibroblasts.

Although a link between aging and ECM dysregulation was proposed previously (8, 17, 30), to our knowledge no studies investigated the link between an accelerated aging phenotype and fibroblast dysfunction in COPD yet. We observed a correlation between higher levels of cellular senescence and lower *DCN* gene expression in both COPD and SEO-COPD-derived fibroblasts and confirmed this association in our PQ-induced senescence model and in lung tissue as well. Decorin is a proteoglycan that binds many growth factors and their receptors, including transforming growth factor beta (TGF-β), thereby inhibiting their activity (14, 37, 48). TGF-β is known to be consistently up-regulated in COPD (15). Stimulation with TGF-β *in vitro* induces ECM protein production via the SMAD pathway, while in contrast TGF-β inhibits the production of decorin (35, 50). Decorin also binds to collagen fibrils, providing structural support for the ECM. In emphysema lower gene expression and protein levels of proteoglycans, including lower decorin, have been detected (35, 47) and lower decorin levels have been linked to skin aging as well (28, 36). It has been proposed that lower *DCN* expression in the small airway contributes to loss of fiber organization in the airway walls contributing to airway obstruction (1, 6, 47). Thus, lower *DCN* expression in senescent lung fibroblasts as observed in our study may affect the ECM structure in the peripheral lung and contribute to lung tissue remodeling and small airway obstruction in COPD. In addition to *DCN*, we also measured gene expression of multiple other ECM proteins. However, we did not find significant differences between COPD and non-COPD control-derived fibroblasts at both time-points (Figure S5 & S6). So, the observed decrease in decorin is likely not preceded by changes in gene expression of the other ECM proteins. We observed a significant correlation between *DCN* gene expression and protein secretion, but we could not confirm the differences between COPD- and control-derived fibroblasts on decorin protein secretion. This may be due to overcorrection for the differences in cell number, since we assessed the cell numbers at the end of culture, while secreted proteins were accumulated over 4 days. The decorin secretion levels were indeed significantly lower in COPD-derived cultures when we did not correct for cell numbers (Figure S3). The fact that we confirmed the correlation between lower *DCN* expression and higher p16 in lung tissue further supports the link between cellular senescence and ECM dysregulation *in vivo*.

Most ECM gene expression changes upon PQ-induced senescence were observed after 4 days, when the cells are senescent (SA-β-gal positive). Since, p21 expression was increased after 24 hours, it is likely that the ECM gene expression changes develop as a result of the senescence induction and not as a direct effect of the PQ treatment. Opposite to the decreased expression of the majority of ECM genes upon PQ-induced senescence, we observed increased *VCAN* gene expression. Interestingly, most of these ECM gene expression changes are in the same direction as changes in lung tissue from COPD patients (1, 6), including higher versican protein levels (20). Versican inhibits the synthesis and regeneration of elastic fibers and is believed to be a negative regulator of elastin (20, 32). Indeed, together with increased *VCAN* expression upon PQ-induced senescence, we observed decreased *ELN* expression after 4 days. Elastin dysregulation plays an important role in COPD and lower elastin protein levels have been shown in small airway walls from COPD patients (19). Together, our findings support the notion that cellular senescence of lung fibroblasts can lead to ECM dysregulation in COPD and contribute to aberrant tissue remodeling.

Remarkably, previous studies using senescent fibroblasts from idiopathic pulmonary fibrosis patients have shown higher ECM gene expression, including *ACTA2* and collagen (38, 49), which is different from our findings in senescent fibroblasts from COPD patients. These differences suggest that the effect of cellular senescence on fibroblasts and their function is context dependent and can be different between cell origin and diseases.

Another interesting finding of our study is that we found differences in the response towards senescence induction between COPD and control-derived fibroblasts. The reduction in DNA damage repair markers Ku70 and Ku80 upon PQ-induced senescence was more pronounced in fibroblasts from COPD patients, which is in line with previous findings of lower protein levels of Ku80 in lung tissue from COPD patients (9). In addition, expression of the oxidative response genes *FOXO3* and *MGST1* upon PQ-induced senescence was more induced in fibroblasts from control subjects than from COPD patients. *FOXO3* is a well-known anti-aging and anti-oxidant protein, and protein levels were shown to be lower in lung tissue from smokers and COPD patients previously (24). Together, these results suggest that COPD-derived fibroblasts are less capable to respond to aging-related damage.

One apparent different result between lung fibroblasts and lung tissue from COPD patients was higher p16 expression in fibroblasts, while p21 expression was higher in lung tissue. Both are markers of cellular senescence and important cell cycle inhibitors. Oxidative stress and DNA damage can induce p53, which activates p21 downstream, while p16 can be activated by multiple stressors (4, 34). In addition, p21 has been implicated in

the early stage of senescence and p16 in the latter stage of senescence (44). The differences between fibroblasts and lung tissue can be explained by differences in cell composition and cell responses, or potential effects of prolonged cell culture of fibroblasts being outside of their diseased microenvironment, since in lung tissue oxidative stress may still be present. Importantly, because we observed higher levels of cellular senescence in lung fibroblasts and lung tissue from COPD patients, our data indicates an accumulation of senescent cells in lungs of COPD patients. This accumulation may contribute to impaired tissue function in lungs (5, 34).

In conclusion, this is the first study showing a link between cellular senescence and deregulated ECM gene expression in COPD, including SEO-COPD. Future studies on the functional consequences of senescent lung fibroblasts may lead to a better understanding of the pathogenesis of accelerated aging in COPD, with respect to lung tissue remodeling. Ultimately, this knowledge might lead to novel therapeutic targets for COPD patients, including SEO-COPD patients, which is important since no treatment is available to cure the disease or to stop or delay the progression of the disease.

DISCLOSURES

Supplementary data

The online supplement is available online at
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CHAPTER 5

COPD-derived fibroblasts secrete higher levels of senescence associated secretory phenotype proteins

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ABSTRACT

COPD-derived fibroblasts have increased cellular senescence. Senescent cell accumulation can induce tissue dysfunction by their senescence-associated secretory phenotype (SASP). We aimed to determine the SASP of senescent and COPD-derived lung fibroblasts, including severe, early-onset (SEO-)COPD. SASP protein secretion was measured after Paraquat-induced senescence in lung fibroblasts using Olink Proteomics and compared between (SEO-)COPD and control-derived fibroblasts. We identified 124 SASP proteins of senescent lung fibroblasts, of which 42 were secreted at higher levels by COPD and 35 by SEO-COPD-derived fibroblasts compared to controls. Interestingly, the (SEO-)COPD-associated SASP included proteins involved in chronic inflammation, which may contribute to (SEO-)COPD pathogenesis.

INTRODUCTION

Accelerated lung ageing has been postulated to contribute to the pathogenesis of chronic obstructive pulmonary disease (COPD) [1]. Several mechanisms of accelerated ageing have been identified in COPD [1,2], of which cellular senescence is most extensively described to be increased in lung tissue and structural cells from COPD patients [3]. Cellular senescence is an irreversible cell cycle arrest that prevents cell death [4]. Senescent cells secrete (pro-inflammatory) proteins, called the senescence-associated secretory phenotype (SASP), to recruit immune cells for their clearance. However, on accumulation of senescent cells, high levels of SASP proteins can have detrimental effects on the surrounding tissue, by inducing chronic inflammation and tissue dysfunction [5]. The SASP is cell type specific and its potential (negative) impact on surrounding cells largely depends on the composition and level of secretion of these SASP proteins. Examples of previously described SASP proteins include interleukins, chemokines, growth factors, and proteases [6,7].

Recently, we demonstrated higher levels of cellular senescence in lung fibroblasts and lung tissue from older, mild-moderate COPD and severe, early-onset (SEO-) COPD patients compared to their matched controls [8]. SEO-COPD patients develop very severe COPD at a relatively early age with relatively low numbers of pack-years. Thus, accelerated lung ageing, including cellular senescence, may contribute to SEO-COPD. The SASP of senescent primary lung fibroblasts and COPD-derived fibroblasts is not defined yet and thus the potential impact of senescent fibroblasts on the surrounding lung tissue is unclear. Therefore, we aimed to first identify SASP proteins of senescent primary human lung fibroblasts and secondly to determine which of these SASP proteins are secreted at higher levels by COPD-derived fibroblasts, including SEO-COPD, compared to their matched non-COPD control-derived fibroblasts.

METHODS

Cell culture supernatants from lung fibroblasts from 10 SEO-COPD patients and 11 older, mild-moderate COPD patients and respectively 9 and 10 matched non-COPD controls were used (Table 1), which were collected as previously described [8] (A detailed description of the methods can be found in the online supplement). Briefly, cellular senescence was induced in fibroblasts from all subject groups by Paraquat (PQ) treatment (250 µM for 24 hours), which by occupational exposure is a risk factor for COPD, and can induce senescence specifically via mitochondrial ROS production [9,10]. Senescence induction was confirmed by a 40% increase in SA-β-gal positive cells and a 7-fold increase in p21 expression [8]. Cell culture supernatants were collected four days after senescence induction. The highly sensitive Olink Proteomics (Olink Proteomics, Uppsala, Sweden) panels *Inflammation* and *Cardiovascular III*, were used to measure the secretion of 184 proteins, whereof 165 proteins passed QC. Since cell number at the end of culture were

significantly different between COPD and control and between PQ and untreated (Figure S1), levels of secreted proteins were corrected for these cell numbers. Significant differences between PQ treated and untreated cells were tested using Wilcoxon signed-rank test adjusted for multiple testing using Benjamini-Hochberg. Proteins were defined as SASP protein when a significant (FDR <0.05) ≥3-fold increase in secretion was observed after PQ treatment. Next, statistical differences in SASP protein secretion between untreated COPD- and control-derived fibroblasts were tested using Mann-Whitney U. FDR P<0.05 was considered statistically significant. Finally, pathway analysis of COPD-associated SASP proteins was performed using the STRING database (v11.0) to provide more insight into the function of the SASP proteins and their potential role in COPD, while it should be noted that the selected panels may have caused a bias in the analysis.

Table 1: Subject characteristics of fibroblasts of combined groups and subgroups

Variable	Control	COPD	P-value	Variable	Control (SEO-COPD-matched)	SEO-COPD	P-value	Control (Older COPD-matched)	Older, mild-moderate COPD	P-value
Number	19	21		Number	9	10		10	11	
Age, mean years (range)	61 (42-81)	62 (44-81)	0.844	Age, mean years (range)	52 (42-59)	50 (44-55)	0.349	70 (65-81)	73 (66-81)	0.176
Male/female, N	9/10	12/9	0.548	Male/female, N	1/8	2/8	0.556	8/2	10/1	0.500
Pack-years	34 (28-40)	30 (15-50)	0.627	Pack-years	32 (28-35)	26 (14-30)	0.673	43 (28-51)	49 (19-53)	0.823
Stop-months,	120 (30-240)	78 (36-96)	0.337	Stop-months	84 (18-168)	78 (63-93)	0.677	186 (81-252)	66 (27-96)	0.421
non-COPD, N	19	-		non-COPD, N	9	-		10	-	
COPD, N	-	21		COPD, N	-	10		-	11	
GOLD 1	-	-		GOLD 1	-	-		-	-	
GOLD 2	-	7		GOLD 2	-	-		-	7	
GOLD 3	-	4		GOLD 3	-	-		-	4	
GOLD 4	-	10		GOLD 4	-	10		-	-	
FEV_1 % pred	88.1 (82.5-98.0)	38.8 (17.1-66.7)	**0.000**	FEV_1 % pred	87.0 (83.5-92.0)	16.5 (14.3-22.7)	**0.000**	90.7 (82.2-104.0)	66.7 (43.4-70.5)	**0.000**
FVC % pred	90.3 (83.0-107.5)	77.9 (44.2-83.5)	**0.005**	FVC % pred	92.8 (84.6-101.0)	42.6 (37.9-68.1)	**0.000**	89.5 (76.7-107.5)	83.5 (79.7-98.8)	0.647
FEV_1/FVC	73.6 (71.8-77.7)	41.8 (28.4-50.0)	**0.000**	FEV_1/FVC	75.9 (73.3-79.0)	27.6 (26.0-38.5)	**0.000**	72.1 (70.3-75.1)	50.0 (41.7-59.0)	**0.000**

- *Data are presented as medians with interquartile ranges unless otherwise stated.*
- *Significant differences between groups were tested using Mann–Whitney U-tests or unpaired T-tests. P-values are stated.*
- *Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage based on FEV_1 % pred.*
- *FEV_1: forced expiratory volume in one second, FVC: forced vital capacity.*
- *% pred: % predicted.*

RESULTS

First, the secretion of 124 proteins was significantly increased ≥3-fold after senescence induction by PQ and these proteins were thus defined as SASP proteins of senescent primary lung fibroblasts (top-50 is shown in Figure 1A, see Table S1 for all SASP proteins). We compared our SASP composition with the recently published *SASP Atlas [7]* and other literature and included the overlap in Table S1. From the 124 found SASP proteins 70 were previously described, including GDF-15 and CCL-3 (Figure 1B). In addition, our approach revealed 54 potentially novel SASP proteins, including GDNF and TGF-α (Figure 1C). We validated the Olink proteomics platform by measuring IL-8 using ELISA. A similar increase in IL-8 secretion was detected by ELISA after PQ-induced senescence with a significant positive correlation with IL-8 levels measured by Olink Proteomics (Figure 1D).

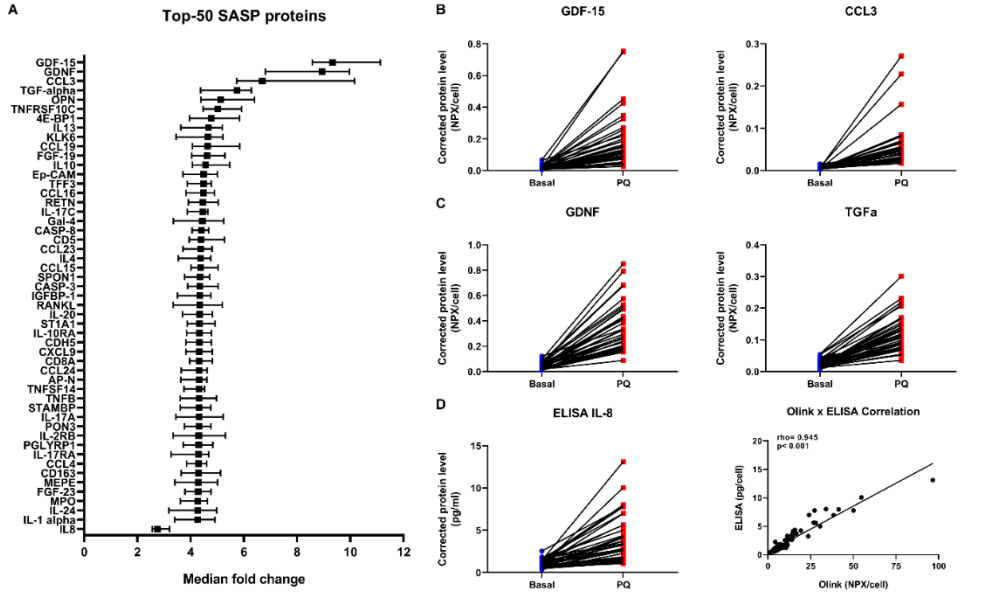

Figure 1: SASP of senescent primary lung fibroblasts. *Graph showing top-50 of 124 significant SASP proteins with highest median fold change and IL-8, sorted on fold change (A). Significant differences were tested using Wilcoxon signed-rank tests (n=40). Benjamini-Hochberg adjusted FDR<0.05 was considered statistically significant. Medians with 95% CI are plotted. Examples of two previously described SASP proteins, i.e. GDF-15 and CCL3 (B) and two not previously described SASP proteins, i.e. GDNF and TGF-α (C) with the highest median fold change are plotted in dot plots (for more details see Table S1). Blue = basal and red = Paraquat (PQ) treatment (both n=40). Protein levels are depicted as Olink NPX values corrected for total cell numbers. IL-8 protein levels were validated using Human DuoSet ELISA (R&D Systems, Abingdon, United Kingdom) (D) and correlated with Olink IL-8 levels (B, right panel). Spearman rho and p-value are plotted in the graph. FDR: false discovery rate. IL: interleukin. SASP: senescence-associated secretory phenotype.*

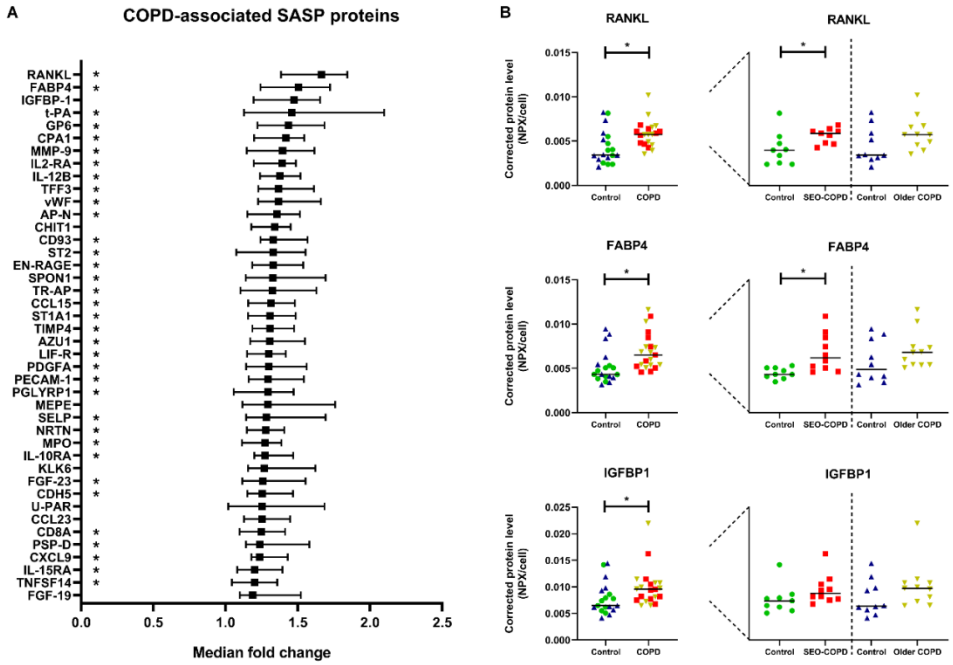

Figure 2: Higher levels of SASP protein secretion by COPD-derived fibroblasts. *Graph showing all 42 significant SASP proteins with higher protein secretion in COPD-derived fibroblasts (n=21) compared to non-COPD controls (n=19), sorted on fold change (A) (for more details see Table S2). Significant differences were tested using Mann-Whitney U tests. Benjamini-Hochberg adjusted FDR<0.05 was considered statistically significant. Medians with 95% CI are plotted. The SEO-COPD associated SASP proteins are indicated with a star in the graph behind the protein names. No older, Mild-moderate COPD associated SASP proteins were found. The 3 COPD-associated SASP proteins with the highest fold change in medians are plotted in dot plots (B). Green = SEO-COPD-matched controls (n=9), red = SEO-COPD (n=10), blue = older, mild-moderate COPD-matched controls (n=10), yellow = older, mild-moderate COPD (n=11). Protein levels are depicted as Olink NPX values corrected for cell numbers. Lines represent medians. SASP: senescence-associated secretory phenotype. SEO: severe, early-onset. FDR: false discovery rate.*

Next, the secreted levels of these 124 defined SASP proteins were evaluated in untreated cell culture supernatants from COPD patients compared to their matched control-derived fibroblasts. We observed higher levels of 42 SASP proteins in supernatants from COPD-derived fibroblasts (Figure 2A, see Table S2 for a detailed overview). The 3 proteins with the highest median fold change were RANKL, FABP4, and IGFBP-1 (Figure 2B). Several of the COPD-associated SASP proteins were previously found to be higher expressed at the transcription level in COPD-derived lung tissue compared to controls, including vWF, CHIT1, SPON1, TR-AP, TIMP4, PECAM1, CDH5, PSP-D, IL-15RA [11]. Furthermore, several

COPD-associated SASP proteins were associated with ageing in lung tissue at the transcription level, including t-PA, CHIT1, SPON1, IL-10RA, and CXCL9 [12]. On subgroup analyses, 35 of the 42 COPD-associated proteins were secreted at higher levels by fibroblasts from SEO-COPD patients compared to their matched controls (Table S2), whereas this was not the case for the older, mild-moderate COPD patients compared to their matched controls

Finally, STRING pathway analysis revealed that responses to stimuli, immune responses, and cytokine-related pathways are associated with the COPD-associated SASP proteins (data not shown). COPD-associated SASP proteins include cytokines (IL12B, TNFSF14, and RANKL) and chemokines (CCL15, CCL23, and CXCL9) that are known to be involved in inflammatory processes. These findings suggest that the SASP proteins that are secreted at higher levels by COPD-derived fibroblasts might be involved in the chronic inflammatory response in COPD.

CONCLUSION

By using a proteomic-based approach, we provide insight into the SASP of primary human lung fibroblasts. Interestingly, 42 of the 124 identified SASP proteins were secreted at higher levels by fibroblasts from COPD patients compared to matched controls. The COPD-associated SASP proteins include proteins that have been implicated in chronic inflammation, and thus may contribute to disease pathology in COPD. Remarkably, 35 of these 42 COPD-associated SASP proteins are secreted at higher levels by SEO-COPD patients compared to their matched controls, whereas none were significantly different between older, mild-moderate COPD patients compared to their matched controls. This lack of significance is likely due to higher biological variation in these older subgroups as the fold changes are comparable (Table S2) and the interquartile ranges are higher in these groups (Figure S2). These results suggest a role for these SASP proteins in COPD. The fact that both cellular senescence and SASP protein secretion were higher in COPD-derived lung fibroblasts compared to their matched controls suggests that senescence accumulation is involved in the pathogenesis of COPD. It should be noted that until now it is unknown whether the higher senescence observed in COPD is driven by acute exposures or chronic exposures, which may result in a different SASP profile. In addition, different senescence-inducing stimuli may result in a different SASP profile as well. The identified (COPD-associated) SASP proteins of primary lung fibroblasts can be used for further studies to understand the role of senescent cell accumulation and its potential detrimental impact in (SEO-)COPD pathogenesis.

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ONLINE SUPPLEMENT

Complete methods

Subjects

Primary lung fibroblasts from subjects undergoing lung transplantation or tumour resection surgery were used. Resected lung tissue was isolated distal from the tumour and was macroscopically and histologically normal. Primary parenchymal lung fibroblasts were isolated and cultured as described before [1]. Briefly, parenchymal lung tissue was cut into small cubes and cultured in 12-wells plates in Ham's F12 medium supplemented with 10% foetal calf serum (FCS), 2mM L-Glutamine, 100µg/ml Streptomycin and 100U/ml penicillin at 37°C and 5% CO_2. Medium was refreshed every week and after four weeks fibroblasts were trypsinized and placed into 25 cm^2 flasks. When cultures reached confluency, fibroblasts were frozen and stored in liquid nitrogen. The following inclusion criteria were used:

1) SEO-COPD patients; FEV_1/FVC <70% and FEV_1 <30% pred measured at an age <53 (according to [2]) and with age <56 at time of lung transplant surgery

2) non-COPD control subjects (SEO-COPD-matched); FEV_1/FVC >70%, age <60 at time of surgery

3) Older, mild-moderate, COPD patients; FEV_1/FVC <70% and FEV_1 30-80% pred, age >65 at time of surgery

4) non-COPD control subjects (Older COPD-matched); FEV_1/FVC >70%, age >65 at time of surgery

None of the COPD patients was alpha-1 antitrypsin deficient. To get sufficient SEO-COPD-matched non-COPD control subjects, subjects at an age <60 at the time of surgery were included, taken into account the age-matching with the SEO-COPD group.

The study protocol was consistent with the Research Code of the University Medical Centre Groningen and national ethical and professional guidelines ("Code of conduct; Dutch federation of biomedical scientific societies", htttp://www.federa.org). Lung fibroblasts and lung tissues used in this study are derived from left-over lung material after lung surgery and transplant procedures. This material was not subject to the act on medical research involving human subjects in the Netherlands and therefore an ethics waiver was provided by the Medical Ethical Committee of the University Medical Centre Groningen (METc UMCG). All samples and clinical information were de-identified before experiments were performed.

Primary parenchymal lung fibroblast culture

The primary parenchymal lung fibroblasts were defrosted and cultured in batches of four, including fibroblasts from each subgroup in equal numbers, as described before [1]. At passage 5, 25000 fibroblasts were seeded in Ham's F12 medium + 5% FCS in 12-well plates and after two days treated with or without 250 µM Paraquat dichloride hydrate (PQ) (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 24 hours to induce cellular senescence [3]. After 24 hours, PQ was removed and cells were kept in culture for four days in Ham's F12 medium + 5% FCS. These time-points were carefully chosen based on pilot study results.

Olink Proteomics

The highly sensitive Olink Proteomics (Olink Proteomics, Uppsala, Sweden) panels *Inflammation* and *Cardiovascular III*, were used to measure the secretion of 184 proteins, whereof 165 proteins passed QC. The Olink Proteomics analysis uses an antibody-based method called Proximity Extension Assay technology. Briefly, oligonucleotide-labelled antibody pairs bind the target protein and when oligonucleotides are in close proximity, these hybridize and get extended by a DNA polymerase. This created DNA barcode is amplified and quantified by qPCR. A full explanation about this analysis can be found on their website: https://www.olink.com/data-you-can-trust/technology/. Levels of secreted proteins were corrected for total cell numbers four days after senescence induction.

Secreted protein analyses

Cell-free supernatants were harvested four days after PQ removal and stored in -80°C prior to analyses. Secreted IL-8 levels were measured using Human DuoSet ELISA (R&D Systems, Abingdon, United Kingdom). As the numbers of cells were different at the end of culture between COPD and control-derived fibroblasts, and between untreated and PQ-treated, we corrected the secreted protein levels for cell numbers counted at the end of culture.

Statistical analyses

SPSS software was used for the statistical analyses. Significant differences between PQ treated and untreated cells were tested using Wilcoxon signed-rank test adjusted for multiple testing using Benjamini-Hochberg. Proteins were defined as SASP protein when a significant (FDR <0.05) ≥3-fold increase in secretion was observed after PQ treatment. Next, statistical differences in SASP protein secretion between untreated COPD- and control-derived fibroblasts were tested using Mann-Whitney U. FDR $P<0.05$ was considered statistically significant.

Supplementary tables and figures

Table S1: Overview of all 124 defined SASP proteins

PROTEIN	Fold change	P-value	FDR	Described or novel?
GDF-15	9.331	5.255E-08	7.226E-08	SASP Atlas
GDNF	8.946	5.255E-08	7.226E-08	Potentially novel
CCL3	6.695	5.683E-08	7.442E-08	SASP Atlas
TGF-ALPHA	5.737	5.255E-08	7.226E-08	Potentially novel
OPN	5.129	5.255E-08	7.226E-08	Potentially novel
TNFRSF10C	5.017	5.253E-08	7.226E-08	Previously described
4E-BP1	4.776	5.255E-08	7.226E-08	SASP Atlas
IL13	4.668	5.255E-08	7.226E-08	Previously described
KLK6	4.651	5.253E-08	7.226E-08	Potentially novel
CCL19	4.636	5.253E-08	7.226E-08	SASP protein family
FGF-19	4.621	5.253E-08	7.226E-08	SASP protein family
IL10	4.564	5.255E-08	7.226E-08	Previously described
EP-CAM	4.490	9.008E-07	1.047E-06	Potentially novel
TFF3	4.486	5.255E-08	7.226E-08	Potentially novel
CCL16	4.484	5.255E-08	7.226E-08	Previously described
RETN	4.459	5.255E-08	7.226E-08	Potentially novel
IL-17C	4.455	5.255E-08	7.226E-08	Previously described
GAL-4	4.435	5.255E-08	7.226E-08	Potentially novel
CASP-8	4.409	5.255E-08	7.226E-08	Potentially novel
CD5	4.387	5.255E-08	7.226E-08	Potentially novel
CCL23	4.379	5.253E-08	7.226E-08	SASP protein family
IL4	4.373	5.255E-08	7.226E-08	Previously described
CCL15	4.370	5.253E-08	7.226E-08	SASP protein family
SPON1	4.359	5.255E-08	7.226E-08	Potentially novel
CASP-3	4.351	5.253E-08	7.226E-08	Previously described
IGFBP-1	4.350	5.255E-08	7.226E-08	SASP protein family
RANKL	4.346	5.255E-08	7.226E-08	Potentially novel
IL-20	4.335	5.255E-08	7.226E-08	SASP protein family
ST1A1	4.332	5.255E-08	7.226E-08	Potentially novel
IL-10RA	4.331	5.255E-08	7.226E-08	SASP protein family
CDH5	4.330	5.255E-08	7.226E-08	Potentially novel
CXCL9	4.328	5.255E-08	7.226E-08	SASP protein family

CD8A	4.322	5.253E-08	7.226E-08	Potentially novel
CCL24	4.321	5.255E-08	7.226E-08	SASP protein family
AP-N	4.320	5.255E-08	7.226E-08	SASP Atlas
TNFSF14	4.316	5.255E-08	7.226E-08	SASP protein family
TNFB	4.316	5.255E-08	7.226E-08	SASP protein family
STAMBP	4.311	5.253E-08	7.226E-08	Potentially novel
IL-17A	4.309	5.253E-08	7.226E-08	Previously described
PON3	4.309	5.255E-08	7.226E-08	Potentially novel
IL-2RB	4.308	5.255E-08	7.226E-08	SASP protein family
PGLYRP1	4.305	5.255E-08	7.226E-08	Potentially novel
IL-17RA	4.302	5.255E-08	7.226E-08	SASP protein family
CCL4	4.301	5.255E-08	7.226E-08	SASP protein family
CD163	4.301	5.255E-08	7.226E-08	Potentially novel
MEPE	4.287	5.255E-08	7.226E-08	Potentially novel
FGF-23	4.278	5.251E-08	7.226E-08	SASP protein family
MPO	4.271	5.255E-08	7.226E-08	Previously described
IL-24	4.269	5.255E-08	7.226E-08	SASP protein family
IL-1 ALPHA	4.262	3.782E-07	4.588E-07	Previously described
PSP-D	4.249	5.255E-08	7.226E-08	Potentially novel
CCL28	4.247	5.255E-08	7.226E-08	SASP protein family
SELP	4.239	5.255E-08	7.226E-08	Potentially novel
LIF-R	4.225	5.253E-08	7.226E-08	Potentially novel
TNFRSF14	4.224	5.255E-08	7.226E-08	SASP protein family
VWF	4.217	5.255E-08	7.226E-08	Potentially novel
SIRT2	4.214	5.253E-08	7.226E-08	Potentially novel
AZU1	4.212	5.253E-08	7.226E-08	Potentially novel
FGF-21	4.211	5.255E-08	7.226E-08	SASP protein family
CD6	4.190	5.255E-08	7.226E-08	Potentially novel
MMP-9	4.183	5.255E-08	7.226E-08	SASP Atlas
CCL25	4.182	5.255E-08	7.226E-08	Previously described
SCGB3A2	4.179	5.253E-08	7.226E-08	Potentially novel
TR	4.175	5.253E-08	7.226E-08	SASP Atlas
CPA1	4.172	5.253E-08	7.226E-08	Potentially novel
CD244	4.168	5.255E-08	7.226E-08	Potentially novel
PECAM-1	4.166	5.255E-08	7.226E-08	Potentially novel
TNF	4.166	5.251E-08	7.226E-08	Previously described

NOTCH 3	4.159	5.253E-08	7.226E-08	Potentially novel
IL-22 RA1	4.153	5.255E-08	7.226E-08	SASP protein family
OSM	4.151	5.251E-08	7.226E-08	Potentially novel
TR-AP	4.141	5.255E-08	7.226E-08	Potentially novel
IL-20RA	4.129	5.255E-08	7.226E-08	SASP protein family
IL-1RT2	4.125	5.255E-08	7.226E-08	SASP protein family
EN-RAGE	4.121	4.070E-07	4.831E-07	Potentially novel
NRTN	4.114	5.255E-08	7.226E-08	Potentially novel
IL2	4.105	5.253E-08	7.226E-08	Previously described
ADA	4.097	5.253E-08	7.226E-08	Potentially novel
IFN-GAMMA	4.095	5.255E-08	7.226E-08	Previously described
U-PAR	4.093	5.255E-08	7.226E-08	SASP Atlas
ICAM-2	4.090	5.255E-08	7.226E-08	Potentially novel
AXIN1	4.089	5.255E-08	7.226E-08	Potentially novel
TIMP4	4.081	5.253E-08	7.226E-08	SASP protein family
CHIT1	4.078	5.255E-08	7.226E-08	Potentially novel
CPB1	4.068	5.255E-08	7.226E-08	Potentially novel
GP6	4.050	5.255E-08	7.226E-08	Potentially novel
ARTN	4.048	5.255E-08	7.226E-08	Potentially novel
VEGFA	4.047	5.255E-08	7.226E-08	Previously described
IL18	4.025	9.669E-07	1.101E-06	SASP Atlas
DNER	4.018	5.255E-08	7.226E-08	Potentially novel
TSLP	3.994	5.255E-08	7.226E-08	Potentially novel
IL33	3.989	5.255E-08	7.226E-08	SASP protein family
IL5	3.985	5.255E-08	7.226E-08	SASP protein family
PDGFA	3.950	5.255E-08	7.226E-08	Previously described
SHPS-1	3.948	5.255E-08	7.226E-08	Potentially novel
CD93	3.944	5.253E-08	7.226E-08	Potentially novel
ST2	3.938	5.255E-08	7.226E-08	SASP protein family
IL2-RA	3.912	5.253E-08	7.226E-08	SASP protein family
LTBR	3.896	5.255E-08	7.226E-08	Potentially novel
PCSK9	3.847	5.255E-08	7.226E-08	Potentially novel
SELE	3.833	5.251E-08	7.226E-08	Potentially novel
IL-18BP	3.785	5.255E-08	7.226E-08	SASP protein family
IL-15RA	3.781	5.255E-08	7.226E-08	SASP protein family
EPHB4	3.756	5.253E-08	7.226E-08	Potentially novel

TNFRSF9	3.736	5.255E-08	7.226E-08	SASP protein family
TLT-2	3.680	5.255E-08	7.226E-08	Potentially novel
FABP4	3.667	5.255E-08	7.226E-08	Previously described
NT-PROBNP	3.666	5.255E-08	7.226E-08	Potentially novel
GAL-3	3.548	5.253E-08	7.226E-08	SASP Atlas
CX3CL1	3.547	5.683E-08	7.442E-08	Previously described
BETA-NGF	3.487	5.255E-08	7.226E-08	Previously described
IL-10RB	3.474	5.255E-08	7.226E-08	SASP protein family
SCF	3.449	5.255E-08	7.226E-08	Previously described
CCL20	3.442	1.196E-06	1.351E-06	Previously described
IL-18R1	3.440	5.255E-08	7.226E-08	SASP protein family
T-PA	3.424	5.683E-08	7.442E-08	SASP Atlas
CXCL11	3.302	5.255E-08	7.226E-08	Previously described
TNF-R2	3.263	5.253E-08	7.226E-08	Previously described
IL-12B	3.259	5.253E-08	7.226E-08	SASP protein family
PD-L1	3.166	5.255E-08	7.226E-08	Potentially novel
CTSZ	3.100	5.255E-08	7.226E-08	SASP Atlas
FGF-5	3.042	5.255E-08	7.226E-08	SASP protein family
CXCL16	3.029	5.255E-08	7.226E-08	SASP protein family
CD40	3.011	4.070E-07	4.831E-07	Previously described

- *Fold change: Median of fold changes between PQ treated and untreated primary lung fibroblasts.*
- *P-value: tested using Wilcoxon signed-rank tests.*
- *FDR: P-value adjusted for multiple testing using Benjamini-Hochberg correction.*
- *Last column describes overlap with SASP Atlas [4], PubMed search for previous described, and protein families of described SASP proteins [5].*

Table S2: Overview of all 42 COPD associated SASP proteins

	COPD vs Controls			SEO-COPD vs matched controls			Older, MM COPD vs matched controls		
Protein	**FC**	**P-value**	**FDR**	**FC**	**P-value**	**FDR**	**FC**	**P-value**	**FDR**
RANKL	1.6630	0.0054	**0.0379**	1.4804	0.0193	**0.0379**	1.6704	0.0783	0.1820
FABP4	1.5049	0.0018	**0.0379**	1.4286	0.0071	**0.0375**	1.3885	0.1809	0.2111
IGFBP-1	1.4742	0.0113	**0.0450**	1.1928	0.0500	0.0568	1.5197	0.0910	0.1820
t-PA	1.4575	0.0132	**0.0450**	1.6420	0.0114	**0.0375**	1.1056	0.3981	0.4180
GP6	1.4362	0.0051	**0.0379**	1.4196	0.0179	**0.0379**	1.4577	0.0910	0.1820
CPA1	1.4189	0.0011	**0.0379**	1.3138	0.0033	**0.0375**	1.4426	0.0573	0.1820
MMP-9	1.3942	0.0122	**0.0450**	1.5136	0.0043	**0.0375**	1.1451	0.4813	0.4931
IL2-RA	1.3935	0.0030	**0.0379**	1.3822	0.0143	**0.0375**	1.3723	0.0573	0.1820
IL-12B	1.3780	0.0070	**0.0379**	1.5486	0.0243	**0.0379**	1.2342	0.1590	0.1964
TFF3	1.3685	0.0076	**0.0379**	1.2113	0.0338	**0.0443**	1.4524	0.0671	0.1820
vWF	1.3678	0.0076	**0.0379**	1.3410	0.0222	**0.0379**	1.2822	0.1590	0.1964
AP-N	1.3555	0.0047	**0.0379**	1.3294	0.0222	**0.0379**	1.4039	0.0573	0.1820
CHIT1	1.3418	0.0047	**0.0379**	1.2939	0.0500	0.0568	1.4140	0.0411	0.1820
CD93	1.3304	0.0008	**0.0379**	1.2934	0.0143	**0.0375**	1.3839	0.0242	0.1820
ST2	1.3296	0.0030	**0.0379**	1.4317	0.0114	**0.0375**	1.1581	0.1213	0.1960
EN-RAGE	1.3288	0.0169	**0.0500**	1.3847	0.0305	**0.0427**	1.2412	0.2050	0.2265
SPON1	1.3282	0.0076	**0.0379**	1.3119	0.0222	**0.0379**	1.3587	0.0910	0.1820
TR-AP	1.3258	0.0055	**0.0379**	1.3555	0.0338	**0.0443**	1.3781	0.0783	0.1820
CCL15	1.3137	0.0060	**0.0379**	1.2205	0.0275	**0.0412**	1.3429	0.0910	0.1820
ST1A1	1.3074	0.0024	**0.0379**	1.4220	0.0118	**0.0375**	1.3526	0.0671	0.1820
TIMP4	1.3072	0.0070	**0.0379**	1.2771	0.0143	**0.0375**	1.3158	0.1590	0.1964
AZU1	1.3067	0.0039	**0.0379**	1.3340	0.0143	**0.0375**	1.2909	0.1213	0.1960
LIF-R	1.3004	0.0145	**0.0450**	1.3056	0.0152	**0.0375**	1.3207	0.1213	0.1960
PDGFA	1.2988	0.0033	**0.0379**	1.3515	0.0412	**0.0495**	1.4048	0.0346	0.1820
PECAM-1	1.2950	0.0036	**0.0379**	1.2810	0.0179	**0.0379**	1.5383	0.0671	0.1820
PGLYRP1	1.2943	0.0097	**0.0445**	1.4064	0.0222	**0.0379**	1.2557	0.1590	0.1964
MEPE	1.2928	0.0132	**0.0450**	1.3013	0.0864	0.0864	1.2737	0.0783	0.1820
SELP	1.2824	0.0036	**0.0379**	1.3386	0.0114	**0.0375**	1.2940	0.0783	0.1820
NRTN	1.2803	0.0064	**0.0379**	1.1821	0.0305	**0.0427**	1.4130	0.0573	0.1820
MPO	1.2742	0.0097	**0.0445**	1.2814	0.0143	**0.0375**	1.3080	0.1392	0.1964
IL-10RA	1.2725	0.0059	**0.0379**	1.2351	0.0118	**0.0375**	1.3135	0.1053	0.1960
KLK6	1.2679	0.0122	**0.0450**	1.4624	0.0604	0.0634	1.1514	0.1590	0.1964

FGF-23	1.2570	0.0157	**0.0474**	1.2329	0.0118	**0.0375**	1.3974	0.2313	0.2491
CDH5	1.2547	0.0036	**0.0379**	1.2409	0.0090	**0.0375**	1.4702	0.0910	0.1820
U-PAR	1.2530	0.0142	**0.0450**	1.4439	0.0864	0.0864	1.2140	0.0486	0.1820
CCL23	1.2522	0.0076	**0.0379**	1.2071	0.0576	0.0621	1.2669	0.0486	0.1820
CD8A	1.2480	0.0124	**0.0450**	1.2829	0.0380	**0.0469**	1.2830	0.1590	0.1964
PSP-D	1.2365	0.0122	**0.0450**	1.6285	0.0025	**0.0375**	1.0308	0.5262	0.5262
CXCL9	1.2358	0.0145	**0.0450**	1.2279	0.0380	**0.0469**	1.3673	0.1590	0.1964
IL-15RA	1.2023	0.0124	**0.0450**	1.2376	0.0243	**0.0379**	1.1905	0.2050	0.2265
TNFSF14	1.2016	0.0114	**0.0450**	1.1898	0.0243	**0.0379**	1.2558	0.1809	0.2111
FGF-19	1.1882	0.0145	**0.0450**	1.1634	0.0576	0.0621	1.3390	0.1213	0.1960

- *FC (Fold change): Fold change in medians of COPD compared to control-derived fibroblasts.*
- *P-value: tested using Mann-Whitney U tests.*
- *FDR: P-value adjusted for multiple testing using Benjamini-Hochberg correction. Boldfaced when significant.*

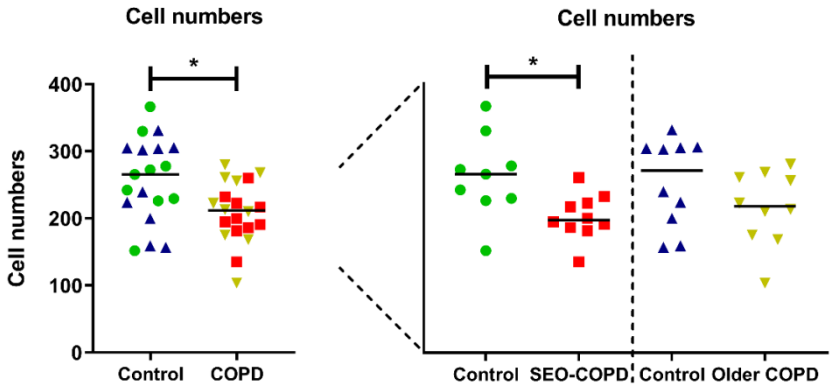

Figure S1: Cell number differences between fibroblasts from COPD patients and controls at baseline. *Dot plots show total cell number at the end of culture of all 4 patient groups. Green = SEO-COPD-matched control, red = SEO-COPD, blue = older COPD-matched control, yellow = older, mild-moderate, COPD. Lines represent medians. Significant differences tested with Mann-Whitney U tests. * P-value < 0.05.*

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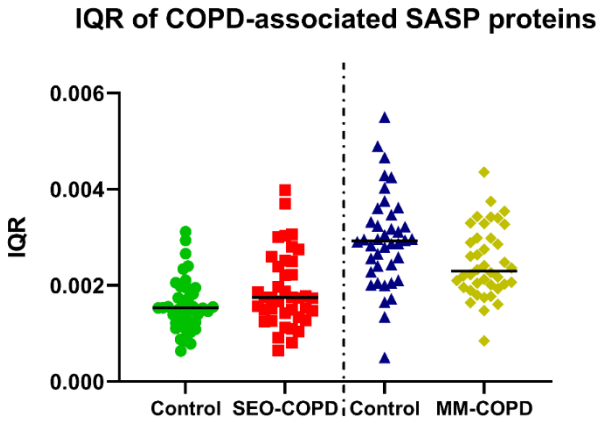

Figure S2: Interquartile ranges of COPD-associated proteins per subgroup*. Interquartile ranges (IQR) of the 42 COPD-associated SASP proteins per subgroup. Green = SEO-COPD-matched controls, red = SEO-COPD, blue = older, mild-moderate COPD-matched controls, yellow = older, mild-moderate COPD.*

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CHAPTER 6

High levels of cellular senescence in airway smooth muscle cells; no increase in COPD

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ABSTRACT

Accelerated aging is recognized to play a role in the pathogenesis of COPD. Recently, extracellular matrix (ECM) dysregulation has been proposed as additional hallmark of lung aging. The airway smooth muscle (ASM) in COPD airways is thickened, accompanied by abnormal ECM homeostasis. We previously demonstrated higher levels of cellular senescence in COPD-derived fibroblasts, which was associated with altered ECM gene expression. Since it is unknown whether ASMCs are more senescent in COPD and whether this also affects ECM expression, we compared cellular senescence levels of ASMCs between COPD patients and non-COPD controls and assessed the association with ECM expression.

Cellular senescence markers and ECM gene expression were measured in ASMCs from COPD patients at baseline and upon Paraquat-induced senescence. COPD-derived ASMCs (n=10) were compared to lung fibroblasts from the same patients (n=6) and to non-COPD control-derived ASMCs (n=11).

Percentages of SA-β-gal positive cells were higher in ASMCs compared to lung fibroblasts from the same patients (35% vs 13% respectively). Paraquat-induced senescence resulted in small ECM changes in ASMCs. Cellular senescence levels were similar in COPD-derived ASMCs compared to non-COPD.

We demonstrated that cellular senescence levels at baseline are higher in ASMCs compared to lung fibroblasts, but are not different between ASMCs from COPD patients compared to non-COPD. We found no link between cellular senescence and ECM gene expression in COPD-derived ASMCs. These results indicate that, in contrast to lung fibroblasts, higher levels of senescence in ASMCs do not appear to play a major role in COPD pathology.

INTRODUCTION

Accelerated aging has been postulated to play a role in the pathogenesis of chronic obstructive pulmonary disease (COPD). Aging is defined as a progressive loss of normal homeostasis, and when abnormal can result in disease or death (1, 2). Normal lung aging includes lung function decline, airspace enlargement, and loss of elasticity (3, 4). In COPD patients, lung aging is accelerated and may contribute to disease development and progression (5, 6). As the pathogenesis of COPD is still largely unknown, more knowledge of this process and the role of accelerated aging is needed.

Multiple studies have found higher levels of several aging markers in COPD patients compared to individuals without COPD (5-8). The aging marker most extensively described in COPD is cellular senescence, which is an irreversible cell cycle arrest that prevents cell death and tumor development. Accumulation of senescent cells can lead to tissue dysfunction by causing chronic inflammation and extracellular matrix (ECM) changes (2). Higher levels of cellular senescence have been found in multiple structural cells derived from COPD patients compared to non-COPD controls, including alveolar and bronchial epithelial cells, pulmonary vascular endothelial cells, pulmonary artery smooth muscle cells, and lung fibroblasts (9-14).

Another proposed hallmark of lung aging is ECM dysregulation (7). The ECM plays an essential role in the function and structure of the lungs and is important in healthy lung tissue repair and remodeling. Age-related ECM changes can affect normal homeostasis and healthy tissue function (15, 16). In general, ECM changes upon aging include an increase in fibrosis and loss of elasticity, which also has been observed in both normal and accelerated lung aging (3, 5, 6, 17, 18). Lung fibroblasts are major producers of ECM and regulate ECM homeostasis. Recently, we found that higher levels of cellular senescence in COPD-derived lung fibroblasts were associated with lower levels of the ECM protein decorin (19). In addition, upon induction of cellular senescence in primary lung fibroblasts using Paraquat (PQ), we found differential gene expression of several ECM proteins, including reduced decorin expression.

The airway smooth muscle cell (ASMC) is another cell type that can play an important role in lung tissue remodeling by regulation of ECM homeostasis (20-22). In COPD pathology, the large and small airway walls are thickened, including a thickening of the ASM, leading to airflow limitation (23-25). In lung tissue from COPD patients, an increase in smooth muscle mass has been observed compared to individuals without COPD (26-28). This increase in smooth muscle mass is accompanied by altered ECM deposition, including dysregulated collagen structure, decrease in elastic fibers, and an increase in fibronectin, and tenascin (20, 22, 29, 30). Whether cellular senescence of ASMCs has an effect on ECM gene expression and plays a role in COPD pathology is unknown. Furthermore, higher levels of cellular senescence in ASMCs from COPD patients compared to non-COPD have, in contrast to other structural lung cells (8), not been shown yet. Therefore, we assessed whether cellular senescence in ASMCs is accompanied by changes in ECM gene expression and assessed whether levels of cellular senescence are

different in COPD-derived ASMCs compared to non-COPD controls. Furthermore, cellular senescence levels of ASMCs and lung fibroblasts from the same donors were compared.

METHODS

A detailed description can be found in the online supplement.

Subjects

Primary airway smooth muscle cells (ASMCs) and primary lung fibroblasts from subjects undergoing lung transplantation or tumor resection surgery were isolated as previously described (31, 32). All included COPD patients were Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV patients, defined as FEV_1/FVC ratio <70% and FEV_1 % pred <30%. Non-COPD control subjects were included when the FEV_1/FVC ratio was >70%. All subjects were ex-smokers and were matched for age, sex, and smoking history (Table 1). None of the COPD patients was alpha-1 antitrypsin deficient. Ethical approval for all experiments with primary lung cells was provided by the Human Ethics Committees (IRB) of the Sydney South West Area Health Service and St Vincent's Hospital IRB, and written informed consent was obtained.

Table 1: patient characteristics of ASMCs from COPD patients and non-COPD controls

Variable	Control	COPD	P-value
Number	11	10	
Age, mean years (range)	63 (53-75)	58 (44-64)	0.168
Male/female, N	7/4	4/6	0.290
Smoking history	All ex-smokers	All ex-smokers	
Pack-years	40 (26-60)	30 (20-50)	0.359
Stop-months	13 (2-48)	95 (53-138)	0.355
FEV_1 % predicted	92 (75-100)	20 (18-22)	**0.001**
FVC % predicted	94 (77-101)	69 (49-86)	0.079
FEV_1/FVC (%)	78 (77-83)	25 (22-26)	**0.001**

- *Data are presented as medians with IQRs unless otherwise stated*
- *Significant differences between groups were tested using Mann–Whitney U-tests. P-values are boldfaced when significantly different*
- *FEV_1: forced expiratory volume in one second, FVC: forced vital capacity*

Primary cell culture

Primary ASMCs and primary lung fibroblasts were cultured in DMEM (Gibco) supplemented with 5% fetal bovine serum (FBS), 25 mM Hepes buffer (Gibco), and 1% antibiotic-antimycotic (Gibco) at 37°C/5% CO2 as described before (31, 32). For the direct comparison of the two cell types from the same patients, freshly isolated cells were used, which had not been stored in liquid nitrogen yet. For both cell types, 40,000 cells were seeded in 6-well plates and treated after two days with or without 250 µM Paraquat dichloride hydrate (PQ) (Sigma-Aldrich) for 24 hours to induce cellular senescence (19, 33). After 24 hours, PQ was washed away and cells were kept in culture with DMEM + 5% FBS for another 24 hours (for RNA collection) or four days (for RNA collection, SA-β-gal staining, and secreted proteins). These time-points were carefully chosen based on pilot study results. All cells were used at passage five unless otherwise stated.

Senescence-associated β-Galactosidase staining

Cellular senescence was assessed with standard Senescence-associated β-Galactosidase (SA-β-gal) staining as described before (19, 34). A detailed description can be found in the online supplement.

Gene expression analyses

To analyze gene expression, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using TaqMan gene expression assays (Life Technologies). A detailed description can be found in the online supplement.

Secreted protein analyses

Cell-free supernatants were harvested four days after PQ removal and stored at -20°C prior to ELISA analysis. Secreted IL-6 and IL-8 levels were measured using Human DuoSet ELISA (R&D Systems). As the numbers of cells were significantly different at the end of culture between untreated and PQ treated, we corrected the secreted protein levels for cell numbers counted at the end of culture.

Western Blot analyses

Western blot analyses were used to assess protein levels of the senescence markers p16 and p21. A detailed description can be found in the online supplement.

Statistical analyses

SPSS Statistics 26 software (IBM) was used for statistical analyses. The effect of PQ treatment compared to untreated and the differences between ASMCs compared to lung fibroblasts from the same patients were tested using paired analyses with Wilcoxon signed-rank tests. Mann-Whitney U-tests were used to test differences between COPD patients and non-COPD controls. $P<0.05$ was considered statistically significant.

RESULTS

Higher percentages of SA-β-gal positive cells in airway smooth muscle cells compared to lung fibroblasts

Since the percentages of SA-β-gal positive cells at baseline seemed already higher in airway smooth muscle cells (ASMCs) than we (19) and others previously found in lung fibroblasts (14, 35-37), we decided to do a direct comparison between both cell types derived from the same patients. ASMCs had a lower proliferation rate compared to lung fibroblasts with significantly lower population doublings from passage 5 onwards (Fig. 1A). The percentages of SA-β-gal positive cells at baseline were significantly higher in ASMCs (35%) compared to lung fibroblasts (13%) at passage 5 (Fig. 1B), which was also observed at passage 4 and 6 (Fig. E1B in the online supplement). No differences in p16 and p21 gene expression (Fig. 1C+D) were observed between ASMCs and fibroblasts. P16 and p21 protein levels at passage 5 were not significantly different either (Fig. 1E+F). Secretion of the well-known senescence-associated secretory phenotype (SASP) proteins IL-6 and IL-8 (38-40) was assessed as well. IL-6 secretion seems higher in ASMCs compared to lung fibroblasts at multiple cell passages (Fig. E1G), but this was not significantly different (Fig. 1G). IL-8 secretion was also not significantly different between the two cell types (Fig. 1H).

More SA-β-gal positive cells upon Paraquat-induced senescence in airway smooth muscle cells compared to lung fibroblasts

To compare the levels of cellular senescence of ASMCs and lung fibroblasts, senescence was induced using Paraquat (PQ). Upon PQ treatment, percentages of SA-β-gal positive cells increased similarly in both cell types with higher percentages of SA-β-gal positive cells in ASMCs (83%) compared to lung fibroblasts (65%) at passage 5 (Fig. 2A) and passage 6 (Fig. E3A). Upon PQ-induced senescence, the total cell numbers were decreased in both cell types, while the levels of decrease were not significantly different between the two cell types (Fig. 2B). PQ increased p21 gene expression at similar levels in both cell types (Fig. 2C+D) but did not affect p16 gene expression (Fig. 2E+F). IL-6 and IL-8 secretion were both induced after PQ treatment in ASMCs and fibroblasts with similar levels of increase (Fig. 2G+H), while a trend ($p=0.094$) towards higher IL-6 secretion post PQ treatment in ASMCs compared to lung fibroblasts (Fig. 2G).

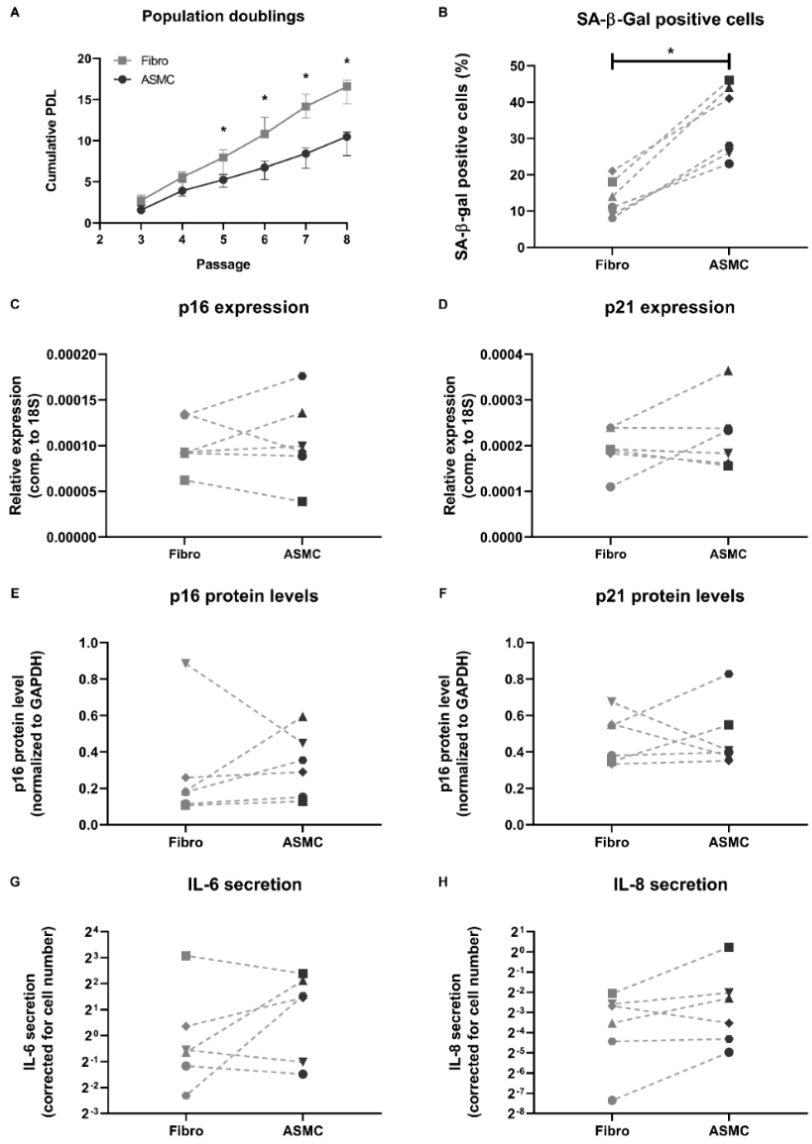

Figure 1: Higher percentages of SA-ß-gal positive cells in airway smooth muscle cells compared to lung fibroblasts. *Dot plots show baseline levels of cellular senescence markers of ASMCs (light grey) and lung fibroblasts (dark grey) from the same 6 patients (same symbols represent the same patients). Population doublings of both cell types at multiple passages are shown in the line graph (A). Percentages of SA-ß-gal positive cells (B) following 4 days of growth in vitro at passage 5. Gene expression of p16 (C) and p21 (D) following 24h of growth in vitro at passage 5. Protein levels of p16 (E) and p21 (F) normalized for GAPDH protein levels following 4 days of growth in vitro at passage 5. Examples of the blots are shown in Fig. E2. IL-6 (G) and IL-8 (H) secretion following 4 days of growth in vitro corrected for total cell numbers at passage 5. Fibro= fibroblasts. Differences between ASMCs and fibroblasts from the same patients were tested using Wilcoxon tests. * means P-value <0.05.*

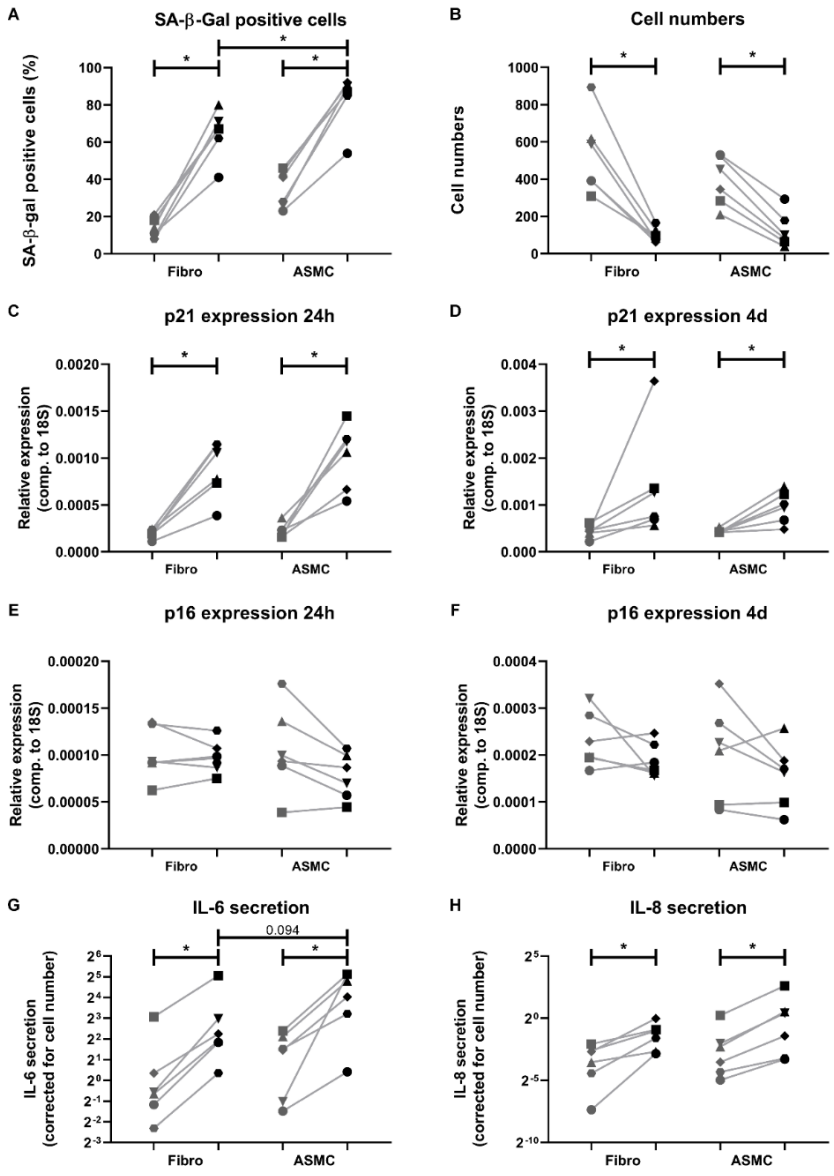

Figure 2: More SA-β-gal positive cells after Paraquat-induced senescence in airway smooth muscle cells compared to lung fibroblasts. *Dot blots show senescence markers at baseline (grey) and upon PQ-induced senescence (black) in ASMCs and lung fibroblasts from the same 6 patients (same symbols represent the same patients). Percentages of SA-β-gal positive cells (A) and total cell numbers (B) at baseline and 4 days post PQ removal at passage 5. Gene expression of p16 (C+D) and p21 (E+F) at baseline and 24h (C+E) and 4 days (D+F) post PQ removal at passage 5. IL-6 (E) and IL-8 (F) secretion levels at baseline and 4 days post PQ removal corrected for total cell numbers at passage 5. Fibro = fibroblasts. Differences between ASMCs and fibroblasts from the same patients were tested using Wilcoxon tests. * means P-value <0.05.*

No differences in cellular senescence in COPD-derived airway smooth muscle cells compared to non-COPD controls

Next, we assessed whether levels of cellular senescence were higher in COPD-derived ASMCs compared to non-COPD-derived ASMCs. The proliferation rate of COPD-derived ASMCs was lower compared to non-COPD controls with population doublings significantly lower at passage 5 and 6 (Fig. 3A). Cellular senescence was not significantly different between COPD and non-COPD-derived ASMCs at baseline, as no differences were found in SA-β-gal positive cells (Fig. 3B), p16, and p21 gene expression (Fig. 3C+D). The secretion of IL-6, but not IL-8, was higher in COPD-derived ASMCs compared to non-COPD (Fig. 3E+F). No differences in *DCN*, *FBLN5*, and *ACTA2* gene expression were observed between COPD-derived ASMCs and non-COPD (Fig. 3G-I).

Similar induction in cellular senescence by Paraquat in airway smooth muscle cells from COPD patients and non-COPD controls

When comparing senescence markers upon PQ-induced senescence between COPD and non-COPD, no significant differences were found in the increase in SA-β-gal positive cell percentage (Fig. 4A and Fig. E4A). Upon PQ treatment, the cell numbers of COPD-derived ASMCs were lower compared to non-COPD (Fig. 4B), but the level of decrease was similar (Fig. E4B). There was a trend ($p=0.051$) for higher gene expression of p21 after PQ treatment in COPD-derived ASMCs and compared to non-COPD-derived ASMCs at 24h (Fig. 4C), but not at 4d (Fig. 4D). Significantly higher fold changes in p21 gene expression were found in COPD-derived ASMCs compared to non-COPD after 24 hours and four days of PQ treatment (Fig. 4E+F). After higher baseline secretion of IL-6 in COPD-derived ASMCs (Fig. 3E), also higher levels after PQ treatment were observed (Fig. 4G), while the fold change was not significantly different between COPD and non-COPD (Fig. E4C). IL-8 secretion was increased at similar levels and not also not significantly different upon PQ treatment between COPD and non-COPD (Fig. 4H & Fig. E4D).

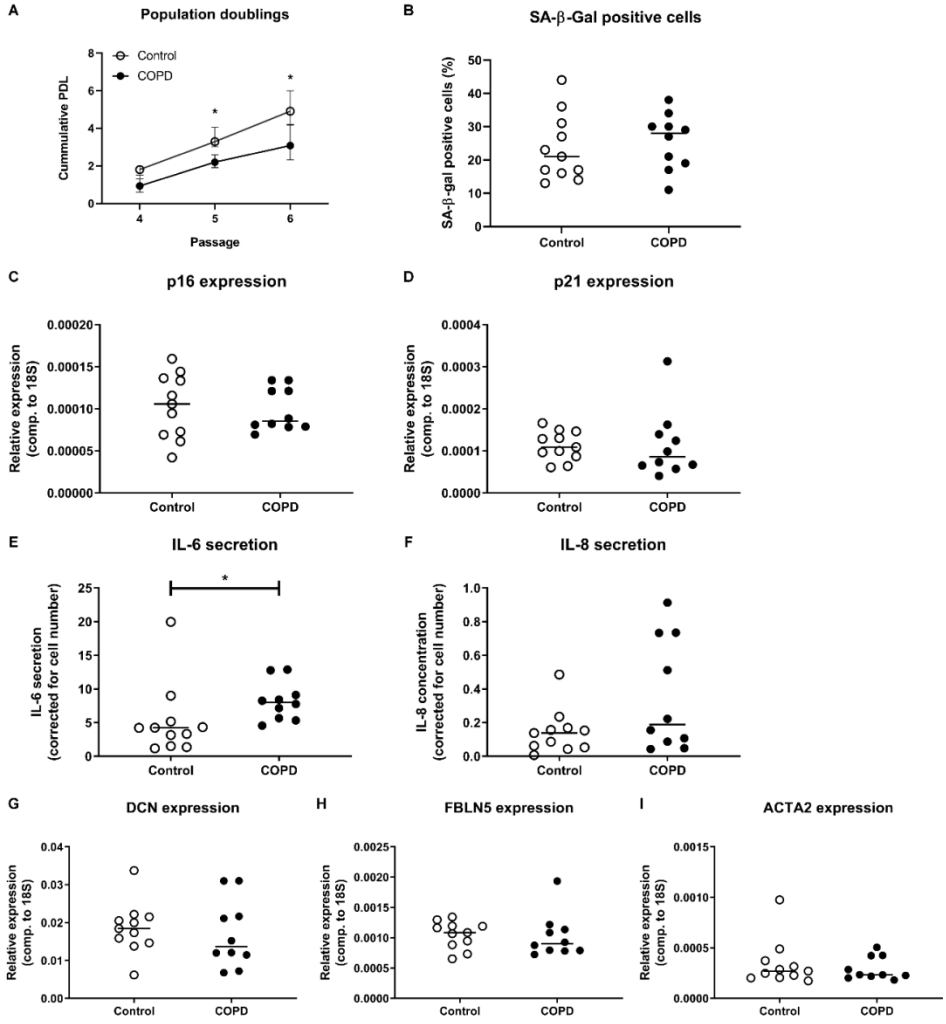

Figure 3: No differences in cellular senescence in COPD-derived airway smooth muscle cells compared to non-COPD controls. *Line graph and dot plots show baseline of cellular senescence markers and ECM gene expression of ASMCs from COPD patients (n=10) and non-COPD controls (n=11). Population doublings of ASMCs from COPD patients and non-COPD controls at multiple passages are shown in the line graph (A). Percentages of SA-β-gal positive cells (B) following 4 days of growth in vitro. Gene expression of p16 (C) and p21 (D) following 24h of growth in vitro. IL-6 (E) and IL-8 (F) secretion following 4 days of growth in vitro corrected for total cell numbers. Gene expression of the ECM proteins DCN (G), FBLN5 (H), and ACTA2 (I) are shown following 4 days of growth in vitro. Differences between COPD and non-COPD control were tested using Mann–Whitney U tests. * means P-value <0.05.*

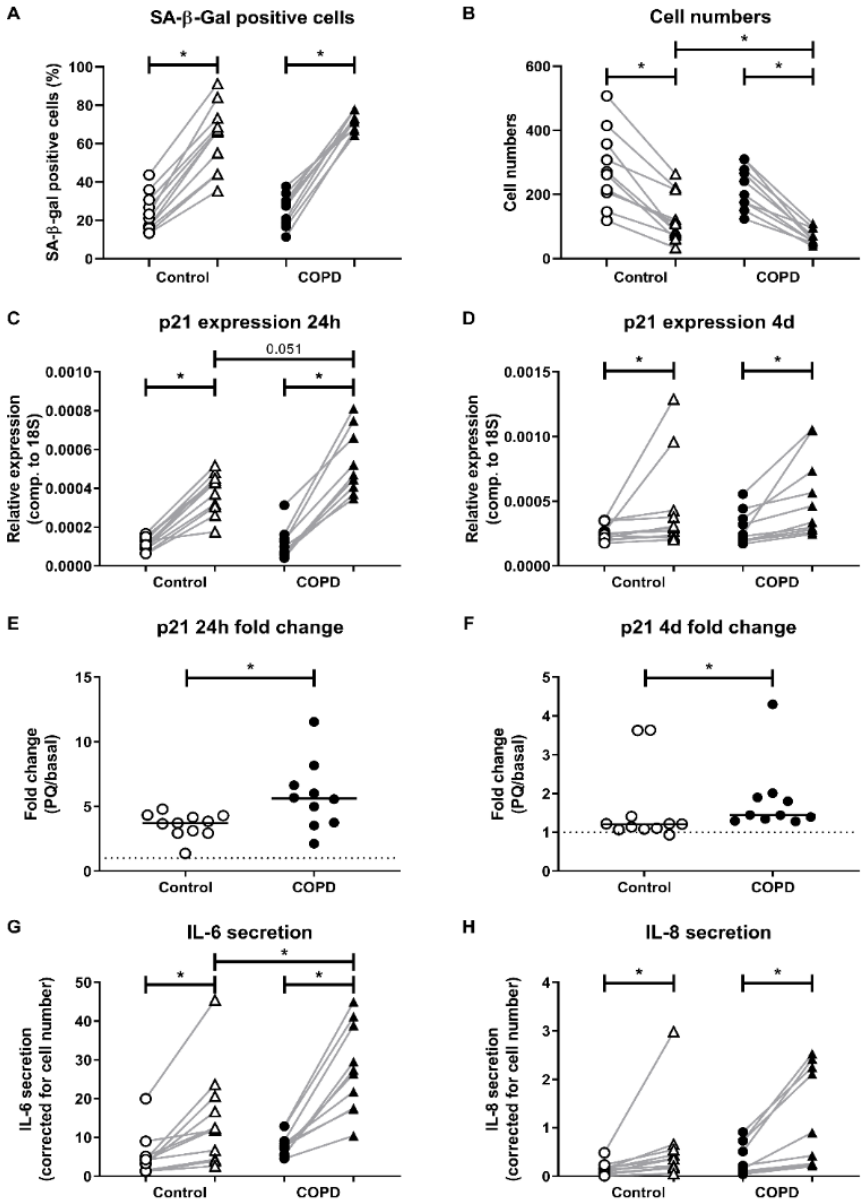

Figure 4: Cellular senescence induction by paraquat in ASMCs from COPD patients and non-COPD controls. *Dot plots show cellular senescence markers of ASMCs from COPD patients (n=10, solid symbols) and non-COPD controls (n=11, open symbols) at baseline (circles) and upon PQ treatment (triangles). Percentages of SA-β-gal positive cells (A) and total cell numbers (B) 4 days post PQ removal. Gene expression of p21 24h (C) and 4 days (D) and p21 fold changes 24h (E) and 4 days (F) post PQ removal. IL-6 (G) and IL-8 (H) secretion 4 days post PQ removal corrected for total cell numbers. Differences between PQ and baseline were tested using Wilcoxon tests. Differences*

*between COPD and non-COPD control were tested using Mann–Whitney U tests. * means P-value <0.05.*

Similar ECM changes upon Paraquat-induced senescence in airway smooth muscle cells from COPD patients and non-COPD controls

Finally, we assessed whether senescence induction in ASMCs affects gene expression of extracellular matrix (ECM) proteins and the fibrotic marker *ACTA2*, as we previously found a strong decrease in *DCN*, *FBLN5*, and *ACTA2* gene expression in senescence-induced lung fibroblasts (19). Upon PQ-induced senescence, gene expression of the ECM protein *DCN* was reduced in ASMCs from both COPD patients and non-COPD controls (Fig. 5A). *FBLN5* gene expression was significantly reduced by PQ in ASMCs from non-COPD, while a similar trend (p=0.084) was observed in COPD-derived ASMCs (Fig. 5B). No significant effect of PQ on *ACTA2* gene expression was observed in ASMCs from both COPD patients and non-COPD (Fig. 5C). The fold changes in ECM and *ACTA2* gene expression upon PQ-induced senescence were not significantly different between ASMCs from COPD patients compared to non-COPD (Fig. 5D-F).

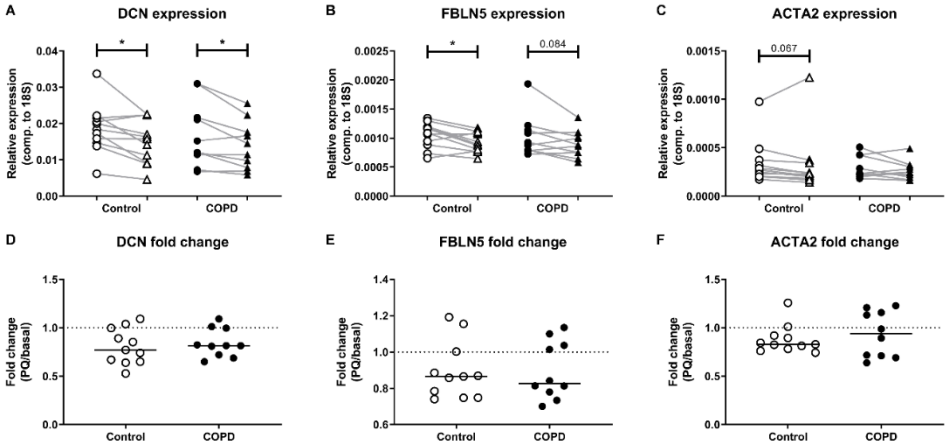

Figure 5: Small ECM changes upon Paraquat-induced senescence in airway smooth muscle cells. *Dot plots show ECM gene expression of ASMCs from COPD patients (n=10, solid symbols) and non-COPD controls (n=11, open symbols) at baseline (circles) and upon PQ treatment (triangles). Gene expression of the ECM proteins DCN (A), FBLN5 (B), and ACTA2 (C) are shown 4 days post PQ removal. Differences between PQ and baseline were tested using Wilcoxon tests. Fold changes in DCN (D), FBLN5 (E), and ACTA2 (F) gene expression 4 days post PQ removal. Differences between COPD and non-COPD controls were tested using Mann–Whitney U tests. * means P-value <0.05.*

DISCUSSION

This is the first study assessing the cellular senescence levels of airway smooth muscle cells (ASMCs) and to compare this to lung fibroblasts, and between COPD patients and non-COPD controls. Percentages of SA-β-gal positive cells, one of the widely used cellular senescence markers, were higher at baseline in ASMCs compared to lung fibroblasts over multiple cell passages. Cellular senescence levels were not significantly different between cells from COPD patients compared to non-COPD, as measured by SA-β-gal positivity, cell proliferation inhibition, and p16 and p21 gene expression, although p21 gene expression increased more upon PQ treatment in COPD compared to non-COPD derived fibroblasts. PQ-induced senescence in ASMCs resulted in small changes in the expression of extracellular matrix (ECM) genes, while we previously found strong ECM changes in senescence-induced lung fibroblasts (19). The observed ECM changes upon PQ-induced senescence in ASMCs were not different between COPD patients and non-COPD controls.

We observed that ASMCs have a more senescent-like phenotype than lung fibroblasts, as the proliferation rate was lower and percentages of SA-β-gal positive cells were higher in ASMCs compared to lung fibroblasts. However, no differences were observed in the gene expression of the senescence markers p16 and p21. The p16 and p21 protein levels of multiple donors (five and four out of six, respectively) seemed higher in ASMCs, but this was not significantly different. This lack of difference might be caused by low protein levels overall. Although the baseline percentages of SA-β-gal positive cells were already higher in ASMCs, PQ was able to further induce these percentages to a higher level at the end compared to lung fibroblasts with a similar level of induction. Similar to baseline, after PQ-induced senescence, no differences were found for p16 and p21 gene expression. The discrepancy between p16 and p21 expression and SA-β-gal positive cells might be explained by the fact that we assessed global gene and protein levels in whole cultures, which might lead to an underestimation of cellular senescence levels, whereas for SA-β-gal the percentages of positive cells were quantified. Another explanation for this discrepancy might be that higher baseline percentages of SA-β-gal positive cells are driven by a p16 and p21 independent pathway, for example by a chromatin-dependent pathway or GATA4-dependent pathway as have been demonstrated in human fibroblast cell lines (41, 42).

A lower proliferation rate was found in ASMCs from COPD patients compared to non-COPD controls, while no differences in senescence markers were found. The lower proliferation rate in COPD-derived ASMCs may be caused by lower mitochondrial activity, as a previous study demonstrated lower mitochondrial membrane potential in COPD patients compared to healthy controls (43). In COPD lungs both ASM hypertrophy and hyperplasia have been observed, where the higher numbers of ASMCs in COPD, might explain that cellular senescence levels are not different between COPD and non-COPD. Higher secretion of the pro-inflammatory cytokine IL-6 was observed in COPD-derived

ASMCs compared to non-COPD, which was unrelated to cellular senescence (Fig. E5A in the online supplement). Pro-inflammatory mediators, including IL-6, have previously been found to be higher in COPD-derived ASMCs at baseline conditions (43), and a greater increase in IL-6 was demonstrated upon TGF-β treatment in COPD-derived ASMCs (44). Furthermore, no differences in ECM and *ACTA2* gene expression were found between ASMCs from COPD patients compared to non-COPD at baseline. In addition, no association between cellular senescence and ECM gene expression was found (Fig. E5B-D in the online supplement). So although previous studies demonstrated ECM changes in ASM from COPD patients (30, 45), we did not observe a role for cellular senescence in ECM regulation in ASM from COPD patients.

Upon PQ-induced senescence, we found a similar increase in senescence markers in ASMCs from COPD patients and non-COPD controls for all markers, except for p21 gene expression, which was more increased in COPD-derived ASMCs compared to non-COPD. Upon PQ-induced senescence, *DCN* gene expression was decreased in ASMCs from both COPD patients and non-COPD, but *FBLN5* gene expression was only significantly decreased in non-COPD control-derived ASMCs. However, the fold changes in both *DCN* and *FBLN5* gene expression upon PQ-induced senescence were similar in ASMCs from COPD patients and non-COPD. So, while PQ-induced senescence resulted in a stronger p21 increase in COPD-derived ASMCs compared to non-COPD, this did not lead to a stronger decrease in ECM gene expression. These results confirm our conclusion that cellular senescence does not play a major role in ECM regulation by ASM from COPD patients.

The decreases in *DCN*, *FBLN5*, and *ACTA2* gene expression in ASMCs were smaller than the strong decreases we found before in lung fibroblasts (19), which are the genes with the strongest decrease upon senescence induction in fibroblasts. The fold changes in ASMCs versus fibroblasts were for *DCN*: 1.26-fold vs 2.81-fold, for *FBLN5*: 1.15-fold vs 1.51-fold, and for *ACTA2*: 1.15-fold vs 3.50-fold. The only previous published study that assessed ECM regulation upon senescence induction in ASMCs found enhanced gene expression of the ECM protein COL1A1 and the ECM proteases MMP-2 and MMP-12 upon hyperoxia-induced senescence (46). None of the ECM genes measured overlapped with our study and these findings were demonstrated in fetal primary ASMCs, whereas we used adult primary ASMCs. So, although PQ induced cellular senescence similarly in ASMCs and lung fibroblasts with eventually higher percentages of SA-β-gal positive cells in ASMCs, the decreases in *DCN* and *FBLN5* gene expression found here in ASMCs were small compared to the strong decreases that we previously observed in fibroblasts.

In vitro growth of primary patient-derived cells favors selection and outgrowth of proliferative cells and thereby reduces the proportion of senescent cells. This may result in an underestimation of the percentages of senescent cells. Therefore, we assessed the differences in cellular senescence between the two cell types at multiple cell passages and confirmed the higher percentages of SA-β-gal positive cells in ASMCs compared to lung

fibroblasts at different passage numbers. As only non-senescent cells will proliferate *in vitro* this also indicates that ASMCs have greater induction of cellular senescence.

In conclusion, cellular senescence is not higher in ASMCs from COPD patients compared to non-COPD controls and there is no link between cellular senescence and *DCN*, *FBLN5*, and *ACTA2* gene expression in COPD-derived ASMCs. These results indicate that, in contrast to lung fibroblasts, higher levels of senescence in ASMCs do not appear to play a major role in COPD pathology. It remains to be elucidated whether ASMCs display other aging features and whether these play a role in aberrant tissue remodeling in COPD.

DISCLOSURES

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Woolcock Institute of Medical Research[1]

Authors' contributions:
Conception and design: RRW, CAB, BGGO
Acquisition and analysis of data: RRW, JB, CAB, BGGO
Interpretation of data: RRW, MdV, IHH, MvdB, WT, CAB, BGGO
Drafting the manuscript: RRW, CAB, BGGO
All authors reviewed, edited, and approved the final manuscript.

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ONLINE SUPPLEMENT

Detailed methods

Subjects

Primary airway smooth muscle cells (ASMCs) and primary lung fibroblasts from subjects undergoing lung transplantation or tumor resection surgery were isolated as previously described (1, 2). All included COPD patients were Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV patients, defined as FEV_1/FVC ratio <70% and FEV_1 % pred <30%. Non-COPD control subjects were included when the FEV_1/FVC ratio was >70%. All subjects were ex-smokers and were matched for age, sex, and smoking history (Table 1). None of the COPD patients was alpha-1 antitrypsin deficient. Ethical approval for all experiments with primary lung cells was provided by the Human Ethics Committees (IRB) of the Sydney South West Area Health Service and St Vincent's Hospital IRB, and written informed consent was obtained.

Table 1: patient characteristics of ASMCs from COPD patients and non-COPD controls

Variable	Control	COPD	P-value
Number	11	10	
Age, mean years (range)	63 (53-75)	58 (44-64)	0.168
Male/female, N	7/4	4/6	0.290
Smoking history	All ex-smokers	All ex-smokers	
Pack-years	40 (26-60)	30 (20-50)	0.359
Stop-months	13 (2-48)	95 (53-138)	0.355
FEV_1 % predicted	92 (75-100)	20 (18-22)	**0.001**
FVC % predicted	94 (77-101)	69 (49-86)	0.079
FEV_1/FVC (%)	78 (77-83)	25 (22-26)	**0.001**

- *Data are presented as medians with IQRs unless otherwise stated*
- *Significant differences between groups were tested using Mann–Whitney U-tests. P-values are boldfaced when significantly different*
- *FEV_1: forced expiratory volume in one second, FVC: forced vital capacity*

Primary cell culture

Primary ASMCs and primary lung fibroblasts were cultured in DMEM (Gibco) supplemented with 5% fetal bovine serum (FBS), 25 mM Hepes buffer (Gibco), and 1% antibiotic-antimycotic (Gibco) at 37°C/5% CO2 as described before (1, 2). For the direct comparison of the two cell types from the same patients, freshly isolated cells were used, which had not been stored in liquid nitrogen yet. For both cell types, 40,000 cells were

seeded in 6-well plates and treated after two days with or without 250 µM Paraquat dichloride hydrate (PQ) (Sigma-Aldrich) for 24 hours to induce cellular senescence (3, 4). After 24 hours, PQ was washed away and cells were kept in culture with DMEM + 5% FBS for another 24 hours (for RNA collection) or four days (for RNA collection, SA-β-gal staining, and secreted proteins). These time-points were carefully chosen based on pilot study results. All cells were used at passage five unless otherwise stated.

Senescence-associated β-Galactosidase staining

Cellular senescence was assessed with standard Senescence-associated β-Galactosidase (SA-β-gal) staining as described before (4, 5). Briefly, cells were fixed with 2% formaldehyde + 0.2% glutaraldehyde in PBS for 3 minutes. After fixation, cells were incubated with the described staining solution for 18 hours (in a dry incubator) at 37°C. After incubation, the staining solution was washed away and cells were covered with 70% glycerol in PBS for storage. DAPI was used for nuclear staining. Four random images of each well were taken using a Nikon microscope at a total magnification of 100x. SA-β-gal positive cells and total cells (DAPI) were scored blindly to calculate the percentage of SA-β-gal positive cells.

Gene expression analyses

To analyze gene expression, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used. RNA samples were collected after 24 hours and four days of PQ removal in RLY buffer (Bioline) + 1% 2-Mercaptoethanol and RNA was extracted using an ISOLATE II RNA Mini Kit (Bioline). cDNA was synthesized using a SensiFAST cDNA Synthesis Kit (Bioline). Gene expression was measured using SensiFAST Probe Hi-ROX Kit (Bioline) and TaqMan gene expression assays (Life Technologies) on a StepOnePlus Real-Time PCR System (Applied Biosystems). 18S rRNA (18S) (Applied Biosystems) was used as an endogenous control. Samples including a no template control as negative control were run in triplicate and 2(-ΔCT) was calculated for relative gene expression levels.

Secreted protein analyses

Cell-free supernatants were harvested four days after PQ removal and stored at -20°C prior to ELISA analysis. Secreted IL-6 and IL-8 levels were measured using Human DuoSet ELISA (R&D Systems). As the numbers of cells were significantly different at the end of culture between untreated and PQ treated, we corrected the secreted protein levels for cell numbers counted at the end of culture.

Western Blot analyses

Cells were lysed in protein lysis buffer including proteases and phosphatase inhibitors four days after PQ removal and after ten minutes of incubation, samples were collected and stored at -20°C. Proteins were separated on a 15% SDS-PAGE gel and transferred on a PVDF 0.2µm membrane using standard wet transfer conditions. Membranes were blocked with 5% BSA (p16) or 5% nonfat dry milk (p21) and incubated overnight at 4°C with

antibodies against p16 (1:1000, Cell Signaling Technology) and p21 (1:1000, Cell Signaling Technology), and GAPDH (1:5000, Merck Millipore) as a loading control. Secondary antibodies conjugated with HRP (Dako) were incubated for one hour at RT and were visualized with Clarity Enhanced Chemiluminescence substrate (Bio-Rad Laboratories) using a ChemiDoc Imager (Bio-Rad Laboratories). Image Lab software (Bio-Rad Laboratories) was used for densitometry (with local background subtraction method) of detected protein bands and protein levels were normalized for GAPDH protein levels.

Statistical analyses

SPSS Statistics 26 software (IBM) was used for statistical analyses. The effect of PQ treatment compared to untreated and the differences between ASMCs compared to lung fibroblasts from the same patients were tested using paired analyses with Wilcoxon signed-rank tests. Mann-Whitney U-tests were used to test differences between COPD patients and non-COPD controls. $P<0.05$ was considered statistically significant.

Supplementary figures

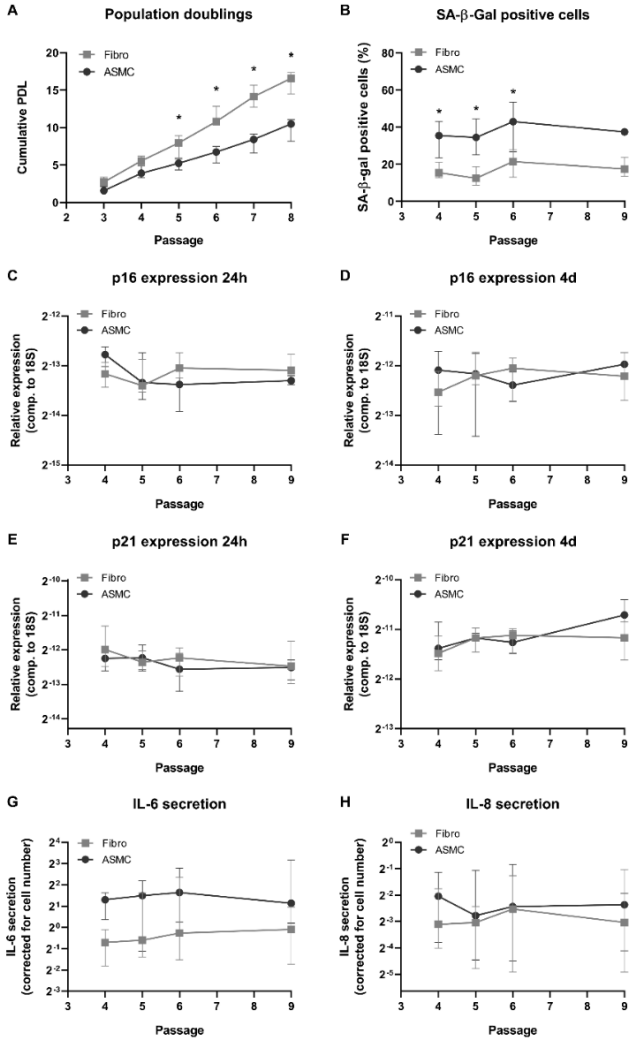

Figure E1: Cellular senescence levels of ASMCs and lung fibroblasts at multiple passages. *Dot plots show baseline levels of cellular senescence markers of ASMCs (circles) and lung fibroblasts (squares) from the same 6 patients at multiple passages. Population doublings of both cell types at multiple passages are shown in the line graph (A). Percentages of SA-β-gal positive cells (B) following 4 days growth in vitro at multiple passages. Gene expression of p16 (C+D) and p21 (E+F) following 24h and 4 days growth in vitro at multiple passages. IL-6 (G) and IL-8 (H) secretion following 4 days growth in vitro corrected for total cell numbers at multiple passages. Fibro = fibroblasts. Passage numbers are indicated on the X-axis of the graphs. We did not have enough cells from 2 donors at passage 4 and 9 (n=4). Differences between ASMCs and fibroblasts from the same patients were tested using Wilcoxon tests. * means P-value <0.05.*

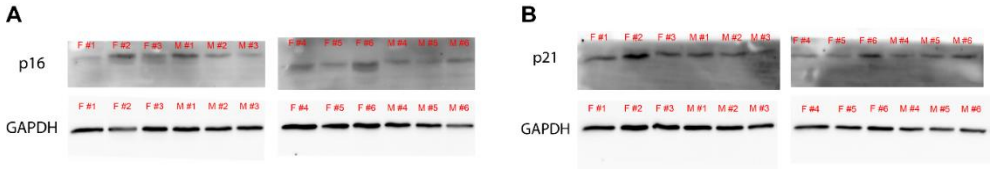

Figure E2: Protein levels of p16 and p21 in lung fibroblasts and ASMCs. *Images of Western Blots for p16 (A) and p21 (B) are shown with samples from ASMCs and lung fibroblasts from the same 6 patients at baseline. GAPDH was used as loading control. Quantifications are shown in Fig. 2E+F.*

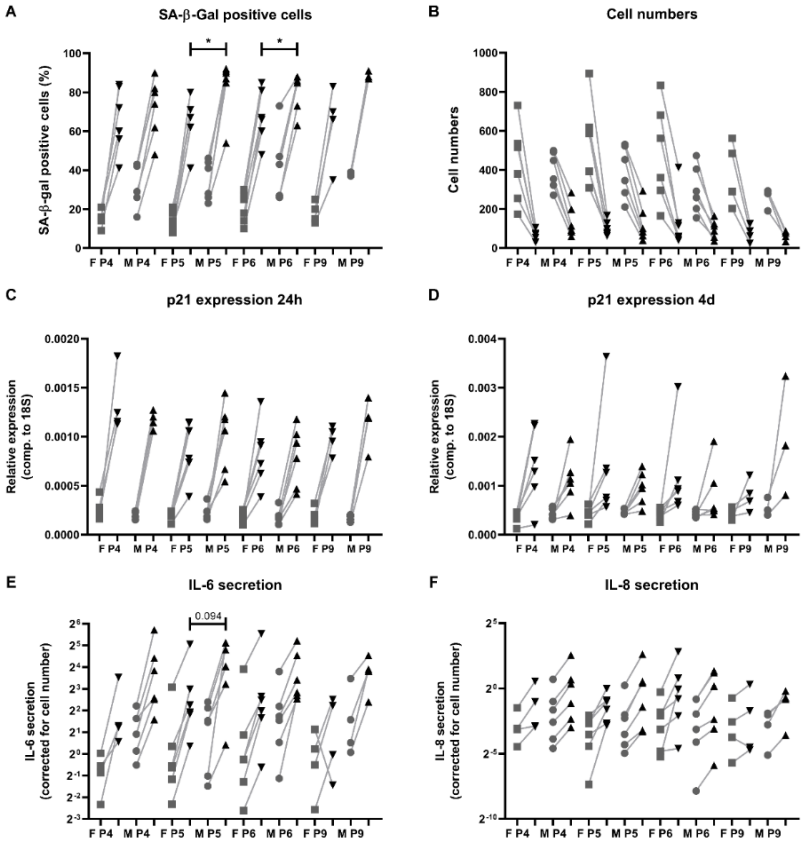

Figure E3: Paraquat-induced senescence of ASMCs and lung fibroblasts at multiple passages. *Dot blots show senescence markers at baseline levels (squares and circles) and upon PQ-induced senescence (triangles) in ASMCs and lung fibroblasts from the same 6 patients at multiple passages. Percentages of SA-β-gal positive cells (A) and total cell numbers (B) at baseline and 4 days post PQ removal at multiple passages. Gene expression of p21 at baseline and 24h (C) and 4 days (D) post PQ removal at multiple passages. IL-6 (E) and IL-8 (F) secretion levels at baseline and 4 days post PQ removal corrected for total cell numbers at multiple passages. F = fibroblasts, M = ASMCs. Differences between ASMCs and fibroblasts from the same patients were tested using Wilcoxon tests. * means P-value <0.05.*

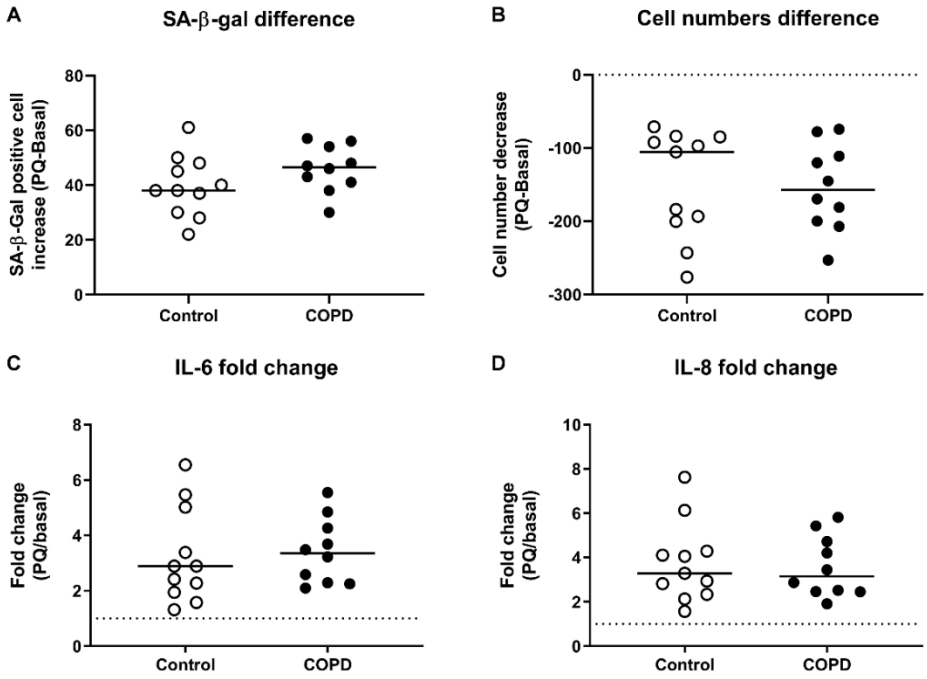

Figure E4: Fold changes upon PQ-induced senescence in airway smooth muscle cells from COPD patients and non-COPD controls. *Dot blots show fold changes upon PQ treatment in ASMCs from COPD patients (n=10) (solid circles) compared to non-COPD control (n=11) (open circles). Increase in percentages of SA-β-gal positive cells (A) and a decrease in total cell numbers (B) 4 days post PQ removal. Fold changes in IL-6 (C) and IL-8 (D) secretion 4 days post PQ removal corrected for total cell numbers. Differences between COPD and non-COPD controls were tested using Mann–Whitney U tests. * means P-value <0.05.*

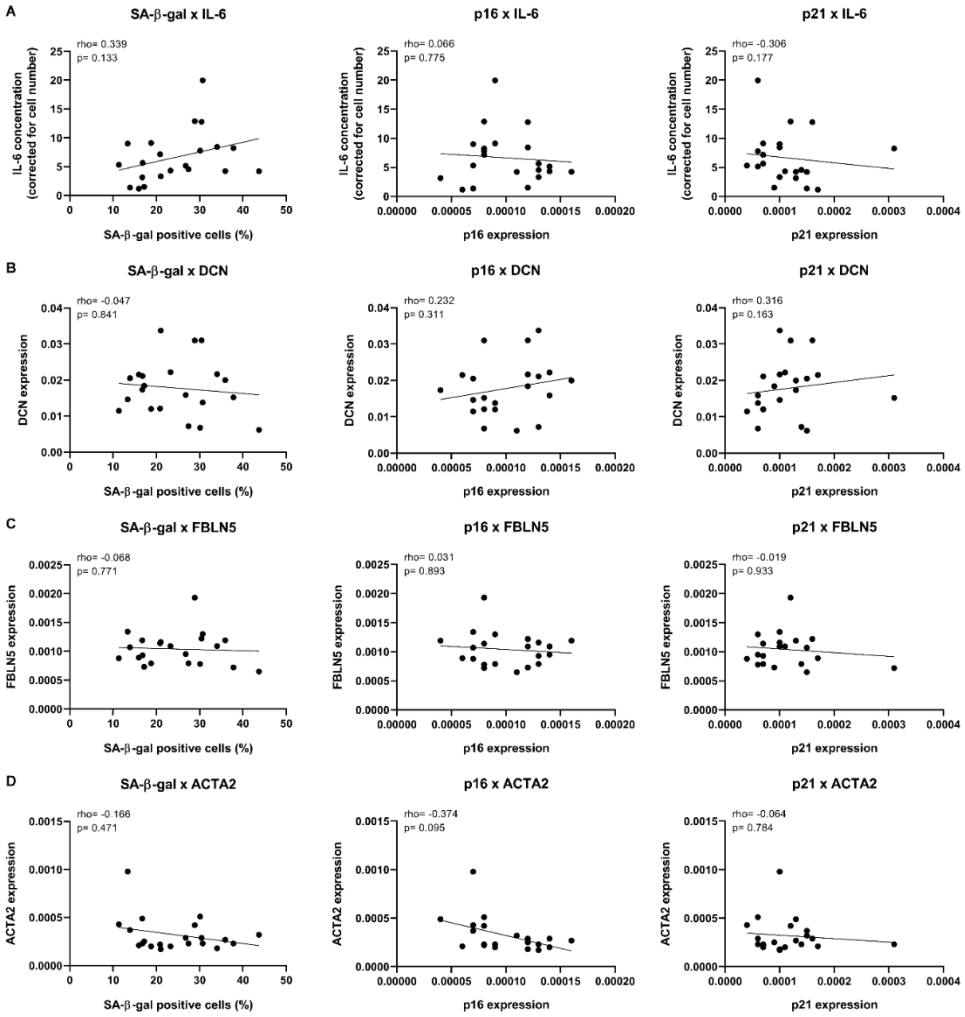

Figure E5: Correlation between cellular senescence and IL-6 or ECM gene expression. *Dot plots show correlations between cellular senescence markers (SA-β-gal, p16 & p21) and IL-6 secretion (A), DCN (B), FLBN5 (C), or ACTA2 (D) gene expression. Significant correlations were tested using Spearman's tests. Spearman's Rho and p-value are indicated in the left top corner of the graphs.*

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CHAPTER 7

E-cigarette vapour induces cellular senescence in lung fibroblasts and may contribute to lung pathology

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Research letter submitted

To the editor,

INTRODUCTION

COPD is a progressive inflammatory lung disease caused by exposure to noxious gases, in particular cigarette smoke. Chronic exposure to cigarette smoke (CS) causes chronic inflammation and eventually leads to lung tissue damage. Ultimately, these pathologic events lead to airway fibrosis and airflow limitation, i.e. chronic bronchitis, and the alveolar breakdown and lack of tissue repair in the parenchyma, i.e. emphysema (1).

Cellular senescence has been recognized to play a role in the pathophysiology of COPD (2). Cellular senescence is defined as an irreversible cell cycle arrest to prevent cell death or abnormal growth. Induction of cellular senescence can be caused by multiple mechanisms, including oxidative stress and DNA damage, both known to result from chronic CS exposure (3). Accumulation of senescent cells in lung tissue can result in chronic inflammation and tissue dysfunction (4) and as such contribute to COPD pathology.

Electronic cigarettes have been proposed as a safer alternative to cigarettes. Evidence of the harms related to E-cigarette use is growing. Similar to CS exposure, E-cigarette exposure causes a pro-inflammatory response after acute and chronic exposure in multiple structural lung cells *in vitro*, *in vivo* in mouse lungs and in human clinical studies (5, 6), suggesting prolonged use may also contribute to COPD. Furthermore, E-cigarette vapour exposure has been shown to induce DNA damage and reduce DNA damage repair in lung epithelial cell lines (5). Oxidative stress from E-cigarette vapour exposure has been studied to a greater extent *in vitro* and *in vivo,* with the majority demonstrating a high oxidative burden from E-vapour exposure. Although E-cigarette use may upregulate known senescence inducers, DNA damage and oxidative stress, it is unknown whether E-vapour can actually induce cellular senescence. Therefore, we investigated whether E-vapour exposure induces cellular senescence in primary lung fibroblasts and whether this affects their tissue repair function.

METHODS

Primary parenchymal lung fibroblasts (n=11) were grown in DMEM (Gibco) supplemented with 5% foetal bovine serum (FBS) as described previously (7). At passage 5-6, cells were seeded on 6 well plates (for collection of supernatants, RNA & SA-β-gal staining) and on 12 well plates (for wound healing assay). Cells were serum-starved in 0.5% FBS DMEM after 48 hours, and 24 hours later cells were stimulated with 250 μM Paraquat (PQ; positive control for senescence induction), 5% cigarette smoke extract (CSE), 1.5% (Lo) or 2% (Hi) nicotine-containing (18mg/ml) tobacco-flavoured E-cigarette Vapour extract (EV), or 1.5% (Lo) or 2% (Hi) nicotine-free tobacco-flavoured E-cigarette Vapour extract (NF EV) as described previously (8). A cytotoxic dose for CSE (10%) and both EV (5%) was used as a

positive control for the stimuli (data not shown). Supernatants (for IL-8 ELISA) and RNA extracts were collected after 24 hours of stimulation, whilst remaining plates were refreshed to 5% FBS DMEM to enable cell proliferation for 3 days. Cellular senescence was assessed by SA-β-gal staining, cell proliferation inhibition, and p16 and p21 gene expression as described previously (9). Wound healing assays were performed 4 days after stimulation as described in the figure legend.

RESULTS

To confirm the stimulatory response of the different stimuli on primary lung fibroblasts, IL-8 secretion was measured, which was significantly induced by all stimuli compared to untreated fibroblasts (Fig. 1A). Cellular senescence was induced by PQ and CSE with an increase in p21 expression (Fig. 1B) and percentages of SA-β-gal positive cells (Fig. 1C), and reduced cell proliferation (Fig. 1D). For both no increase in p16 expression was found (data not shown). CS is a known risk factor for COPD and both stimuli are known to induce senescence, confirming our model's validity.

Upon stimulation with nicotine-containing E-vapour extract (EV), cellular senescence was induced with significant differences in the same senescence markers as PQ and CSE, which was dose-dependent (Fig. 1B-D). This induction in cellular senescence by E-vapour appeared to be nicotine-independent as stimulation with nicotine-free E-vapour extract (NF EV) also increased p21 expression and the percentages of SA-β-gal positive cells, and reduced cell proliferation. Only upon the low dose of NF EV stimulation, no significant p21 increase was observed (Fig. 1B). Similar to PQ and CSE, no increase in p16 expression was found after NF EV and EV stimulations (data not shown).

Upon senescence induction by PQ and CSE, impaired repair in a wound healing model occurred after 48 hours and 72 hours (Fig.2). Low dose EV impaired wound healing capacity with reduced wound closure after both 48 and 72 hours, which again appeared to be nicotine-independent as similar results were found in NF EV treated cells. No significant reduction in wound closure was found upon EV Hi stimulation, due to one donor with improved wound closure upon stimulation

To confirm our in vitro results *in vivo*, gene expression of p21 was measured in a previously performed mouse model (10), where p21 expression seemed higher upon exposure compared to non-exposed controls (mean fold change + SEM: 1.3+0.09 (EV) and 1.2+0.2 (NF EV) vs SHAM). However, no significant differences were found as this pilot study was underpowered, thus larger *in vivo* studies should be done to confirm our *in vitro* findings.

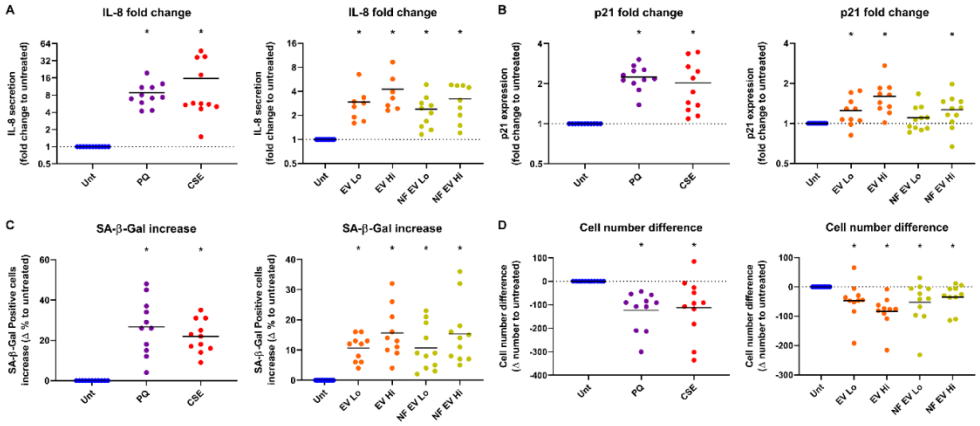

Figure 1: Cellular senescence induction upon stimulation with known senescence-inducers and upon E-vapour extract stimulation. *Differences in IL-8 secretion (A) in cell culture supernatants, p21 gene expression (B), percentages of SA-β-gal positive cells (C) and total cell numbers (D) are shown compared to untreated (Unt, blue) upon stimulation with 250µM paraquat (PQ, purple) 5% cigarette smoke extract (CSE, red) and high and low doses of nicotine-containing (EV Lo and Hi, orange) and nicotine-free E-vapour (NF EV Lo and Hi, yellow) extracts. N=11 per group. Lines represent means and dotted lines represent levels of untreated. Lo= low dose: 1.5% and Hi= high dose: 2%. Significant differences between stimulated and untreated fibroblasts were tested using One-Way ANOVA with Fisher's LSD tests, * means P-value < 0.05 compared to untreated.*

DISCUSSION

This study is the first to identify E-cigarette vapours' potential to induce cellular senescence in primary lung cells, which is a known contributing factor to disease in COPD (2). The findings of our study further add to the identified risks of E-cigarette use (5). E-cigarette harms are often compared to cigarettes in relation to harm reduction, but this study focused on the standalone risk for E-cigarette users. These risks are not isolated to COPD patients with other E-cigarette users like young never-smokers, more likely to develop lung pathology from long-term use.

In the current study, we did not investigate the mechanisms of senescence induction by E-vapour extract, but we hypothesize that DNA damage and oxidative stress may be involved as previous studies demonstrated that E-cigarette vapour exposure can result in DNA damage and oxidative stress (5). Future studies should elucidate these mechanisms involved in EV-induced senescence and whether particular components of E-liquids are directly causing up-regulation of pathological processes in the lung.

E-cigarettes' potential to induce cellular senescence, alongside other previously identified risks, should serve as a warning to avoid use as a safe alternative to cigarette smoking or as a cessation device. Considering senescence induction was dose-dependent indicates excessive and long-term use should be avoided.

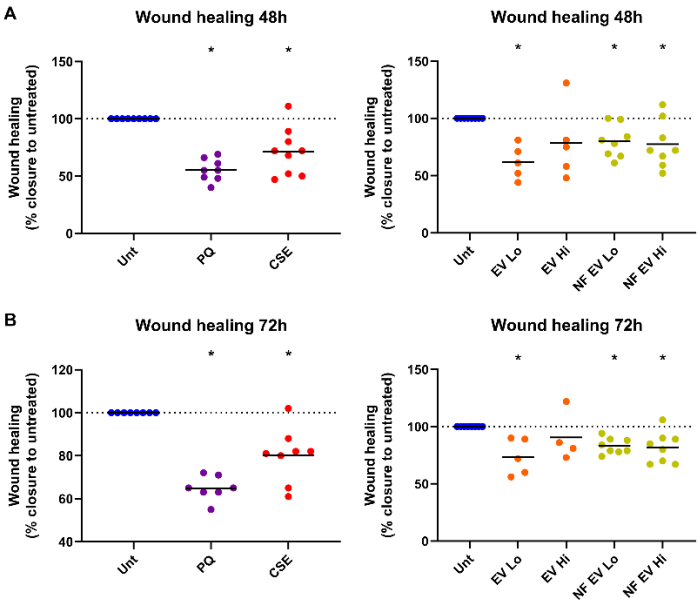

Figure 2: Cellular senescence induction resulted in an impaired wound healing capacity. *Wound healing capacity was assessed 4 days after stimulation using the scratch assay by measuring wound closure after 48 and 72 hours of scratching stimulated and untreated fibroblasts. A wound in the cell layers was made by scratching with a p200 pipet tip in the middle of the well from top to bottom, wells were washed twice with Hanks buffer and DMEM + 0.5% FBS was added to enable wound closure, which was captured on a Nikon Eclipse Ti microscope at total magnification of 40x. Percentages of wound closure after 48 hours (A) and 72 hours (B) compared to untreated (Unt, blue) are shown upon stimulation with 250µM paraquat (PQ, purple) 5% cigarette smoke extract (CSE, red) and high and low doses of nicotine-containing (EV Lo and Hi, orange) and nicotine-free vapour (NF EV Lo and Hi, yellow) extracts. N=11 per group. Lines represent means and dotted lines represent levels of untreated. Lo= low dose: 1.5% and Hi= high dose: 2%. Significant differences between stimulated and untreated fibroblasts were tested using One-Way ANOVA with Fisher's LSD tests, * means P-value < 0.05 compared to untreated.*

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Drafting the manuscript: RRW, JB
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CHAPTER 8

Summary, general discussion and future perspectives

SUMMARY

Since the pathogenesis of COPD is unclear and no treatment options are available to reduce disease progression, novel insights into the pathogenesis of COPD are urgently needed, such as elucidating the role of accelerated ageing in COPD. I hypothesized that accelerated ageing contributes to the pathogenesis of COPD, especially in the relatively young, severe, early-onset COPD (SEO-COPD) patients. These SEO-COPD patients represent a subgroup of COPD with a significant proportion of hospital admissions and healthcare costs. Recently, dysregulation of extracellular matrix (ECM) homeostasis has been described as an additional hallmark for lung ageing, although it is unknown whether accelerated ageing contributes to ECM dysregulation in COPD. Therefore, the overall aim of this thesis was to elucidate the role of accelerated ageing specifically in lung tissue repair and remodelling in COPD.

In **chapter 2**, the overlap between lung ageing and COPD was described and all data on ageing in COPD as available at the start of this thesis was reviewed. This comprehensive review demonstrates that most ageing hallmarks are observed in lung tissue from COPD patients, indicating that accelerated ageing may play a role in COPD. However, this overview also identified a lack of evidence as to whether accelerated ageing occurs in lung fibroblasts and airway smooth muscle cells from COPD patients and whether this impacts their functional roles in repair, remodelling and ECM regulation. Moreover, since no data were available including relatively young patients or SEO-COPD, this thesis is more focused on this group of patients with the hypothesis that accelerated ageing may especially play a role in SEO-COPD.

In **chapter 3**, potential gene-miRNA interactions were discovered that may play a role in normal ageing of the airways. Interestingly, genes that had a lower expression with age were part of pathways involved in three ageing hallmarks, including genomic instability, cellular senescence and altered intercellular communication, implicating a particular role for these ageing hallmarks in normal lung ageing.

In **chapter 4**, COPD-derived fibroblasts displayed features of accelerated ageing compared to non-COPD controls, with higher levels of cellular senescence, DNA damage and oxidative stress. Interestingly, some of these effects were most pronounced in fibroblasts from SEO-COPD patients. The observed increase in cellular senescence was correlated with lower gene expression of the ECM protein decorin (*DCN*) in COPD-derived fibroblasts. In addition, Paraquat-induced cellular senescence resulted in changes in ECM gene expression, including decreased *DCN* expression. Our study showed a clear link between cellular senescence and ECM dysregulation in COPD.

To give more insight into the potential consequences of accelerated ageing in lung fibroblasts, the senescence-associated secretory phenotype (SASP) of senescent COPD-derived lung fibroblasts was assessed in **chapter 5**. 124 SASP proteins from primary

lung fibroblasts upon senescence induction were identified. 42 of these proteins were secreted at higher levels by COPD-derived fibroblasts compared to non-COPD controls, and 35 were secreted at higher levels by SEO-COPD-derived fibroblasts compared to their matched non-COPD controls. Interestingly, multiple COPD-associated SASP proteins have been implicated in chronic inflammation, and as such might contribute to COPD pathogenesis.

In **chapter 6**, cellular senescence levels at baseline were higher in airway smooth muscle cells (ASMCs) compared to lung fibroblasts, but were not different between ASMCs from COPD patients compared to non-COPD controls. No link between cellular senescence and ECM gene expression in COPD-derived ASMCs was found. These results indicate that, in contrast to lung fibroblasts, higher levels of senescence in ASMCs do not appear to play a major role in COPD pathology.

Finally, in **chapter 7**, the potential of E-cigarettes, commonly used by COPD patients, to induce cellular senescence was assessed. E-vapour exposure induced cellular senescence in primary human lung fibroblasts. In addition, senescence induction by E-vapour exposure, similar to cigarette smoke exposure, and paraquat treatment resulted in an impaired wound healing capacity. Hence, E-cigarette vaping appears not a safe alternative for cigarette smoking and might even contribute to accelerated lung ageing and pathology.

A summary overview of all main findings in the studies of this thesis is depicted below in Figure 1.

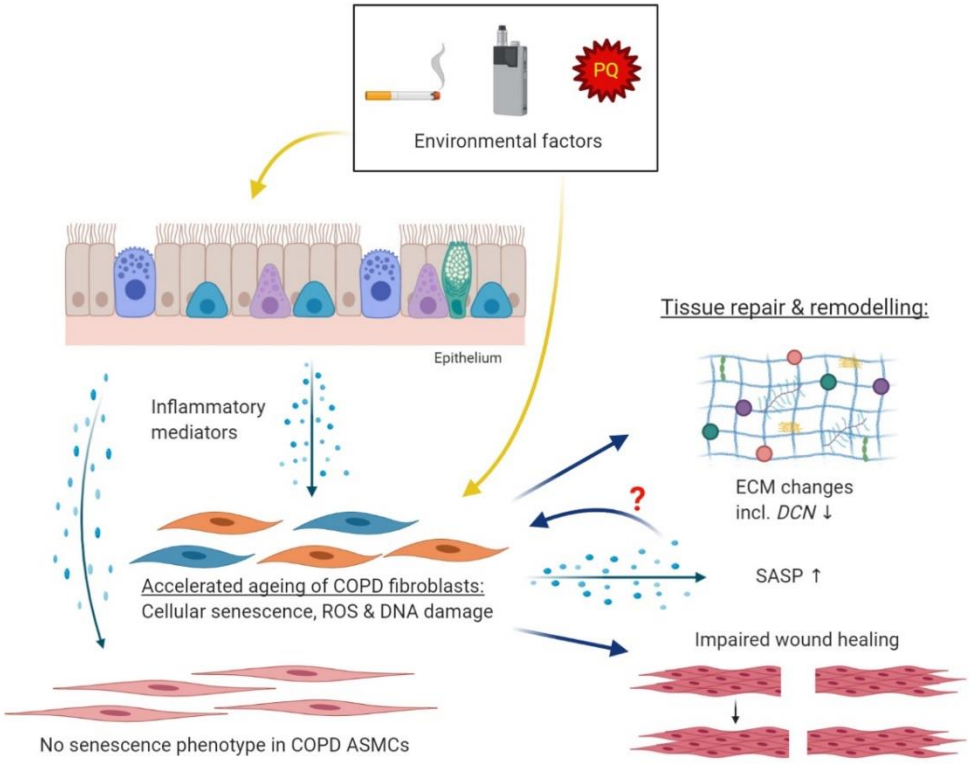

Figure 1: Overview of the role of accelerated ageing in lung tissue repair and remodelling in COPD. *Environmental factors, including cigarette smoke, E-cigarette vapour and Paraquat (PQ), can induce features of accelerated ageing (cellular senescence) in lung fibroblasts. COPD-derived fibroblasts have features of accelerated ageing with higher levels of cellular senescence, reactive oxygen species (ROS) and DNA damage. This accelerated ageing may be a direct result of environmental factors or indirect via induction of a pro-inflammatory environment in the lungs, for example, by secretion of inflammatory mediators by epithelial cells. Higher levels of cellular senescence resulted in extracellular matrix (ECM) changes, including lower DCN expression, higher SASP protein secretion and impaired wound healing capacity, and thus affect the tissue repair and remodelling functions of lung fibroblasts. These functional consequences have been implicated to play a role in COPD pathogenesis. Created with BioRender.com*

GENERAL DISCUSSION & FUTURE PERSPECTIVES

Cellular senescence in COPD

Cellular senescence can be induced by multiple factors, including environmental and lifestyle factors that are of relevance in COPD. Most of these factors induce cellular senescence via an increase in oxidative stress, mitochondrial dysfunction or DNA damage. Cigarette smoke exposure, the major risk factor of COPD, has been extensively described to induce oxidative stress, DNA damage, telomere dysfunction and cellular senescence *in vitro* in multiple structural lung cells (as reviewed in **chapter 2**) and also *in vivo* in mouse lungs (1-4). Both oxidative stress and DNA damage were higher in (SEO-) COPD-derived fibroblasts (**chapter 4**), which suggests that one or both of these stress factors may have caused the higher levels of cellular senescence observed in these cells. Future studies should assess whether one or both caused the induction in senescence by specifically inducing these stress factors separately, for example by radiation (DNA damage), and specifically reducing these stress factors separately, for example by anti-oxidants (oxidative stress), and assess the effects on senescence induction.

E-cigarettes are used as smoking cessation or replacement device and assumed to be a safer alternative than smoking cigarettes. Previous studies have already demonstrated that *in vitro* in multiple structural lung cells and *in vivo* in mouse lungs exposure to E-cigarette vapour can induce inflammation, oxidative stress and DNA damage (5, 6). In **chapter 7**, we showed that E-cigarette vapour exposure induces cellular senescence in primary human lung fibroblasts. The induction of senescence by E-cigarettes appeared to be nicotine-independent, but further studies should be done to elucidate the mechanisms that are involved in the induction of senescence, for example, oxidative stress and DNA damage. Moreover, future studies should assess the chronic effects of E-cigarettes as limited data on chronic use is available.

Only a few studies have been done to assess senescence induction by air pollution like particulate matter. Particulate matter has been demonstrated to induce inflammation and oxidative stress *in vivo* in mouse lungs (7-9). Till now, induction of cellular senescence has only been shown in fine particulate matter (PM2.5) treated corneal epithelial cells and keratinocytes (10, 11). Hence, future studies need to be done to assess the senescence inducing potential of air pollution in the lungs.

Finally, the inflammatory and fibrotic environment of COPD lungs can enhance the senescence phenotype as well, for example TGF-β, one of the best-known COPD-associated proteins (12, 13), has been demonstrated to be able to induce senescence (14, 15).

In summary, most of the environmental and lifestyle factors that are risk factors for COPD have the potential to induce cellular senescence in structural lung cells, which eventually may contribute to COPD pathogenesis. An important question that remains to

be answered, is whether the induction of cellular senescence in COPD-derived lung fibroblasts is caused directly by the environmental and lifestyle factors or by the pro-inflammatory environment in the lungs, for example, by inflammatory mediators secreted by epithelial cells. Therefore, experiments with co-cultures of exposed epithelial cells with lung fibroblasts or treatments of lung fibroblasts with conditioned media from exposed epithelial cells should be performed. It would also be of interest to assess whether exposed epithelial cells from COPD patients and non-COPD smokers provoke a different response in co-cultured or conditioned media treated lung fibroblasts.

Accumulation of senescent fibroblasts in lung tissue may be caused by an enhanced induction of cellular senescence and/or reduced clearance of senescent cells by immune cells and may have detrimental effects on the surrounding lung tissue. In **chapter 4**, higher levels of cellular senescence were observed in both lung tissue and lung fibroblasts from COPD patients, indicating that in COPD lung tissue senescent cells accumulate, including senescent fibroblasts. In this study, it could not be determined whether this accumulation is caused by an enhanced senescence induction or reduced senescence clearance or both. Till now, data assessing the capacity of senescence clearance by immune cells in COPD patients is very limited. Immune cells that can clear senescent cells by phagocytosis are macrophages, and cells that can induce apoptosis of senescent cells are NK cells and T cells, but the exact mechanisms are still not clear (16, 17). Numbers of macrophages are higher in lung tissue from COPD patients compared to smokers without COPD, which was associated with disease severity (18, 19), while levels of macrophage attractant MCP-1 were found to be higher in sputum (20) and BAL fluid (21) from COPD patients. Despite higher numbers of macrophages, the phagocytosis capacity of pathogens of COPD-derived alveolar macrophages was found to be reduced compared to non-COPD controls (22-25). Furthermore, levels of the senescence marker p21 are increased in macrophages from smokers (26), which indicates an impaired function of these cells upon smoking, but higher levels of senescence in COPD-derived macrophages have not been demonstrated yet. These studies suggest that clearance of senescent cells might be reduced in lung tissue from COPD patients as well, but future studies should assess the potential of senescent cell clearance of COPD-derived immune cells. These studies should include co-culture models of senescent cells (fibroblasts) with COPD-derived immune cells like macrophages to answer this question.

Functional consequences of senescence accumulation in COPD lung tissue

Cellular senescence is an important homeostatic mechanism, but accumulation of senescent cells can lead to pathology. Transient occurrence and clearance of senescent cells are part of normal physiology by contributing to embryogenesis, tissue development, and normal tissue repair and remodelling (27-30). The detrimental effects of cellular

senescence are caused by an accumulation of senescent cells. These effects may be the result of altered functions of the senescent cell itself and by the detrimental effects of the SASP. Cellular senescence in lung fibroblasts resulted in differential ECM expression (**chapter 4**) and an impaired wound healing capacity (**chapter 7**), which both are implicated in COPD pathogenesis. While little was known about ECM regulation in senescent cells in the lungs, several studies have found altered ECM and matrix metallopeptidase (MMP) regulation in senescent fibroblasts derived from other tissues like dermal and foreskin fibroblasts. Most of these studies have shown an increase in MMP secretion (31-33), which can degrade ECM proteins, and in general a decrease in expression of collagen genes (32-36). Therefore, this suggests that cellular senescence can directly affect the repair and remodelling function of fibroblasts. As cultures of senescent cells include non-senescent cells as well it cannot be excluded that the observed differences might be driven by the effect of senescent cells on neighbouring non-senescent cells. Sorting a pure population of senescent cells may answer this question. Since most of the current studies only describe an association between senescence and ECM changes or an effect of senescence on gene expression instead of ECM protein deposition and structure, future studies need to be done to elucidate the exact effect of cellular senescence in lung fibroblasts on the matrix biology in COPD lungs. It would be of interest to assess whether newly formed ECM from senescent lung fibroblasts is different in levels and biomechanical properties compared to non-senescent fibroblasts. In addition, whether these senescent fibroblasts change the existing ECM would be of interest as well.

The potential detrimental effects of SASP proteins on surrounding lung tissue include a wide range of processes; ECM interference, chronic inflammation, paracrine senescence, epithelial-mesenchymal transition (EMT) and tumorigenesis (27, 29, 37). In **chapter 5**, multiple SASP proteins were identified in senescent primary lung fibroblasts and COPD-associated SASP proteins that have been implicated in these detrimental processes. Firstly, multiple enzymes and proteases like MMPs that were identified as SASP proteins can have a direct effect on lung ECM, including MMP-2, -3, -9, -10. Proteases that were found to be secreted at higher levels by COPD-derived fibroblasts compared to non-COPD controls include MMP-9 and t-PA. Both have been implicated in ECM degradation, where MMP-9 can degrade collagen (38, 39) and decorin (40, 41), while tPA activates plasminogen and MMPs and thereby provokes ECM breakdown (42). Secondly, the SASP of senescent lung fibroblasts and the SASP of COPD-derived fibroblasts contained various cytokines and chemokines that have been implicated in inflammation. Several of our identified SASP proteins are COPD-associated inflammatory mediators, including the COPD-associated SASP proteins CCL15 and CXCL9 (43-45). Thirdly, single SASP proteins and also the full SASP profile can induce paracrine senescence, where the full SASP has been demonstrated to induce senescence in surrounding cells *in vitro* in multiple cell lines (46,

47) and even *in vivo* in mouse lungs (48, 49). Although paracrine senescence was observed in multiple tissues including liver, stroma, colon and muscle, paracrine senescence by the SASP has not been demonstrated in lungs yet. Fourthly, multiple studies that co-cultured senescent cells with non-senescent cells or treated cells with conditioned media of senescent cells found that SASP can induce cell growth (27, 50-54) and characteristics of tumorigenesis, including morphogenesis, migration, invasion and angiogenesis (51, 53, 55-57). Whether the SASP can play a role in tumorigenesis in the lungs is still unknown and thus it remains to be elucidated whether the SASP may have a potential role in the COPD-lung cancer overlap. Finally, the SASP has also been demonstrated to cause EMT (58), which leads to a loss of epithelial function, where senescent fibroblasts and conditioned media from senescent fibroblasts caused EMT in human and mouse breast cancer cells (27, 51). Whether SASP protein secretion by COPD-derived lung fibroblasts can cause EMT of epithelial cells in COPD lungs has not been studied yet. In summary, the SASP has been demonstrated to induce detrimental effects on the surrounding tissue in multiple organs, but studies on the detrimental effects of the SASP (from senescent fibroblasts) on surrounding lung tissue are limited. Therefore, future studies should focus on the detrimental effects of the SASP proteins on the different structural lung cells and whether these effects are driven by a particular group of proteins or by the complete SASP composition.

In preliminary studies, I studied the potential autocrine effect of the SASP proteins secreted by senescence-induced primary lung fibroblasts. Conditioned media containing the SASP of senescent lung fibroblasts was used to treat naïve lung fibroblasts. Very low to no effects of senescent fibroblast-derived conditioned media were observed, with a small increase in cellular senescence in some donors and no effect on inflammation, ECM gene expression and wound healing capacity. Based on the preliminary findings, I expect that the SASP of senescent lung fibroblasts is more likely to have detrimental effects in a paracrine manner affecting its surrounding cells. For future studies, the effect of senescent fibroblasts on surrounding lung tissue needs to be addressed by co-culture models and treatment of different lung-derived cells with conditioned media from senescent fibroblasts. Furthermore, the effect of single COPD-associated SASP proteins, which are secreted at the highest levels, on lung-derived cells should be assessed to get more insight into the paracrine effect of senescent lung fibroblasts on COPD lung tissue.

Accelerated ageing in COPD

Although accelerated ageing has widely been described to contribute to COPD pathogenesis, causality has not been demonstrated and remains challenging. The fact that features of ageing were observed at a relatively young age (SEO-COPD) already, supports the hypothesis that accelerated ageing is involved in the disease. Since many

ageing markers are increased upon cigarette smoking as well, like oxidative stress, DNA damage, and cellular senescence, proving that accelerated ageing contributes to COPD pathogenesis or is a result of the disease is difficult. To minimize the effect of smoking, ex-smoking COPD patients were compared to ex-smokers who did not develop COPD in the studies in this thesis. However, this does not exclude that features of ageing may be a result of disease and not the cause of disease. To get more insight into this, our study and some other studies compared COPD patients with different Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages, showing that cellular senescence, telomere length, oxidative stress, mTOR activity and loss of proteostasis were associated with disease severity (59-65). In accordance, correlations between disease severity (FEV_1 % predicted) and cellular senescence, oxidative stress, and DNA damage were found in **chapter 4**. These results suggest that these ageing processes may contribute to disease progression.

An important question is whether accelerated ageing can be a driver of the development of COPD. Since in our studies samples from end-stage of disease were used, this question could not be answered. In mouse models, spontaneous emphysema occurs upon telomerase knockout and knockout of the anti-ageing gene Klotho (66-68). Genetic alterations of age-related repair mechanisms can enhance emphysema development in combination with cigarette smoke exposure, including a knockout of the anti-oxidant gene *NRF2* and knockdown of anti-ageing gene *SIRT1* (69, 70). Whether these age-related mechanisms can drive COPD development in humans is not known yet. So, while many studies suggest that accelerated ageing may contribute to the progression of the disease, it remains unclear whether accelerated ageing can be a driver of the development of COPD. Exposure to cigarette smoke and other noxious gases has been recognized to cause chronic inflammation and lung tissue damage in the development of COPD, but as not all smokers develop COPD it remains unclear which processes are involved in the onset of disease in combination with the exposures. Accelerated ageing might be one of these driving processes in COPD development. Confirming this hypothesis is difficult because most studies are done in patients that already developed symptoms. Therefore, longitudinal studies should be done in patients with no clinical disease yet who are at risk to develop COPD, because of the exposure to known COPD inducing stimuli, to assess ageing markers before the onset of disease. However, these studies also have their downsides in that they take a long time to finish, sample collection for example by bronchoscopy is invasive and large number of smoking individuals need to be included as not all smokers develop COPD and this is unpredictable. Studying the role of accelerated ageing in cohort studies including families with smokers who are diagnosed with COPD and families with smokers without COPD may help to reduce the study duration and to include relevant subjects. Examples of such studies are the COPDGene study (71), the

ECLIPSE study (72), and a multicentre COPD susceptibility study (73, 74), which are studies of interest to assess the role of accelerated ageing in COPD development.

Why (SEO-) COPD-derived fibroblasts display more features of accelerated ageing remains an important unanswered question. On a cellular level, accelerated ageing is thought to be the result of environmental and inflammatory damages and impaired repair mechanisms (75, 76). Cigarette smoke causes cellular damages via oxidative stress, mitochondrial dysfunction, DNA damage, and telomere dysfunction (2, 67, 77-80). In **chapter 4**, higher levels of oxidative stress and DNA damage in COPD-derived fibroblasts compared to non-COPD controls were observed. Since not all smokers develop COPD, an impairment in cellular repair and maintenance mechanisms has been hypothesized to result in a predisposition for accelerated ageing (81-83). The causes of these impaired mechanisms in COPD are largely unknown, but postulated to be influenced by genetic and epigenetic backgrounds (83, 84). Previous studies observed a reduction in protective repair mechanisms in lung tissue and epithelial cells from COPD patients, including reduced DNA damage repair, reduced telomerase activity, loss of proteostasis (autophagy and mitophagy), and reduced anti-oxidant as reviewed in **chapter 2**. In **chapter 4**, some markers of DNA damage repair and autophagy in COPD-derived fibroblasts were assessed, but no differences were observed between COPD and non-COPD at baseline. The limitations of this study were that only a few markers were selected to assess these repair mechanisms, measured at a single time-point, and that isolation and *in vitro* culture may have caused a loss of difference between COPD and non-COPD at baseline. Upon stimulation with Paraquat, which is a COPD risk factor by occupational exposure, COPD-derived fibroblasts were less capable to respond to the damage with lower up-regulation of the oxidative stress response genes *FOXO3* and *MGST1* compared to non-COPD derived fibroblasts. These results support the hypothesis that impaired repair mechanisms may cause a predisposition for accelerated ageing in COPD, but which exact mechanisms are involved needs to be elucidated. The genetic and epigenetic background of these patients may cause this predisposition. A chronic exposure *in vitro* model with cells derived from healthy smokers and COPD smokers may reveal repair and maintenance mechanisms that are involved in accelerated ageing, for example, autophagy, mitophagy or DNA damage repair. Upon unravelling these exact mechanisms that are involved in impaired repair in COPD-derived fibroblasts, therapeutic targets that restore these repair functions can be discovered.

Clinical implications and potential therapies

First of all, smoking cessation is at the moment the best option to prevent COPD development as cigarette smoke is a major source of oxidative stress and inducer of cellular senescence. E-cigarette vaping is becoming more popular as an alternative for cigarettes and as a cessation device, while younger individuals also start vaping without

having smoked cigarettes before. In **chapter 7**, E-cigarette vapour appeared to be not harmless and might contribute to COPD pathology as it induces cellular senescence. Thus, E-cigarette use should be avoided as well. Since smoking cessation may be challenging for addicted smokers and 25-45% of COPD patients are non-smokers (85), therapeutics to stop the disease progression need to be developed as well. Therefore, interfering in the processes of accelerated ageing may be a promising approach for COPD patients. Currently, many studies are assessing the efficacy of anti-ageing drugs to improve healthy ageing and to prevent and treat age-related diseases. Targeting senescent cells by senostatics that prevent induction of cellular senescence and senolytics that specifically kill senescent cells, seem promising approaches for multiple age-related diseases including COPD.

Senostatics are drugs that improve cellular repair mechanisms to prevent an accumulation of damage, which eventually prevents cellular senescence induction. The repair mechanisms that can or may be improved are DNA damage repair, telomerase activity, the proteostasis, mitochondrial function and normal nutrient sensing. Limited studies have been done to investigate the potential of improving DNA damage repair, telomerase activity and the proteostasis in COPD. Only one study showed that activation of an autophagy transcription factor reduced cigarette smoke-induced oxidative stress, cellular senescence and emphysema features in mice (86). Tested therapies that improve mitochondrial function are mainly anti-oxidants to reduce oxidative stress, but these are not clinically effective in COPD yet (87). Therapeutic approaches that are studied in more detail and seem to be beneficial are reduction of nutrient-sensing activity and activation of anti-ageing regulators. Inhibition of mTOR via rapamycin, AMPK activation via metformin and SIRT1 activation have all been found to reduce cellular senescence *in vitro* and *in vivo* and improve the lifespan of mice (88-92), but beneficial treatments in human clinical trials have not been demonstrated yet, while rapamycin has major side-effects (91). Regarding COPD, only AMPK activation reduced the mortality rate in patients with diabetes, but not in COPD patients without diabetes (93). So, senostatics may potentially be beneficial for COPD patients, but limited treatment options are currently available and only limited studies have been done in COPD. Furthermore, since the mechanisms that contribute to the predisposition of accelerated ageing in COPD are unclear yet, these need to be unravelled first to identify the specific mechanisms to target in COPD. More importantly, it is questionable whether severe COPD patients will benefit from these therapies as they already present features of accelerated ageing including senescence accumulation. Therefore, senostatics might be more beneficial for mild-moderate COPD patients to prevent disease progression. Hence, it is also important to know whether senescent cells continue to accumulate after smoking cessation, which still is critical to stop disease progression. For COPD patients that never smoked and thus are likely exposed to other

environmental or occupational factors, senostatics may be especially beneficial to stop disease progression.

The other promising approach, senolytics, eliminate senescent cells by inducing apoptosis specifically in senescent cells and not in functional non-senescent cells. The first study to demonstrate the potential of senolytics found a delay in age-related disorders upon clearance of p16 positive senescent cells in mice (94). Since then, multiple senolytic treatments have been developed that eliminate senescent cells *in vitro* leading to reduced SASP protein secretion and reduced inflammation (95-97). Several mouse models in which treatment with senolytics was tested showed an extended lifespan and improved physical activity and lung function (98-103). Interestingly, a recent study has demonstrated that senolytic CAR T cells that target uPAR, eliminated senescent cells *in vitro* and *in vivo* and extended the lifespan of mice (104), which is of particular interest as in **chapter 5** uPAR was found in the SASP of COPD-derived fibroblasts. The senolytic cocktail Dasatinib and Quercetin (D+Q) has been demonstrated to eliminate senescent foetal and senescent primary lung fibroblasts and thereby reducing fibrosis, improving pulmonary and physical health upon bleomycin-induced lung injury (105, 106). The D+Q cocktail has been used in a phase 1 clinical trial to treat idiopathic pulmonary fibrosis patients and did improve physical function without causing severe side-effects and thus may be a feasible treatment that needs further clinical trials (107). Currently, more clinical trials are being performed to assess the benefits of D+Q and other senolytics in various age-related diseases, but not yet including COPD. So, future studies should assess the potential benefits of senolytic treatments in COPD-derived structural cells, COPD mouse models and eventually in clinical trials in COPD patients.

Finally, another suggested anti-ageing therapeutic approach is targeting the pathways and release of SASP proteins, because SASP proteins can have multiple detrimental effects on lung tissue as discussed above. Since cellular senescence and the SASP have been recognized to be involved in COPD pathogenesis, targeting the SASP might be a potential therapeutic approach (108). However, activation pathways of SASP proteins are similar to the COPD-related inflammatory pathways, including NF-κB, p38 and JAK/STAT, and targeting these pathways have been demonstrated to be poorly effective and lead to major side-effects (44). Glucocorticoids have been demonstrated to suppress the SASP in irradiation-induced senescence (109). Although they reduce exacerbation risks and severity, they have proven to not reduce COPD progression (110). Therefore, targeting specific SASP proteins may be a more promising therapeutic approach, for example by targeting specific miRNAs (110). In **chapter 3**, 29 age-related miRNAs were identified, which might be involved in the regulation of ageing processes. The expression of these miRNAs was not assessed in the lungs of COPD patients yet. Hence, elucidating the role of miRNAs in accelerated ageing and COPD may reveal novel specific therapeutic

targets. Ultimately, miRNA-based therapies with miRNA inhibition or miRNA activation might be a potential therapeutic approach to target accelerated ageing in COPD.

Since cellular senescence has multiple beneficial functions in healthy physiology, targeting cellular senescence may also lead to side-effects related to these normal functions. First of all, cellular senescence is a mechanism that prevents abnormal growth of a cell and thereby prevents tumour development. Thus, targeting cellular senescence may promote tumorigenesis. Furthermore, cellular senescence has also been demonstrated to play an important role in normal wound healing. Therefore, too low levels of cellular senescence can affect wound healing and thereby cause impaired tissue repair. Moreover, COPD is a heterogeneous disease, so a homogenous therapeutic approach might have more detrimental side-effects than beneficial effects. Hence, more selective, site-targeted therapies should be developed. More studies to determine the healthy balance of cellular senescence levels need to be done to enable more site-targeted therapies, which may prevent these side-effects. Another opportunity to limit these side-effects and to help restore normal lung tissue would be combining senolytic treatments with lung tissue regeneration therapies, which recently are being studied more intensively and show promising benefits (111-113), but no clinical trials are done yet. So future *in vitro* and *in vivo* studies should assess the potential of such combination treatments.

Overall conclusion

The studies in this thesis support the hypothesis that accelerated ageing may play a role in aberrant tissue repair and remodelling in COPD and thereby contribute to disease pathology. Therefore, therapeutics that target the mechanisms of accelerated ageing may be a potential therapeutic approach in COPD. Future studies should unravel the exact mechanisms that lead to accelerated ageing in COPD to discover therapeutic targets and to develop therapies more specifically. These therapies can also be beneficial for other age-related diseases, which of the majority are comorbidities of COPD.

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CHAPTER 9

Dutch summary / Nederlandse samenvatting

De chronische obstructieve longziekte COPD is een progressieve longziekte die vooral wordt veroorzaakt door blootstelling aan schadelijke stoffen, waarvan sigarettenrook de belangrijkste is. De blootstelling hieraan veroorzaakt een ontstekingsreactie in de longen wat uiteindelijk resulteert in longweefsel schade en vernauwing van de luchtwegen door verdikking van de luchtwegwand. In de luchtwegen zorgt dit ervoor dat de instroom van lucht wordt beperkt en dat slijm overmatig wordt aangemaakt, wat chronische bronchitis wordt genoemd. Dieper in de longen is er schade aan de kleine luchtwegen en longblaasjes. Als de longblaasjes kapotgaan wordt dit longemfyseem genoemd, dit resulteert in verminderde zuurstof opname. Deze verminderde functies van de longen veroorzaken benauwdheid en hoesten, en dit heeft een grote impact op het dagelijks leven van COPD-patiënten. De ziekte en ook de symptomen ontwikkelen zich langzaam en komen pas tot uiting op latere leeftijd van ongeveer 65 of ouder. De diagnose wordt bepaald door een combinatie van het ziektebeeld en een longfunctie test genaamd spirometrie. Hiermee wordt de totale hoeveelheid geforceerd uitgeademde lucht (FVC) gemeten en de geforceerde uitgeademde lucht in 1 seconde (FEV_1). De diagnose COPD wordt gesteld bij een verhouding van de FEV_1/FVC minder dan 70%. Aan de hand van spirometrie wordt ook de ernst van de ziekte bepaald, namelijk door de FEV_1 (zie tabel 1). Deze wordt uitgedrukt in een percentage van de gemiddelde waarde die voorspeld wordt op basis van leeftijd, geslacht en lengte.

Naar schatting waren er in 2016 wereldwijd 251 miljoen mensen met de ziekte COPD. Op dit moment is COPD de op twee na meeste voorkomende doodsoorzaak wereldwijd met in 2015 een dodental van 3 miljoen. Naar verwachting zal dit aantal de komende jaren verder toenemen gezien de veroudering van de populatie wereldwijd. In Nederland is COPD de 6^{e} meest voorkomende doodsoorzaak en de ziektelast wordt op de 4^{e} plek ingeschaald. In 2017 zijn 33.735 ziekenhuis opnames van COPD-patiënten geweest en waren de totale zorgkosten 912 miljoen. Patiënten met ernstige COPD met meer symptomen vormen het grootste deel van sterfgevallen, opnames en zorgkosten.

Ondanks dat roken de grootste risico factor is voor COPD hebben naar schatting 25% van de COPD-patiënten nooit gerookt en daarbij ontwikkelen niet alle rokers COPD. Daaruit blijkt dat de genetische achtergrond ook een belangrijke rol speelt in de risico op en in de ontwikkeling van COPD. Er zijn enkele gen varianten bekend die de kans op COPD vergroten, maar hoe deze varianten bijdragen aan risico, ontstaan en variatie in ontwikkeling van COPD is nog grotendeels onbekend. Mede daarom zijn er nog geen effectieve medicijnen beschikbaar om het ziekteproces te vertragen of om te keren. Op dit moment zijn de behandelingen met medicijnen vooral gericht op het verbeteren en voorkomen van symptomen. De belangrijkste methode op dit moment voor zowel verbetering van klachten en beperken van verder achteruitgang is het voorkomen van de blootstelling aan de giftige stoffen, en dus stoppen met roken. E-sigaretten zijn recent op de markt gekomen als alternatief voor tabaks-sigaretten en als hulpmiddel voor het

stoppen met roken, maar de veiligheid hiervan is grotendeels onbekend en wordt steeds meer betwijfeld. Als COPD zich tot een zeer ernstige vorm heeft ontwikkeld is uiteindelijk de enige behandelmogelijkheid een longtransplantatie. Om die ernstige vorm te voorkomen en veel vroegtijdiger effectief te kunnen ingrijpen zijn nieuwe behandelmogelijkheden nodig en daarvoor is meer kennis nodig over de processen die betrokken zijn bij het ziekteproces van COPD.

Tabel 1: Classificatie van COPD GOLD stadia aan de hand van

GOLD stage	Ernst van de ziekte	FEV_1 % voorspeld
I	Mild	> 80 %
II	Gematigd	50 – 80 %
III	Ernstig	30 – 50 %
IV	Zeer ernstig	< 30 %

COPD is gediagnostiseerd bij FEV_1/FVC <70%.
FEV_1: Geforceerde expiratie volume in 1 seconde. Gemeten door spirometrie.
FVC: Geforceerde vitale capaciteit. Gemeten door spirometrie.

De levensverwachting wereldwijd neemt toe en daarom is er steeds meer interesse in de rol van veroudering in gezondheid en ziekte. Veroudering is de progressieve afname van het in evenwicht houden van de normale processen en functies in het lichaam, genaamd homeostase. Het verliezen van dit evenwicht zorgt ervoor dat de kans op ziektes en overlijden toenemen. Recentelijk zijn negen kenmerken van veroudering beschreven die onderverdeelde kunnen worden in de oorzaken van schade, de reactie op schade en de uiteindelijke veranderingen die optreden bij veroudering.

Bij normale long veroudering neemt de longfunctie af en vergroten de longblaasjes, wat de zuurstof opname vermindert. Deze normale veroudering wordt veroorzaakt door verlies van longelasticiteit en wordt seniel of ouderdoms-emfyseem genoemd. Dit is in zeker mate vergelijkbaar met het pathologische emfyseem in COPD, maar in COPD wordt dit veroorzaakt door de directe schade van rook of andere gassen en de chronische ontsteking, en hierbij is de schade ook erger. Recentelijk zijn meerdere overeenkomsten aangetoond tussen verouderde longen en longen van COPD-patiënten, waaronder cellulaire senescentie. Cellulaire senescentie is een onomkeerbare stop van de groei van cellen, wat wordt veroorzaakt door allerlei soorten schade aan cellen en verminderde reparatie van deze schade. Deze senescente cellen hebben niet meer hun originele functie en kunnen weefselschade in het omliggende weefsel veroorzaken, onder andere door de eiwitten die ze uitscheiden. De uitscheiding van deze eiwitten wordt

senescence geassocieerde secretie fenotype (SASP) genoemd. Aangezien meerdere verouderingskenmerken aangetoond zijn in COPD-patiënten vergeleken met ex-rokers zonder COPD van dezelfde leeftijd, wordt COPD ook wel gezien als ziekte met versnelde longveroudering.

Een groep van COPD-patiënten die vooral relevant is met betrekking tot versnelde longveroudering zijn de relatief jonge COPD-patiënten met de meest ernstige vorm van COPD, ook wel 'severe, early-onset COPD' genoemd (SEO-COPD). Deze patiënten hebben zeer ernstig COPD ontwikkeld rond een leeftijd van 50-55 jaar ondanks een vaak relatief korte geschiedenis van roken. Deze SEO-COPD patiënten lijken dus extra vatbaar om COPD te ontwikkelen en op dit moment begrijpen de nog niet goed waarom. Mogelijk speelt versnelde longveroudering juist een belangrijke rol bij deze groep COPD-patiënten.

Recentelijk zijn abnormale veranderingen in het bindweefsel (extracellulaire matrix (ECM)) beschreven als aanvullend kenmerk voor long veroudering. ECM vormt de structuur van de longen en is belangrijk voor de stevigheid en elasticiteit. Bij normale veroudering verandert de ECM met vooral een toename van bindweefselvorming wat leidt tot stijfheid en een verlies aan elasticiteit. Een bekend zichtbaar voorbeeld hiervan is het rimpelen van de huid. In COPD-longen is de ECM sterk veranderd met ook een toename in bindweefselvorming in de luchtwegwand, maar afbraak van ECM in de longblaasjes. Onlangs hebben we aangetoond dat de mate van activiteit van de genen die coderen voor de ECM-eiwitten veranderd met veroudering in longweefsel. We vonden hierbij dat de veranderingen in de bindweefsel genen groter was in COPD-patiënten vergeleken met controles. De longcellen die vooral belangrijk zijn voor de productie van ECM en de regulatie ervan zijn long fibroblasten. Een ander celtype die ECM-eiwitten aanmaken zijn gladde spiercellen in de luchtwegwand. Deze twee celtypen spelen dus een belangrijke rol bij het vormen, in stand houden en herstellen van de ECM van de longen. In COPD is er een verdikking in de gladde spiercel laag in de luchtwegen en is er verhoogde ECM-productie in de luchtwegwanden. De ECM in longen van COPD-patiënten is veranderd met verhoogde productie van de ECM-eiwitten collageen en versican en verlaagde productie van elastine, decorine en perlecan. Ook na stimulatie van long fibroblasten met sigaretten rook extract is de ECM-productie veranderd. Het is dus bekend dat er ECM-veranderingen optreden tijdens veroudering en bij COPD-patiënten, maar wat de rol is van versnelde veroudering in COPD in relatie tot longweefsel herstel en ECM veranderingen is tot nu toe onbekend.

Het doel van dit proefschrift was om vast te stellen wat de rol is van versnelde long veroudering in COPD met betrekking tot longweefsel herstel en ECM-veranderingen.

Om inzicht te krijgen in de overlap van long veroudering en COPD, hebben we in **hoofdstuk 2** een overzicht gegeven van de studies die verouderingskenmerken hebben aangetoond in COPD. Dit laat zien dat in COPD-longweefsel veel verouderingskenmerken aanwezig zijn wat onderschrijft dat versnelde veroudering een rol speelt in COPD. Dit overzicht heeft ook aangetoond dat er nog weinig bekend was over veroudering in long fibroblasten en luchtweg gladde spiercellen van COPD-patiënten. Deze cellen spelen een belangrijke rol in longweefsel herstel en ECM-veranderingen. Wat ook duidelijk werd in dit overzicht was dat er nog bijna geen gegevens zijn over veroudering in de specifieke jonge groep patiënten met SEO-COPD. Daarom was **hoofdstuk 2** de basis en aanleiding voor de studies die zijn beschreven in dit proefschrift.

In **hoofdstuk 3** hebben we gekeken naar veranderingen op mRNA en miRNA niveau met normale veroudering in luchtwegbiopten van gezonde individuen. Het mRNA in cellen wordt omgezet in eiwit, genaamd translatie, wat uiteindelijk het functionele effect in cellen heeft. MiRNAs zijn kleine RNA-moleculen die kunnen binden aan specifieke mRNAs en daardoor deze mRNA afbreken of de translatie blokkeren. Door naar de veranderingen hiervan te kijken met veroudering probeerden we meer inzicht te krijgen in welke genen (mRNA) en welke gen regulatoren (miRNA) een rol spelen in normale veroudering. We hebben 285 mRNAs en 27 miRNAs gedetecteerd die waren veranderd met veroudering. Uit dit onderzoek bleek dat de genen met verlaagde expressie met veroudering betrokken zijn bij bepaalde signaal routes die onderdeel uitmaken van 3 van de 9 algemene verouderingskenmerken; genomische instabiliteit, cellulaire senescentie en veranderde intercellulaire communicatie. Deze resultaten suggereren dat deze verouderingskenmerken een rol spelen in normale longveroudering.

Om te kijken naar het effect van versnelde veroudering op normaal longweefsel herstel in COPD-patiënten hebben we in **hoofdstuk 4** onderzocht of long fibroblasten van ex-rokers met COPD meer kenmerken van veroudering laten zien dan fibroblasten van ex-rokers zonder COPD. Hierbij werd speciaal aandacht besteed aan de jonge SEO-COPD patiënten aangezien onze hypothese is dat versnelde veroudering vooral in deze patiënten een rol kan spelen. Deze verouderingskenmerken werden geanalyseerd na het kweken van long fibroblasten geïsoleerd uit longen van COPD-patiënten en uit ex-rokers zonder COPD als controles. Uit deze studie bleek dat meerdere kenmerken van veroudering hoger zijn in COPD-patiënten in vergelijking met de controles met ook hogere waarden van cellulaire senescentie, DNA-schade en oxidatieve stress. Deze effecten waren vooral zichtbaar in de fibroblasten van SEO-COPD patiënten vergeleken met de controles van dezelfde leeftijd. In relatie tot ECM vonden we dat hogere waarden van cellulaire senescentie geassocieerd waren met lagere genexpressie van het ECM-eiwit decorine. Deze combinatie vonden we vervolgens ook in het totale longweefsel van COPD-patiënten vergeleken met controles. Vervolgens hebben we cellulaire senescentie geïnduceerd met Paraquat, wat een bekend model hiervoor is. Paraquat werd vroeger gebruikt als onkruidverdelger en kan daardoor door beroepsmatige blootstelling een risico factor zijn

voor COPD. Na inductie van cellulaire senescentie vonden we veranderingen in genexpressie van meerdere ECM-eiwitten waaronder verlaagde decorine expressie, in overeenstemming met hier bovenstaande resultaten. Dit bevestigt dat hogere waarden van cellulaire senescentie kunnen resulteren in ECM-veranderingen in COPD.

Om meer inzicht te krijgen in de potentiële consequenties van versnelde veroudering in long fibroblasten van COPD-patiënten hebben we in **hoofdstuk 5** onderzocht of deze cellen hogere waarden van de SASP (senescentie geassocieerde secretie fenotype) eiwitten uitscheiden in vergelijking met fibroblasten van controles zonder COPD. Eerst hebben we met Paraquat cellulaire senescentie geïnduceerd in long fibroblasten en de eiwitten die meer dan 3 keer zoveel worden uitgescheiden dan niet-gestimuleerde fibroblasten gedefinieerd als SASP-eiwitten. Na senescentie inductie hebben we 124 SASP eiwitten geïdentificeerd. Van deze 124 eiwitten werden 42 eiwitten in hogere mate uitgescheiden door fibroblasten van COPD-patiënten vergeleken met die van controles. Van deze 42 COPD-geassocieerde eiwitten werden 35 eiwitten uitgescheiden in hogere mate door fibroblasten van SEO-COPD patiënten vergeleken met controles van dezelfde leeftijd. Na analyse van de signaalroutes van deze eiwitten bleek dat veel van deze eiwitten betrokken zijn bij chronische ontstekingen en daarom zouden kunnen bijdragen aan het ziekteproces van COPD.

Aangezien luchtweg gladde spiercellen ook een rol spelen bij de regulatie van ECM-productie hebben we in **hoofdstuk 6** onderzocht of deze cellen van COPD-patiënten ook kenmerken hebben van veroudering vergeleken met de gladde spiercellen van ex-rokers zonder COPD als controles. Luchtweg gladde spiercellen en long fibroblasten werden geïsoleerd uit longen van COPD-patiënten en ex-rokers zonder COPD. Ten eerste bleek dat de waarden van cellulaire senescentie hoger lagen in luchtweg gladde spiercellen dan in long fibroblasten van dezelfde patiënten. Echter, cellulaire senescentie was niet hoger in luchtweg gladde spiercellen van COPD-patiënten vergeleken met controles. Daarnaast was er ook geen associatie tussen cellulaire senescentie en genexpressie van enkele ECM-eiwitten, inclusief decorine. Deze resultaten suggereren dat, in tegenstelling tot in long fibroblasten, hogere waarden van cellulaire senescentie in luchtweg gladde spiercellen geen grote rol spelen in COPD.

In **hoofdstuk 7** hebben we onderzoek gedaan naar de schadelijke effecten van E-sigaretten op long fibroblasten. E-sigaretten worden gebruikt als vervanging voor tabak roken en als hulpmiddel bij het stoppen van roken aangezien deze minder schadelijk worden geacht. Daarom worden ze ook vaak gebruikt door COPD-patiënten. Om te onderzoeken of E-sigaretten effect kunnen hebben op longveroudering hebben we long fibroblasten behandeld met kweekmedium waar de damp, ook vape genoemd, van E-sigaretten doorheen is gestroomd en hetzelfde deden we met rook van tabaks-sigaretten. Nadat eerder was aangetoond dat door behandeling met sigaretten rook cellulaire senescentie geïnduceerd kan worden in long fibroblasten, zagen wij dit ook na

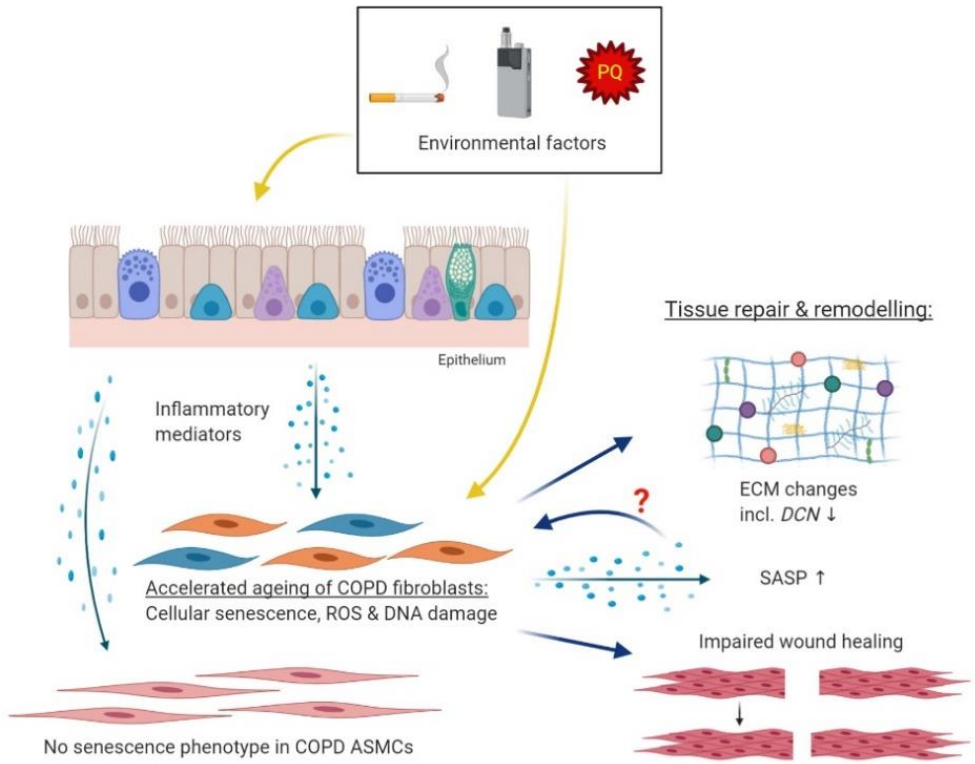
behandeling met E-sigaretten vape. Daarnaast bleek dat na inductie van cellulaire senescentie door E-sigaretten vape, en ook door tabaks-sigaretten rook en Paraquat, de herstelfunctie van deze fibroblasten was verminderd. Uit deze resultaten blijkt dat het onwaarschijnlijk is dat vapen van E-sigaretten een veilig alternatief is voor tabaks-sigaretten en dat ook dit dus zou kunnen bijdragen aan longveroudering en long ziekten, inclusief COPD.

In Figuur 1 is een overzicht weergegeven van de belangrijkste bevindingen in dit proefschrift. Natuurlijk zijn er nog veel verdere vragen te beantwoorden en ook nieuwe vragen voortgekomen uit ons onderzoek. Zo moet er verder nog onderzoek gedaan worden om de exacte oorzaak van de versnelde veroudering van de longfibroblasten van COPD-patiënten beter op te helderen. Ditzelfde geldt voor het effect van de versnelde veroudering van de fibroblasten op het omliggende longweefsel. Dit zou kunnen resulteren in longweefsel disfunctie door chronische ontstekingen, inductie van cellulaire senescentie in omliggende cellen en veranderingen in de ECM. In hoofdstuk 4 vonden we een associatie tussen verhoogde cellulaire senescentie en ECM-regulatie, maar of dit effect direct of indirect is en wat het functionele effect op de ECM-productie, structuur en organisatie is, is nog onbekend. Vervolgstudies zouden deze vragen over cellulaire senescentie in long fibroblasten van COPD-patiënten moeten beantwoorden.

Andere belangrijke vragen met betrekking tot versnelde veroudering in COPD die nog onbeantwoord zijn, zijn wat de exacte oorzaak van deze versnelde veroudering in het totale longweefsel van COPD-patiënten is en of dit aan COPD bijdraagt of dat dit juist het gevolg is van de ziekte. Sommige studies probeerden deze vraag te beantwoorden door versnelde veroudering te veroorzaken in muis modellen en dan te onderzoeken of deze muizen COPD ontwikkelen en in enkele gevallen bleek dit zo te zijn. Echter is nog veel onbekend hierover en zal verder onderzoek gedaan moeten worden door bijvoorbeeld mensen met een verhoogd risico voor COPD over langere tijd te volgen in een zogenaamd longitudinale studie.

Nu dit proefschrift en meerdere andere studies aantonen dat versnelde longveroudering een belangrijke rol kan spelen in COPD-patiënten zou het interessant kunnen zijn om te interfereren in de processen van veroudering. Op het moment worden vooral twee strategieën onderzocht in relatie met veroudering en versnelde veroudering, genaamd senostatics en senolytics. Met senostatics wordt geprobeerd om veroudering tegen te gaan door vooral de oorzaken van cellulaire senescentie te remmen met behulp van het verbeteren van cellulaire reparatie mechanismen. Door middel van senolytics wordt specifieke celdood veroorzaakt in alleen senescente cellen. Beide strategieën laten veelbelovende resultaten zien in celkweek experimenten en diermodellen. Op dit moment zijn ook enkele klinische studies bezig om de potentie van zulke therapieën te onderzoeken. Het nadeel van het tegen gaan van versnelde veroudering is dat dit kan

leiden tot bijwerkingen, met vooral risico op ontwikkeling van kanker. Cellulaire senescentie is namelijk ook een belangrijk proces bij het remmen van abnormale celgroei. Het aanpakken van cellulaire senescentie kan daarom zorgen voor abnormale celgroei en tumor vorming. Om deze reden is het belangrijk om de gezonde balans hierin te vinden en om specifieke cel-gerichte therapieën te ontwikkelen. Ondanks dat anti-verouderingstherapieën veelbelovend lijken, is nog veel onderzoek nodig om dit te kunnen gebruiken in ziektes met versnelde veroudering, zoals COPD.



Figuur 1: Overzicht van de rol van versnelde veroudering in long fibroblasten van COPD.
Schadelijke stoffen, zoals sigarettenrook, E-sigaretten en Paraquat (PQ) kunnen kenmerken van versnelde veroudering induceren in long fibroblasten. Long fibroblasten van COPD-patiënten hebben kenmerken van versnelde veroudering, inclusief hogere cellulaire senescentie, oxidatieve stress (ROS) en DNA-schade. Deze versnelde veroudering kan een direct effect zijn van de schadelijke stoffen of indirect, bijvoorbeeld via het epitheel. Hogere waarden van cellulaire senescentie resulteerden in ECM-veranderingen, inclusief verlaagde decorine expressie, hogere uitscheiding van SASP-eiwitten en een verlaagde longweefsel herstel functie. Deze drie functionele consequenties zijn betrokken bij het ziekteproces van COPD. Gecreëerd met BioRender.com

APPENDIX 1

Acknowledgements / dankwoord

Iets meer dan 4 jaar geleden ben ik in het avontuur van mijn 2+2 PhD project gestapt. De eerste 2 jaar in Groningen en de laatste 2 jaar als extra avontuur in Sydney, Australië. In het begin dacht ik 2 jaar klinkt heel ver en 4 jaar zelfs nog verder. Maar nu zijn deze jaren alweer voorbijgevlogen en heb ik naast het harde werken er ook zoveel mogelijk van geprobeerd te genieten. Nu is het tijd om deze 4 jaar rond te maken met dit proefschrift. In deze 4 jaar heb ik natuurlijk veel te danken al alle mensen die mij hebben begeleid, geholpen, ondersteund en/of bijgestaan.

As first I would like to thank my promotors who helped me with everything and made it possible to develop myself to the researcher I am now. About 4,5 years ago I heard that a PhD project was available at the department of Pathology and Medical Biology at the UMCG. I contacted Corry-Anke and Irene about the project and told them I was keen on doing the project as I did an internship at this department during my HBO. However, without knowing that it was a 2+2 project with the last 2 years in Sydney, Australia. After hearing this I was directly interested, but without realizing the big changes it included. However, I have no regrets at all and am really happy with the experience in living overseas in such a beautiful country as Australia. So, I would like to start with the people that made all this possible and that supported and helped me the whole way long, my promoters **Prof. Corry-Anke Brandsma**, **Prof. Irene Heijink, Prof. Wim Timens** and **Prof. Brian Oliver**.

Ten eerste mijn eerste promotor en dagelijkse supervisor; **Corry-Anke**, vanaf begin af aan kon ik bijna altijd bij je terecht. Als ik vragen had of advies nodig was, hoe klein ook, stond de deur bijna altijd open en kreeg ik snel antwoord van jou. Als supervisor probeerde je altijd ervoor te zorgen dat alles soepel liep en geregeld was, en daarbij vooral denkend aan de dingen die niet als eerste voor de hand liggen. Vooral de praktische zaken waar ik zelf nog niet aan had gedacht, zoals introducties in technieken, beschikbaarheid van mensen waarvan ik hulp nodig was en andere formaliteiten. Qua planning ben jij een expert en daar heb ik veel van kunnen leren. Ondanks jouw drukke schema kreeg ik bij alles wat ik opstuurde voor feedback een reactie van jou binnen een paar dagen en soms zelfs dezelfde dag. Vooral de laatste maanden heeft dit er enorm aan bijgedragen dat ik mijn proefschrift kon indienen binnen de termijn van 4 jaar. Zonder jou was dit zeker niet gelukt, dank daarvoor. Daarnaast zorgde je er altijd voor dat de prioriteiten op de juiste aspecten lagen en kan je goed duidelijk maken op welke manier alles zo gestroomlijnd mogelijk loopt. Wat betreft onderzoeksideeën zaten we vaak op 1 lijn en was al jouw input zeer waardevol en ik denk dat ik bijna al jouw feedback direct kon toepassen. Toen je in November 2019 bij ons langskwam in Sydney voor een congres vertelde je mij over de mogelijkheid van een Postdoc positie als jij de Aspasia beurs toegewezen kreeg. Ik ben dan ook erg blij dat dit uiteindelijk is gelukt en dat ik nu bij jou als Postdoc aan de slag kan. Deze zekerheid heeft me zeer goed gedaan en dit heeft de

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Irene, jij bent bijna altijd aanwezig geweest tijdens al mijn wekelijkse en maandelijkse besprekingen en je kwam altijd met waardevolle suggesties. Jouw blik over hoe alles in de cellen te werk gaat of eventueel zou werken en wat dit dan betekent heeft mij gefascineerd. Jij kan binnen en paar seconden helemaal inzoomen op de moleculaire werking in cellen en bepaalde pathways om dan weer volledig uit te zoomen wat dit dan betekent voor de cel, het weefsel en uiteindelijk de patiënt. Hiermee heb je mij geleerd om het overzicht te houden maar ook om te realiseren te kijken naar het complete proces in de cellen en niet naar één bepaalde marker. Helaas vielen het project met geconditioneerd medium van met rook behandelde epitheelcellen tegen. Hierdoor heeft jouw kennis hiervan en ervaring hiermee niet direct geresulteerd in een hoofdstuk. Echter heb jij een grote bijdrage kunnen leveren aan alle hoofdstukken. Jouw feedback was erg nuttig aangezien je soms met een ander oog naar de resultaten keek dan de rest. Daarnaast werden mijn hoofstukken beter te begrijpen door jouw duidelijke en complete manier van schrijven. Ik ben je daarom erg dankbaar voor al jouw input tijdens mijn PhD en in mijn proefschrift.

Wim, jouw kennis over longen en natuurlijk de pathologie daarvan is ongelofelijk. Elke bespreking die we hadden heb ik hierover wel iets geleerd van jou. De manier dat jij de biologie en de pathologie in de longen kan uitleggen is vaak zo duidelijk dat bijna iedereen en zelfs een leek dit zou kunnen begrijpen. De bijdrage die je hiermee aan mijn project hebt geleverd is daarom enorm. Daarnaast was ik vaak weinig introductie nodig om te vertellen waar we stonden in de projecten. Na een paar zinnen wist je precies wat we ook alweer gedaan hadden en waarom en daardoor kon je direct bijdragen aan de discussie. Deze bijdragen waren altijd erg waardevol. Ondanks jouw enorme kennis liet je veel ruimte om mijn eigen ideeën in te brengen en zorg je ervoor om niet terughoudend te zijn in het stellen van vragen. Daarnaast kan jij ook uitstekend prioriteiten aangeven en daarmee bijdragen aan een logische planning. Ook was je altijd in voor een praatje voor of na de besprekingen als de tijd dit toe liet. Heel erg bedankt voor jouw bijdrage aan mijn project en dit proefschrift.

Brian, I will start with the day I first met you. We were at the ERS congress in Paris and we arranged a meeting to discuss the ongoing projects, which resulted in some useful input. But, after that first meeting you invited me for a breakfast at McDonalds where we had some great conversations, not about science but about life. I think this partly tells how easy-going you are. However, only partly, as I realized after spending the last 2 years of my PhD at your lab. At least twice a week, but sometimes every day you came by our office just for a chat, and again not about science most of the times.

Frequently, you had some good tips to visit in Sydney. However, when I need some help or input for my project, your door was always open, literally. And when sending a manuscript for feedback most of the times you provided it within 24 hours, which surprised me every time. You also gave me a lot of freedom to develop my own ideas and to give direction to my own projects with always very useful suggestions. Therefore, you are a great supervisor that supports independence and the development as a scientist. In addition, you always approached every comment or suggestion from the positive side. Even after a rejection or major revisions, you remained positive and ensured me that it would get accepted in the end, and it did. All of this contributed to the great experience I've had in your lab. I would like to thank you very much for this and thanks for all your contributions to my thesis.

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Roy R Woldhuis

APPENDIX 2

List of publications

PUBLICATIONS

1. de Vries M, Faiz A, **Woldhuis RR**, Postma DS, de Jong TV, Sin DD, Bosse Y, Nickle DC, Guryev V, Timens W, van den Berge M, Brandsma CA. Lung tissue gene-expression signature for the ageing lung in COPD. Thorax 2017.

2. Brandsma CA, de Vries M, Costa R, **Woldhuis RR**, Konigshoff M, Timens W. Lung ageing and COPD: is there a role for ageing in abnormal tissue repair? Eur Respir Rev 2017;26:10.1183/16000617.0073-2017. Print 2017 Dec 31.

3. Folkerts H, Wierenga AT, van den Heuvel FA, **Woldhuis RR**, Kluit DS, Jaques J, Schuringa JJ, Vellenga E. Elevated VMP1 expression in acute myeloid leukemia amplifies autophagy and is protective against venetoclax-induced apoptosis. Cell Death Dis 2019;10:421-019-1648-4.

4. Ong J, **Woldhuis RR,** Boudewijn IM, van den Berg A, Kluiver J, Kok K, Terpstra MM, Guryev V, de Vries M, Vermeulen CJ, Timens W, van den Berge M, Brandsma CA. Age-related gene and miRNA expression changes in airways of healthy individuals. Sci Rep 2019;9:3765-019-39873-0.

5. **Woldhuis RR**, de Vries M, Timens W, van den Berge M, Demaria M, Oliver BGG, Heijink IH, Brandsma CA. Link between increased cellular senescence and extracellular matrix changes in COPD. Am J Physiol Lung Cell Mol Physiol 2020.

6. **Woldhuis RR**, Heijink IH, van den Berge M, Timens W, Oliver BGG, de Vries M, Brandsma CA. COPD-derived fibroblasts secrete higher levels of senescence-associated secretory phenotype proteins. Thorax 2020.

PUBLISHED ABSTRACTS

1. **Woldhuis RR**, Heijink IH, van den Berge M, Timens W, Oliver B, de Vries M, Brandsma CA. Higher Secretion Levels of Senescence Associated Secretory Phenotype (SASP) Proteins by COPD-Derived Fibroblasts Compared to Control-Derived Fibroblasts. *American Journal of Respiratory and Critical Care Medicine 2020;201:A4416.*

2. Bozier JE, Xenaki D, **Woldhuis RR**, Oliver B. Combined Cigarette Smoke Extract and E-Vapour Extract Stimulation Has a Synergistic Effect on Inflammatory Mediator Production. *American Journal of Respiratory and Critical Care Medicine 2020;201:A7685.*

3. **Woldhuis RR**, Heijink IH, van den Berge M, Timens W, Oliver B, de Vries M, Brandsma CA. COPD-derived fibroblasts secrete higher levels of senescence associated secretory phenotype (SASP) proteins. *ERJ Open Research 2020 6: 28.*

4. Kruk D, **Woldhuis RR**, Brandsma CA, Timens W, Ten Hacken NHT, Heijink IH. Late Breaking Abstract - Mesenchymal stromal cells of the lung: Can we finally tell them apart? *European Respiratory Journal 2019 54: OA2117.*

5. **Woldhuis RR**, de Vries M, Timens W, van den Berge M, Oliver B, Heijink IH, Brandsma CA. Cellular Senescence in Lung Fibroblasts from COPD Patients Is Associated with Altered Extracellular Matrix Regulation. *American Journal of Respiratory and Critical Care Medicine 2019;199:A3763*

6. **Woldhuis RR**, Ong J, Boudewijn IM, van den Berg A, Kluiver J, Kok K, Terpstra MM, Guryev V, de Vries M, Vermeulen CJ, Timens W, van den Berge M, Brandsma CA. Age-related gene and microRNA expression changes in the airways of healthy individuals. *European Respiratory Journal 2018 52: PA4230.*