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Remodeling of the extracellular matrix in COPD

a process of accelerated lung ageing?

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Abstract
Chronic obstructive pulmonary disease (COPD) is a chronic, life-threatening lung disease characterized by the chronic obstruction of lung airflow, interfering with normal breathing. The main risk factor for COPD is the inhalation of noxious gases, which causes damage to airway epithelial cells by increasing reactive oxygen species levels. This, in turn, is thought to contribute to chronic inflammation and aberrant repair processes leading to remodeling of the extracellular matrix. It has often been suggested that COPD is a disease of accelerated lung ageing. In this thesis we wanted to investigate whether the remodeling of the ECM seen in COPD is similar to remodeling of the ECM seen in ageing and whether it occurs much faster than in ageing, possibly enabling us to conclude whether COPD is indeed a disease of accelerated lung ageing. The main components of the ECM surrounding the lung epithelium consist of collagen, elastin, fibronectin, laminin, glycosaminoglycans, proteoglycans, matrix metalloproteinases and tenascin-C. In different areas (small airways and parenchyma) of the lung, different levels of each components were expressed. Upon investigating the changes in individual components of the ECM in COPD and in ageing, we found that for both collagen protein expression was increased, elastin protein expression was decreased and tenascin-C protein expression was increased in both the small airways and the parenchyma. For the other ECM components discussed, no comparison could be made, due to conflicting or lack of data. It was also found that there are high similarities between the processes involved in the remodeling of the ECM in COPD in ageing. In both ageing and COPD chronic inflammation, caused by oxidative stress, results in a profibrotic phenotype and emphysema. This ultimately causes aberrant tissue repair and ECM remodeling. The main difference between the two was the speed with which this remodeling occurs. It is therefore plausible to state that remodeling of ECM seen in COPD is a process of accelerated lung ageing. To fully understand the changes in individual components of the ECM in COPD and ageing, more uniform studies should be performed, with the same subject criteria and experimental set-up.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic, life-threatening lung disease characterized by the chronic obstruction of lung airflow, interfering with normal breathing. The disease is currently the fourth leading cause of death, with increasing morbidity and mortality and 3 million people dying as a consequence of COPD every year [1–4].

The main risk factor for COPD is the inhalation of noxious gases. While smoking is the main source of these gases, other causes like job-related exposures to these gases, air pollution and genetic susceptibility can cause COPD in non-smokers[5]. When inhaled, these noxious gases first encounter the epithelial barrier of the lungs. This barrier is designed to keep foreign and toxic particles and gases out of the human body. However, the epithelial barrier can be damaged by the inhalation of these toxic particles. This damage occurs through the production of reactive oxygen species (ROS) by the epithelial cells of the barrier. In healthy tissue the production of ROS is countered by the production of antioxidants like superoxide dismutase (SOD) and vitamins[6]. However, patients with COPD have a constant inhalation of these noxious gases, with induces high levels of ROS in the epithelial cells. The damaged cells, in turn, are not able to produce enough antioxidants which causes a disbalance in ROS and antioxidants levels. Constant oxidative stress in the cell causes it to be heavily damaged, eventually leading to cellular senescence. Cellular senescence is a process in which cells irreversibly stop proliferating and differentiating[7,8] Damaged and senescent cells produce factors that promote the recruitment of inflammatory cells, which clear out debris of damages cells and possible infiltrating toxins, and stimulate fibroblast to start producing extracellular matrix (ECM). In healthy tissue, this inflammation and repair process would halt as soon as the damaged tissue was repaired. However, the constant damage done by noxious gases causes a chronic inflammation and aberrant tissue repair. This, in turn, leads to remodeling of the lung epithelium by processes such as emphysema in the lung parenchyma and fibrosis in the airways, all together resulting in the loss of FEV_1 (forced expiratory volume in 1 second). FEV_1 is used as the main parameter for severity of COPD. GOLD (Global Initiative for Chronic Obstructive Lung Disease) distinguishes 4 stages of severity in COPD[1,2,5] (figure 1):

1. Mild, with a FEV_1 ≥80% of predicted. At this stage, the patient may not be aware that their lung function is abnormal.
2. Moderate, with a FEV_1 between 50 -80% of predicted. Symptoms usually progress at this stage, with shortness of breath typically developing on exertion.
3. Severe, with a FEV_1 between 30-50% of predicted. Shortness of breath typically worsens at this stage and often limits patients' daily activities. Exacerbations are especially seen beginning at this stage.
4. Very severe, with a FEV_1 <30% of predicted. At this stage, quality of life is very much impaired and exacerbations may be life-threatening.

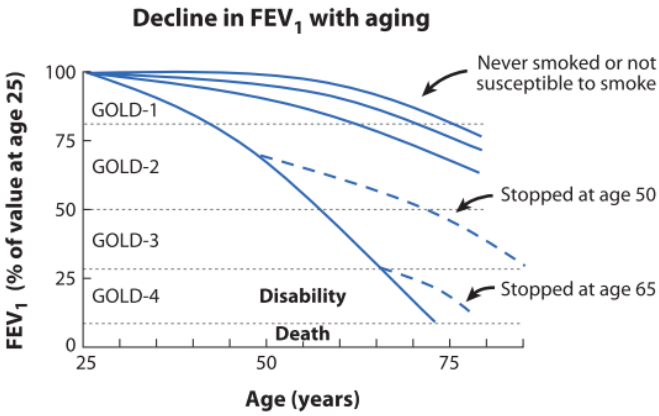

Figure 1 the Fletcher curve. This curve compares the decline in FEV_1 in smoking and non-smoking individuals and shows the FEV_1 areas of the different GOLD-stages [3]

It has been shown that lung tissue changes in COPD show high similarity with lung changes occurring in ageing. Ageing is a process characterized by a progressive decline in tissue homeostasis over time, which leads to an increasing risk of degenerative diseases and death[7,9]. In normal, healthy tissue, ROS is produced as a byproduct of aerobic respiration in the mitochondria and kept at low levels in the cell by producing antioxidants[7]. However, over time, ROS levels increase and oxidative damage in the cells accumulates, eventually leading to an aged phenotype. The aged phenotype is characterized by nine hallmarks of ageing, with cellular senescence being one of them. The other eight hallmarks of ageing are genomic instability, telomer attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, stem cell exhaustion and altered intercellular communication[10]. In the lungs this ageing phenotype is characterized by the loss of lung elasticity, increase of tissue stiffness and a decrease of FEV1[11]. This phenotype strongly resembles the phenotype seen in COPD. However, in COPD, these changes occur much earlier in live and are much more extreme than in aged tissue. This is why COPD is suggested to be a disease of accelerated lung ageing[7,12,13].

Understanding the mechanisms that drive a disease is very important in potentially finding a therapeutic target against the disease. One process that is suggested to show a form of accelerated ageing in COPD is the remodeling of the ECM. In COPD the remodeling of the ECM leads to the characteristic loss of elasticity and thickening and stiffening of the airway walls. If COPD is indeed a disease of accelerated ageing, it is important to know which specific processes in COPD and ageing are similar, but also which are not. In this thesis we therefore want to investigate whether the remodeling of the ECM seen in COPD is similar to remodeling of the ECM seen in ageing. Subsequently this thesis aims to investigate if the remodeling of ECM in COPD can be classified as a process of accelerated ageing. To determine whether this is the case first the basics will be described; what is the composition of the healthy ECM and what is its main function? Then we investigate how the remodeling of the ECM in COPD is established and what the causes are. After this, we study how the remodeling of the ECM seen in ageing is established and what the causes are. Lastly, the remodeling of COPD and ageing is compared to determine whether ECM remodeling in COPD is a process of accelerated lung ageing.

Chapter 1 The extracellular matrix of the lung

The extracellular matrix (ECM) of any tissue is defined as the connective tissue in which cells reside. It provides biochemical and biomechanical stimuli and acts as a physical scaffold causing a dynamic microenvironment for every cell. It regulates, for example, when cells undergo differentiation or are in homeostasis[14,15].
There are two places in the lungs were ECM can be found. Firstly, the ECM is found as a thin layer between the epithelial cell layer and the endothelial cell layer, the mesenchymal cell layer and the epithelial connective tissue. This layer of ECM is called the basement membrane[16,17]. Secondly, ECM can be found in the interstitial spaces of the lung, between the alveoli and the blood vessels, called the interstitium. Fibroblasts, which are responsible for the production of most ECM components, also reside in the interstitium[16]. The ECM of the lung has to protect the cells against the constant stretch and strain of breathing, but also needs to be effective in the exchange of oxygen and carbondioxide[18]. The main components of the ECM consists of collagen and elastin, which are both fibrous proteins, fibronectin and laminin, which are both adhesive proteins, and proteoglycans and glycosaminoglycans, which form a hydrated polysaccharide gel in which all other components are embedded (see figure 2)[14,18,19,17].

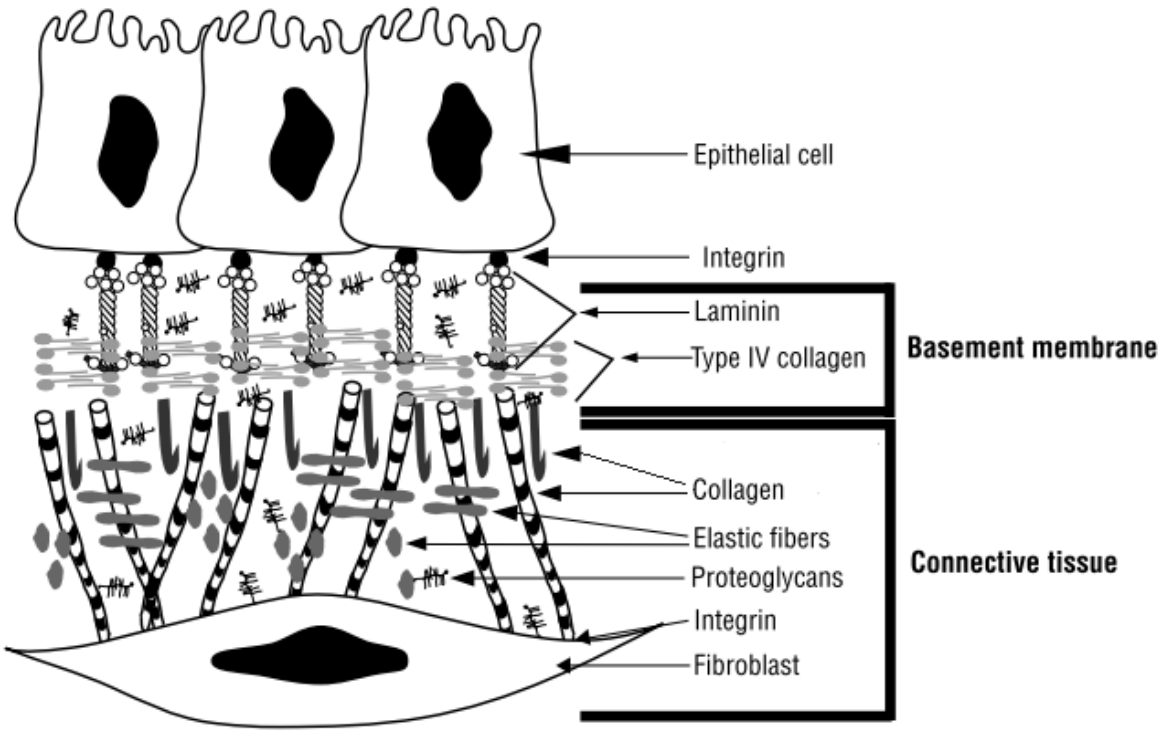

Figure 2 A schematic overview of the main molecules of the matrix and associated cells of the lungs[17]. Not shown, but mentioned in the text, are fibronectin, the MMPs, tenascin-C and airway smooth muscle cells.

Collagen

Collagen fibers are the main components of the ECM. They are produced by fibroblasts and are responsible for the tensile strength of the ECM. There are 28 types of collagens, but the main subtypes of collagen found in the lung ECM are types I, II, III, IV,V and VI. Each type has a distinct characterization; while collagen type I is for instance a thick and rigid fiber, type III is a more flexible collagen[18]. Although collagens are mainly addressed as collagen fibers, not all collagen types are actually fibrous. Collagen type I-III are fibrous collagens and IV-VI are non-fibrous collagens. All types are expressed heterogenous and together they form a mesh

of collagen. Which collagen types are expressed differs per ECM type. Collagens type I and III are mainly seen in the interstitial spaces, while type IV is a key component of the basement membrane. However, expression of different collagen types does not only differ in ECM layer, but also in tissue region in the lung. For example, the large bronchioles consists mainly of type I and III, while the alveoli's consist of III, IV and V[14,15,18,19].

Elastin

The protein elastin is formed by the cross-linking of its precursor tropoelastin, which is synthesized by smooth muscle cells and fibroblasts residing in the lung ECM. Together with microfibrils, the elastin proteins in turn cross-link into elastin fibers. Elastin fibers are highly flexible and resilient and are responsible for the intrinsic recoil property of the lung tissue[14,15,18,19].

Fibronectin and laminin

While fibronectin and laminin are classified as fibrous proteins, their main function in the lungs is not to give strength to the ECM, but rather to regulate the adhesion of cells to the ECM. They are mainly produced by fibroblasts. Fibronectin functions as a extracellular mechano-regulator; it has binding sites for the cells of the epithelium (integrin binding sites), ECM components (like collagen, fibrin and proteoglycans) and secreted molecules (like growth factors). By attaching to cell and matrix and being able to stretch out multiple times its size, it can signal cells for environmental changes and causing the cells to, for example, change their morphology and motility[14,15,20]. Laminin has a similar function to fibronectin. It attaches cells to their basement membrane[18,19].

Glycosaminoglycans and proteoglycans

Glycosaminoglycans (GAGs) are composed of repeating disaccharides forming long polysaccharides. There are two types of GAGs; non-sulfated and sulfated. The most abundant non-sulfated GAG is hyaluronic acid. It is synthesized by mesenchymal cells and it is an important stabilizer of the loose ECM[18]. Important sulfated GAGs are heparan sulfate (and its modified form heparin), chondroitin sulfate, keratin sulfate and dermatan sulfate. Sulfated GAGs are able to form proteoglycans (PGs) by attaching to a protein core[21].

While proteoglycans are mainly responsible for maintaining the tissue hydration, there are different types of PGs, which all have their own specific function. In lung tissue, there are three main PGs distinguished based on the GAGs that are formed around the core protein [18,19,21]:

- Versican is a PG which contains chondroitin sulfate. It is mainly located in the interstitium in regions where there are no collagen and elastin fibers. Its exact function is unclear, but it is thought to be important in tissue hydration and cell-matrix interactions.
- Perlecan is a PG containing heparan sulfate. It is mainly located in the vascular basement membrane. Perlecan functions as a filtration barrier by interacting with collagen IV. This barrier limits the flow of macromolecules and cells between tissues. It also regulates ECM repair and remodeling processes by being able to bind to growth factors, cytokines and proteinases.
- Decorin is a PG containing dermatan sulfate. It is located in both the interstitium and the epithelial basement membrane. It plays a role in tissue repair and remodeling by their ability to bind to the growth factor TGF-β (see chapter 2).

Tenascin-cytotactin

Tenascin-cytotactin or tenascin-C is a large hexametrical glycoprotein, comprised of multiple binding domains. These domains are able to bind to cells, ECM components and pathogens.

In the lungs, tenascin-C is expressed upon injury of the epithelium and is responsible for the migration of fibroblasts towards the site of injury. By binding to fibroblasts as well as different ECM components like fibronectin it is able to change cellular adhesion, contractility and motility and organize ECM remodeling. This drives fibroblast migration and activation at the injury site and promotes tissue repair. After the epithelium is repaired, tenascin-C is degraded and secretion is stopped[22–24].

Matrix metalloproteinases

To maintain tissue homeostasis, ECM molecules do not only have to be produced, but they also have to be broken down. Degradation of ECM molecules is done through cleaving by proteases. The main type of proteases in the ECM are the matrix metalloproteinases (MMPs), which is produced by fibroblasts and inflammatory cells. Their activity has to be is highly regulated, considering that too much MMPs causes a lot of tissue damage by excessive ECM degradation. MMPs are secreted in latent form and are only activated by proteolytic cleaving. They are not stored and activity is inhibited by tissue inhibitors of metalloproteinases (TIMPs). MMPs and TIMPs bind in a 1 on 1 manner and balance between these two is essential for ECM homeostasis[18,19,25].

Epithelial barrier and epithelial mesenchymal trophic unit

The ECM does not only provide strength and support for cells, but also interacts with them. While cells secrete signals (cytokines and growth factors) that regulate the composition of the ECM, the ECM in turn regulates cellular processes (proliferation, differentiation, motility etc.) by binding to the cells (via for example integrins). In the lungs the ECM plays an important role in maintaining the epithelial barrier. The epithelial barrier can be divided into two types according to function. The epithelial barrier of the airways (trachea, bronchi and bronchioles) is responsible for warming and moistening the air, clearing the air of possible toxic particles (by mucociliary clearance) and preventing toxic particles from entering the human body through forming a physical and immunological barrier. It is composed of different types of epithelial cells, such as ciliated cells, mucous-producing cells, Clara cells and undifferentiated basal cells[26,27]. The epithelial barrier of the alveoli is mainly responsible for gas exchange and is composed out of type I and type II alveolar epithelial cells or pneumocytes. Pneumocytes type I are responsible for the gas exchange, while type II ensure tissue integrity by secreting pulmonary surfactant, ensuring efficient gas exchange. It is a progenitor cell of type I pneumocytes[26,28,29].

Underneath the epithelium there is a thin layer of fibroblasts in the ECM that are in close contact with the epithelial cells. This layer of epithelium, ECM and fibroblast is called the epithelial mesenchymal trophic unit (EMTU) and was first described by Evans and colleagues. They suggested that the close bidirectional communication between cells and matrix in the EMTU are responsible for keeping airway homeostasis[30]. This close contact allows them to efficiently and quickly control inflammatory reactions and repair and remodeling processes when an injury occurs. [30–32].

Apart from the epithelial cells and fibroblast, airway smooth muscle cells (ASM) are also found in the airway tissue. They have important functions in the constriction and dilution of the bronchi, but are also regulators of the airways homeostasis by producing ECM components and factors like cytokines, growth factors and inflammatory mediators[33,34].

Chapter 2 ECM airway remodeling in COPD

As mentioned in the introduction, the exposure to noxious gases leads to remodeling of the ECM. In this chapter it will be discussed in detail which pathways are responsible for the ECM remodeling, what this remodeling consists of and what this means for the function of the airway. While we will only focus on the pathways that lead to ECM remodeling, it is important to point out that there are many more processes involved in the pathology of COPD.

Tissue repair upon damage to the epithelial barrier

Upon encountering noxious gases, the epithelial cells of the airways are damaged by increasing ROS levels in the cell[8]. This causes the epithelial cells to initiate a wound healing process (see figure 3). Epithelial cells release proinflammatory cytokines, like interleukin-8 (IL-8/CXCL8), tumor necrosis factor α (TNFα), IL-1α and IL-1β[3,35,36], which attract inflammatory cells like macrophages and neutrophils to the site of injury, and growth factors like transforming growth factor-β (TGF-β), that induce tissue repair. Platelets are also attracted to the site of injury. They promote blood vessel dilation and increase permeability, allowing the inflammatory cells to infiltrate the damaged tissue. The migrated inflammatory cells (mainly macrophages and neutrophils) start to clear tissue of damaged cells and infiltrating toxins, cause ECM remodeling by producing MMPs and increase the recruitment of specific inflammatory cells (like leukocytes) by producing ROS. Together with the damaged epithelial cells, they also release factors that induce tissue repair, like TGF-β[37,38]. These factors cause proliferation and recruitment of cells that transdifferentiate into myofibroblasts[39,38]. Two cell types that are known to be able to transdifferentiate into myofibroblasts are the resident fibroblasts present in the ECM of the epithelium and the fibrocytes (fibroblastic stem cells) recruited from the circulation. It is believed that there are two more cell-types able to undergo this

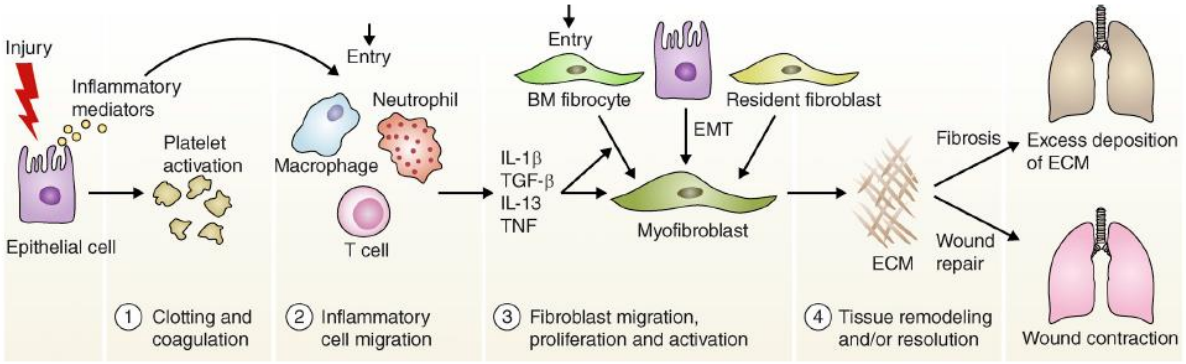

Figure 3 Schematic view of tissue repair of epithelial cells. Upon injury the epithelial cells react by producing inflammatory mediators that attract platelets and inflammatory cells. Platelets increase permeability of the blood vessel allowing the inflammatory cells to migrate toward the site of injury. Epithelial cells and inflammatory cells release factors promoting the fibroblast to myofibroblast transdifferentiation of resident fibroblasts, fibrocytes, epithelial cells (through EMT) and ASM (trough SMT). Activated myofibroblast are able to produce ECM components which are needed for the injury to be healed. After tissue is repaired myofibroblast undergo apoptosis and inflammatory cells migrate back into the blood vessels (not shown). However, in aberrant tissue repair, myofibroblasts and inflammatory cells stay active causing an excess deposition of ECM leading to impaired function of the lungs.

transdifferentiation into myofibroblast, namely epithelial cells (by epithelial-to-mesenchymal-transition or EMT) and smooth muscle cells (by smooth muscle to mesenchymal transition or SMT). However, while in theory this transition is possible, it is not yet proven. [34,40,41].
Before fibroblast differentiate into myofibroblast, they differentiate into proto-myofibroblasts[42]. Proto-myofibroblasts are cells that form cytoplasmic actin stress fibers

causing them to contract. This contraction is essential for the tissue repair of the epithelial barrier. It causes the site of injury to decrease in size so smaller surface needs to be repaired. Subsequently, proto-myofibroblasts transdifferentiate further into myofibroblasts under the influence of TGF-β and IL-1β secreted by damaged epithelial cells and attracted inflammatory cells. Myofibroblasts are cells characterized by the expression of α-smooth muscle actin (α-SMA) and show an increased expression of cytoplasmic actin stress fibers, which causes them to have an even higher contractile force. Fully differentiated myofibroblasts start regulating secretion of ECM components that are needed to repair damaged tissue[3,38,42]. When the epithelial cell layer is repaired and the ECM components are back to their normal composition, the inflammatory cells migrate back into the bloodstream. Myofibroblast have been shown to undergo apoptosis when the mechanical stress caused by the injury is lost[42,43].

Aberrant tissue repair in COPD

In COPD remodeling of the ECM after injury is disturbed. While normally repair processes in tissue would cease once the tissue is healed, this does not happen in COPD[34,42]. This is because there is a constant supply of noxious gases that cause injury to the tissue of the airways. In patients developing COPD this leads to a constant and chronic activation of the repair mechanisms and inflammatory processes, causing aberrant tissue repair. However, not everybody who is constantly exposed to noxious gases develops COPD. This is because there are many more factors that contribute to the development of COPD, like age, gender, genetic predisposition and lung growth and development[1].

In patients with COPD the aberrant tissue repair does not manifest in one specific phenotype throughout the lungs. As mentioned earlier, the composition of the epithelium and underlying ECM is different in different parts of the lungs. The biggest distinction is made between the airway walls (bronchi and bigger bronchioles) and the parenchyma (alveoli and small bronchioles). Because the composition is different, the aberrant tissue repair seen in COPD is also different per region. In the bronchi and bronchioles there is excessive ECM deposition which causes airway wall fibrosis, whereas in the lung parenchyma ECM degradation by proteolysis is occurs which is not sufficiently repaired. What causes this difference in remodeling processes is poorly understood[34,39,44]. The mechanisms are discussed in more detail below.

EMTU disruption & fibrosis

As mentioned earlier, the EMTU controls the responses of the epithelium and underlying fibroblast to environmental changes such as injury via close, bidirectional communication between cells and matrix[32]. Research of Behzad and colleagues showed that there is contact between the epithelium and fibroblast in the EMTU through small cytoplasmic extensions, causing them to be able to communicate. They also showed that in early stages of COPD there is a reduced contact between the epithelium and fibroblasts, caused by damage through oxidative stress in the epithelial cells, which leads to a decrease of intercellular communication[31]. They suggested that this loss of contact liberates the fibroblast from inhibitory control of the epithelium to secrete ECM molecules. Liberated fibroblasts are in turn more susceptible to other factors controlling them. The damaged epithelium and inflammatory cells recruited to the site of injury produce factors like TGF-β and IL-1α, which cause fibroblast to transdifferentiate into myofibroblast and start to produce an excessive amount of ECM components, mainly collagen and fibronectin. This uncontrolled deposition of ECM components can eventually lead to airway wall fibrosis. This fibrosis in COPD is characterized by a thickening and stiffening of airway wall tissue leading to impaired function [3,45].

Emphysema caused by proteolysis

Emphysema is characterized by the destruction of alveolar wall structure due to extensive inflammation and ECM degradation, resulting in enlarged airspaces in the parenchyma. This leads to loss of elastic recoil, hyperinflation of the lung and airway collapse, altogether resulting in a loss of FEV_1 [34,44,46,47].

In healthy tissue there is a balance between proteases, such as the MMPs (see chapter 1) and neutrophil elastase (a protease specifically cleaving elastin) and antiproteases, such as TIMPs and α-1- antitrypsin (a antiprotease inhibiting neutrophil elastase)[18,47,48]. However, in COPD there is an imbalance, with the amount of proteases exceeding the amount of antiproteases present. This imbalance is caused by two processes. First of all the noxious gases, which cause tissue damage, are able to inhibit antiprotease activation in the airways[48]. Secondly, myofibroblasts and inflammatory cells migrated towards the site of injury produce proteases (myofibroblast and macrophages mainly produce MMPs, while neutrophils produce neutrophil elastase) to help get rid of debris and unwanted toxins. This leads to an overproduction of proteases, while antiprotease function is inhibited. This overload and constantly activated proteases causes degradation of (healthy) ECM components, especially elastin, which ultimately leads to emphysema[34,39,48].

Changes of ECM components in COPD

The aberrant tissue repair processes seen in COPD lead to the remodeling of the ECM by excessive ECM deposition in the airways (leading to airway fibrosis), as well as ECM degradation in the lung parenchyma (leading to emphysema). To fully understand the phenotype seen in COPD it is important to look further into the details of the ECM remodeling. To do this it is necessary to investigate which specific ECM components are overexpressed or degraded in which part of the lung. It is also important to look at the transcriptional levels of these components, because it could well be that for one of the components mRNA levels are highly increased, but that there is an even faster degradation of the protein in the ECM, which eventually could still be causing a decrease in total protein deposition. Lastly, it is important to distinguish in which GOLD stage the data was found, considering each stage could have a different phenotype. All studies found were performed using human lung samples, either from biopsies taken from COPD patients or healthy subjects.

Collagen

Eurling et al. showed that the overall deposition of collagen (all types) was increased in all GOLD stages of COPD in both the small airways and the parenchyma[49]. However, as described earlier, there are six specific collagen types found in the lungs. Studies assessing specific collagen types found varying results Table 1 shows the changes in deposition of collagen type I, III and IV in COPD compared to healthy subjects. No data was found on collagen type II, V and VI. While it is often assumed that chronic inflammation and fibrosis in the small airways leads to the excessive deposition of collagen I and III, this cannot be concluded when looking at table 1, as the data is very inconsistant[17,50–52]. This could be attributed to the way the research was performed. For example, while Harju et al. looked at the expression level of the precursor proteins for collagen I and III, Annoni et al. looked at the fractional areas (amount of deposition) of ECM components in different lung compartments[53,54].

Collagen type	Small airways		Lung parenchyma	Airway*
	Mild/moderate	Severe	Mild/moderate	Mild to severe
I	↓[54] ; ↑[50,53]	↓[53]	↑[54]	↑[55]
III	↓[53] ; ↔[54]	↓[53]	↔[54]	↑[50]
IV	↔[54]		↔[54]	↑[50]

*Table 1 Changes in expression levels of collagen type I, III & IV. *in these articles it was not defined which part of the lung was used for this analysis. References according to the numbers; 47= Kranenburg et al.,2006;50= Harju et al.,2010; 51= Annoni et al.,2012; 52= Pini et al., 2014*

Elastin

Data regarding changes of elastin deposition in COPD is also discordant. Most studies investigating the changes of elastin in COPD find that there is a decrease in protein expression, in both the airways and the parenchyma and in all stages of GOLD (mild to severe)[49,56,57]. However, Annoni et al. saw no changes in elastic fiber content in the airways and the parenchyma in COPD patients compared to healthy controls[54]. One study showed that there was an increase of mRNA expression of elastin in severe COPD. However, they also showed that the elastin fibers were disorganized and that this possibly contributes to the progressive loss of elastic recoil seen in the lungs. They suggested that the increased mRNA expression of elastin and the disorganized elastin fibers were caused by failed attempts of the tissue to repair elastin levels [58].

Fibronectin and laminin

Kranenburg et al. and Annoni et al. showed that there were increased deposition levels of fibronectin in the small airways in mild and moderate COPD, possibly contributing to airway obstruction, but not in the parenchyma[50,54]. However, Gosselink et al. showed that there was a decrease of gene expression of fibronectin in tissue surrounding the small airways in severe COPD[59]. There was not much data found on the difference in laminin levels in COPD compared to controls. Kranenburg et al. showed that the deposition of laminin was increased in the small airways in mild and moderate COPD, again possibly contributing to airway obstruction[50]. Liesker et al. and van Straatten et al. showed no change in laminin protein expression in the small airways in moderate COPD compared to controls[60,61].

Glycosaminoglycans and proteoglycans

As mentioned in chapter 1 the most abundant non-sulfated glucosamineglycans is hyaluronic acid. Eurlings et al. found an overall increase in hyaluronic acid deposition in the parenchyma and the small airways in patients with mild to severe COPD[49]. Dentener et al. showed an increase in hyaluronic acid levels in the sputum of patients with mild to moderate COPD compared to controls contributing to alveolar and small airway wall remodeling[62].

Chapter 1 also described that there are three main proteoglycans in the airways consisting of different types of sulfated glycosaminoglycans and a core protein. Table 2 shows the differences found in protein expression of these four proteoglycans in COPD patients compared to healthy subjects.

Proteoglycan	Small airways		Lung parenchyma		Airway *
	Mild/moderate	Severe	Mild/moderate	Severe	Moderate/Severe
Versican	↔[54]	↑[63]	↓[54];↑[57]	↑[63]	↑[55]
Perlecan					↓[63]; ↑[64,65]
Decorin	↔[54]	↓[44,61]	↔[54,57]	↓[44,61]	↔[55]

*Table 2 Changes in expression levels of versican, perlecan, syndecan and decorin. *in these articles it was not defined which part of the lung was used for this analysis. References according to the numbers; 41= Zandvoort et al., 2006; 49= Annoni et al.,2012; 50= Pini et al., 2014; 52= Merrilees et al., 2008; 56= Hallgren et al., 2010; 57= Krimmer et al., 2012; 58= Ichimaru et al., 2012*

While there was no difference found in expression of decorin in mild and moderate COPD by Annoni et al. and Pini et al., there was found a decrease in decorin levels when patients had severe COPD by Zandvoort et al[44,54,55]. Furthermore, Tjin et al. showed that there was a correlation between the loss of decorin expression and the morphology of collagen fibrils. They found that collagen fibrils of COPD patients were immature and less organized than those of healthy controls[66].

For versican a clear increase in protein expression levels was found in patients suffering from moderate to severe COPD, compared to healthy subjects. However, data was inconsistent when comparing expression levels of versican in mild to moderate CODP with controls[54,55,57,63] Merrilees et al stated that high levels of versican led to a decrease in number of elastin fibers, as versican would inhibit the protein that assembles the elastin fibers, elastin binding protein (EBP), by its chondroitin sulphate chain (which is a sulfated GAG)[57].

There were contradictory data found for perlecan expression. Ichimaru et al. showed that there was an increased expression of perlecan by airway smooth muscle cells under influence of TGF-β1 and Krimmer et al. showed there was an increased deposition of perlecan by human lung fibroblasts[64,65]. However, Hallgren et al. found that there was a decrease in expression levels of perlecan by hum lung fibroblasts of the central airways in severe COPD[63].

Tenascin-C

For tenascin-C there was found an increase in protein expression in the small airways in mild to moderate COPD[54,60,67]. No difference between healthy subjects and COPD patients was found in protein expression levels in the parenchyma[54].

Matrix metalloproteinases

An increase of protein expression MMP subtypes -2,-9 and -12 by macrophages was found[57,68]. Lagente et al stated that MMP levels were increased by the inflammatory processes in COPD, while TIMP levels remained the same. This imbalance of MMP/TIMP causes degradation of elastin. This degradation leads to elastin fragments, which in turn cause an increase in recruitment of inflammatory cells to clean up this debris, creating a vicious circle leading to emphysema[48,68].

Chapter 3 ECM airway remodeling in ageing

As stated earlier, ageing is a process characterized by a progressive decline in tissue homeostasis over time, which leads to an increasing risk of degenerative diseases and death[7,9]. There are nine hallmarks of ageing, all describing processes that change in ageing[10]. However, Meiners et al. proposed that for lung ageing there should be one more hallmark; the dysregulation of extracellular matrix[11]. In this chapter we will first briefly describe the overall changes seen in the ageing lung, after which we will focus on the changes seen in the ECM components of the ageing lung.

Ageing of the lung

As the lung ages, more and more stressors from the outside environment damage the cells. This causes an increase of ROS levels and a loss of effectiveness of antioxidant defenses. This ultimately leads to cellular senescence. While senescent cells are in permanent cell cycle arrest, they remain metabolically active, with an altered protein expression profile. This altered expression profile is called the senescence-associated secretory phenotype (SASP) and mainly results in the excessive production of MMPs, cytoskeletal proteins, growth factors and inflammatory mediators[6,12]. This SASP leads to multiple changes contributing to the ageing of the lung. First of all, it leads to systemic immune dysfunction characterized by inflamm-ageing and immunosenescence. The increase of inflammatory mediators and MMPs leads to activation of the immune system, while there is no real immunological threat. The recruitment and activation of inflammatory cells in turn leads to more ROS and MMP-secretion. It is believed that inflamm-ageing therefore contributes to the loss of elasticity and destruction of lung parenchyma, leading to emphysema. Parallel to inflamm-ageing, immunosenescence occurs, which is a blunted or absent reaction of the immune system to clear the tissue of stressors (bacteria, viruses, toxins etc.) and damaged, apoptotic/necrotic or senescent cells[6,10,69]. All together this leads to an even bigger, chronic inflammatory response, as the remaining damaged and senescent cells keep producing inflammatory mediators. Secondly, the SASP leads to a profibrotic phenotype in ageing lungs[70]. The increase in production and secretion of TGF-β by senescent cells leads to the increase of fibroblast to myofibroblast transdifferentiation. Furthermore, when ageing, fibroblasts show a decrease in proliferation and migration and an increase in myofibroblast differentiation[71]. Altogether, the SASP causes remodeling of ECM. The inflamm-ageing, immunosenescence and increased myofibroblast transdifferentiation lead to an altered expression of ECM components through changes in expression of factors that stimulate matrix production like TGF-β, but also in factors that stimulate degradation of matrix molecules like MMPs[70,72].

Changes of ECM components in ageing

Not much data is found on the expression of ECM components in ageing, especially in human subjects. This is not that surprising, considering that the need to study healthy, ageing subjects is not that high, because they do not need of any therapies or treatment. Studies discussed in this part were mainly performed in animals (rats or mice) and data was summarized.

Collagen

All studies investigating collagen deposition in ageing showed an increase of collagen deposition with age, however, none of the studies specifically stated which area of the lungs (parenchyma or airways) was investigated. Calhoun et al. was the only study found which compared humans. They found an increase in collagen deposition in aged humans (50-60 years old) in comparison to younger humans (20-30 years old) was found. Furthermore, they showed an increase in cross-linked and newly synthesized collagen (overall, no specific type was

tested) deposition in mice[12]. Godin et al. found an increase in collagen deposition in older mice, compared to young ones[73]. Sueblinvong et al. showed an increased mRNA expression and deposition of collagen I in aged mice compared to younger mice when injury was induced[74]. Calabresi et al. found that collagen type I and III protein accumulation was increased in old rats. Interestingly, while there was an increase in mRNA expression for collagen type I, there was not for type III. Calabresi hypothesized that the accumulation of collagen type III protein was not due to more synthesize, but rather to a lower degradation of this collagen[75].

Elastin
All found studies showed a decrease of elastin and elastin fibers. Godin et al. found an overall decrease in elastin protein and mRNA levels in old mice[73]. Huang et al. showed there was a significant decrease in elastin fibers in the parenchyma of old mice. They also showed an increase of collagen deposition. However, they showed that the degradation of elastin fibers occurs as one of the earliest remodeling processes in the parenchyma, while the increase of collagen occurs later[76].

Fibronectin & Laminin
Data found in animal studies for both fibronectin and laminin expression were conflicting. While Godin et al. found a decrease in protein expression and mRNA levels fibronectin with increasing age, Calabresi showed no change in fibronectin protein expression[73,75]. For laminin, Calhoun and colleagues found an upregulation of laminin deposition in aged lungs, while Godin et al. showed a decreased in mRNA levels and deposition of laminin[12,73].

Glycosaminoglycans and proteoglycans
The only data found on glycosaminoglycans was in a study done by Godin et al. They showed that there was an increase of sulfated GAGs in aged lungs of mice, but they did not characterize the different type of GAGs[73]. No data was found on expression of proteoglycans.

Tenascin- C
The only data found on tenascin-C expression was in a study of Calhoun et al. They showed an increase of tenascin-C in the lungs (mainly in the parenchyma) of old mice compared to younger mice[12].

Matrix metalloproteinases
Contradiction data was found when studying MMPs. Calabresi et al. found a significant decrease in MMP-1 and -2 protein expression and a small (but not significant) decrease in MMP-9 levels in ageing rats. They also found that the inhibitors of MMPs, TIMP-1 and -2, were increased. They suggested that the fibrosis seen in old animals was due to the collagen accumulation and increase in MMP inhibitors[75]. Sueblingvong et al. found an increase in MMP-2, MMP-9 and TIMP-2 mRNA expression in lungs of old mice . No change in TIMP-1 expression was found. They suggested that the increase of MMPs led to a fibrotic phenotype, considering MMPs stimulate the production of TGF-beta, which in turn causes fibroblasts to produce collagens[74].

It is important to note that all changes found in ECM compositions are subtle and they do not cause extreme remodeling of the lung tissue. Changes in matrix composition lead to alterations in tissue mechanics (stiffness and elastic etc.), which drives cells of the epithelial barrier to change gene expression, for example upregulation of TGF-beta[6]. While these changes do not cause a strong decline in lung function, they slowly damage cells and alter immune reactions and repair mechanisms[70,72]. This, in turn, leads to the slow, but progressive decline in lung function seen in ageing[70].

Discussion and conclusion

In this thesis we wanted to investigate whether the remodeling of the ECM seen in COPD is similar to the remodeling of the ECM seen in ageing. To determine this, we first described the main components of the ECM of healthy lungs and their function. We found that the main components of the airway ECM are collagens, elastin, fibronectin, laminin, glycosaminoglycans, proteoglycans, MMPs (and their inhibitors) and tenascin-C. Their main function is to provide a physical scaffold for the cells in the epithelial barrier and fibroblast and ASMs residing in the ECM and to maintain tissue homeostasis of the lung. The ECM is also found to play a major role in tissue repair. Damaged epithelial cells and inflammatory cells produce factors that lead to the production of ECM components by fibroblasts, promoting tissue repair. However, in patients with COPD aberrant tissue repair and chronic inflammation occur, leading to remodeling of the ECM. When looking at changes in specific components of the ECM in COPD patients compared to controls, studies overall showed an increase in total collagen deposition and a decrease in total elastin deposition in the small airways as well as the parenchyma. The proteoglycans showed a changed protein expression when patients had severe COPD; versican levels were increased, decorin levels were decreased. The expression of MMPs, subtypes 1,9 and 12, by macrophages was increased, while the expression of their inhibitors, the TIMPs, did not change. This imbalance in MMP/TIMP was suggested to cause elastin degradation leading to emphysema. Tenascin-C protein expression was found to be increased, but only in the small airways. Data for fibronectin, laminin and perlecan levels (protein levels, as well as gene expression levels) were contradicting.
When looking at the changes in ECM compositions in ageing animal lungs compared to young animals studies showed an overall increase of collagen deposition and decrease of elastin deposition throughout the whole lungs. Tenascin-C expression in the parenchyma of ageing mice was increased. Data for fibronectin, laminin and the MMPs levels (protein levels, as well as gene expression levels) were conflicting and no data was found on the expression of proteoglycans in aged lungs.
When comparing the specific ECM components of COPD lungs (human) with ageing lungs (mice/rat), we found that for collagen (increased deposition), elastin (decreased deposition) and tenascin-C (increased deposition) the same changes in expression occurred. For the other ECM components discussed no comparison could be made, due to conflicting or lack of data. However, when comparing the processes involved in the ECM remodeling seen in COPD with the processes involved in the ECM remodeling seen in ageing, similarities are found. In both ageing and COPD chronic inflammation, caused by oxidative stress, results in a profibrotic phenotype and emphysema. This ultimately causes aberrant tissue repair and ECM remodeling. The biggest difference between the COPD and ageing seems to be the speed of which the processes occur. In COPD the chronic insult of noxious gasses causes constant oxidative damage to epithelial cells, which, in turn, cause chronic activating inflammatory cells like macrophages, neutrophils and leukocytes and constant transdifferentiation of fibroblasts to myofibroblasts. This ultimately leads to aberrant tissue repair and ECM remodeling, resulting in small airway fibrosis and emphysema in the alveoli. In ageing, all processes mentioned above establish much slower. Over time, built-up oxidative damage done to the epithelium by lifelong stressors form the outside environment causes cellular senescence. Senescent cells have an altered expression profile (SASP), which results in inflamm-ageing and immunosenescence. This causes a chronic activation of the immune system, while there is no real immunological threat, leading to increased production of MMPs, ROS, interleukins and growth factors. This eventually results in a pro-fibrotic phenotype and emphysema.

Thus, the oxidative stress levels in epithelial cells in COPD build up fast by a chronic increase of ROS by noxious gases, while the oxidative stress in epithelial cells in ageing build up slowly, considering the ROS levels increase by sporadic interactions with toxic particles.

As mentioned above a lot of contradicting data was found. A possible explanation for this could be that the ECM remodeling seen in COPD and ageing is a highly diverse process. However, it is more likely that the contradicting data are the result of different study designs. For example, in COPD studies different criteria can be used for patients and healthy subjects. Differences can also be explained by the way data was collected; while some studies collect data out of qPCR experiments, which produces data in the form of gene expression profiles, others use stainings, which indicate protein deposition levels. Increase in gene expression profiles does not automatically mean that there also is an increase in protein deposition. For example, it could be that there is an increase of protein expression of MMP, but still a decrease of protein deposition, because there is also a high production of the antiprotease TIMP, which degrades the MMP proteins. Furthermore, many studies did not specify which area of the lungs was tested, however, there are differences in epithelial barrier and ECM composition in different areas of the lungs. Lastly, while COPD studies mainly used human biopsies to study ECM changes, all ageing studies found were done in ageing lungs of rat or mice.

In conclusion, while comparing the individual ECM component changes in COPD and ageing was difficult due to contradicting and lack of data, it was found that there are high similarities between the processes involved in the remodeling of the ECM in COPD in ageing. However, data suggests the difference between the two is the speed with which this remodeling occurs. It is therefore plausible to state that remodeling of ECM seen in COPD is a process of accelerated lung ageing. To fully understand the changes in individual components of the ECM in COPD and ageing, more uniform studies should be performed, with the same subject criteria and experimental set up. We propose a study should be done comparing changes in ECM components in young subjects, old subjects and COPD patients, to get a clear and complete picture of the changes and processes involved in ECM remodeling in ageing and COPD.

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