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Cigarette smoke-induced oxidative stress in COPD

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CHAPTER 1

General Introduction

Pathogenesis of Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a chronic disease predominantly affecting the lungs, with an increasing rate in morbidity and mortality, especially in the elderly, and high healthcare costs (43). Estimates from the world health organization (WHO) suggests that 65 million people worldwide have moderate to severe COPD. It is currently the fourth leading cause of death (39, 43), and it is estimated to be the third leading cause of death worldwide by 2030 (WHO, 2014 http://www.who.int). COPD is characterized by chronic lung inflammation, resulting in aberrant lung tissue repair and remodelling, not fully reversible airflow limitation and accelerated lung function decline. COPD includes chronic bronchitis, with increased deposition of structural proteins, thickening of the small airway walls and mucus production on one hand, and emphysema, with degradation of structural proteins in the alveoli, loss of alveolar tissue and loss of lung elastic recoil on the other. These features can occur separately and within the same patient (6, 8). The most prominent risk factors for COPD are cigarette smoking, environmental cigarette smoke exposure or exposure to other noxious particles and gases, including other tobacco smoke, fuels and wood smoke (42, 43). Tobacco smoking is a major problem in the western world and upcoming industrialized countries and reaches an almost epidemic proportion. The `World Health organization` (http://www.who.int/tobacco/global_report/2011/en/) reports that worldwide, the use of tobacco causes more than 5 million deaths per year, and current trends show that tobacco smoke will cause more than 8 million deaths annually by 2030. Also, smoking is the fourth most common risk for developing other serious diseases, including various cancers, stroke and cardiovascular disease. Crucially, non-tobacco related risk factors of COPD include genetic deficiencies, in particular alpha1-antitrypsine deficiency, and occupational exposure to dusts and chemicals. Important to note is that 95% of the COPD patients are smokers, but only 10-15% of the smokers actually develop COPD, indicating that genetic susceptibility contributes to the development of COPD (6, 15, 28, 39, 42, 43, 56, 57, 68).

Inhaled cigarette smoke induces an aggravated inflammatory response and abnormal tissue damage and repair responses in the lungs of COPD patients. The chronic inflammatory response in the airways of COPD patients is characterized by infiltration of inflammatory cells, e.g. CD8$^+$T-cells, neutrophils and macrophages. These cells release a variety of proteases, including neutrophil elastase, known to be responsible for the degradation of the elastin and thus emphysema development (8, 41, 70). Inhaled cigarette smoke first encounters the airway epithelium, continuously lining the airways and forming a tightly regulated barrier against environmental insults. The airway epithelium is also a source of pro-inflammatory cytokines, especially when damaged. Inhaled cigarette smoke induces oxidative stress and epithelial damage, leading to activation of epithelial cells in the lungs and the release of several cytokines and chemokines, including Interleukin 6 (IL-6), CXC-chemokine ligand 1 (CXCL1), CC-chemokine ligand 2 (CCL2), Chemokine C-X-C-Motif Ligand 8 (CXCL8) and

Granulocyte-macrophage colony-stimulating factor (GM-CSF) (7, 8, 10, 18, 29, 36, 49). CCL2, CXCL1 and CXCL8 attract monocytes and neutrophils to the lungs, while GM-CSF promotes the survival, proliferation and differentiation of neutrophils (7). In addition, epithelial cells produce CXCL9, CXCL10 and CXCL11, which are responsible for the recruitment of T helper 1 (Th1) cells and type 1 cytotoxic T (Tc1) cells to the lungs (7, 10, 18). Th1 cells produce cytokines that promote the recruitment and activation of inflammatory cells, while the production of proteolytic enzymes by Tc1 cells induces apoptosis or necrosis of epithelial cells (7).

Next to chronic inflammation of the lungs, systemic alterations have been observed in COPD patients, with systemic inflammation, a decrease in body weight and loss of skeletal muscle mass (wasting) as common manifestations of the disease. It is well known that skeletal muscle cells of COPD patients are more dependent on the glycolytic metabolism than oxidative metabolism. As a result, less adenosine triphosphate (ATP) is produced per mole of glucose and more rapid acidification occurs in muscle tissue of COPD patients (24, 59, 60). This phenomenon is thought to be an indication for a mitochondrial dysfunction in these cells.

Treatment of COPD: Glucocorticoid responsiveness

Current treatment with long-acting bronchodilators and inhaled glucocorticoids (IGC) provides relatively little therapeutic benefit in COPD, despite the broad anti-inflammatory effects of IGC. They reduce exacerbations, but do not effectively change the course of neither the disease nor the tissue damage that results from chronic airway inflammation. The GLUCOLD study demonstrated beneficial glucocorticoid (GC) effects on airway wall inflammation and decline in lung function; however, there were great inter-individual differences (38). The poor response of COPD patients to IGC may stem from the development of GC insensitivity, in which oxidative stress has been implicated (5, 19).

GCs are the first choice of medication used to suppress an inflammatory response and belong to a class of steroid hormones that bind to and activate the cytosolic glucocorticoid receptor (GR) α, which is present in almost all eukaryotic cells. Upon its activation, GR translocates to the nucleus to regulate the expression of anti-inflammatory genes with a glucocorticoid response element (GRE) in their promoter. In addition, GR activation induces recruitment of histone deacetylases, leading to reduced access of various transcription factors, e.g. nuclear factor (NF)-κB, to promoter regions in pro-inflammatory genes (5, 63).

In vitro studies have shown that the ability of dexamethasone to suppress cytokine release (e.g. CXCL8) from alveolar macrophages is impaired in COPD patients compared to healthy smokers (21). Furthermore, macrophages from healthy smokers are more resistant to GCs than alveolar macrophages from non-smokers (62). We have previously shown that GC insensitivity also exists in bronchial epithelial cells from COPD patients, and may be the result of excessive oxidative stress (29), leading to reduced suppressive effects of IGC on pro-inflammatory cytokine production. Indeed, oxidative stress has been linked to GC unresponsiveness. Oxidative stress can induce PI3K-dependent post-translational histone deacetylase 2 (HDAC2) modifications, leading to reduced expression and activity of HDAC2 (1, 32, 45, 61). In addition to the regulation of NF-κB response elements, HDAC2 can deacetylate the GRα (31–33). In line with a role for reduced HDAC2 expression in the observed GC unresponsiveness in COPD, reduced HDAC2 expression has been observed in the lungs and alveolar macrophages of COPD patients and has been implicated in GC insensitivity in COPD (32, 33). This suppression may also play a role in reduced GC responsiveness of the airway epithelium, which is in first contact with cigarette smoke, inducing oxidative stress (60, 74). GC insensitivity may be gradually acquired by smoking, and because of the increased burden of oxidative stress in COPD patients, we propose that smokers with COPD are more prone to develop GC insensitivity than smokers with normal lung function.

Mitochondria and oxidative stress

Mitochondria are thought to play an important role in pathologies associated with oxidative stress, including COPD. Mitochondria are oval to rod shaped cellular organelles that have a double membrane structure, where the internal membrane is lobular shaped to increase its surface area for proper energy production. Mitochondria are present in most eukaryotic cells with a wide variety in numbers ranging from a none (mature erythrocytes) to several thousand mitochondria per cell (liver and muscle cells), depending on the cell type. Lung cells contain relatively high numbers of mitochondria, although exact mitochondrial numbers are hard to determine per cell type because their occurrence correlates with the cell's level of metabolic activity. The mitochondrial inner membrane is composed of lipids, including cardiolipin similar to those found in prokaryotic cells and it is the cell's only organelle that contains its own circular shaped DNA (mitochondrial DNA; mtDNA) (14, 26). The specified lipid and DNA profile in mitochondria led to the `endosymbiotic theory`, which proposes that prokaryotic cells were taken up inside a eukaryotic cell as an endosymbiont, and that most of the prokaryotic genes have been transferred to the host cell genome over time (25). Mitochondria are often referred to as the `energy factories` of the cell and they are composed of various compartments that carry out multiple specialized functions involving energy metabolism. These compartments include the outer membrane, the inter-membrane space, the inner membrane, the cristae and matrix (Figure 1). The matrix of the mitochondrion contains enzymes of the tricarboxylic acid cycle (TCA) or Krebs cycle and other structures and compounds including ribosomes, matrix granules and mitochondrial DNA. Energy is derived from oxidative phosphorylation (OXPHOS), a process involving electron transfer over the inner-membrane and proton pumps that drive the synthesis of chemical energy in the form of ATP. Next to producing energy in the form of ATP, the mitochondria are responsible for programmed cell death, calcium storage and signaling, roles in cellular proliferation and differentiation, hormone and haem synthesis and cellular signaling processes (4). Together, this makes the mitochondria key regulators of cellular function, which is pivotal for cellular homeostasis in healthy tissue. Depending on the cell type, mitochondrial dysfunction can lead to the loss of various crucial processes, including ATP production, apoptosis regulation, cellular proliferation, differentiation and signaling processes, calcium storage and signaling.

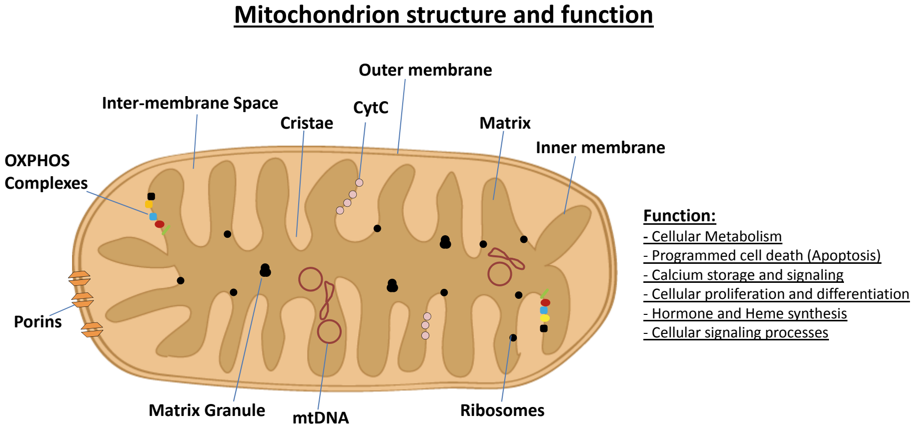

Mitochondrion structure and function
Outer membrane
Inter-membrane Space
CytC
Cristae
Matrix
Inner membrane
OXPHOS Complexes
Porins
Matrix Granule
mtDNA
Ribosomes
Function:
- Cellular Metabolism
- Programmed cell death (Apoptosis)
- Calcium storage and signaling
- Cellular proliferation and differentiation
- Hormone and Heme synthesis
- Cellular signaling processes

Figure 1: Overview of a mitochondrion and important matrix located components which all serve various important functions in the cells. Cytochrome C; CytC, Oxidative Phosphorylation; OXPHOS, Mitochondrial DNA; mtDNA.

For the production of ATP, ~85% of the cell's oxygen is consumed, while a small fraction of this is converted to superoxide ($O2^{-}$), a highly reactive radical that needs to be neutralized as quickly as possible (55, 66). Thus, while electron leakage and subsequent oxidant production is a normal consequence of the OXPHOS process, oxidative stress should be controlled. Mitochondria have their own quality control and mechanisms to ensure a healthy mitochondrial population within the cell, including endogenous anti-oxidant defense mechanisms and exchange of mtDNA through fission and fusion processes with neighboring mitochondria. When anti-oxidant enzymes, e.g. superoxide dismutase, catalase and glutathione cannot neutralize the superoxide radical to H_2O fast enough, oxidative damage to components will occur and accumulate within the mitochondria (23, 55). Mitochondria that are damaged beyond repair are normally cleared through a process called mitophagy. This process is initiated by the recruitment of the proteins peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (*PPARGC1α*), PTEN induced putative kinase 1 (*PINK1*) and *Parkin,* proteins involved in mitochondrial biogenesis, repair or clearance (71). In addition, telomerase, which was initially thought only to have a nuclear function, has been shown to have an protective effect on mitochondrial DNA as it migrates to the mitochondria during times of excessive oxidative stress (27). Furthermore, the energy metabolism protein Glycogen-synthase kinase (GSK3)β, has recently been shown to be present in mitochondria, protecting the mitochondria against oxidant induced responses (27, 54). Increased oxidative stress, e.g. upon long-term cigarette smoking and/or impaired anti-oxidant responses, can cause mtDNA mutations and damage, excessive electron leakage and increased production of reactive oxygen species (ROS). In turn, mitochondrial dysfunction may lead to excessive ROS production in a vicious circle. ROS are known to stimulate inflammation by activating the pro-inflammatory transcription factor NF-κB. Indeed, it has been shown that mitochondrial dysfunction, induced by different chemical compounds, can increase or induce inflammatory responses by increasing the expression of CXCL8 and cyclooxygenase 2 (COX-2) (77, 78). Additionally, mitochondrial dysfunction may lead to cellular damage and impaired regeneration responses. Mitochondrial dysfunction has been linked to cellular ageing, which also results in accumulation of mtDNA mutations and impairment of oxidative phosphorylation. Importantly, cigarette smoke has been shown to induce oxidative stress in mitochondria of lung epithelial cells (74, 75), and may thus induce mitochondrial dysfunction in these cells, potentially leading to accelerated ageing, impaired repair and ongoing pro-inflammatory responses (Figure 2).

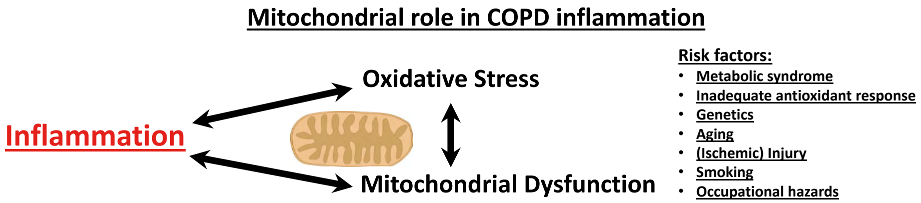


Figure 2: Possible model of a vicious circle sustaining inflammatory response in COPD. Various risk factors may increase the production of ROS and/or induce mitochondrial dysfunction, stimulating pro-inflammatory responses and accelerating the onset of the disease.

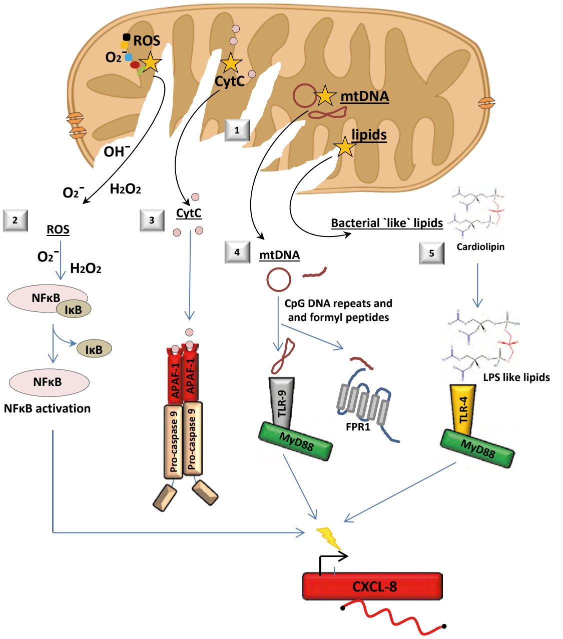
Mitochondria and disease

As outlined above, mitochondrial dysfunction can hamper multiple functions within the cell and depending on the cell type, this can give rise to the onset of various (often age-related) diseases, including diabetes, Alzheimer`s and Parkinson`s disease. Mutations in mtDNA, impaired energy supply, Ca^{2+} buffering, increased ROS production and dysregulation of cellular apoptosis by mitochondria may all contribute to the progressive decline in longevity of the cell (71). The cell types that are often affected by mitochondrial dysfunction are those that relies the most on proper mitochondrial function and often have the most mitochondria. Cells of the muscle, heart, brain, liver, kidneys, nerves, pancreas, eyes and lungs have high numbers of mitochondria in their cytosol and rely strongly on proper mitochondrial function. Known inherited conditions in which a (partial) mitochondrial dysfunction has been implicated include diseases that result either from mutations/high levels of heteroplasmy in the mtDNA or mutations in the genomic DNA (gDNA) encoding for important mitochondrial components (55, 64, 72). All these inherited conditions affect the multitude of the previous mentioned organs and include Kearns–Sayre syndrome, Leber hereditary optic neuropathy, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like syndrome, Myoclonic epilepsy and ragged-red fibers, Leigh syndrome sub-acute sclerosing encephalopathy, neuropathy, ataxia, retinitis pigmentosa, and ptosis and myoneurogenic gastrointestinal encephalopathy (12, 37, 55, 64, 65, 72, 76). Therefore, most research is focused on these diseases, while relatively little is known about mitochondrial (dys)function and its contribution to lung disease.

Role of mitochondrial dysfunction in COPD

The role of mitochondria in lung disease has not yet intensively been investigated, although several studies have already demonstrated that cigarette smoke can disrupt the mitochondrial membrane potential ($\Delta\psi_m$) in macrophages of the lungs and epithelial cells (2, 74, 75). Cigarette smoke can directly damage mitochondria in lung cells, however, excessive damage to mitochondria can also occur by reactive oxygen species (ROS) generated by the mitochondria themselves, for instance when their antioxidant response is insufficient (55). Of interest, the production of various anti-oxidant enzymes, including Mn-SOD, catalase and glutathione peroxidase (GPx), is reduced in cells/lung tissue of COPD patients (35, 59, 61). Recently, polymorphisms in Mn-SOD have been linked to the development of bronchial hyperresponsiveness in COPD (67). Possibly, cells from COPD patients are deficient in their compensatory mechanisms that protect from mitochondrial damage. When these mechanisms fail, this may result in excessive oxidative damage, which may also extend to mtDNA and proteins of the OXPHOS system, eventually hampering mitochondrial function. As described above, mitochondrial damage can lead to increased production of ROS and activity of NF-κB, thus sustaining an inflammatory response seen in COPD (Figure 2).

Mitochondria that are damaged beyond repair are cleared through a process called autophagy, an intracellular degradation process. Mitophagy is an autophagic degradation pathway specific for mitochondria, a process initiated by the recruitment of the proteins PINK1 and Parkin (17, 22, 53), as described above. There are indications that the autophagy processes is disrupted in COPD patients (13, 48). In mitophagy deficient cells or when the mitochondrial clearance capacity is exhausted, defective mitochondria will accumulate (3, 34). In this case, mitochondria can fall apart, leading to the release of their lipid and protein components, which are capable to trigger a localized (lung) inflammatory or apoptotic response (Figure 3).



Mitochondrial damage associated molecular patterns

ROS
O2⁻
CytC
mtDNA
lipids
1
OH⁻
O2⁻
H2O2
2
ROS
O2⁻
H2O2
NFκB
IκB
IκB
NFκB
NFκB activation
3
CytC
APAF-1
APAF-1
Pro-caspase 9
Pro-caspase 9
4
mtDNA
CpG DNA repeats and
and formyl peptides
TLR-9
MyD88
FPR1
Bacterial `like` lipids
5
Cardiolipin
LPS like lipids
TLR-4
MyD88
CXCL-8

Figure 3: Model of mitochondrial damage associated with various inflammatory and apoptotic responses. 1) When extensive mitochondrial damage occur various proteins, nucleic acids, reactive oxygen species (ROS) and lipid compounds will be released into the cytosol which all can contribute to a pro-inflammatory response and cell death. 2) ROS can activate the pro-inflammatory transcription factor. 3) Binding of cytochrome-C can lead to the activation of caspase-9 and start the induction of apoptosis. 4) The release of CpG DNA repeats and formyl peptides can trigger Toll like receptor (TLR)-9 and formyl peptide receptor 1 (FPR1) and induce a pro-inflammatory response through MyD88 signaling leading to the transcription of pro-inflammatory genes (e.g. CXCL8). 5) Lipids of the inner-membrane, which are similar in composition to bacterial lipids, can also induce a pro-inflammatory response through TLR-4-MyD88 signaling.

These components are also referred to as mitochondrial damage associated molecular patterns (mDAMPs), acting on so-called pattern recognition receptors on innate immune cells to induce inflammatory responses. In this respect, CpG DNA (mtDNA) or N-formyl-peptides released from mitochondria can activate Toll-like receptor (TLR)9 and Formyl Peptide Receptor 1 on neutrophils and epithelial cells. This induces the activation of pro-inflammatory signaling molecules, e.g. MyD88, leading to the release of pro-inflammatory mediators like CXCL8 (58). In addition, release of ROS from damaged mitochondria has been proposed to induce the activation of NF-κB by degrading its inhibitor (40). It has also been suggested that the secretion of the pro-inflammatory cytokine IL-1β, which is elevated in the airways of COPD patients (6), can be induced by cytosolic release of mtDNA in autophagy deficient cells (3, 11, 52). Lipids released from mitochondria, such as cardiolipin which especially in oxidized form resembles bacterial lipopolysaccharide (LPS), can trigger an inflammatory responses through the activation of TLR4 (3, 46, 73). Both TLR4 and TLR9 activation can lead to CXCL8 production and secretion, resulting in neutrophil accumulation (50, 51). Thus, besides from impaired respiration, damaged mitochondria and circulating mitochondrial components can inflict inflammatory responses in the lungs. In addition to these inflammatory responses, the sudden release of high quantities of cytochrome-C from the mitochondria upon mitochondrial damage can initiate (3, 46, 73) apoptosis through the activation of caspase 9 (3).

Oxidative stress induces signaling events leading to mitochondrial dysfunction in COPD

As described above, oxidative stress may induce mitochondrial dysfunction, with important consequences for COPD. Various signaling processes may contribute to oxidative stress induced mitochondrial dysfunction. In particular, the regulation of the redox-responsive kinase GSK3β activity may be of interest for mitochondrial dysfunction in COPD. GSK3β is considered to be predominantly localized in the cytosol of the cell, however, localization in the nucleus and mitochondria has been reported and is linked to a higher activity of the enzyme (9). The exclusive function of mitochondrial GSK3β activity is not yet fully understood. There are studies indicating an important role in apoptosis regulation by GSK3β, changing the mitochondrial outer membrane permeabilization by destabilization of Myeloid Cell Leukemia 1 MCL-1 (47). Another group has shown that GSK-3β is able to mediate phosphorylation of Dynamin-1-like protein (DRP1), which is an important member of superfamily that regulate mitochondrial fission, inducing an elongated mitochondrial morphology against oxidative stress (16). Furthermore, GSK3β mediates phosphorylation of the Voltage-Dependent Anion Channel (VDAC), which controls the outer mitochondrial membrane permeability (44).

In all cases, GSK3β activity is subject to dynamic regulation by various kinases, which are commonly involved in oxidant-mediated responses, including Akt, indicating that GSK3β may represent an important downstream effector of oxidant-mediated signaling. Akt-mediated phosphorylation of GSK3β at Ser9 inhibit its activity (20). In cardiomyocytes, phosphorylation of GSK3β at Ser9 protects against oxidant induced apoptosis (54). In addition, GSK3β activity has been implicated in glucocorticoid-induced apoptosis in lymphoma cells, its inactivation resulting in GC insensitivity (69). Two other studies showed that pharmacological inhibition of GSK3β by SB216763 in a guinea pig model of LPS-induced pulmonary inflammation did not affect inflammatory cell influx, but prevented small airway remodeling and LPS-induced muscle mass decline and myofiber atrophy, which is normally observed in COPD (30, 79). Thus, inactivation of GSK3β by phosphorylation at Ser9 may be involved oxidant-induced GC insensitivity, although it may also exert beneficial effects and protect from thickening of the airway walls. Together, we hypothesize that alterations in GSK3β may have important implications for mitochondrial function and development of airway inflammation in COPD.

Scope of this thesis

We hypothesize that the damaging effects of cigarette smoke-induced oxidative stress in lung epithelial cells affect the mitochondria. We speculate that these dysfunctional mitochondria contribute to the (onset) of the disease, leading to pro-inflammatory responses of epithelial cells and impaired regeneration upon damage. In addition, oxidative stress and mitochondrial dysfunction may lead to GC insensitivity of airway epithelial cells. In ***chapter 2***, we aimed to assess the dose depend effects of long-term cigarette smoke exposure on mitochondria in human bronchial epithelial cells. In particular, we studied the effect of long-term exposure to cigarette smoke extract on mitochondrial morphology, function, fission and fusion in the human bronchial epithelial cell line BEAS-2B. Additionally, we explored whether and how mitochondrial morphology and function is altered in bronchial epithelial cells from COPD patients. In ***chapter 3***, we studied the mechanisms involved in cigarette smoke-induced GC insensitivity, a pinnacle in the treatment of COPD patients, and whether GSK3β is involved in this effect. This was studied in human bronchial epithelial 16HBE cells as well as monocytes. Furthermore, we compared steroid responsiveness in primary bronchial epithelial cells from non-smokers, smoking controls and COPD patients. In ***chapter 4*** of this thesis, after demonstrating the influence of long-term cigarette smoke extract on mitochondria, we investigated whether mitochondrial dysfunction leads to changes in epithelial pro-inflammatory responses, repair and GC (in)sensitivity. In order to do so, we used A549 Rho-0 cells, which are depleted of mitochondrial DNA (mtDNA), and studied effects on IL-8 release, wound healing capabilities and GC responsiveness. In ***chapter 5***, we explored the potential of lipidomics to monitor cigarette smoke-induced, and/or disease related-changes in sputum from COPD patients, which we compared to sputum from smokers without COPD and never-smokers. In ***chapter 6***, we studied whether similar changes were observed in lipid compounds of long-term cigarette-smoke-exposed bronchial epithelial BEAS-2B cells that were used in chapter 2. With these studies, we aimed to improve insight in the role of cigarette smoke-induced mitochondrial dysfunction in the development of **COPD,** and whether mitochondria can serve as future therapeutic target.

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