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Direct cigarette smoke exposure to 3D epithelial cell cultures by an aerosol exposure system

Development and evaluation of an in vitro model for COPD with direct cigarette smoke exposure on small airway epithelial cells in the air-liquid interface by the Vitrocell smoking system

Master's thesis in Biomedical Engineering

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DEPARTMENT OF BIOLOGY AND BIOLOGICAL ENGINEERING

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Development and evaluation of an in vitro COPD model for direct whole cigarette smoke exposure on small airway epithelial cells in the air-liquid interface.
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Abstract

Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory and progressive lung disease that causes obstructed airflow from the lungs. *The Global Burden of Disease Study* reports a prevalence of 251 million cases of COPD globally in 2016 and classify the disease as the third most common causes of deaths worldwide. Cigarette smoke exposure is one of the major risk factors for developing COPD. Up to 40% of smokers develop COPD over time, and continuous smoking drives the development and progression of the disease.

The aim of this project was to develop and evaluate an in vitro model for direct whole cigarette smoke exposure on small airway epithelial cells (SAECs) with an automated aerosol system (Vitrocell smoking system). SAECs from three COPD donors and two healthy donor, cultured in the air-liquid interface (ALI), were used. To examine the effects of cigarette smoke on airway epithelium, cells were exposed to either one or three non-toxic doses of smoke, and samples were collected 24 and 48 hours post exposure. Cytotoxicity, barrier integrity, ciliary beat frequency, gene expression and cytokine expression were investigated. Cigarette smoke effectively increased the amount of inflammatory mediators IL-6 and IL-8 in supernatants from smoke-exposed samples, and up-regulated inflammatory gene expression of IL-8, and gene expression of antioxidants SRXN1 and HMOX1.

This project was performed at AstraZeneca in the Bioscience COPD IPF Department within the Early Respiratory and Immunology R&D unit located in Gothenburg, Sweden.

Keywords: COPD, Small airway epithelial cells, cigarette smoke, Vitrocell system, RT-PCR, air liquid interface, ciliary beat frequency.

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Lastly, I want to thanks my family and friends for all their support during these hectic and rewarding weeks of doing my master thesis.

This thesis is dedicated to my grandfather Rolf Andersson, who suffered from severe COPD and was diseased eight days before the start of this project. You are forever loved and remembered.

Veronika Knudsen, Gothenburg, June 2021

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Acronyms

AB/PAS Alcian Blue Periodic Acid-Schiff. 23
ALI Air-liquid interface. 2, 9–11, 16, 47, 51

CBF Cilia beating frequency. 9, 11, 12, 19, 26, 27, 32–35, 47, 51, 60
CF Cystic Fibrosis. 1
COPD Chronic Obstructive Pulmonary Disease. vii, 1–3, 7–9, 15, 16, 47, 48

HCI Health Canada Intense. 17, 47

LDH Lactase dehydrogenase. 12, 18, 47

PBS Phosphate-Buffered Saline. 11, 18, 22

RT-qPCR Real Time Quantitative Polymerase Chain Reaction. 13, 48

SAEC Small airway epithelial cells. 2, 9–11, 15, 16, 29, 42, 46–48, 51, 59, 60

TEER Trans epithelial electrical resistance. 11, 18, 19, 27, 47, 49

1

Introduction

This chapter describes the background behind the project and the issues to be investigated, including the objective, limitations, and ethical considerations. A short description of the in vitro model and the methodologies used in the project are presented.

1.1 Background

Respiratory diseases are some of the most common medical conditions worldwide, causing suffering and death for millions of people [1]. Among the most prevalent respiratory diseases are Chronic Obstructive Pulmonary Disease (COPD), asthma, pulmonary hypertension and Cystic Fibrosis (CF). COPD is a chronic, inflammatory and progressive lung disease that causes obstructed airflow from the lungs [2]. *The Global Burden of Disease Study* reports a prevalence of 251 million cases of COPD globally in 2016 and classify the disease as the third most common cause of deaths worldwide [3]. In 2015, approximately 3.17 million deaths were caused by the disease [4], representing 6% of the total deaths worldwide [5].

The human airway is lined with a mucociliated, polarized, pseudostratified epithelium which concise mainly of four cell types; ciliated cells, goblet cells, basal cells, and club cells. One of its essential functions is to act as a barrier between the external environment and the underlying tissue. It also carries a regulatory and pro-inflammatory role by releasing neuropeptide degrading enzymes, TGF-β endothelin, and cytokines, among others [6]. In COPD, the airway epithelium is subject to significant molecular and morphological changes. Studies suggest that the structural and functional changes to the airway epithelium are correlated to both disease initiation and progression in COPD. These changes cause alterations in the immune/inflammatory response, host defense, airway milieu and affect the repair mechanisms [7]. Hyperplasia of basal cells and goblet cells and metaplasia of squamous cells are known characteristics of the disease. Other aspects include; epithelial barrier dysfunction, decreased ciliated cell differentiation, altered goblet cell differentiation, and reduced cilia function [8, 9]. The mechanisms for these malfunctions and alterations are not entirely understood, which is why novel models on the airway epithelium are needed to assess disease stages associated with respiratory diseases as COPD, and to find novel therapeutic strategies [7].

Cigarette smoke exposure does not only pose a considerable health risk but is also the primary cause of COPD [5, 10, 11]. Up to 40% of smokers develop COPD over time, and continuous smoking drives the development and progression of the disease [12, 13]. Cigarette smoke has been shown to damage the epithelial barrier and increase permeability [13]. In addition, the number of ciliated cells is reduced in smokers, and the protection against the effects of inhaled cigarette smoke is reduced. The mechanisms for the loss of ciliated cells are not well-understood [10]. Ciliary beating is crucial for mucociliary clearance, and in COPD patients, this function is decreased. To examine the effects of cigarette smoke on the epithelium, direct cigarette exposure on epithelial cells in the Air-liquid interface (ALI) can be used. Previous studies have been conducted with cigarette smoke-extract and condensate diluted and mixed in with cell culture media, and then exposed to submerged cells on the basal side [14–17]. By using ALI cultured epithelial cells exposed to direct cigarette smoke, a more *in vivo* like environment can be obtained [9, 13, 18].

COPD remains a disease with considerable unmet medical needs [19, 20]. A lot more can be learned about susceptibility and pathogenesis, especially during early and prediagnostic stages of disease [12]. Earlier diagnosis and novel treatments for the disease are necessary to halt or reverse progression and achieve lung regeneration [19]. Therefore, new and refined in vitro models are crucial for the development of respiratory treatments and pharmaceuticals.

Today, no protocols for direct cigarette exposure of ALI cultured Small airway epithelial cells (SAEC) by a direct aerosol system are established. This project aims to establish a protocol for direct exposure on SAEC, cultured in ALI, to cigarette smoke by the Vitrocell smoking machine. The SAECs are cultured at an ALI for 28 days when cell differentiation has occurred. The cells are at this time be able to express mucins and functional cilia, which are of relevance to assess the disease progression of COPD. The ALI SAEC will provide an *in vitro* environment similar to the *in vivo* setting. By creating an *in vitro* setting reflecting the *in vivo* state, respiratory diseases, like COPD, can more easily be understood.

1.2 Aim

The objective of this project is to establish and evaluate a protocol for exposure of ALI cultured small airway epithelial cells to direct whole cigarette smoke by the Vitrocell® VC 10® S-type smoking machine. By instituting a smoke protocol for cigarette smoke exposure, the cell culture damaged caused by cigarette smoke can be more distinctively investigated. In addition, using small airway epithelial cells cultured at the air-liquid interface, a more in vivo-like model can be obtained. The development of novel in vitro models is crucial for developing new drugs and treatments for respiratory diseases.

The main objective was broken down into three focus areas:

1. Establishing non-toxic smoke dilution rates for the smoke exposure and evaluating the consistency of the smoke delivery to the cells.

2. Examine if the model generates a COPD-like response, by examining barrier dysfunction, ciliary dysfunction, inflammatory response, oxidative stress response, and mucus hypersecretion.

3. Examine if the model is robust and reproducible by examining the consistency of cigarette smoke delivery and statistically examining the difference in results between independent smoke experiments.

In addition, the smoking protocol should not take more than one week to perform, not including analytical assessments of the samples. The time frame for the protocol is to ensure the usage of the protocol in different projects. It is preferable if the protocol doesn't require more than one person to perform.

1.3 Limitations

Developing COPD can take several years of continuous smoking. In the project, the lung tissue samples from COPD patients are only exposed to direct cigarette smoke one or three times a week. The model is thus a simplified model for cigarette exposure. Secondly, only small airway epithelial cells will be examined. Other cell types are affected in COPD patients, but they will not be studied in this thesis. Lastly, the lung tissue samples provided by Lonza are from three different COPD donors. The donors differ in age and the progression of the disease. There might therefore be a prominent donor variation that poses a limitation in establishing a broad-spectrum protocol.

1.4 Ethical Approvals

The SAECs are isolated from normal human lung tissue and COPD-damaged lung tissue from the distal portion of the lung in the 1 mm bronchiole area. The cells are bought from Swiss pharmaceutical and biotech company Lonza. No additional approval by the Swedish Research Ethical Committee in Gothenburg is needed.

1.5 Thesis structure

The content of the following chapters in this thesis is divided into *theory*, *analytical procedure*, *materials and method*, *experimental design*, *result*, *discussion* and *conclusion*. The theory chapter will give the reader an introduction about the human airways and the epithelium, an overview of COPD and existing in vitro COPD models. The chapter describing analytical procedures will briefly describe the different analytical methods used in this thesis. Material and method describe the material used, and a short description of the analytical procedures is given. The chapter about experimental design describes the different time points of the experiments and the number of samples collected for the different readouts. This chapter is followed by the result, a discussion, and finally, a conclusion.

2

Theory

2.1 Respiratory system

The respiratory system consists of a network of organs and tissue involved in respiration. Its primary function is to act as an interface between the external environment and the systemic circulation where the exchange of O_2 and of CO_2 occur [21]. The respiratory system is divided into two major parts, the upper- and lower respiratory tract. The upper respiratory tracts comprise the nose, including the nasal cavity and sinuses, and pharynx. The lower tract consists of the larynx, trachea, right and left bronchi, and lungs [22], see Figure 2.1.1.

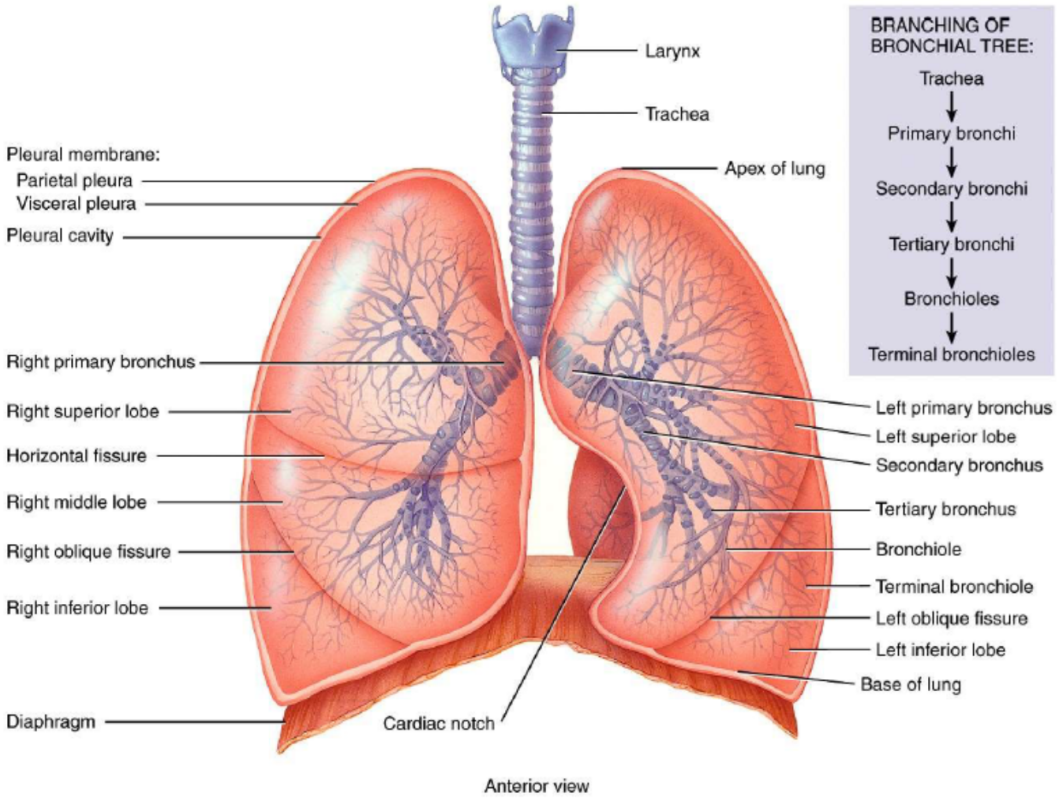


Figure 2.1.1: Illustration of the lower respiratory tract from the larynx to the terminal bronchioles. [21]

As seen in Figure 2.1.1, the bronchial tree branches out into the right and left primary bronchus, secondary bronchus for each lobe, tertiary bronchus for each segment, conducting bronchiole, terminal bronchiole, respiratory bronchiole, alveolar duct, and alveoli [23]. The lower respiratory tract is divided into a conducting zone and a respiratory zone. The conduction zone is where the inhaled and exhaled air passes and includes the trachea, bronchi, and terminal bronchioles. The respiratory region consists of the lung parenchyma, including the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. It is the area in which gas exchange between the lung and pulmonary capillary occurs [18, 21]. The respiratory zone is illustrated in Figure 2.1.2.

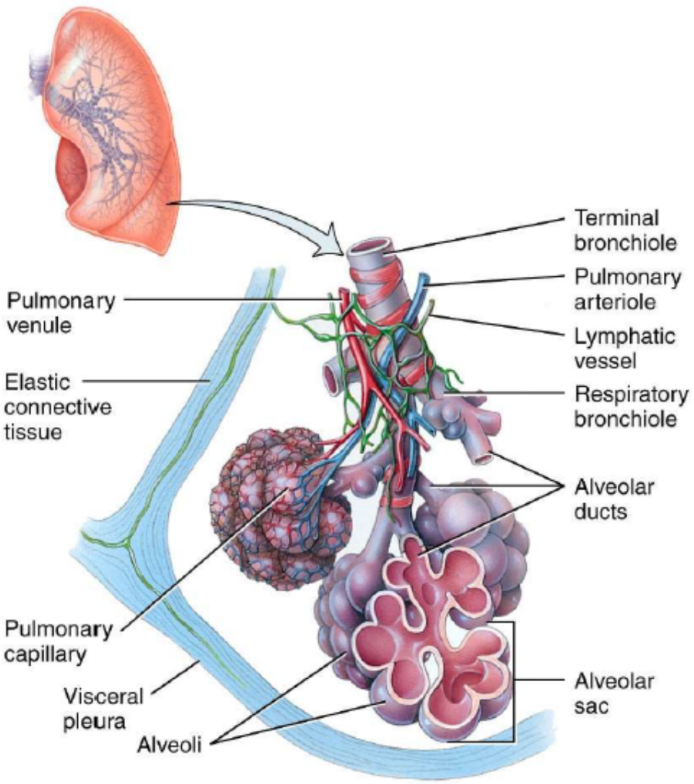


Figure 2.1.2: Illustration of the terminal branchiole containing the respiratory bronchiole ending in alveolar ducts made up of alveoli. [21]

2.1.1 The epithelium

The human airway consists of several different specialized cell types with various functions. The epithelial cells lining the airway differ in cell composition depending on location. In the large airway, the bronchi, the epithelium consists mainly of basal cells, goblet cells, and ciliated cells. The small airways consist mainly of basal cells, club cells, and ciliated cells. The alveoli consist of alveolar type 1 and type 2 cells. In Figure 2.1.3, the epithelium is illustrated with different cell types.

Ciliated cells are columnar epithelial cells responsible for the mucociliary clearance through synchronized cilia beating. Ciliated cells are the most predominant cell

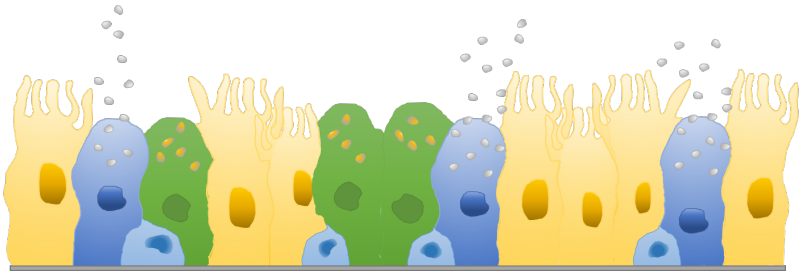


Figure 2.1.3: Pseudostratified columnar epithelium seen in small airway epithelial cells, consisting of three cell types; ciliated cells (yellow), goblet cells (blue, with mucus release), basal cells (green) and club cells (green). The basement membrane is marked in gray.

type of the epithelium, making up about 50 to 80 percent of the epithelium [6]. Goblet cells (MUC5AC), together with the submucosal gland (MUC5B), produce the protective mucus layer lining the lungs [24]. Basal cells are the cells closest to the basement membrane in the epithelium. They are characterized by their triangular shape, with the base closely connected to the basement membrane by desmosomes. They are the progenitor cells of all cells in the respiratory epithelium and constitute about 6-30 % of the epithelium [25].

2.2 Airway diseases

2.2.1 COPD

COPD is a respiratory disease characterized by airflow limitation (emphysema) that is not fully reversible, is usually progressive, and is associated with an abnormal inflammatory response (chronic bronchitis) of the lungs to toxic particles or gases. Cigarette smoke is the primary risk factor for COPD [6]. Cigarette smoke and other pollutants trigger the innate immune response, but the mechanism behind it is unknown [26]. Pathological changes found in the small airways ($<$ 2mm diameter) of COPD patients include an increased number of macrophages, T-lymphocytes, B-lymphocytes, lymphoid follicles, and fibroblasts. As the disease progresses, peribronchial fibrosis and narrowing of the airways occur [27]. Inflammation in the small airway is a normal reaction to inhaled toxins, but the inflammation is amplified in COPD patients. Inflammation destroys the tissue and impairs the repair mechanisms. Inflammation, an imbalance between proteases and antiproteases together with an imbalance between oxidants and antioxidants (oxidative stress) are all involved in the pathogenesis of COPD [27].

Inflammatory mediators increased in COPD are; T-cell chemoattractant, chemotactic factors, chemokines, IL-8, pro-inflammatory cytokines as TNF-alpha, IL $-$ 1β and IL-6, and growth-factor B [27]. Cigarette smoke and inflammation induce oxidative stress, which promotes inflammatory cells to release proteases and at the same time inactivate antiprotease by oxidation. These proteases are mainly produced by neutrophils, macrophages, and different matrix metalloproteases

(MMP-8, MMP-9, and MMP-12) [27].

Pharmaceutical therapies for COPD focus on relieving symptoms for the patients since no long-term treatment that reduces disease progression exists today. The most common treatment is bronchodilators with beta2-antagonist and muscarinic receptor antagonists. These can reduce breathlessness and improve lung function. GOLD criteria are used to assess the severeness of COPD to provide the most suitable treatment. There are four different stages; early COPD (1) to the end-stage (4). In early COPD, no clear sign of illness can be seen. The end-stage (4) entails frequent exacerbations, which can be fatal.

COPD is characterized by chronic inflammation with continuous tissue damage and repair of the small airways, and cytokines play a crucial part in the disease progression. This complicated network of cytokines involved in COPD can be seen in Figure 2.2.1.

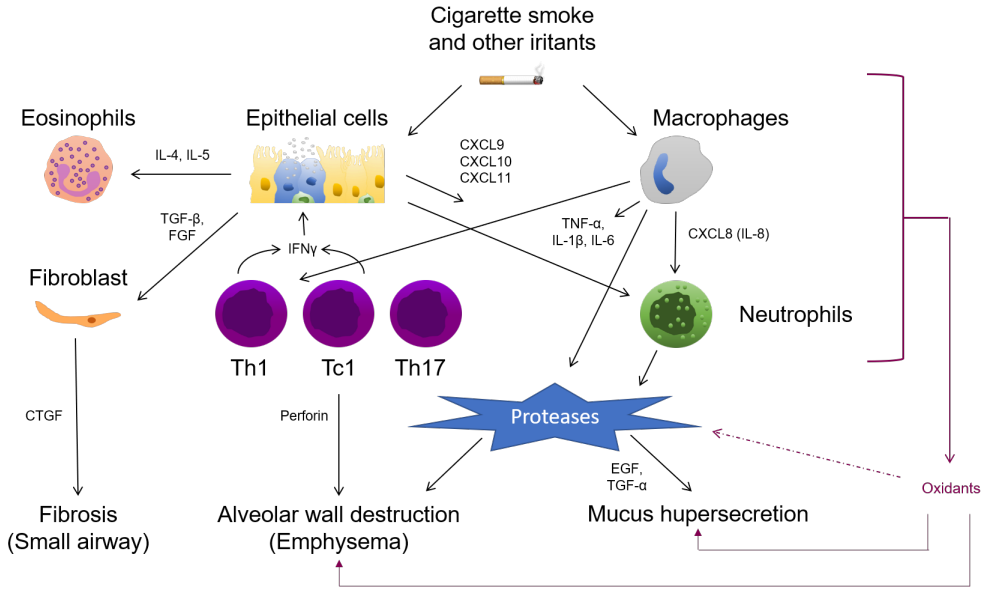


Figure 2.2.1: Network of cytokines in chronic obstructive pulmonary disease (COPD).

Inhaled cigarette smoke or other toxins activates epithelial cells and macrophages. The epithelial cells and macrophages release chemotactic factors that attract inflammatory cells, which result in the gathering of monocytes, neutrophils, T-helper cells, and eosinophils to the lungs. The inflammatory cells, macrophages, and epithelial cells release proteases, growth factors, and inflammatory cytokines. The release may result in fibrosis of the small airways, emphysema, and mucus hyper-secretion. By studying the roles of cytokines in COPD, potential anti-cytokine therapies can be examined [28].

In sputum sampled from COPD patients, a significant increase of growth-related oncogene-α (GROα) and monocyte chemotactic protein-1 (MCP-1) has been observed compared to non-smokers and to healthy smokers [29]. Increased levels of

IL-8, IL-6, IL-1β, and MCP-1 have also been detected in sputum from COPD patients [30]. IL-8 has been found to increase in normal human bronchial epithelial cells (NHBE) exposed to cigarette smoke, whereas no significant increase could be found in COPD-diseased HBECs [31].

The airway epithelium acts as a first line of defense for inhaled toxins. Mucociliary clearance, in which inhaled particles and toxins are cleared from the respiratory tract, is dependent on cilia beating functionality. Cilia of the upper and lower respiratory tract needs to cohesive beating to transport secretions out of the lungs [6]. The ciliated cells are the most predominant cell type in the lung epithelium (50-80%) and especially in the small airways. In COPD, the cilia beating is reduced due to remodeling of the epithelium and mucus hyper-secretion [6, 32]. In addition, cigarette smoke has been shown to reduce the ciliary beat frequency in SAEC [33]. Another study showed that CBF was not affected by smoke [34]. In [33] showed frequency of 7-10 Hz in SAECs, [34] showed a CBF of 14 Hz in healthy nasal epithelial cells with a decrease of 25% in COPD patients.

2.3 In vitro models

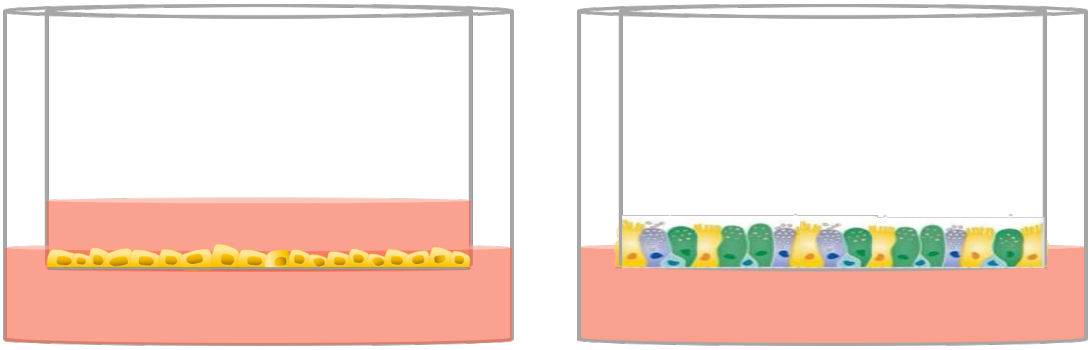
The development of new and refined in vitro models is of great importance to assess disease stages associated with respiratory diseases. In this study, primary small airway epithelial cells from COPD donors and healthy donors are cultured at the air-liquid interface. The cells are exposed to whole cigarette smoke by the Vitrocell smoking machine.

2.3.1 Cell cultures

Different cell models can be used to assess disease stages in vitro from various areas of the respiratory tract. Cells are taken from the nasal, tracheal, bronchial, and alveolar areas. In this study, primary small airway epithelial cells from COPD and healthy donors will be assessed. Primary cells closely resemble the tissue origin and are taken directly from the organ tissue and stay unmodified. Thus, they pose a good representation of the *in vivo* state and exhibit the tissue physiology. They provide an excellent model system for studying the physiology of the cells and how the cells respond to toxins or drugs. Primary epithelial cells are commonly used in research and are targeted in chronic respiratory diseases like COPD. They are heterogeneous initially and can, later on, differentiate, be kept in a beneficial environment, and then differentiate to different cell types. The disadvantage of using primary epithelial cells is that they cannot be maintained in vitro for an unlimited period. Advantages of using primary cells include; fewer ethical objections, allows studying human tissue which could not be studied *in vivo* and they are less costly than animal models. In addition, they provide a more *in vivo* like situation than animal models [35, 36].

For the primary epithelial cells to differentiate into a mucociliated pseudostratified epithelium, the primary human small airway epithelial cells must be cultured in an ALI environment. In the ALI culture, primary cells are cultured in a medium, and

the apical surface is exposed to air. The cells are seeded on a permeable membrane and are exposed to a culture medium on both basal and apical sides. When the cells are confluent, the medium on the apical side is removed, creating the air-liquid interface. The high level of mucociliary differentiation the cells undergo generates an *in vitro* model that closely resembles the *in vivo* human airway [35, 36].



(a) Submerged culture **(b)** Air-liquid interface (ALI) culture

Figure 2.3.1: The cultivation of the human small airway epithelial cells (SAEC). Initially, the cells are cultured in a submerged condition with cultivation media in the basal and apical compartments. When the cells are completely confluent, the apical culture medium is removed and the air-liquid interface is created. The exposure to air promoted differentiation of the epithelial cells into different cell types.

2.3.2 Cigarette smoke exposure

Previous models for cigarette smoke exposure on cell cultures have been performed with different approaches. In some studies, cigarette smoke was diluted and mixed in with the cell culture media, either by cigarette smoke extract and cigarette smoke-condensate and then exposed to the submerged cells on the basal side [14–17].

To assess how the human airway is affected by cigarette smoking, ALI cultured epithelial cells are exposed to direct cigarette smoke. The exposure can be done by using smoking machines as the Vitrocell VC10 smoking robot. There are different predefined smoking regimes used for smoke machines, where the two most common ones are the International Standards Organization (ISO) and Health Canada Intense regime. These regimes include 35 ml and 55 ml of smoke, a 2 s duration, every 30 s or 60 s, respectively. By using a smoking machine for exposure of ALI cultures, a more *in vivo* like condition may be established. The experimental model can be used to assess both chronic and prolonged smoking experiments [37].

More intensive smoking regimes, like the HCI, are less likely to underestimate smoke toxicant intakes by smokers and are recommended to be used in smoke toxicological testing strategies until better smoking regimens are developed [38, 39]. Therefore, the HCI regimen is used in this project for the whole cigarette smoke generation.

3

Analytical Procedure

The cellular changes on the ALI-cultured SAEC due to smoke treatment can be followed through TEER measurements, analysis of gene expression and protein expression, and through CBF analysis. In this section, different available readouts used to assess the cellular behavior throughout the project are presented.

3.1 Trans epithelial electrical resistance

The transepithelial electrical resistance (TEER) technique is non-invasive, rapid, and is measured in real-time. It is a widely accepted quantitative technique to measure the integrity of tight junctions, creating the intracellular barrier between epithelial cells. To measure the resistance, the electrical current traveling across the cell culture is measured [11, 40]. The TEER measurement setup is presented in Figure 3.1.1. The insert with cell culture is transferred to a new well plate and filled with PBS or culture medium on the basolateral side and the apical side. Two electrodes are then transferred into the PBS or culture medium, one in the apical compartment and the basolateral compartment.

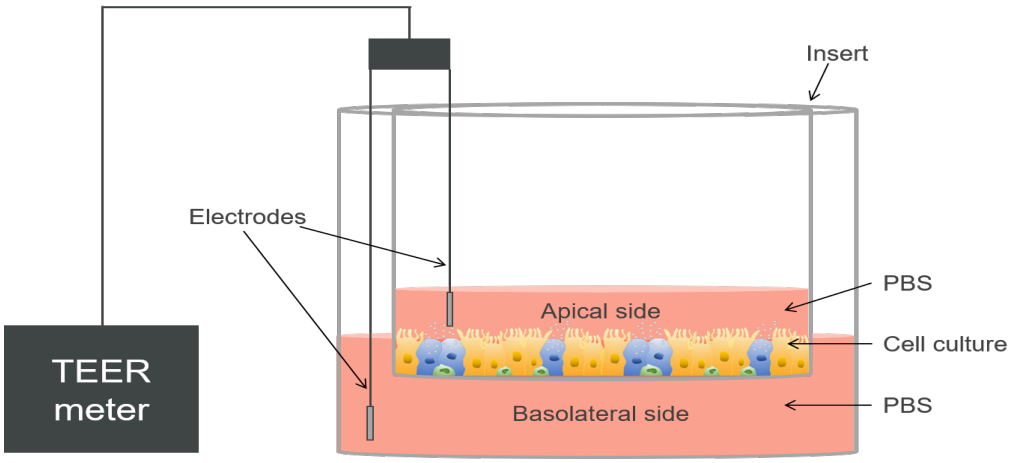


Figure 3.1.1: Illustration of the TEER measurement setup. The insert with the cell culture is submerged in PBS and two electrodes is placed in the PBS, one on the apical side and one on the basolateral side.

3.2 Cytotoxicity analysis

Lactase dehydrogenase is an enzyme present in the cytoplasm of every living cell. It is considered a stable enzyme and is therefore widely used to assess cell damage and cytotoxicity. If the cell membrane is damaged due to external factors such as toxic chemicals, the LDH enzyme is released into the extracellular space. The enzyme will only be present in the culture medium if the barrier integrity is compromised. The LDH release can then be quantified using either a colorimetric or fluorometric LDH cytotoxicity assay [41]. In this project, the colorimetric CyQuant LDH cytotoxicity assay from Invitrogen is used.

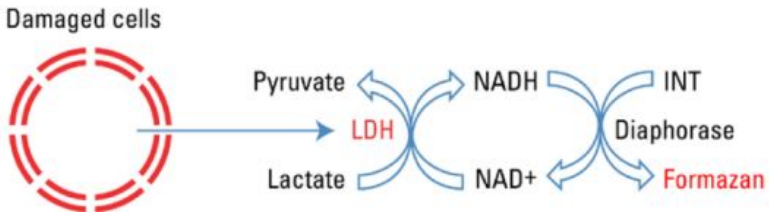


Figure 3.2.1: Schematic overview of the CyQuant LDH cytotoxicity assay mechanism. From [41]

The LDH quantification is based on the enzymatic reaction where LDH catalyzes the conversion of lactate to pyruvate via NAD+ reduction to NADH, see Figure 3.2.1. NADH is oxidized by diaphorase, which leads to the reduction of tetrazolium salt (INT) into formazan. INT is measured spectrophotometrically at 490 nm, and the measured values are directly proportional to LDH release into the culture medium [41]. The absorbance is measured at 490 nm and 680 nm, and the LDH activity is determined by subtracting the 680 nm background signal from the instrument) from the 490 nm absorbance values.

3.3 Ciliary beating functionality

Mucociliary clearance is one of the defense mechanisms of the lungs where mucus and toxins or other harmful substances are removed from the lungs. The ciliated cells of the epithelium are responsible for this mechanism since they possess the necessary force of moving substances in a coordinated beating of their cilia. The ciliated cells are significant for the health and function of the respiratory system. By observing changes in the ciliated cells' ability to transport mucus and other substances from the lungs, assessment in correlation to disease can be examined.

Each sampled was examined at room temperature under a Nikon microscope, and CBF was determined with a high-speed digital camera, connected to software made for CBF measurements.

3.4 Quantification of gene expression

Real-time quantitative polymerase chain reaction (RT-qPCR) is based on the well-established PCR method and is used to investigated gene expression [42]. Figure 3.4.1 describes the TaqMan gene expression assay process.

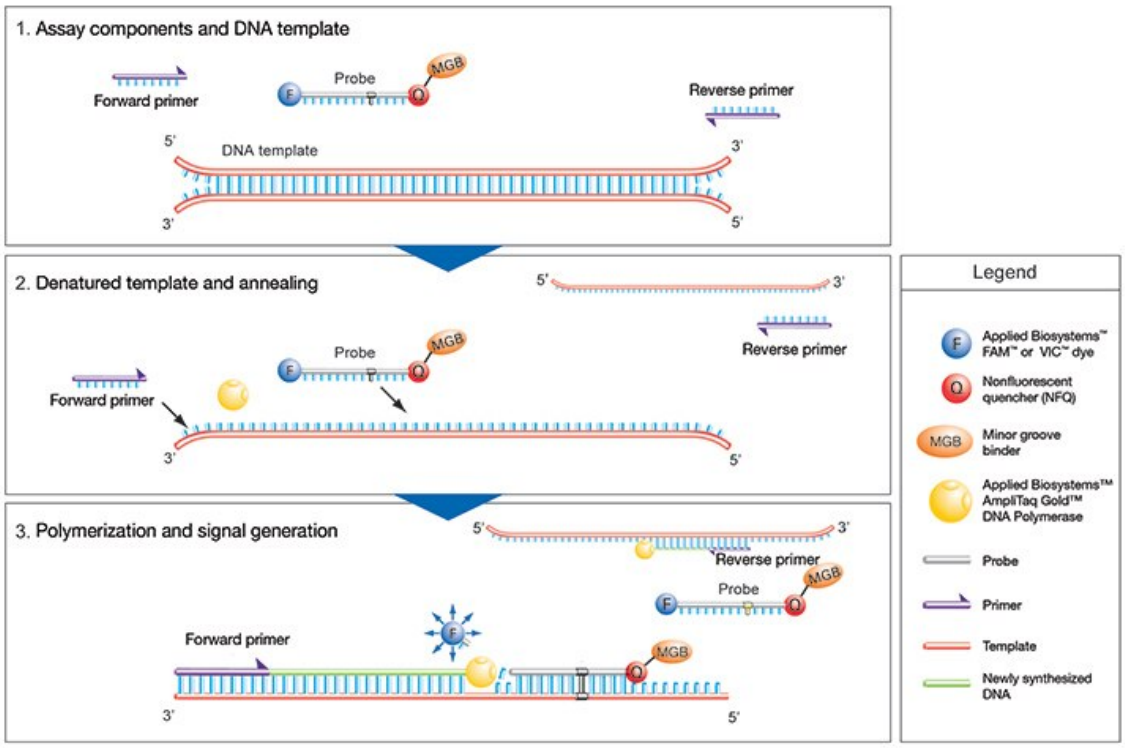


Figure 3.4.1: TaqMan gen expression assay procedure. From [43].

In short, cDNA is used as a template and fluorophore labeled probes specific to the target gene of interest is added. For every PCR cycle, the fluorophores are cleaved and emits light at certain wavelengths. At a certain cycle, the emitted fluorescence light is greater than the background signal. This specific value is referred to as the cycle threshold (C_T). If the target gene is highly expressed in the sample, the emitted light will reach the threshold faster, resulting in a lower C_T value [42].

3.5 Quantification of protein expression

Quantification of cytokine expression was performed with MSD immunoassay. Electrochemiluminescent labels (SULFO-TAG), conjugated to detection antibodies, are used in the panels to measure protein amount in samples. The light intensity is then measured by the Meso Scale Delivery plate reader. An overview of the sandwich immunoassay used can be seen in Figure 3.5.1. Two different panels were used; U-PLEX Biomarker Group 1 (human) multiplex assay and V-PLEX proinflammatory panel (human) IL-8.

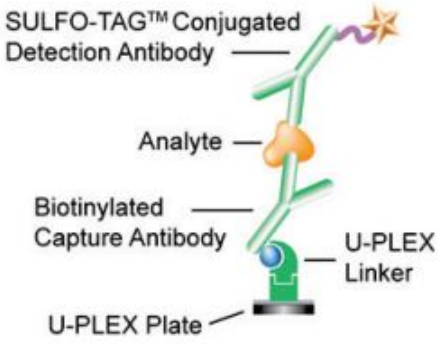


Figure 3.5.1: U-PLEX sandwich immunoassay. From [44]

4

Materials and Methods

This chapter contains information about the cigarettes, equipment, and cells used in this project. It also explains how cigarette smoke is generated and exposed to the cells and how epithelial integrity, cytotoxicity, cilia beat frequency, gene expression, and cytokine expression are assessed.

4.1 Cigarettes

1R6F reference research cigarettes were obtained from the University of Kentucky (Kentucky Tobacco Research & Development Center, University of Kentucky in Lexington). Cigarettes were stored in the original packaging at room temperature, and open packages were sealed with parafilm until further usage. Before each experiment, the cigarette filters were taped with scotch tape.

4.2 Primary human small airway epithelial cells

Primary COPD and healthy human small airway epithelial cells SAEC (Lonza, Basel, Switzerland) are isolated from lung tissue from the distal portion of the lung in the 1 mm bronchiole area and were received at passage 2. All cells are screened for mycoplasma, bacteria, yeast, and fungi and tested negative [45]. Additional information about the donors are presented in Table 4.2.1.

Table 4.2.1: The primary human cell donors used in this study.

Donor	Batch number	Age	Sex	Ethnicity	Smoker	COPD cause of death
D083	0000628083	51	Female	Caucasian	Yes	Yes
D387	18TL234841	52	Male	Hispanic	No	No
D841	18TL186387	69	Female	Hispanic	Yes	No
N065	20TL107065	56	Male	Caucasian	No	N/A
N938	0000501938	56	Male	Hispanic	Unknown	N/A

COPD donor D083 was diagnosed at 50 years old and died one year later of

respiratory causes after 40 years of smoking. Donor D387 suffered from COPD with a chronic cough but did not smoke conventional cigarettes. However, she smoked marijuana daily for approximately 40 years. The third COPD donor, D841, smoked for about 45 years but did not die due to respiratory causes.

4.2.1 Culture conditions

SAEC (Lonza, CC-2547) were expanded to passage two during three days in S-ALI growth media (Lonza, CC-3281). The cells were then seeded onto Costar Transwell Permeable 0.4 µm polyester filter inserts in 24-well plates (Costar, 2470), with a density of approximately 20'000 live cells per well. The cells were kept in this state for four days. Monday following week, the apical medium was removed, and S-ALI differentiation media (Lonza, CC-3282) was added to the basolateral compartment, initiating the Air-liquid interface culture. The SAECs were kept in ALI for four weeks until fully differentiated. See Appendix for complete culture protocol. The medium was changed every third day. Cells from different donors were cultivated on individual plates resulting in 24 samples of one donor on every plate.

4.3 Cigarette smoke generation and exposure

Cigarette smoke is generated and exposed to the cells through the Vitrocell smoking system. The system is fully automated when run according to pre-determined smoking protocol. The cigarettes are inserted, lit, smoked, and disposed of after use. The system consists of three main parts: the VC10 S-type smoking machine, the Vitrocell 24 cultivation and exposure system for 24-well sized cell culture inserts with an integrated sensor-controlled heating plate, and a dose monitoring system (Vitrocell Systems GMBH, Germany) [37]. The machine is set up according to instructions supplied by Vitrocell, and calibration was conducted before each experiment. In Figure 4.3.1, an overview of the system is provided.

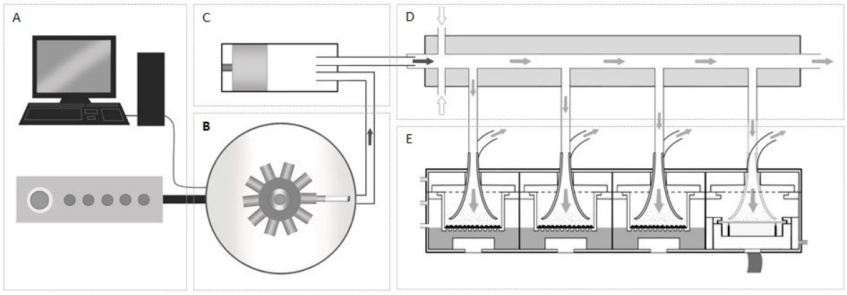


Figure 4.3.1: (a) Computer software in which the smoking regime set. (b) Smoking carousel where cigarettes are loaded, lit and smoked inside an enclosed ventilation hood. (c) Syringe drawing and delivering the cigarette smoke to the smoke dilution system. (d) The dilution bars where the cigarette smoke is diluted with air. (e) Smoke exposure model in which the cells are exposed to the cigarette smoke. From [46].

The cigarettes used in the study are generated from the University of Kentucky (Lexington, Kentucky USA) and are 1R6F research graded. The cigarettes are smoked according to the Health Canada Intense Health Canada Intense (HCI) regimen, as seen in Table 4.3.1, with nine puffs per cigarette.

Table 4.3.1: Health Canada Intense smoke regimen used for generating whole cigarette smoke.

Smoke regime	**Puff volume (mL)**	**Puff frequency (s)**	**Puff duration (s)**	**Ventilation block (%)**
HCI	55	30	2	100

The smoke is guided from the smoking machine to Vitrocells' 6-fold dilution system, connected to the cultivation and exposure system, by a 5 ml/min vacuum flow. Dilution airflows are controlled by mass flow controllers (Bronkhorst High-Tech BV, Ruurlo, The Netherlands), see Figure 4.3.1 [d]. One initial setting for dilution airflow was used to examine smoke delivered to the cells. The smoke assay was then conducted on all cells according to the second dilution airflow setting, seen in Table 4.3.2 and Figure 4.3.2.

Table 4.3.2: Dilution airflow (cigarette smoke condition) set by the flow meter controllers to adjust the exposure dose.

Channel	1	2	3	4	5
Dilution airflow [L/min]	0,1	0,1	0,25	0,25	0,5
Dilution airflow [L/min]	0,85	0,6	0,9	1,5	3,0

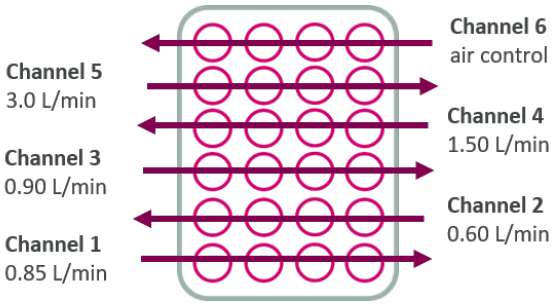


Figure 4.3.2: An overview of the dilution rates and channels in the Vitrocell exposure system.

Before each run, the 24-well base module of the exposure system was filled with 2,75 ml Dulbecco's Modified Eagle Medium (Gibco, 21885-025) in each well. The cell inserts were then placed in the module and transferred onto the preheated base of the exposure system. The dosimetry program and smoke machine were started

and run for 10 minutes. The amount of smoke delivered to the cells in the exposure module was measured with photometers and microbalance sensors. The photometers measure the aerosol particles with an online read-out. One Vitrocell 12/3 unit with two integrated microbalance sensors was used to monitor the mass deposition. The Vitrocell Dosimetry Software was used to observe and record each measurement.

4.4 Epithelial Integrity

The epithelial integrity was assessed by measuring the Trans Epithelial Electrical Resistance (TEER) 24 and 48 hours after cigarette smoke exposure with an EVOM2 resistance meter (World Precision Instruments Inc, Sarasota, USA), according to manufacturers' protocol. The cells were transferred to a 24-well cell culture plate (Greiner, 662160), and 37°C PBS was added to the basolateral side (500 µL) and on the apical side (200 µL). To reduce the effects of resistance from the medium and semipermeable membrane, the resistance of the Transwell insert in the identical testing configuration without cell layer (a blank) was measured and subtracted from the total resistance, according to Equation 4.4.1.

$$\mathrm{R_{Cells}} = \mathrm{R_{Total}} - \mathrm{R_{Blank}} \tag{4.4.1}$$

TEER is normalized to the area of the semipermeable membrane by multiplying the resistance of the cell layer, see Equation 4.4.1, by the membrane area, according to Equation 4.4.2.

$$\mathrm{TEER}\ (\Omega \mathrm{x cm^2}) = \mathrm{R_{Cells}}\ (\Omega)\ \mathrm{x}\ \mathrm{Area_{Membrane}}\ (\mathrm{cm^2}) \tag{4.4.2}$$

4.5 Cytotoxicity analysis

Basolateral supernatants were collected from the plates 24 and 48 hours post final exposure. The cytotoxicity was determined by lactate dehydrogenase LDH release by using the CyQUANT LDH Cytotoxicity Assay kit (Invitrogen, C20300). The cytotoxicity assay was conducted according to the manufacturer's protocol. In short, 12,5 µL of the sample, and positive control from the kit, was transferred to a clear flat-bottom 384-well plate (Greiner, cat no 781101). Each sample was run in triplicates. 12,5 µL of LDH reaction mixture (Invitrogen, C20300) was added to each well. The plates were then incubated at room temperature and protected from light for 30 min. Afterward, the reaction was stopped by adding 12,5 µL stop solution (Invitrogen, C20300). Within 2 hours of adding the stop solution, the absorbance was measured at 490 nm - 680 nm in the SpectraMax iD3 multi-mode microplate reader (Molecular Devices, San Jose, USA).

$$\%\ \mathrm{Cytotoxicity} = \left(\frac{\mathrm{LDH\ activity_{Sample}} - \mathrm{LDH\ activity_{Spontaneous}}}{\mathrm{LDH\ activity_{Maximum}} - \mathrm{LDH\ activity_{Spontaneous}}} \right) \times 100 \tag{4.5.1}$$

4.6 Ciliary beat frequency analysis

Ciliary beat frequency (CBF) was examined by imaging of the apical surface of samples 24 hours after cigarette smoke exposure with an X40 objective with slow-motion capture at 142 fps (frames-per-second). Five captures of each well were performed, as seen in Figure 4.6.1. The image analysis was performed using the Cilia-X software (Epithelix, Switzerland). The software analyses the files in batch mode and provides an analysis report with cilia beating frequency in Hz and active area in %.

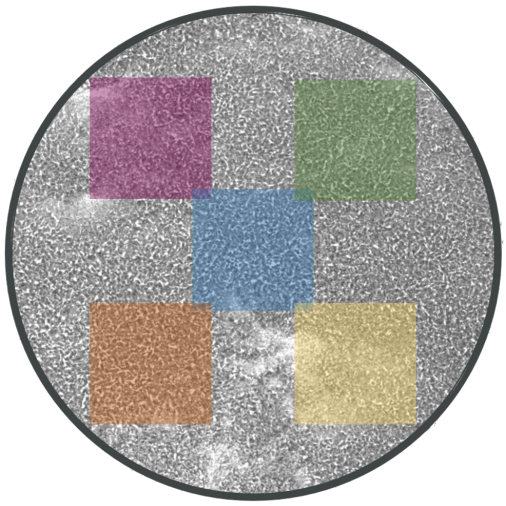


Figure 4.6.1: A schematic figure representing how the five video files were taken in each well. The image is taken from donor N065.

4.7 Gene expression analysis

The method in which the gene expression analysis is performed is presented in this section.

4.7.1 Lysis of cell cultures

After TEER measurement was performed, the cells were lysed with 150 or 200 µL QIAzol Lysis Reagent (QIAGEN, 5660293). The lysis reagent was left to sit for approximately one minute. A pipette tip was then used to scrape the membrane, allowing the cells to detach. The lysed samples were then transferred to Eppendorf tubes and stored in -80°C until RNA purification.

4.7.2 RNA purification

Lysed cell samples were thawed on ice and purified according to the manufacturer's protocol using the RNeasy Mini Kit (QIAGEN, 74104). The RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific,

Waltham, MA, USA). A 1 µL drop of each sample was measured on the Nanodrop spectrophotometer, with RNase-free water as blank. Samples were then stored at -80 °C or used immediately.

4.7.3 cDNA synthesis

The cDNA synthesis was performed according to standard procedure using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368813) (ThermoFisher, Waltham, MA, USA). For each reaction, a 2X RT master mix was prepared on ice with 2.0 µL 10X RT Buffer (Applied Biosystems, 4319981), 0.8 µL 25x dNTP Mix (100 mM) (Applied Biosystems, 362271), 2.0 µL 10x RT Random Primers (Applied Biosystems, 4319979), 1.0 µL MultiScribe Reverse Transcriptase (Applied Biosystems, 4319983) and 4.2 µL nuclease-free water. As a negative control, Multiscribe Reverse Transcriptase was replaced with nuclease-free water. The master mix was prepared according to the number of samples in every run. The master mix was then added to each sample, diluted with nuclease-free water, to a total volume of 40 µL. The samples were then transferred to a non-skirted 96-well PCR plate. cDNA synthesis reaction was performed at 25°C for 10 min, 37°C for 120 min, then 85°C for 5 min followed by hold at 4°C in an MJ Research PTC-200 Thermal Cycler. The cDNA samples were then stored at -20°C.

4.7.4 TaqMan assay

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using the TaqMan Gene Expression Assay (ThermoFisher, Waltham, MA, USA) with TaqMan Fast Advanced Master Mix (4444557) (Applied Biosystems, Foster City, CA, US). Genes used in the TaqMan gene expression assay are presented in Table 4.7.1. Samples were run in duplicates or triplicates.

4.7.5 Data analysis

A C_t-value (cycle threshold) is provided for every sample. It is defined as the number of cycles required for the fluorescent signal to cross the threshold and thereby exceed the background signal. C_t-values are inversely proportional to the amount of target nucleic acid in the sample, which means the higher the C_t-value, the lower amount of nucleic acid in the samples. In general, C_t-values below 29 indicate an abundant amount of nucleic acid in the samples, 30-37 a moderate amount, and a value of 38-40 imply a minimal amount of target. Such high C – t-values can be an indication of contamination or too low a concentration of cDNA sample.

The mean of the C_t-value is calculated. The geometric mean of the C_t-values of the reference genes used in the assay is calculated. In order to normalize the C_t-values, Equation 4.7.1 is used.

$$\Delta C_{T_{target}} = C_{T_{target}} - C_{T_{reference}} \tag{4.7.1}$$

Table 4.7.1: Gene used in the TaqMan analysis.

Gene	Target	Supplier
MUC5AC	Mucin, Goblet cells	Applied Biosystems
MUC5B	Mucin, Goblet cells	Applied Biosystems
MMP-9	Cellular migration	Applied Biosystems
CDKN2A	Senescence	Applied Biosystems
IL-8	Inflammatory response	Applied Biosystems
IL-1β	Inflammatory response	Applied Biosystems
NQO1	Oxidative stress	Applied Biosystems
HMOX1	Oxidative stress	Applied Biosystems
SRXN1	Oxidative stress	Applied Biosystems
DDIT3	ER stress	Applied Biosystems
GAPDH	Reference gene	Applied Biosystems
HPRT1	Reference gene	Applied Biosystems

The change in relation to the control is calculated according to Equation 4.7.2.

$$\Delta\Delta C_{T_{target}} = \Delta C_{T_{target}} - \Delta C_{T_{nullsample}} \tag{4.7.2}$$

The fold change is then calculated according to Equation 4.7.3.

$$2^{\Delta\Delta C_{T_{target}}}(\text{foldchange}) \tag{4.7.3}$$

4.8 Protein expression

Basolateral supernatants were collected from samples 24 hours and 48 hours after cigarette smoke exposure(s) and were immediately frozen and kept at -80 °C until MSD analysis. Two different panels from Meso Scale Discovery were used; U-PLEX Biomarker Group 1 (hu) (K15067L-2) (IL-6, IP-10, and MCP-1) and V-PLEX Pro-inflammatory Panel Human IL-8 (IL-8) (Mesoscale Discovery, Rockville, Maryland, USA). A summary of cytokines used in the panels can be seen in Table 4.8.1.

Table 4.8.1: Cytokines used in the MSD immunoassay.

Cytokine	Target	Supplier
IL-6	Lymphokines, Pro-inflammatory	Meso Scale Diagnostics
IL-8	Pro-inflammatory	Meso Scale Diagnostics
MCP-1	Chemotactic factors for monocytes/macrophages, T cells	Meso Scale Diagnostics
IP-10	Pro-inflammatory	Meso Scale Diagnostics

The cytokine assays were run according to the manufacturer's protocol. Individual U-PLEX linker-coupled antibody solution was created and combined with stop solution after 30 min at room temperature to form the multiplex coating solution. The U-PLEX plates were coated with 50 µl coating solution per well and incubated at room temperature on an orbital shaker for one hour at 800-900rpm. Calibrator standards were prepared in Eppendorf tubes, supernatants from samples were thawed on ice, and detection antibody solution with sulfo-tagged antibodies was prepared. The incubated plates were washed three times, and 25 µl of prepared calibrator standards were added in duplicates and 25 µl of samples according to layout on the MSD plates. The plates were then incubated at room temperature on the orbital shaker for one hour. The plates were washed three times, and 50 µl detection antibody solution was added to each well, followed by another hour of incubation. The plates were then washed, 150 µl read buffer was added to each well, and the plates were analyzed on the MESO SECTOR S 600 (Mesoscale Discovery, Rockville, Maryland, USA). The protocol for the V-PLEX panel is very similar to that of the U-PLEX, except the plates are pre-coated, and the incubation time is two hours instead of one. The samples were also diluted tenfold before being added to the plates in the V-PLEX panel.

4.9 Cell staining

By studying the histology of the human small airway epithelial cells, the morphology, cell composition, and ciliation may be examined. Samples were fixated, embedded in paraffin, sectioned, and then stained with AB/PAS staining.

4.9.1 Cell fixation

24 after post cigarette smoke exposure, SAEC ALI-cultures samples were rinsed with Phosphate-Buffered Saline (PBS) (Gibco, 14040-091) three times and fixated in paraformaldehyde (PFA) (VWR Chemicals, 9713.1000). The PFA was then

removed, and the samples were once again washed three times and then kept at 4°C until embedding and sectioning.

4.9.2 Embedding and sectioning

The membranes were cut loose from the inserts using a scalpel and then put tissue cassettes. The cassettes were then transferred to a high throughput lean tissue processor (Magnus, Milestone Medical Technologies, Kalamazoo, USA), where the membranes were dehydrated in ethanol and xylene followed by paraffin infiltration. The cassettes were then warmed in the HistoStar embedding workstation (Epredia, A81000002), cut in half, and embedded in paraffin blocks. The paraffin blocks were cooled and stored at room temperature until sectioning. The Leica RM2165 microtome (Leica, Wetzlar, Germany) was used to section the paraffin blocks into 4.0 µm thick slices, which were mounted on microscope slides until further examination.

4.9.3 AB/PAS staining

Microscope slides with sectioned samples were stained with Alcian Blue Periodic Acid-Schiff (AB/PAS) according to the protocol in a Leica ST5020. Samples were stained with Alcian Blue/Periodic Acid-Schiff (AB/PAS) and dehydrated in ethanol and xylene, mounted with Pertex mounting medium (Histolab, Göteborg, Sweden), and scanned with an Aperio Scanscope slide scanner (Leica Biosystems, Buffalo Grove, USA).

4.10 Statistical analysis

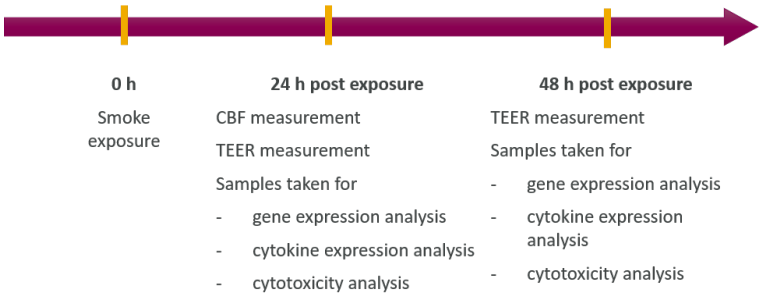
All data analysis was performed using the software GraphPad Prism® v7.04 (GraphPad Software, Inc.). One-way ANOVA test was performed to examine the difference between the smoked samples and the air control. Two-way ANOVA multiple comparison test was used to assess the difference between experiments, where each mean for each group was compares.

5

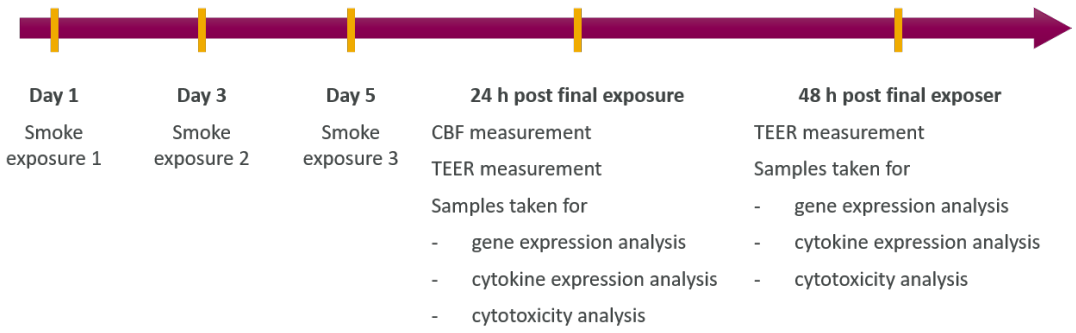
Experimental design

The following section describes the experimental plan for the smoke protocol used in this study. The timeline for the two smoke assays is explained, and the number of samples taken at each time-point for the readouts is presented.

Figure 5.0.1 gives an overview of the two smoke assays. The one-exposure assay requires three workdays, and the three-exposure assay demand five workdays, excluding further analysis. The smoke experiments began on Wednesdays, with sample collection on Thursdays and Fridays for the one-exposure assay. The second and third smoke exposure occurred on Fridays and the Monday the following week for the three-exposure assay.



(a) One cigarette smoke exposure assay.



(b) Three cigarette smoke exposure assay.

Figure 5.0.1: Timeline for the smoke assays used on the SAEC ALI-cultures.

The number of times the two different smoke assays were used on each donor respectively can be seen in Table 5.0.1.

Table 5.0.1: The number of time every donor was exposed according to the one and three cigarette smoke exposure protocols.

	No. cigarette smoke exposure(s)	
Donors	**1**	**3**
D083	3	3
D387	3	2
D841	3	3
N065	1	-
N938	1	1

Six samples (one for every smoke dilution and air control) were collected for each donor at every time-point, after one and three CS exposures. Five readouts were used to examine the effect of direct cigarette smoke on the SAEC ALI-cultures; barrier integrity (TEER measurements), cytotoxicity (LDH assay), ciliary beat frequency, gene expression analysis (TaqMan assay), and cytokine expression analysis (MSD immunoassay). At each time point, TEER was measured on six samples (one sample of each smoke dilution and air control). The cells were then lysed, collected in Eppendorf tubes, and frozen. Supernatants from the samples were collected for cytotoxicity analysis and cytokine expression analysis. In addition, videos were collected for CBF analysis 24 hours post-exposure.

6

Results

This chapter presents the results from the initial experiment, examining different smoke dilutions, which are presented in terms of cytotoxicity and barrier dysfunction. The result from the initial assessment was used as a standard for all subsequent experiments. The results from the TEER measurements, cytotoxicity assay, CBF measurements, gene expression analysis, and cytokine expression analysis performed on the smoke-exposed ALI-cultured SAEC and air control, with the final dilution rates, are presented.

6.1 Analysis of smoke delivery

The 1R6F reference cigarettes were used to generate the cigarette smoke in the Vitrocell exposure module. The deposited smoke was measured with microbalance sensors and monitored through the Vitrocell dosimetry system. The result from the initial cigarette smoke assay can be seen in Table 6.1.1. A high concentration of smoke (low dilution rate) was used in the exposure system on one COPD donor with the one-time cigarette smoke exposure protocol.

Table 6.1.1: Mass deposition registered by the microbalance sensors connected to the Vitrocell dosimetry system.

Channel	1	2	3	4	5
Dilution airflow [L/min]	0,1	0,1	0,25	0,25	0,5
Mass deposition [$\mu g/cm^2$]	109,89	38,46	22,13	11,79	6,13

As seen in Figure 6.2.1, the first three doses affect the physiological barrier with TEER values below $\Omega \times cm^2$. This result is consistent with observations directly after cigarette smoke exposure where the cilia were shown to stop beating, and several wholes were detected in the cell layer. These trends can also be seen in Figure 6.3.1. As a result, the smoke doses were adjusted so that the deposited mass monitored in channel four in the initial cigarette smoke assay was used as the highest dosage in the following experiments.

The dilution rates in the Vitrocell exposure system were then adjusted so that the deposited mass was approximately bisected for each channel, as seen in Table 6.1.2. A stable dose range was achieved by repeated smoke experiments without cells.

Table 6.1.2: Mass deposition registered by the microbalance sensors connected to the Vitrocell dosimetry system.

Channel	1	2	3	4	5
Dilution airflow [L/min]	0,85	0,6	0,9	1,5	3,0
Mass deposition [µg/cm^2]	11,51 ± 0,77	5,76 ± 0,54	3,29 ± 0,33	1,66 ± 0,27	0,88 ± 0,13

Means ± standard deviation.

The deposited mass for three COPD donors and two healthy donors, from all experiments, can be seen in Figure 6.1.1. As seen in the figure, the mass deposition is consistent throughout the different experiments.

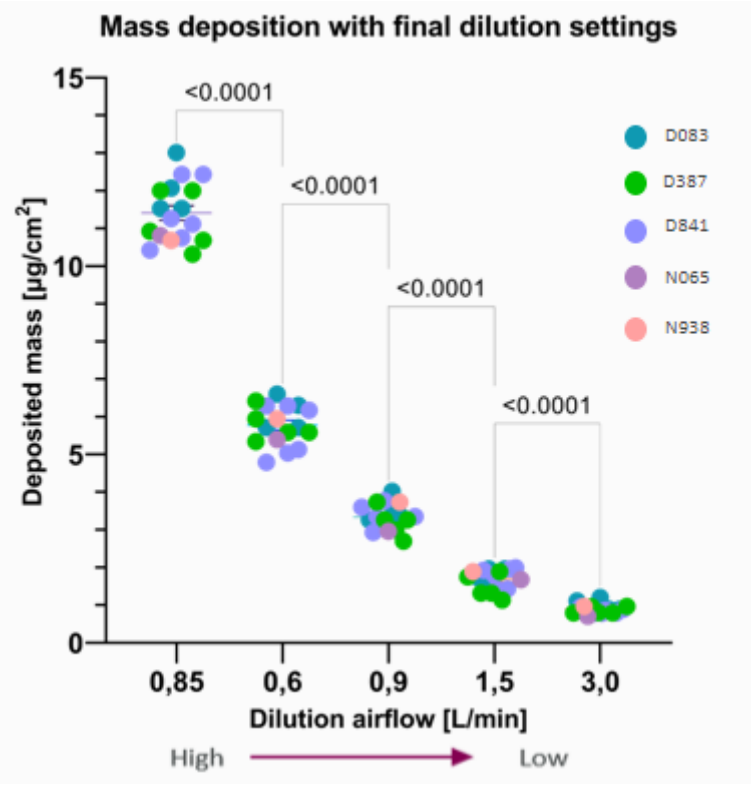
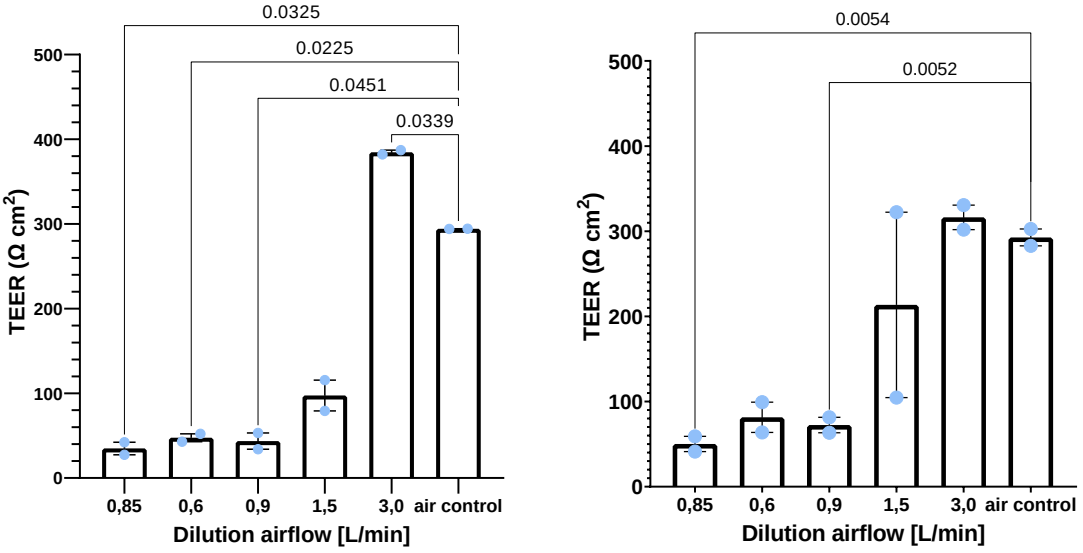


Figure 6.1.1: Mass deposition for three COPD donors and two healthy donors, from all experiments, presented as µg/cm^2 to dilution airflow.

6.2 Physiological barrier

The TEER measurements from the initial cigarette smoke experiment can be seen in Figure 6.2.1. TEER is presented as TEER ($\Omega \times cm^2$) $\pm$ SEM. TEER values measured of sampled exposed to different concentrations of smoke are compared to air control through a one-way ANOVA multiple comparison test. A P value < 0.05 is considered significant.



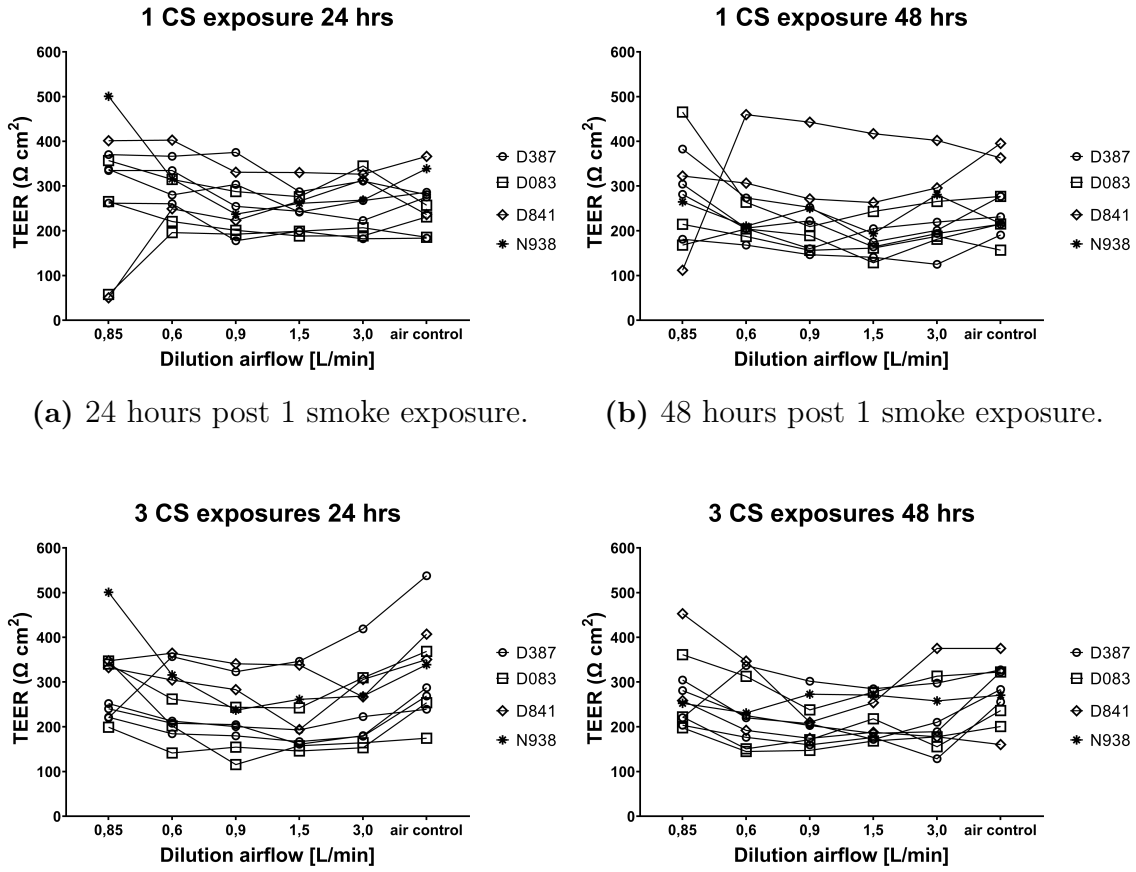
(a) 24 hours post smoke exposure.

(b) 48 hours post smoke exposure.

Figure 6.2.1: TEER measurements on SAEC exposed to five concentrations of smoke, and air control, from COPD donor D387, 24 and 48 hours after exposure.

The TEER value is significantly reduced for the three highest concentrations of smoke compare to air control (P=0.0325, P=0.0225, P=0.0451), as seen in Figure 6.2.1a. The fourth dose (dilution airflow=1.5) lowers the TEER value, but no significant difference is observed compared to air control. The lowest concentration of smoke (dilution airflow=3) significantly raises TEER (P=0.0339). In addition, a difference between the 24 hours and 48 hours samples can be observed. The TEER values are slightly more decreased after 24 hours than after 48 hours. This occurrence can be due to the recovery of the cells, after the acute effect has worn off.

In Figure 6.2.2, TEER measurements of smoked sampled after one and three exposure, 24 and 48 hours after exposure, for three COPD and one healthy donor, are presented.



(a) 24 hours post 1 smoke exposure.

(b) 48 hours post 1 smoke exposure.

(c) 24 hours post 3 smoke exposures

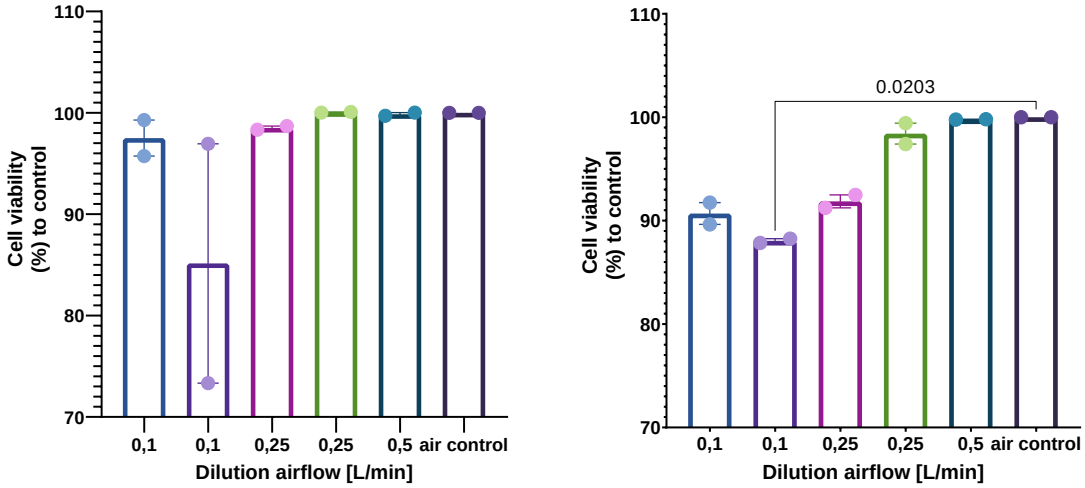
(d) 48 hours post 3 smoke exposures

Figure 6.2.2: TEER measurements of smoke exposed ALI-cultured SAEC compared to non-treated cell cultures. TEER values of COPD donors and healthy cells at two different time-points after one-exposure and three-exposures smoke assays.

There is no difference between cells from COPD donors (D083, D387, and D841) and healthy donor N938. Donor D083 shows lower TEER values than the rest of COPD donors. No significant difference between TEER measurements from sampled one-exposure samples and three-exposure samples can be observed. A slight decline in TEER values can be seen between samples 24 hours and 48 hours post smoke exposure.

6.3 Cytotoxicity/Cell viability

The basolateral supernatants were collected 24 and 48 hours after smoke exposure(s). The level of LDH in the supernatants was used as an indicator of cytotoxicity. Cytotoxicity is presented as cell viability (%) to air control to make the results more easily interpreted. The result from the LDH assay performed with the initial dilution airflow settings was used to assess cytotoxicity. Cell viability from the initial smoke experiment can be seen in Figure 6.3.1. Cell viability is presented in % to air control ± SEM. A one-way ANOVA multiple comparison test was performed to evaluate the difference between smoke-exposed samples and air control. A P value < 0.05 is considered significant.

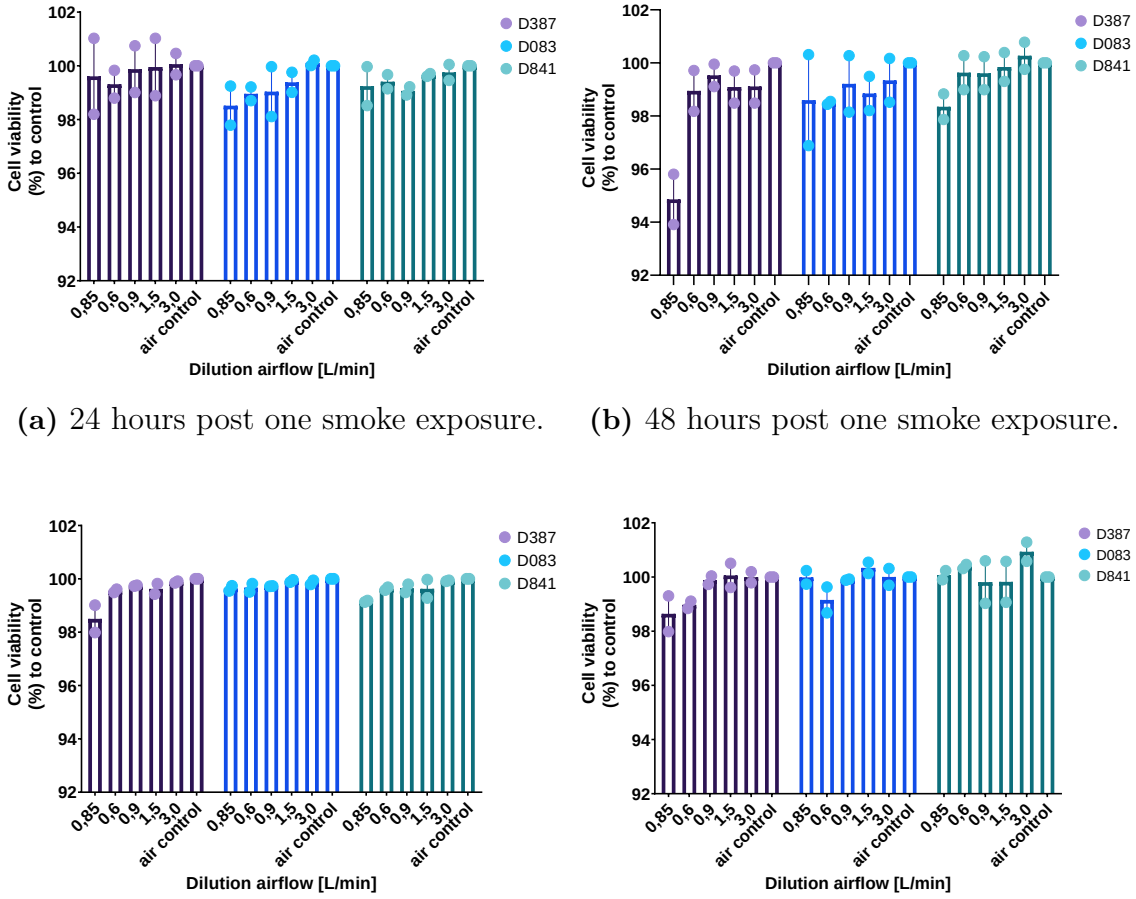


(a) 24 hours post smoke exposure.

(b) 48 hours post smoke exposure.

Figure 6.3.1: Cell viability in % to air control examined by measuring LDH release in cell medium. Cytotoxic effect of cigarette smoke with different dilution airflow on SAECs 24 hours (a) and (b) 48 hours post exposure.

In the 24 hours samples, a minor decline in cell viability is observed for the highest cigarette smoke dose and considerable recession in the second-highest dose. The 48-hour specimens show higher toxicity for the three highest cigarette smoke doses, where a significant difference can be observed for the second-highest dose compared to air control. LDH cytotoxicity assay was performed on all smoked exposed samples to ensure the viability of the cells and membrane integrity. LDH results for all following experiments can be seen in Figure 6.3.2. A minor decline in cell viability can be seen in samples exposed to the highest smoke concentration, but no significant difference to air control is observed.



(a) 24 hours post one smoke exposure.

(b) 48 hours post one smoke exposure.

(c) 24 hours post three smoke exposure.

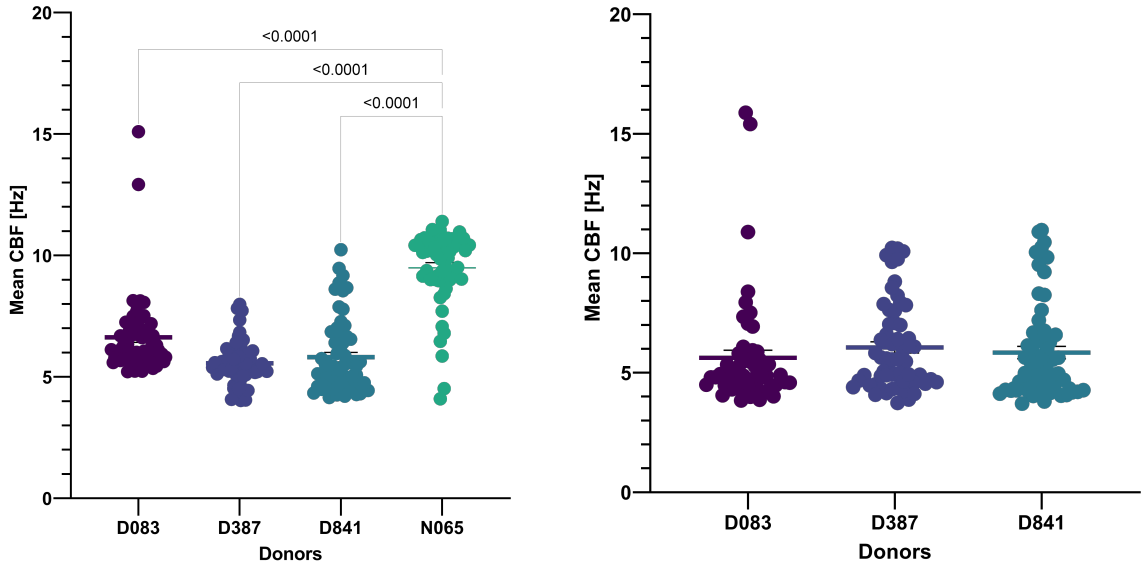
(d) 48 hours post three smoke exposure.

Figure 6.3.2: Cell viability (%) to air control for COPD donors D387, D083 and D841 24 hours and 48 hours post smoke exposure(s).

Based on the result from TEER measurements and cytotoxicity analysis, new dilution airflows were set on the Vitrocell smoking system, as seen in Table 6.1.2. The fourth dilution (11,79 µg/cm^2) in the initial smoke experiment showed no significant difference to air control in TEER measurement or cytotoxicity in the LDH assay. Therefore, a deposited mass of 11,79 µg/cm^2 was used as the highest cigarette smoke dose in the following experiments.

6.4 Ciliary beating frequency

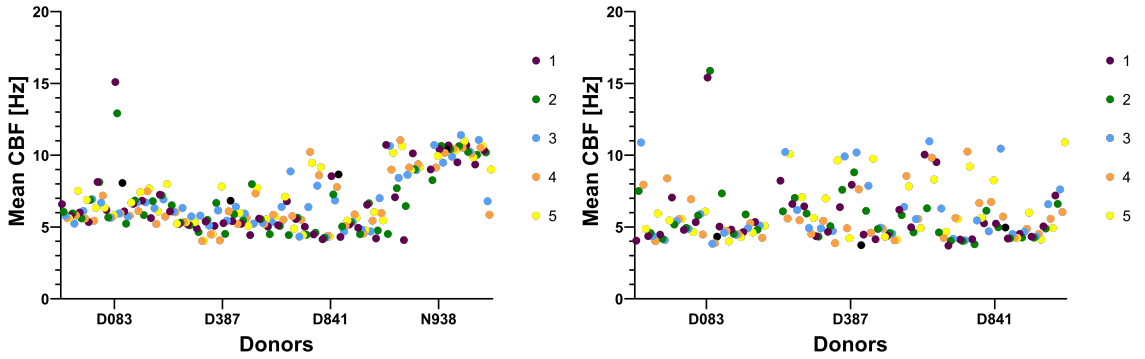
The ciliary beat frequency (CBF) was measured 24 hours post smoke exposure, both after one and three exposures. The mean cilia beating frequency ± standard deviation for all samples are presented in Figure 6.4.1. There is a significant difference between cilia beating frequency values for the COPD donors and the healthy donor with p values < 0.0001. Mean CBF for the COPD donors vary between five and seven. The healthy donor has a significantly higher mean CBF with a mean between nine and ten.



(a) 24h hours post one smoke exposure. **(b)** 24 hours post three smoke exposures.

Figure 6.4.1: Mean CBF for ALI-cultured SAECs sampled 24 hours post one and three smoke exposures according to donor.

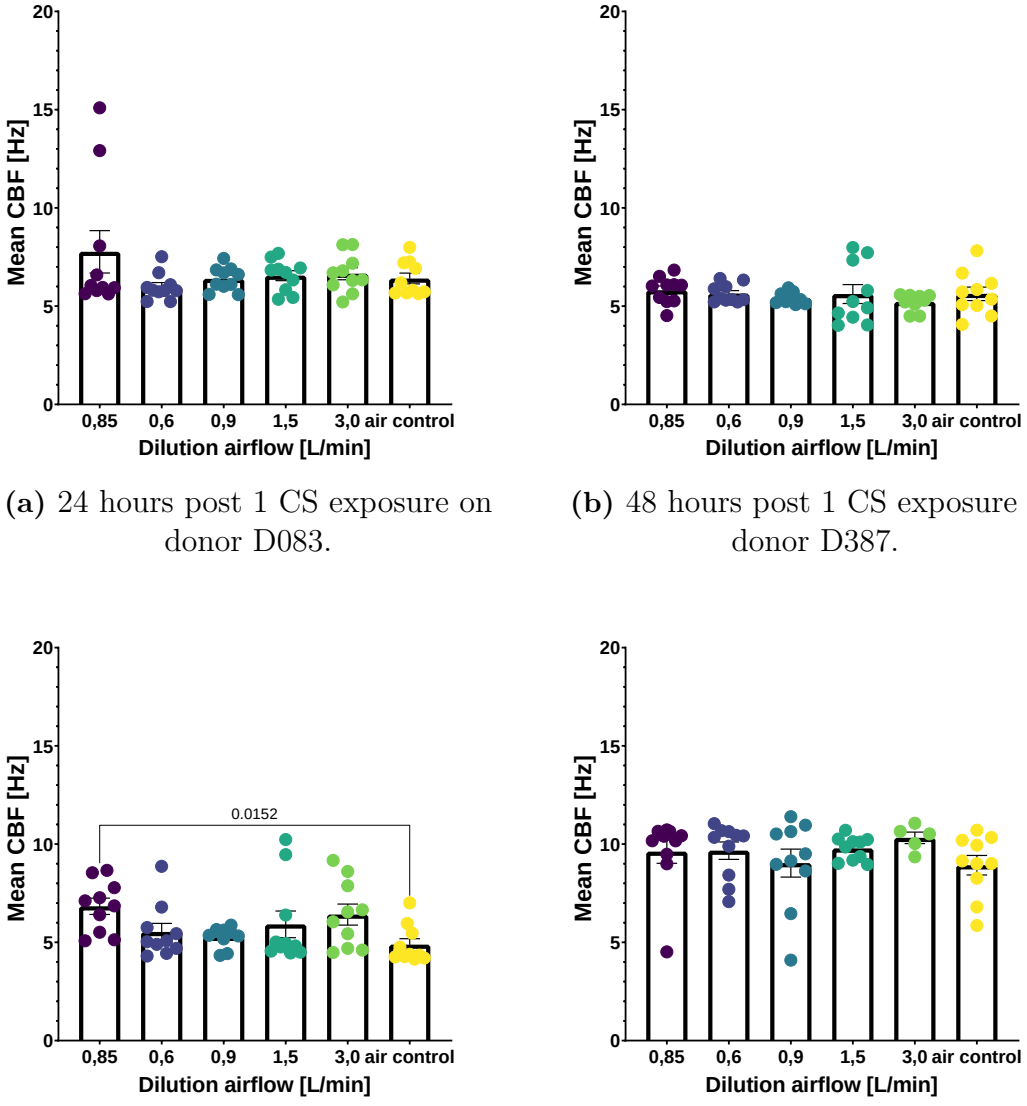
For each sample, five videos at different positions (see Figure 4.6.1) were recorded with the Nikon NIS-Elements Imaging Software, which were then converted to TIF:s (Tagged Image Files) before importing into the Cilia-X software. In Figure 6.4.2, the mean CBF for all donors according to well-position is presented. No significant difference in CBF between well-positions can be observed for any donor. There is no major difference in CBF between the two different time points.



(a) 24 hours post one smoke exposure. **(b)** 24 hours post thee smoke exposures.

Figure 6.4.2: Mean CBF measured at five points on each smoke exposed ALI-cultured SAEC sample, according to donor and number of smoke exposures.

To examine the cigarette smoke effect on CBF, a one-way ANOVA test was performed, as seen in Figure 6.4.3 and Figure 6.4.4. There is no significant difference between the smoked samples and the air control for one exposure, except for donor D841 exposed to the highest dose of smoke with P < 0.0152.



(a) 24 hours post 1 CS exposure on donor D083.

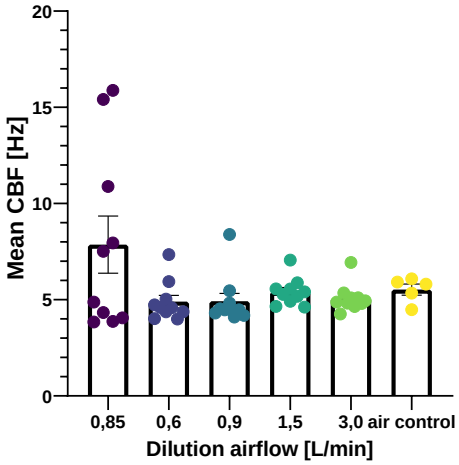
(b) 48 hours post 1 CS exposure on donor D387.

(c) 24 hours post 1 CS exposure on donor D841.

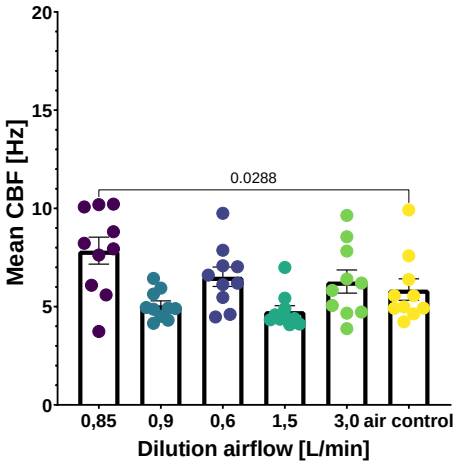
(d) 48 hours post 1 CS exposure on donor N065.

Figure 6.4.3: Mean CBF of smoked-exposed SAECs compared to air control according to smoke dilution airflow for 3 COPD donors and one healthy donor post 1 CS exposure.

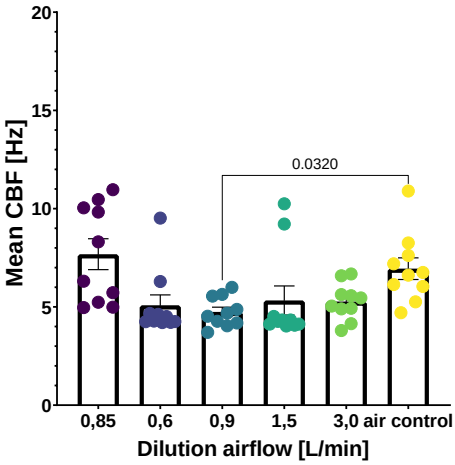
For the three exposure samples no significant difference between the smoked samples and the air control can be seen, except for donor D841 or the third cigarette smoke dose.



(a) 24 hours post 3 CS exposure on donor D083.



(b) 24 hours post 3 CS exposure on donor D387.

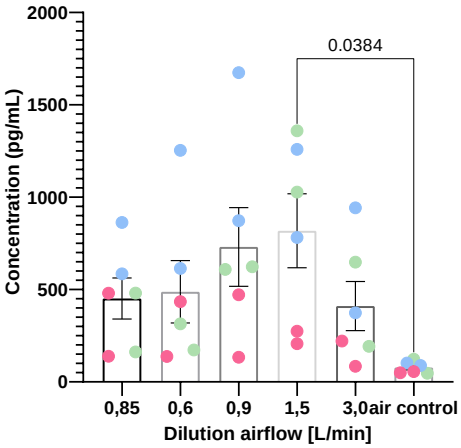


(c) 24 hours post 3 CS exposure on donor D841.

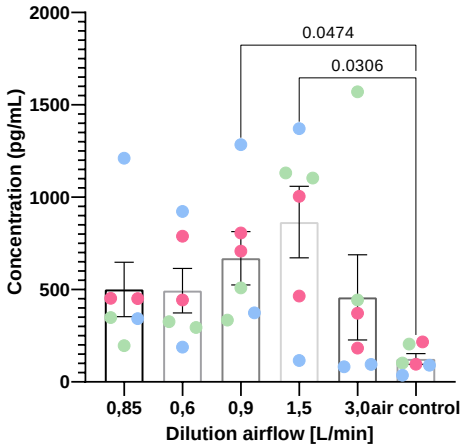
Figure 6.4.4: Mean CBF of smoked-exposed SAECs compared to air control according to smoke dilution airflow for 3 COPD donors and one healthy donor post 3 CS exposure.

6.5 Cytokine expression analysis

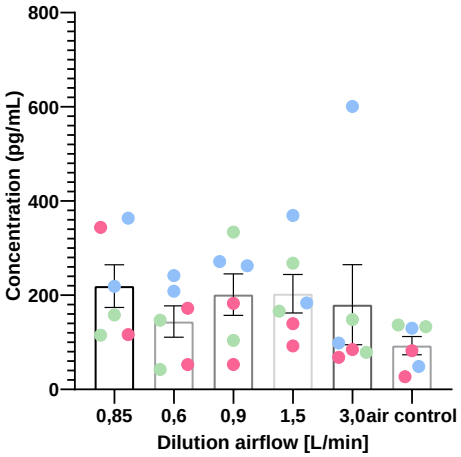
Supernatants from samples were collected 24 and 48 hours post smoke exposure(s) to examine the level of secreted cytokines into the cell medium. The U-PLEX human inflammatory panel (cytokines IL-6, IP-10, and MCP-1) and V-PLEX human panel (IL-8) were used. The difference between smoke-exposed samples and air control was examined with a one-way ANOVA multiple comparison test ($P < 0.05$). Data are presented as mean cytokine concentration ± SEM to dilution airflow (L/min). The three different colors represent the three different COPD donors used in this study.



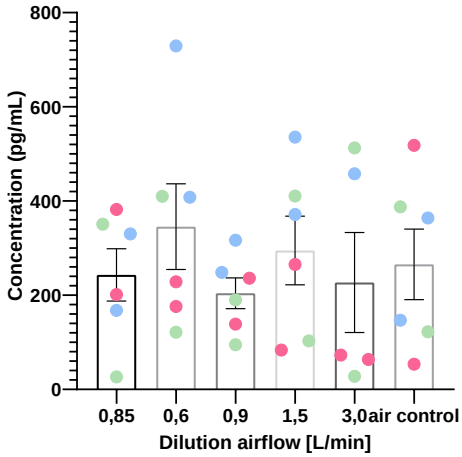
(a) 24 hours post 1 CS exposures.



(b) 48 hours post 1 CS exposures.



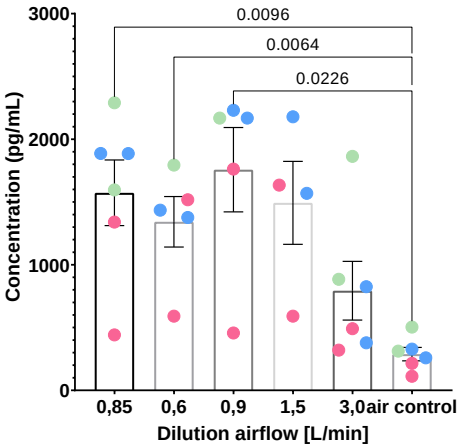
(c) 24 hours post 3 CS exposures.



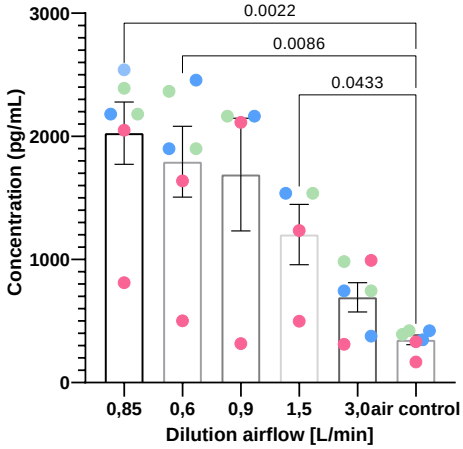
(d) 48 hours post 3 CS exposures.

Figure 6.5.1: Levels of cytokine interleukin (IL) 6 in samples 24h (a) and 48h (b) post one cigarette smoke exposure and 24h (d) and 48h (d) post three cigarette smoke exposures related to air control presented ng/mL. The green points represent donor D387, blue donor D083 and pink donor D841.

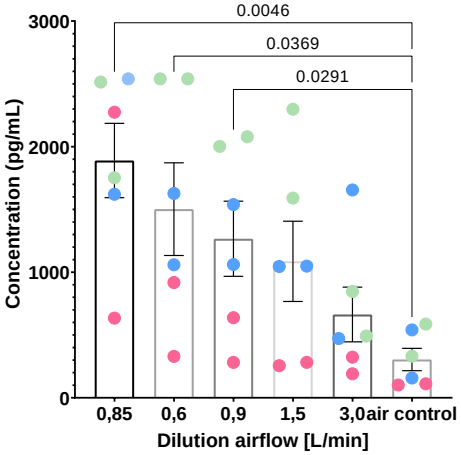
A significant increase in cytokine expression to air control was detected in IL-6, IL-8, and IP-10 secretion. In samples taken after one smoke exposure, 24 hours and 48 hours post-exposure, the third (0,9 L/min) and fourth (1,5 L/min) smoke dose induces an increase in IL-6 expression, as seen in Figures 6.5.1a and Figure 6.5.1b. The most extensive increase is seen in COPD donor D083. No significant change in IL-6 expression is observed in samples exposed to smoke three times, neither 24 nor 48 hours post-exposure.



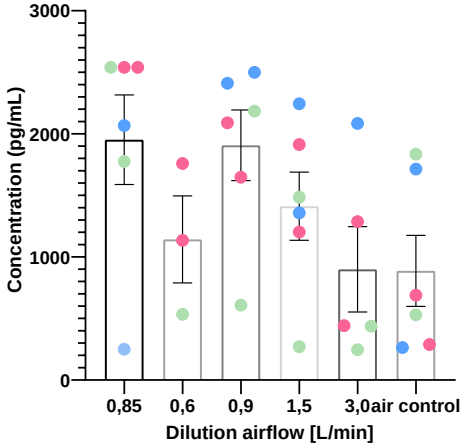
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



(c) 24 hours post 3 CS exposure.



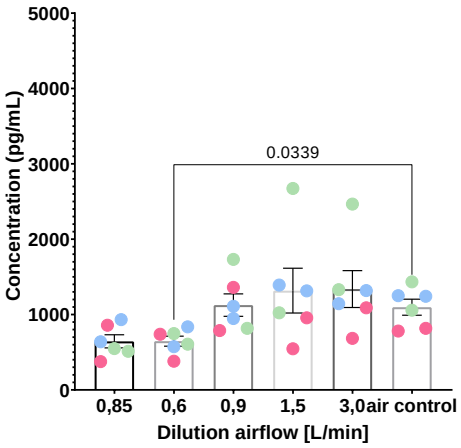
(d) 48 hours post 3 CS exposure.

Figure 6.5.2: Levels of cytokine interleukin (IL) 8 in samples 24h (a) and 48h (b) post one cigarette smoke exposure and 24h (d) and 48h (d) post three cigarette smoke exposures related to air control presented ng/mL. The green points represent donor D387, blue donor D083 and pink donor D841.

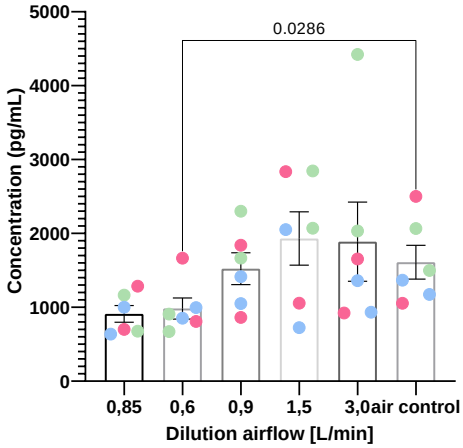
A significant up-regulation in cytokine IL-8 levels compared to air control can be observed in samples from one and three smoke exposures. In Figure 6.5.2a, a significant increase in IL-8 can be seen for the three highest cigarette smoke doses (P=0.0096, P=0.0064, and P=0.0226). The third (0,9 L/min) and fourth (1,5

L/min) smoke dose increase IL-8 concentration the most. In Figure 6.5.2b and Figure 6.5.2c, a clear dose-response pattern can be observed where a higher concentration of smoke up-regulates IL-8 cytokine amount. COPD donor D841 has the lowest concentration of IL-8. The results from three smoke-exposed samples after 48 hours show no significant difference between smoked samples and air control.

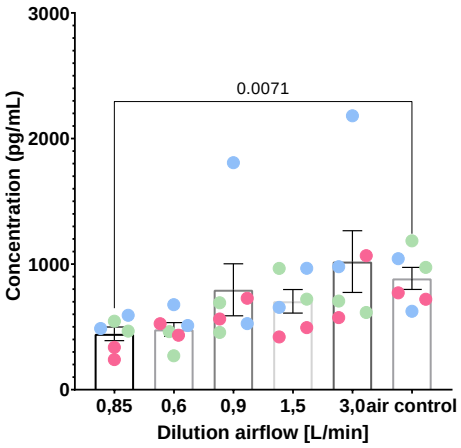
Cytokine IP-10 levels are down-regulated in smoke-exposed SAECs, see Figure 6.5.3. A slight increase can be seen in cells sampled 48 hours post smoke exposure, see Figure 6.5.3b and Figure 6.5.3d. The result shows a smoke-dose-dependent pattern where a higher concentration of smoke decreases cytokine IP-10 levels in samples.



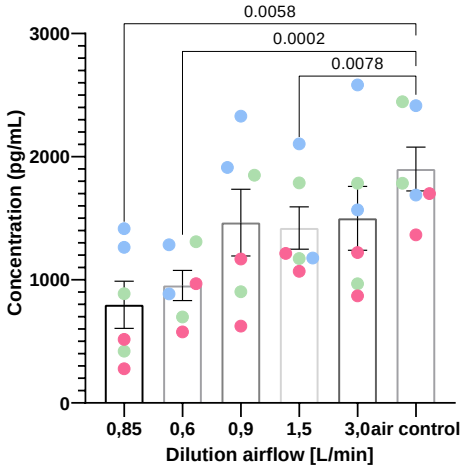
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



(c) 24 hours post 3 CS exposure.



(d) 48 hours post 3 CS exposure.

Figure 6.5.3: Levels of cytokine IP-10 in samples 24h (a) and 48h (b) post one cigarette smoke exposure and 24h (d) and 48h (d) post three cigarette smoke exposures related to air control presented ng/mL. The green points represent donor D387, blue donor D083 and pink donor D841.

6.5.1 Multiple comparison

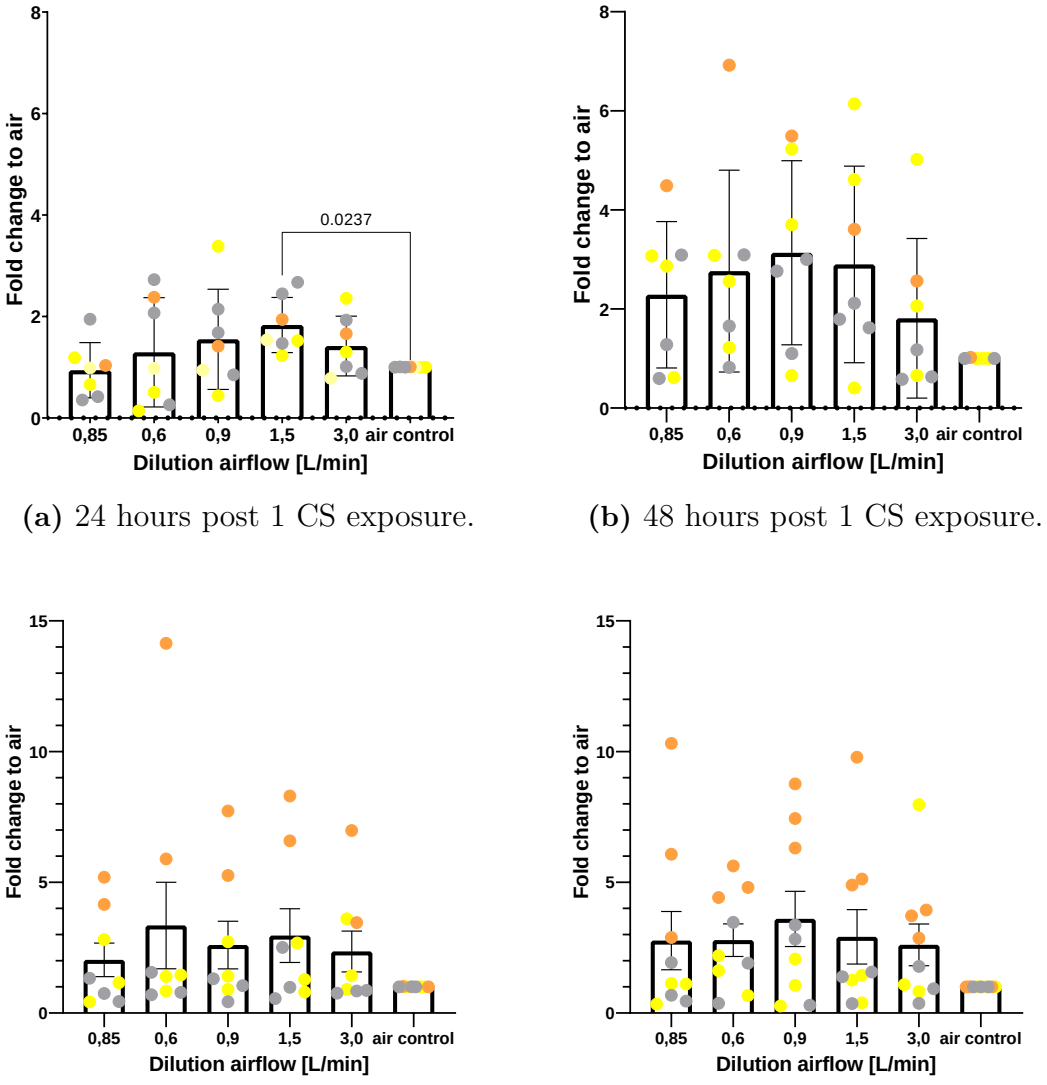
To investigate differences between the results of two (N=2) independent smoke exposure experiments, Sidak's multiple comparison test was used. Adjusted P-values from the tests are presented in Table 6.5.1. $P < 0.05$ was used as an indicator of significant difference. One sample exposed to 0.85 L/min diluted smoke tested for MCP-1 cytokine expression showed significance (P=,0149), marked in red.

Table 6.5.1: Adjusted P values from the multiple comparison test on cytokine expression

		Adjusted P Values			
	Dilution airflow	IL-6	IL-8	IP-10	MCP-1
24hrs post 1 CS exposure	0,85	0,9992	1	>0,9999	0,701
	0,6	0,5281	>0,9999	>0,9999	0,9726
	0,9	0,4815	1	0,9964	0,949
	1,5	>0,9999	0,9	0,694	0,9979
	3,0	>0,9999	0,8	0,1086	0,6981
	air control	>0,9999	1	0,9888	>0,9999
48hrs post 1 CS exposures	0,85	0,9995	>0,9999	0,9963	0,5482
	0,6	0,9998	>0,9999	0,9982	0,2582
	0,9	>0,9999	1	0,9999	0,6851
	1,5	0,9969	>0,9999	0,9969	0,9996
	3,0	0,9817	0,7	0,9997	0,9989
	air control	>0,9999	1	0,7463	>0,9999
24hrs post 3 CS exposure	0,85	>0,9999	>0,9999	0,4266	0,0149
	0,6	>0,9999	1	0,9995	0,395
	0,9	0,9994	0,4	0,9928	0,8071
	1,5	0,8478	>0,9999	0,9972	0,9371
	3,0	0,3887	0,2	0,5347	0,4029
	air control	0,9983	1	0,9819	0,957
48hrs post 3 CS exposures	0,85	>0,9999	1	0,6245	0,9998
	0,6	>0,9999	0,4	0,821	0,6325
	0,9	0,9994	0,7	>0,9999	0,9621
	1,5	0,8478	0,4	0,9996	0,8755
	3,0	0,3887	0,1	0,5917	0,6521
	air control	0,9983	0,2	0,1269	0,217

6.6 Gene expression analysis

24 and 48 hours after smoke-exposure(s), SAECs were lysed and collected for gene expression analysis. The TaqMan Gene Expression kit was used. The genes investigated are listed in table 4.7.1. The difference between smoke-exposed samples and air control was examined with a one-way ANOVA multiple comparison test ($P < 0.05$). Data are presented as fold change ± SEM to air control according to dilution airflow (L/min).



(a) 24 hours post 1 CS exposure.

(b) 48 hours post 1 CS exposure.

(c) 24 hours post 3 CS exposure.

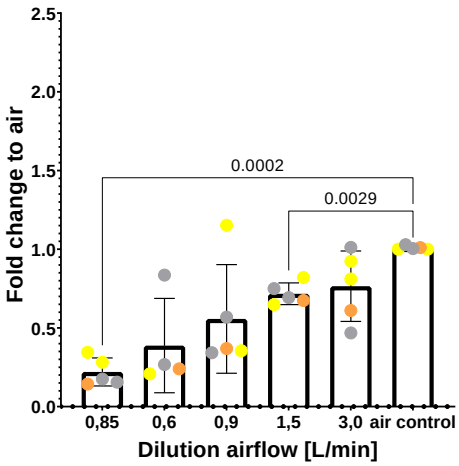
(d) 48 hours post 3 CS exposure.

Figure 6.6.1: MUC5AC gene expression of smoke-exposed SAECs and air control presented as fold change to air control, according to dilution airflow (L/min). Presented as mean ± SEM with $P < 0.05$.

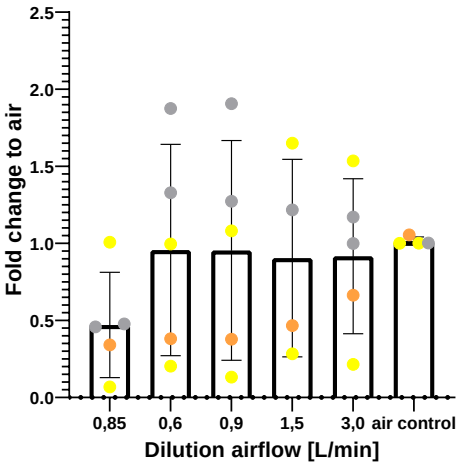
A minor up-regulation can be seen in gene expression of MUC5AC, see Figure 6.6.1, and a slightly higher expression in samples 48 hours post-exposure. No

considerable pattern can be observed, but the highest expression is seen in the moderate concentrations.

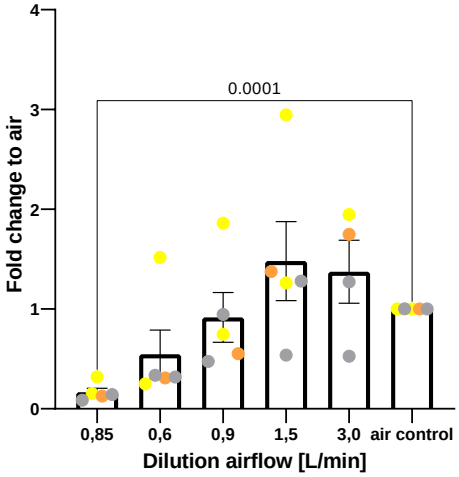
MUC5B expression is down-regulated, in a dose-dependent manner, where a higher cigarette smoke dose induces a decrease in MUC5B gene expression. The first and second cigarette smoke dose shows a significant difference compared to air control. 24 hours after one cigarette smoke exposure, a significant difference in expression to air control can be observed for the highest concentration (dilution airflow=0.85) and the fourth concentration (dilution airflow=1.5). After 48 hours, no considerable pattern or significant difference is observed. The gene expression seems higher after three cigarette exposures than one exposure.



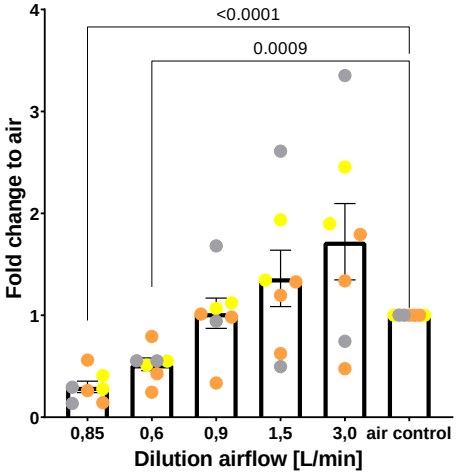
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



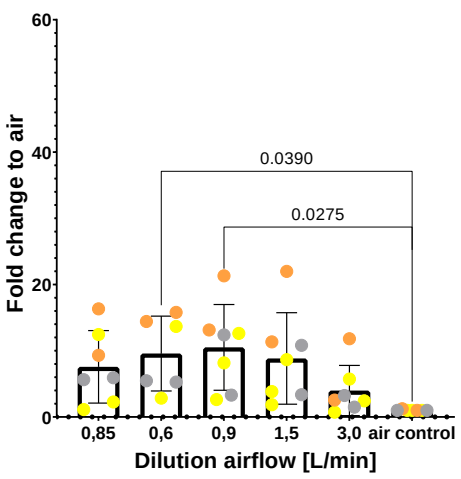
(c) 24 hours post 3 CS exposure.



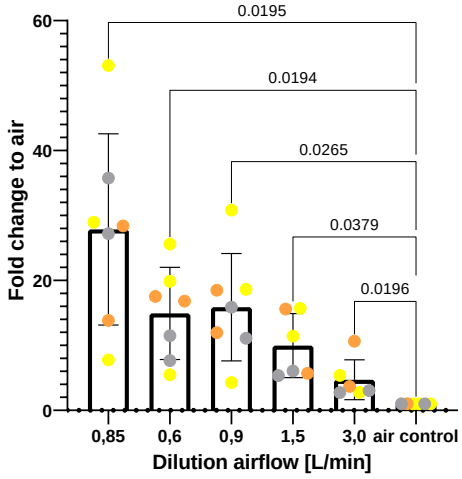
(d) 48 hours post 3 CS exposure.

Figure 6.6.2: MUC5B gene expression of smoke-exposed SAECs and air control presented as fold change to air control, according to dilution airflow (L/min). Presented as mean ± SEM with $P < 0.05$.

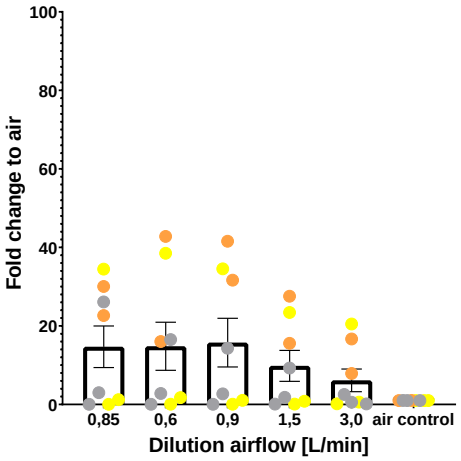
A considerable up-regulation can be seen in IL-1B gene expression, see Figure 6.6.3. 24 hours after one smoke-exposure, a significant difference between the second and third highest smoke dose (dilution airflow=0.6 and 0.9 respectively) and air control. After one cigarette smoke exposure, a dose-dependent relation can be seen. SAEC exposed to higher doses of smoke generate higher levels of gene expressions, especially after 48 hours. No significant difference between smoke-exposed cells and air control can be seen for cells exposed to smoke three times.



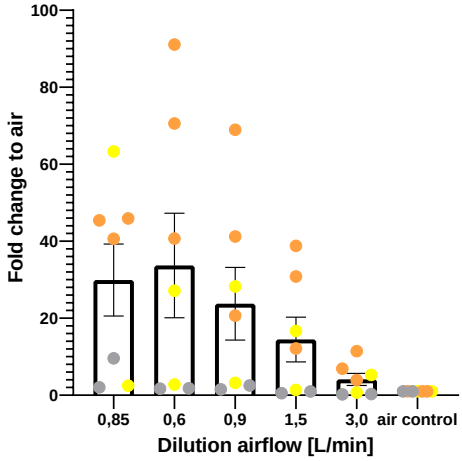
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



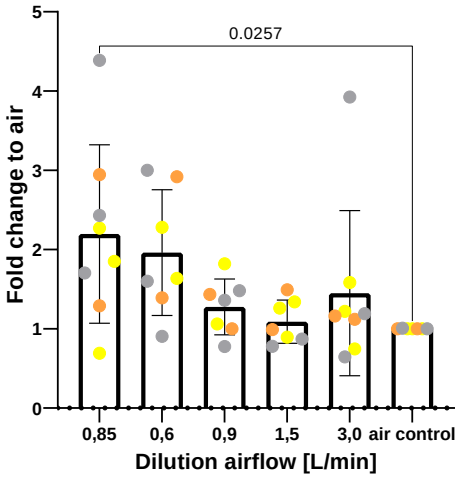
(c) 24 hours post 3 CS exposure.



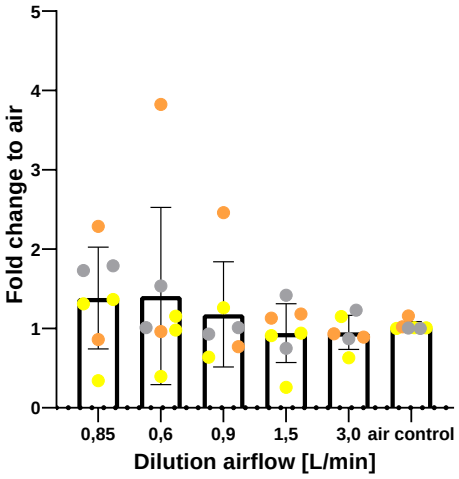
(d) 48 hours post 3 CS exposure.

Figure 6.6.3: IL-1B gene expression of smoke-exposed SAECs and air control presented as fold change to air control, according to dilution airflow (L/min). Presented as mean ± SEM with $P < 0.05$.

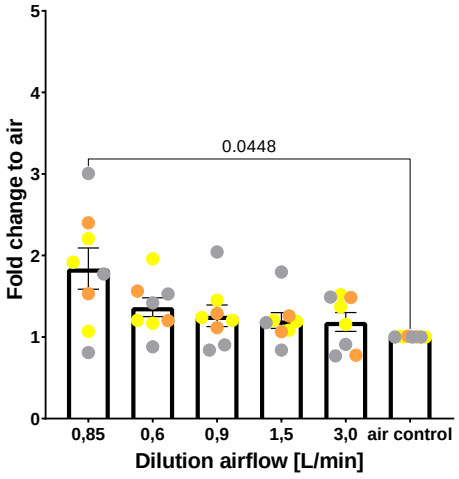
The gene expression analysis of HMOX1 shows no considerable up-regulation for either one nor three smoke exposures, see Figure 6.6.4. A slight up-regulation compared to air control can be seen for the highest dose (dilution airflow=0.85) 24 hours after smoke exposure, both after one and three exposures.



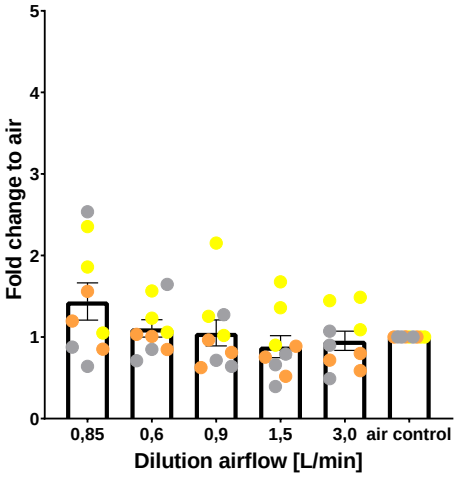
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



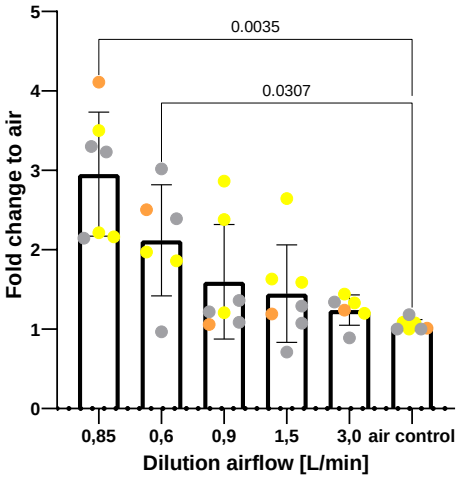
(c) 24 hours post 3 CS exposure.



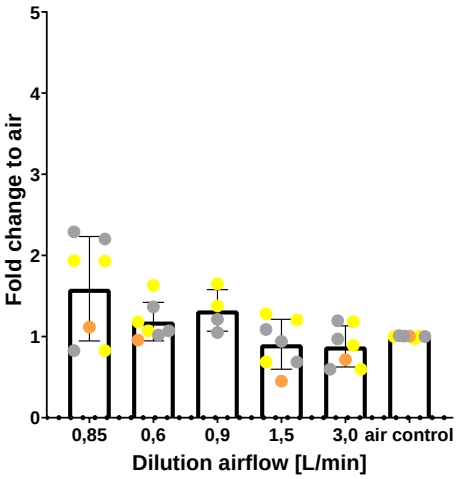
(d) 48 hours post 3 CS exposure.

Figure 6.6.4: HMOX1 gene expression of smoke-exposed SAECs and air control presented as fold change to air control, according to dilution airflow (L/min). Presented as mean ± SEM with $P < 0.05$.

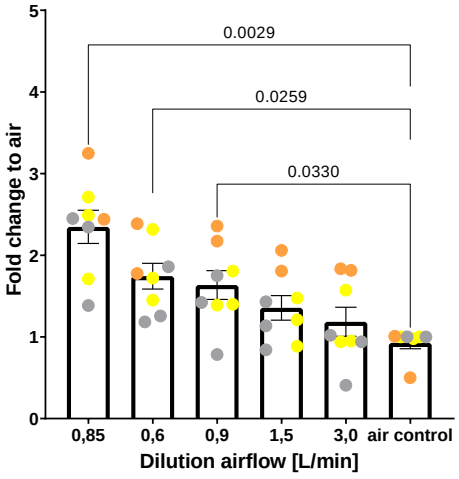
Gene expression of SRNX1 is up-regulated after one and three cigarette smoke exposures 24 hours after exposure. The first three doses (dilution airflow=0.85, 0.6 and 0.9) show significant difference compared to air control 24 hours after exposure. The relation seems to dose-dependent where a higher cigarette dose result in higher gene expression.



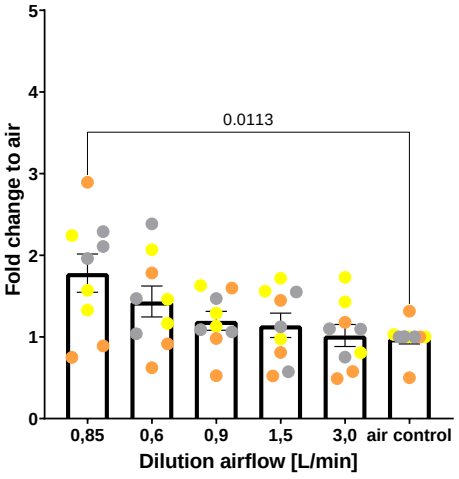
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



(c) 24 hours post 3 CS exposure.



(d) 48 hours post 3 CS exposure.

Figure 6.6.5: SRNX1 gene expression of smoke-exposed SAECs and air control presented as fold change to air control, according to dilution airflow (L/min). Presented as mean ± SEM with $P < 0.05$.

6.6.1 Multiple comparison

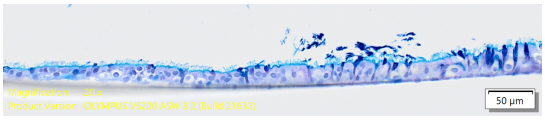
To investigate the difference between results of two (N=2) independent smoke exposure experiments on gene expression, Sidak's multiple comparison test was used. Adjusted P-values from the tests are presented in Table 6.6.1. $P < 0.05$ was used as an indicator of significant difference. No significant difference in gene expression can be observed between independent smoke experiments in any smoke dilution condition or time-points or number of exposures.

Table 6.6.1: Adjusted P values from the multiple comparison test on the gene expression results.

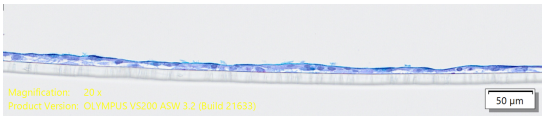
	Adjusted P Values										
	Dilution airflow	MUC5AC	MUC5B	MMP-9	CDKN2A	IL-8	IL-1B	NQO1	HMOX1	SRNX1	DDIT3
24hrs post 1 CS exposure	0,85	0,3413	0,9987	0,9475	0,869	0,5289	0,0091	0,6098	>0,9999	0,5833	0,6028
	0,6	0,0293	0,0049	0,6715	0,759	0,3834	0,2589	0,6443	>0,9999	0,5833	0,6028
	0,9	0,4783	0,0027	>0,9999	0,5357	0,0396	0,2865	0,8589	>0,9999	0,5833	0,6028
	1,5	0,4577	0,9868	0,1993	0,9997	0,009	0,5292	0,7637	>0,9999	0,5833	0,6028
	3,0	>0,9999	0,0577	>0,9999	0,9833	0,9406	0,6877	0,9936	>0,9999	0,5833	0,6028
	air control	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	0,5833	0,6028
48hrs post 1 CS exposures	0,85	0,9998	0,9919	0,1245	0,9923	0,4381	0,3471	0,8983	0,1987	0,8316	0,2327
	0,6	0,9132	>0,9999	>0,9999	0,9768	0,9853	>0,9999	>0,9999	0,1987	0,8316	0,2327
	0,9	0,9996	>0,9999	0,7233	>0,9999	>0,9999	0,937	0,9883	0,1987	0,8316	0,2327
	1,5	0,8792	0,8658	0,6989	0,9886	0,9997	0,9469	>0,9999	0,1987	0,8316	0,2327
	3,0	0,9758	0,9877	>0,9999	0,4193	0,9975	0,9914	>0,9999	0,1987	0,8316	0,2327
	air control	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	0,9881	0,1987	0,8316	0,2327
24hrs post 3 CS exposures	0,85	0,9957	>0,9999	0,3412	0,9996	0,883	0,7142	0,0095	0,0656	>0,9999	0,1567
	0,6	0,1546	0,9843	0,3807	0,9948	>0,9999	0,9865	0,0518	0,0656	>0,9999	0,1567
	0,9	0,8969	0,9801	>0,9999	0,9763	>0,9999	0,9143	0,0987	0,0656	>0,9999	0,1567
	1,5	0,8232	0,6992	0,6674	0,9944	>0,9999	0,9968	0,2627	0,0656	>0,9999	0,1567
	3,0	0,9997	0,0313	0,9972	0,6492	0,9983	0,9995	0,8166	0,0656	>0,9999	0,1567
	air control	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	0,9968	0,0656	>0,9999	0,1567
48hrs post 3 CS exposures	0,85	0,9726	>0,9999	0,9631	>0,9999	0,6647	0,3202	0,048	0,3929	0,9611	0,8873
	0,6	0,9986	>0,9999	>0,9999	>0,9999	0,5664	0,3372	0,124	0,3929	0,9611	0,8873
	0,9	>0,9999	>0,9999	>0,9999	0,9999	0,8673	0,4021	0,6845	0,3929	0,9611	0,8873
	1,5	0,413	0,998	0,9947	0,8068	0,8275	0,8236	0,5519	0,3929	0,9611	0,8873
	3,0	>0,9999	0,9969	0,9996	0,9997	0,991	>0,9999	0,658	0,3929	0,9611	0,8873
	air control	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	0,3929	0,9611	0,8873

6.7 Staining

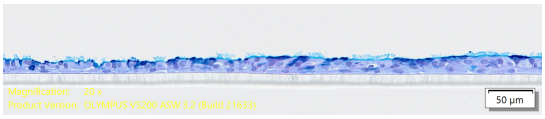
Six samples from COPD cell donor D387 were collected 24 hours post three cigarette smoke exposures to show typical characteristics of pseudostratified epithelium from SAEC. The samples were washed, fixated, embedded, sectioned and stained according to protocol. The results from the staining can be found in Figure 6.7.1.



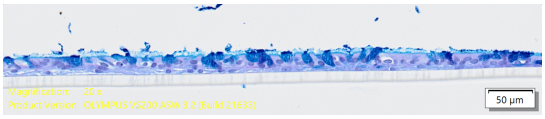
(a) SAECs exposed to direct cigarette smoke with dilution airflow 0.85 [L/min].



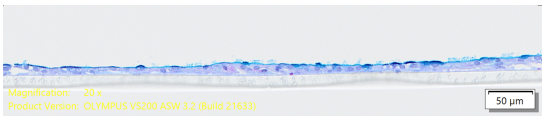
(b) SAECs exposed to direct cigarette smoke with dilution airflow 0.6 [L/min].



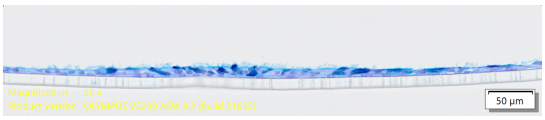
(c) SAECs exposed to direct cigarette smoke with dilution airflow 0.9 [L/min].



(d) SAECs exposed to direct cigarette smoke with dilution airflow 1.5 [L/min]



(e) SAECs exposed to direct cigarette smoke with dilution airflow 3.0 [L/min].



(f) SAEC air control sample.

Figure 6.7.1: Histological sections of SAEC cultures from COPD cell donor D387 exposed to five different doses of cigarette smoke. The sections were stained with AB/PAS staining.

The dark blue stained cell in Figure 6.7.1 are mucus-producing cells. The epithelium difference in thickness, depending on where on the membrane the section is taken. Figure 6.7.1a shows a stained section from the outer rim of the membrane where the cell layer is thicker and consists of more mucus-producing cells. Since only six samples could be spared for staining, no conclusions can be drawn from this analysis. Although, the sections can be used to further analyze morphology of epithelium exposed to direct cigarette smoke in future studies.

7

Discussion

To examine the effect of direct cigarette smoke, generated and exposed by the Vitrocell smoke system, on ALI-cultured SAECs, analytical procedures described in Chapter 4 were used. TEER measurements were performed to ensure barrier integrity of the epithelium after smoke exposure. LDH cytotoxicity assay was used to ensure cell viability after the smoke challenge. CBF was measured to investigate cilia functionality after smoke exposure with Cilia-X software. Gene expression and cytokine expression were determined with TaqMan gene expression assay and MSD immunoassay.

The Vitrocell VC10 S-type smoking robot was used to mimic real-life smoking according to the HCI smoke protocol. The HCI replaces the previous ISO standard protocol to generate a more in vivo like smoke behavior [38]. The 1R6F research cigarettes were used for smoke generation. The cigarettes are research graded and are used to create reproducible and comparable experiments. The Vitrocell smoking robot has been used for in vitro inhalation toxicology research [47] previously, and has shown good interlaboratory reproducibility [38]. As seen in Table 6.1.2, a consistent level of smoke was delivered to the microbalance sensors for all five dilution rates. The smoke dilution settings were based on the results of an initial smoke dose experiment, as seen in Table 6.1.1. The cells exposed to the first smoke conditions were examined 24 and 48 hours post-exposure by measuring TEER, and LDH activity in supernatants. The fourth highest dose (mass deposition = 11.79 $\mu g/cm^2$) showed a non-toxic response and unaffected TEER value, compared to air control, and was used as the highest dose in the final smoke dilution setting. SAECs from three COPD donors and 2 healthy donors were smoked one and/or three times, and samples were taken 24 and 48 hours post-exposure.

Chronic obstructive pulmonary disease (COPD) is characterized by obstructive airflow and abnormal inflammatory response in the innate and adaptive immune system. Inhaled toxic particles, as seen in commercially used cigarettes, cause inflammatory effects in the lungs, but they are amplified in COPD [7, 27]. The pathology of COPD includes mucous hypersecretion (chronic bronchitis), tissue destruction (emphysema), and impaired repair mechanisms leading to inflammation of the small airways and fibrosis [2, 20, 27]. To examine if the direct cigarette smoke model generates a COPD-like response of the small airway

epithelium, inflammatory response, oxidative stress response, mucus production, and cilia function was assessed.

To examine the inflammatory response in SAECs exposed to direct whole cigarette smoke, gene expression of inflammatory genes IL-8 and IL-1B were examined with TaqMan RT-qPCR. The secretions of cytokines IL-6, IL-8, IP-10, and MCP-1 were examined using MSD U-PLEX pro-inflammatory panel and V-PLEX human biomarker panel. Samples taken after one smoke exposure show a significant up-regulation in IL – 1β expression to air control, but not after three exposures. Levels of cytokine IL-6 secretion are increased after one smoke exposure, 24 and 48 hours post-exposure. The highest response was found in the third and fourth smoke dilution. An even higher increase is seen in IL-8 levels, showing a clear dose-response relation to smoke. The increase in IL-8 48 hours after one exposure resembles the response 24 hours after three smoke exposures. Cytokine IP-10 amplifies the inflammatory response in COPD by attracting monocytes and Th1 cells. The amount of IP-10 is usually increased in COPD patients and in response to smoke [28, 48]. However, levels of IP-10 cytokine in supernatants are decreased in this study. The amount of secreted IP-10 cytokines are lowered in high doses of smoke. IP-10 is especially down-regulated after three smoke exposures, 48 hours post-exposure. Increased levels of MCP-1 are usually seen in COPD patients [29, 30]. In this study, MCP-1 is highly expressed, ranging between 20'000 to 10'000 pg/ml in samples. Although, no significant increase in comparison to air control is observed. As seen in cytokine expression of IL-6, MCP-1 levels are increased most by the fourth and fifth smoke dilutions, indicating that a small dose of cigarette smoke is needed to trigger a response. COPD donor D841 (marked in pink) seems to consistently express lower levels of cytokines than donors D083 (blue) and D387 (green). The result from the cytokine expression evaluation is consistent with the results from the gene expression analysis. IL-1b is highly expressed and up-regulated for all smoke doses after one smoke exposure, but no significant increase can be seen after three exposures.

Besides chronic inflammation, COPD is characterized by an imbalance in oxidants and antioxidants, leading to oxidative stress. Oxidative stress poses a considerable factor for the parthenogenesis of COPD [19, 49]. HMOX1, NQO1, and SRXN1 were used to examine the oxidative stress response after smoke exposure. These genes are all regulated by the nuclear factor erythroid 2–related factor 2 (Nrf2). It is a regulator of cellular resistance to oxidants, controlling induced expression of various antioxidants [50]. HMOX1 is an antioxidant reduced by oxidative stress. It acts protectively by suppressing inflammatory properties of monocytes and macrophages [51]. HMOX1 gene expression is slightly up-regulated after one and three exposure, 24 hours post-exposure. A significant difference in expression to air control can be seen for the highest smoke dose. Expression of SRXN1 was up-regulated in smoked samples compared to air control. The highest response was seen in samples exposed to the highest doses, 24 hours post-exposure. The increase in response seems dose-dependent. Although no significant difference, compared to air control, was seen in the gene expression of NQO1, the results from SRXN1 and HMOX1 indicate an oxidative stress response in the SAECs after smoke exposures. Since the highest

response in gene expression was seen 24 hours after smoke exposure, it would be interesting to examine the response even closer to the smoke exposure.

Goblet cell hyperplasia and mucus hypersecretion are seen in the epithelium of COPD patients[28]. Chronic smoking also increases the number of goblet cells in the epithelium, and up-regulation of mucin MUC5AC is seen at the gene level in smokers [6, 32]. To examine smoke-induced change in goblet cells, gene expressions of mucins MUC5AC and MUC5B were used in this study. A minor up-regulation of MUC5AC gene expression was seen, especially in samples exposed to the third and fourth smoke doses. The overall result varies a lot between smoke doses and donors. In gene expression of MUC5B, a down-regulation can be observed instead. As for the results from MUC5AC analysis, the results vary considerably between doses and donors, but a dose-dependent pattern can be observed. A high cigarette smoke dose seems to down-regulate the expression of MUC5B compared to air control, and a significant difference can also be observed for the intermediate dose (fourth highest dose). These findings do not correlate to results found in the literature. Therefore, it would be interesting to examine the gene expression of MUC5AC and MUC5B further at other time points. However, this model with these dilutions and these time points may not be suitable for examining mucus hypersecretion and goblet cell hyperplasia in response to cigarette smoke, based on the inconclusive results.

The results from the gene expression analysis and cytokine expression analysis were used to examine the robustness and reproducibility of the model. Dunn-Sidaks comparison test was used to find a significant difference between the experiments for all genes/cytokines, every dilution rate, and the two time-points when smoked cells and controls were sampled. For the cytokines, no significant difference is seen, except for one case in MCP-1, see table 6.5.1. In gene expression, very few significant differences are seen. These results, together with results from TEER measurements and cytotoxicity analysis, validate that the model is robust.

8

Conclusion

In this project, a model for direct whole cigarette smoke exposure on small airway epithelial cell cultures in ALI is established. The smoke generation and delivery to the cells through the Vitrocell smoking system were shown to be stable and consistent. Different dilutions of cigarette smoke were examined for the model, and five non-toxic doses with minimal barrier disruption of the epithelium were chosen. The second part of the evaluation was to study if the established smoke model generates a COPD response. Thus, inflammatory response, oxidative stress response, mucus hypersecretion, and ciliary dysfunction of the smoke-exposed SAEC were examined.

Cilia functionality was examined by measuring CBF 24 hours post-exposure, and no difference was seen in CBF between smoked cells and air control. The inflammatory effect in response to smoke was examined by gene expression analysis (IL-8 and IL-1B) and cytokine expression analysis (IL-6, IL-8, IP-10, and MCP-1). A significant increase in cytokine expression to air control was seen in IL-6 and IL-8 after one cigarette smoke exposure, 24 and 48 hours post-exposure. Gene expression of IL-1B shows a significant increase after one cigarette smoke exposure. This increase indicates that the model does induce inflammatory response seen in COPD airway epithelium. Expression of HMOX1 and SRXN1 was examined to study the oxidative stress response to smoke exposure. Both gene expressions were up-regulated after one and three smoke exposures 24 hours post-exposure and thereby suggest a smoke-dose-dependence response. The gene expression of mucins MUC5AC and MUC5B shows inconsistent results, suggesting that the protocol might not be suitable for studying mucus hypersecretion or goblet cell hyperplasia.

Overall, the model provides robust results. Results from the gene expression analysis, cytokine analysis, and TEER measurements suggest reproducibility. The response in genes and cytokines related to inflammation are elevated in samples exposed to smoke one time, 24 and 48 hours post-exposure. In oxidative stress regulating genes, no considerable difference was seen between one and three cigarette smoke exposures. Although, the response was mainly seen in samples 24 hours post-exposure in both conditions. Therefore, the smoke exposure model is deemed adequate for the evaluation of inflammatory response and oxidative stress response in smoke-exposed ALI-cultured SAECs.

Even though COPD is a multifaceted and complex disease and one in vitro model alone cannot replicate the entire disease pathogenesis. However, developing smoke exposure models with direct whole cigarette smoke exposure on ALI-cultured epithelium, resembling the in vivo configuration, pose great promise for future COPD research. New and refined in vitro models are essential for earlier diagnosis and novel treatments to halt the progression of the disease in the pursuit of finding a long-term cure.

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A.4.1Conventional staining protocol for AB/PAS staining. VI

A

Appendix - Protocols

In this chapter, protocols regarding cell cultivation, LDH assay, TaqMan assay, and staining is presented. These protocols are not provided by manufacturer.

A.1 Culture protocol PneumaCult for 24w Single Inserts

Primary normal human small airway epithelial cells (SAEC) are obtained from Lonza. Cells are in passage 2. To increase the number of cells, the cells are cultured in conventional (submerged) culture for an additional passage (p3).

A.1.1 SAEC expansion

1. 1 vial of SAEC cells from the P2 generation of one donor is quickly thawed at 37°C. Remove it from the water bath when there is a sliver of ice left.
2. Transfer the thawed cells to 30 mL PneumaCult ExPlus medium.
3. Seed the cells into two T75 flasks. Incubate at 37°C.

Medium is changed with 12 mL the next day and then with 15 ml 3 times a week (Monday, Wednesday and Friday) until reaching about 50-70% confluency, usually after 3-5 days.

A.1.2 Trypsinization and freezing

When the cells reach 50-70% confluency it is time for harvest.

1. Wash cells with 2x 10 mL PBS (RT-37°C) without Mg2+ and Ca2+.
2. Add 3 mL TrypLE (RT-37°C) per flask.
3. Incubate 10-15 min at 37°C (check regularly). Cells can be detached by gently knocking on the flask.
4. Add 3 mL medium per flask and transfer cells to a 50 mL eppendorf tube.

5. Rinse flask 1 with 6 mL medium, transfer to flask 2 and rinse. Transfer wash to above mentioned tube (total volume 18 mL).
6. Centrifuge for 10 min at 1200 rpm ar RT.
7. Resuspend the cell pellets in 5 or 10 mL ExPlus medium depending in confluency.
8. Take out 250 000 cells and seed into one T75 flask, 12 mL medium per flask.
9. Dilute the remaining cells to 10^6 cells/mL in ExPlus medium with 10% DMSO.
10. Add 0.5 mL per cryotube and freeze in a Stratagene box at -80°C for 1-2 days, then move to -150°C freezer for permanent storage.

A.1.3 Seeding for ALI

1. 1 vial of frozen SAECs is quickly thawed at 37°C. Remove them from the water bath when there is a sliver of ice left.
2. Gently resuspend the cells by pipetting the cell suspension up and down a few times. Avoid introducing bubbles.
3. Add the cell suspension into a Falcon tube containing 5 ml of PneumaCult ExPlus medium. Swirl gently.
4. Add 200 µL of cell suspension to the apical side of each insert.
5. Add 800 µL ExPlus medium in the basolateral compartment of each insert.

Cells are preferably seeded on a Thursday morning. No medium change has to be performed until air-lift (Monday). (The cells should be over 50% confluent when air-lifted.)

A.1.3.1 Medium for seeding

To prepare **Complete PneumaCult™Ex Medium Plus:**

1. Thaw PneumaCult™-Ex Plus 50X Supplement at room temperature. Mix gently by inverting the vial; do not vortex.
2. Add 10 mL PneumaCult™-Ex Plus 50X Supplement and 0.5 mL Hydrocortisone Stock Solution (96µg/mL) to 490 mL PneumaCult™-Ex Plus Basal Medium. Mix thoroughly.

Store at 2 - 8°C for up to 4 weeks.

A.1.4 ALI culture

When cells are over 50% confluent (Monday morning if seeded Thursday) remove the apical medium and change the basolateral medium to PneumaCult ALI medium.

The medium is changed every second day with the exception of weekends. Medium leaking through the cell layer the first days after air-lifting is aspirated carefully.

When the cells are 2.5 weeks the first mucus wash is performed. Add 200 µL warm medium or PBS apically on each well. Incubate for a couple of hours and then gently remove it.

A.1.4.1 Medium for ALI-maintenance

To prepare 500 mL **PneumaCult™-ALI Complete Base Medium**:

- Thaw PneumaCult™-ALI 10X Supplement overnight at 8°C Mix gently by inverting the vial, do not vortex.
- Add 50 mL PneumaCult™-ALI 10X Supplement to 450 mL PneumaCult™-ALI Basal Medium. Mix thoroughly.

To prepare 500 mL **PneumaCult™-ALI Maintenance Medium:**

Add:

- 5 mL PneumaCult™-ALI Maintenance Supplement
- 1 mL Heparin (2 mg/mL stock solution)
- 2.5 mL Hydrocortisone Stock Solution (96 µg/mL)

Store at 2 - 8°C for up to 2 weeks.

A.2 LDH Assay Protocol

Mix substrate mix according to instructions and freeze at -20°C after use. It can be freeze-thawed a few times. Plates can be found on KL1. Reader (Spectramax) on AC2 (protocol in Amy's folder).

Run in clear 384-well plates (e.g. Greiner, cat no 781101)

A.2.1 In kit:

- Substrate mix
- Assay buffer 600 µL
- Lysis buffer 2.5 mL
- Stop solution 12 mL (this is used on the cells during the experiment)
- Positive control 6 µL

A.2.2 Preparation

- Thaw stop solution and assay buffer.
- Make substrate stock solution:

  Add 11.4 mL H_2O to substrate mix and mix gently to dissolve
- Make reaction mixture:

  Add the assay buffer to the substrate stock solution. Mix gently and protect from light (save leftovers at -20°C).
- Dilute the LDH positive control by adding 1.5 µl to 1 mL of 1% BSA in PBS (leftovers at -20°C)

A.2.3 Analysis

To a clear flat bottom 384-well plate:

1. Add 12.5 µL of sample (or positive control from the kit) - run duplicates if you like.
2. Add 12.5 µL of reaction mixture and mix well (shake plate and spin it).
3. Incubate plate in the dark at RT for 30 min.
4. Add 12.5 µL of stop solution, mix and centrifuge plate to remove bubbles.
5. Measure absorbance at 490nm-680nm (within 2 hours of adding stop solution).

Subtract 680nm absorbance values from 490nm values, present as such or calculate fold change versus negative control.

A.3 TaqMan protocol

A.3.1 RNA purification

Based on 150 µl QIAzol lysates.

For RNeasy mini kit (RNA of >200 nt isolated)

1. Thaw samples with lysates on ice.
2. Add another 100µl QIAzol (total vol ca 250µl).
3. Vortex tubes for homogenisation for 10 s.
4. Place tubes at RT for 5 min (pre-cool centrifuge to 4°C).
5. Add 50µl chloroform, shake vigorously for 15 s.

6. Place tubes at RT for 2-3 min.
7. Centrifuge for 15 min at 12'000g, 4C (then heat centrifuge to RT).
8. Transfer upper aqueous phase (ca 140µl) to a new tube and add 1 volume (ca 140µl) 70% ethanol and mix by pipetting.
9. Transfer solution (up to 700µl), including any precipitate, to an RNeasy Mini spin column and centrifuge for 15 s at more than 8'000g, discard flowthrough.
10. Repeat step 9 if there's still sample left.
11. Add 700µl RW1 buffer, do another quick spin.
12. Add 500µl RPE buffer, do a quick spin.
13. Add another 500µl RPE buffer, spin for 2 min at same speed.
14. If any remaining flowthrough is on the column, put it in a new tube and spin for 1 min.
15. Transfer column to a 1.5 ml tube and add 35µl water to the membrane, spin for 1 min.
16. Repeat step 15 using the eluate for a second elution.

A.3.2 cDNA Reverse Transciption

For High-Capacity cDNA Reverse Transcr. Kit (Applied Biosystems #4368814):

1. Thaw RT kit components on ice.
2. Measure RNA concentration using NanoDrop.
3. Calculate the volume RNA to use per RT reaction (use maximum 2µg/10µl RNA per 20µl RT reaction) and the total number of reactions (n).
4. Mix buffer (2µl/reaction), dNTPs (0.8µl/reaction), primers (2µl/reaction), enzyme (1µl/reaction) and water (4.2µl/reaction) for n+2 reactions.
5. Add the calculated volume of water (10-vol RNA µl) for n+2 reactions to the RT mix.
6. Aliquot 10+calc. amount of water µl of the mix into PCR strip tubes.
7. Add the calculated volume of RNA to each tube.
8. Seal tubes and spin briefly, keep on ice.
9. Run the reaction at 25°C for 10 min, 37°C for 120 min, then 85°C for 5 min followed by hold at 4°C.

10. Freeze tubes at -80°C.

A.4 Alcian blue/periodic acid–Schiff staining

Table A.4.1 describes the course of action for staining according to the Alcian Blue/Periodic Acid Schiff staining protocol.

Step	Action	Reagent	Time
1-3	Deparaffinize	Xylene	3 min
4-5	Hydration	100% Alcohol	2 min
6	Hydration	80% or 95% Alcohol	1 min
7	Hydration	Deionized Water	1 min
8	Stain	Alcian Blue	30 min
9	Wash	Water	5 min
10	Oxidize	Periodic Acid	5 min
11	Wash	Deionized Water	several changes, 10 sec each
12	Stain	Schiff Reagent	15 min
13	Wash	Lukewarm Water	10 min
14-15	Dehydration	95% Alcohol	2 min
16-17	Dehydration	100% Alcohol	2 min
18-19	Clearing	Xylene	2 min

Table A.4.1: Conventional staining protocol for AB/PAS staining.

The colors of the stained samples ranges from blue to magenta where blue is characteristic for acidic mucins, magenta neutral mucins, deep blue nuclei and a mixture of neutral and acidic mucins are colored blue/purple.

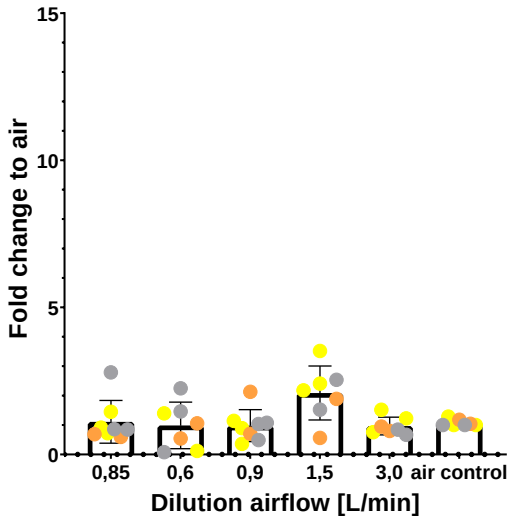
B

Appendix - Results

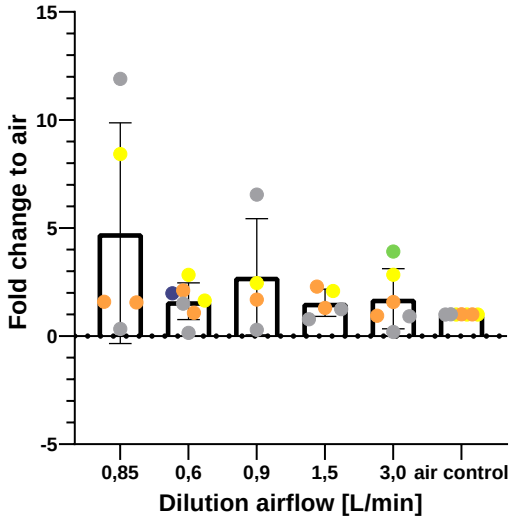
B.1 TaqMan results

In the following section, inconclusive and non-significant results from the gene expression analysis is presented.

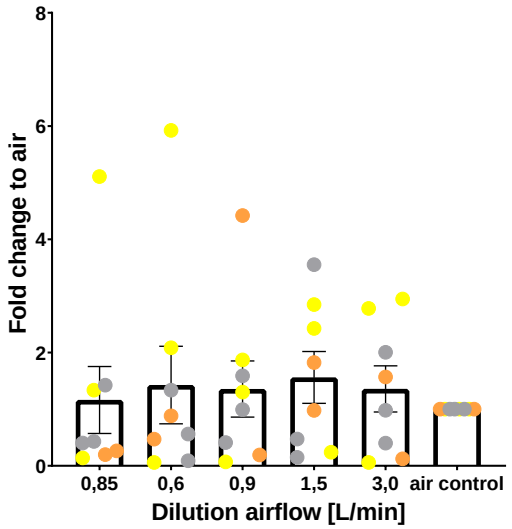
Cellular migration examined by quantification of gene expression of MMP-9.



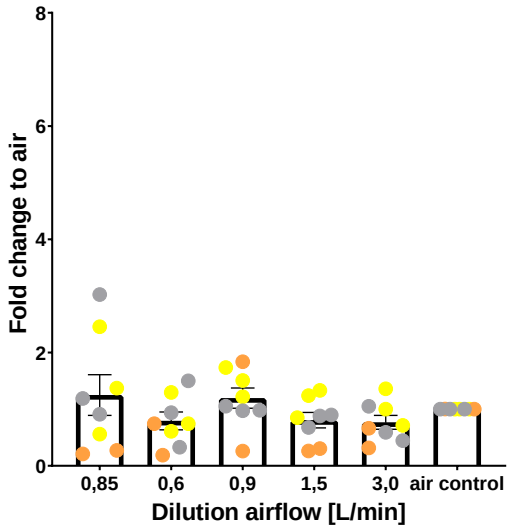
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



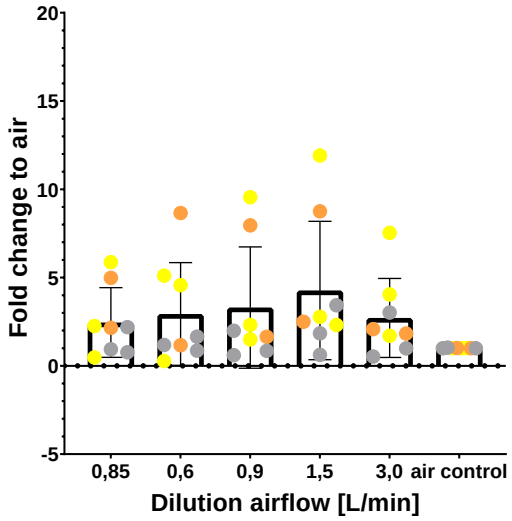
(c) 24 hours post 3 CS exposure.



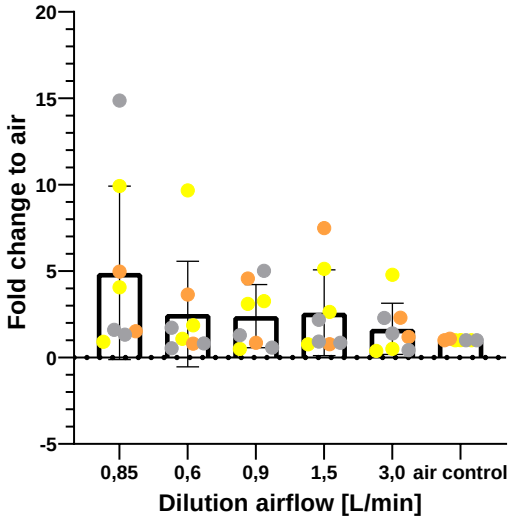
(d) 48 hours post 3 CS exposure.

Figure B.1.1: MMP-9 gene expression of smoke-exposed SAECs and air control presented as fold change to air control, according to dilution airflow (L/min). Presented as mean ± SEM with $P < 0.05$.

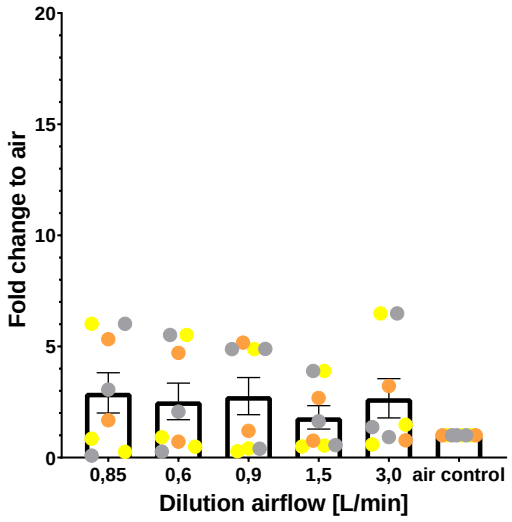
Inflammatory response examined by quantification of gene expression IL-8.



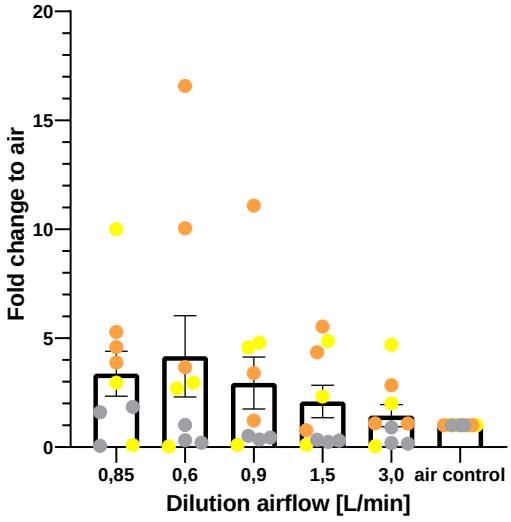
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



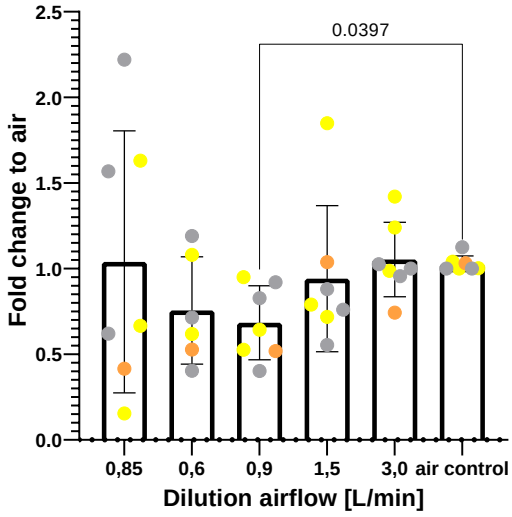
(c) 24 hours post 3 CS exposure.



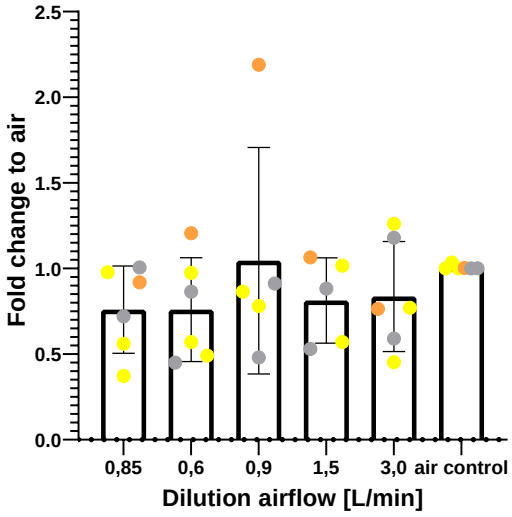
(d) 48 hours post 3 CS exposure.

Figure B.1.2: IL-8 gene expression of smoke-exposed SAECs and air control presented as fold change to air control, according to dilution airflow (L/min). Presented as mean ± SEM with $P < 0.05$.

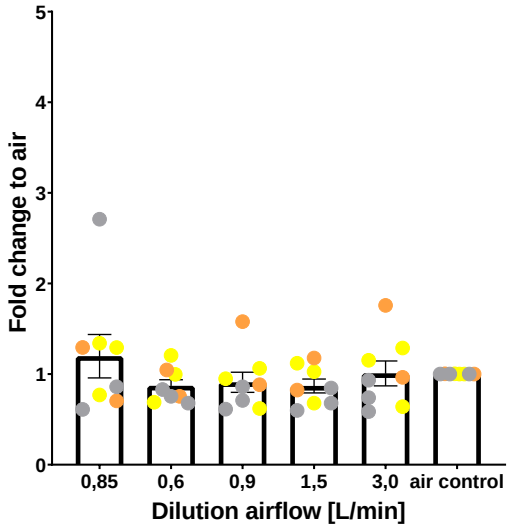
Cellular stress examined by quantification of gene expression of DDIT-3



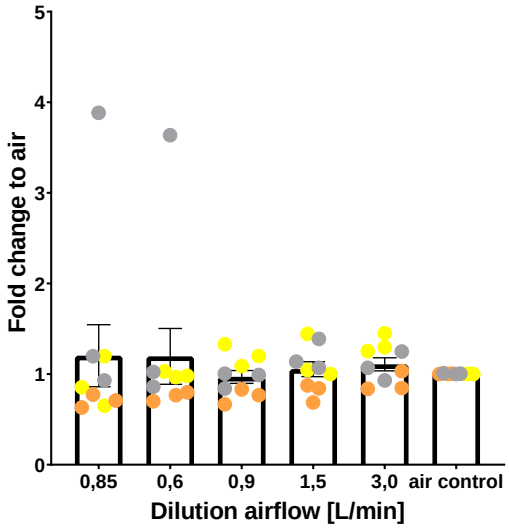
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



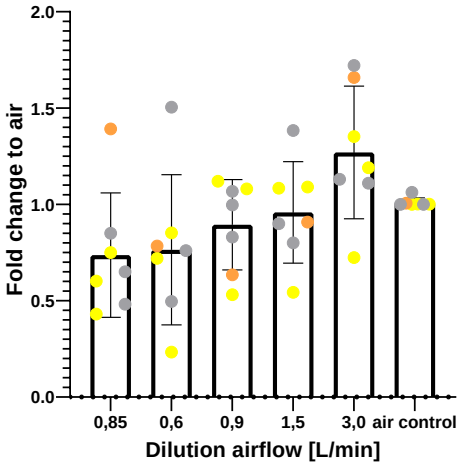
(c) 24 hours post 3 CS exposure.



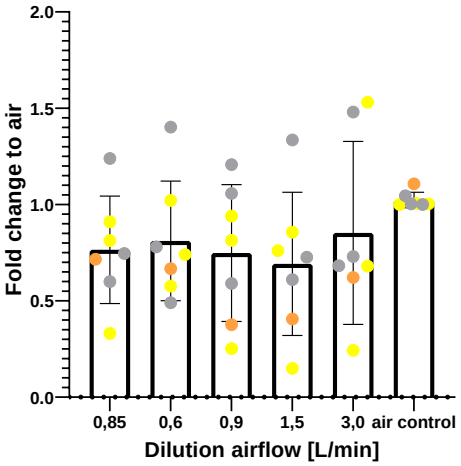
(d) 48 hours post 3 CS exposure.

Figure B.1.3: DDIT3 gene expression of smoke-exposed SAECs and air control presented as fold change to air control, according to dilution airflow (L/min). Presented as mean ± SEM with $P < 0.05$.

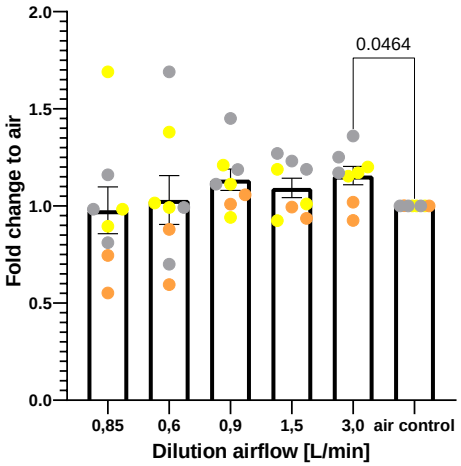
Senescence examined by quantification of gene expression of CDKN2A.



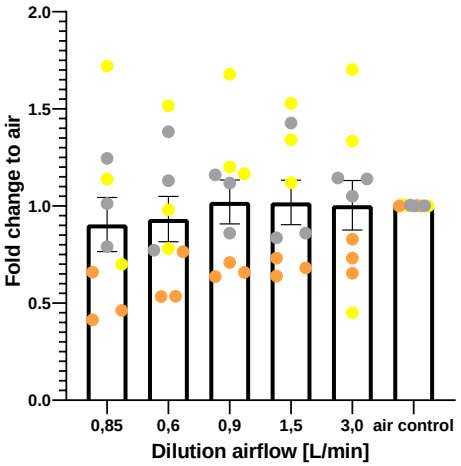
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



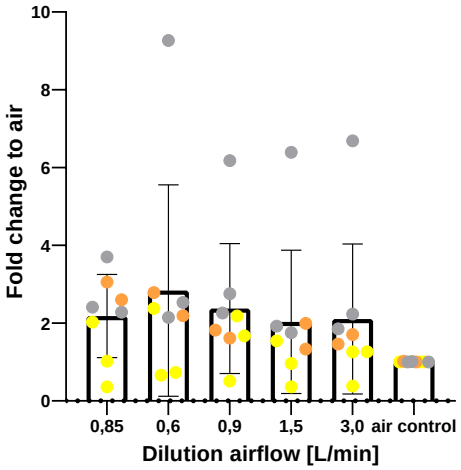
(c) 24 hours post 3 CS exposure.



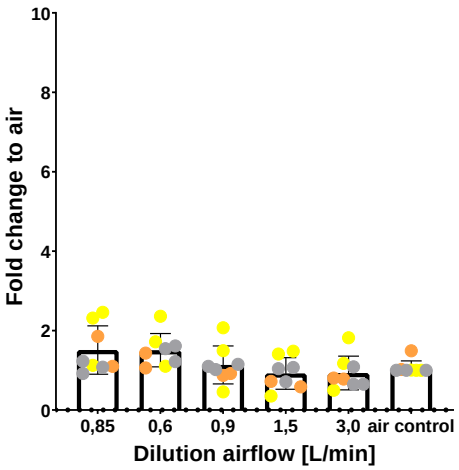
(d) 48 hours post 3 CS exposure.

Figure B.1.4: CDKN2A gene expression of smoke-exposed SAECs and air control presented as fold change to air control, according to dilution airflow (L/min). Presented as mean ± SEM with $P < 0.05$.

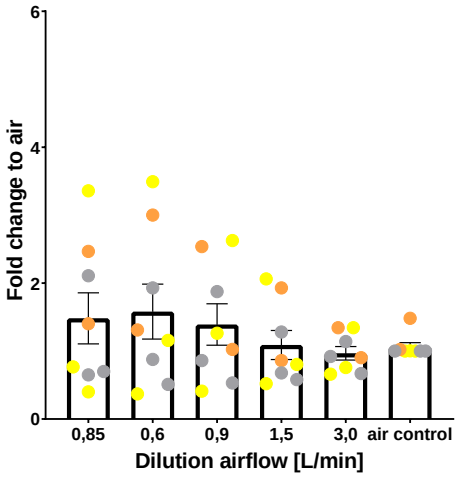
Oxidative stress examined by quantification of gene expression of NQO1.



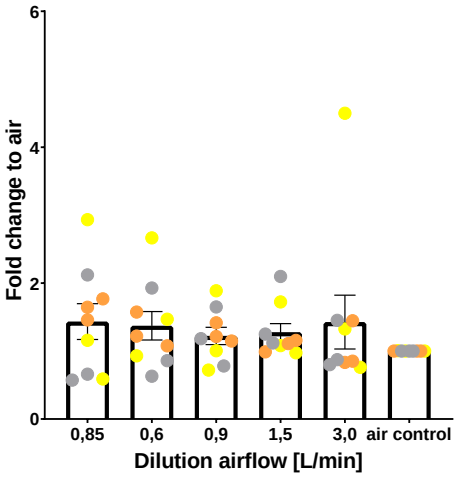
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



(c) 24 hours post 3 CS exposure.

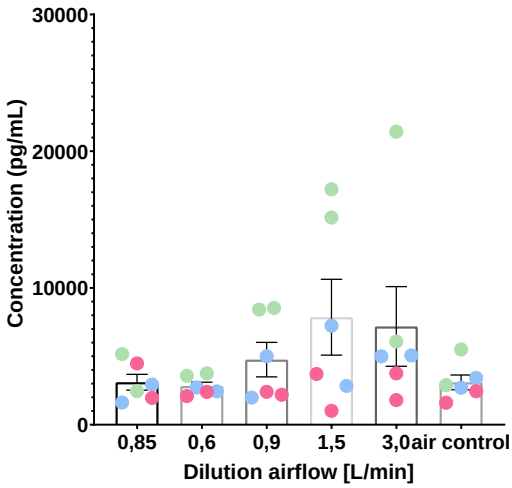


(d) 48 hours post 3 CS exposure.

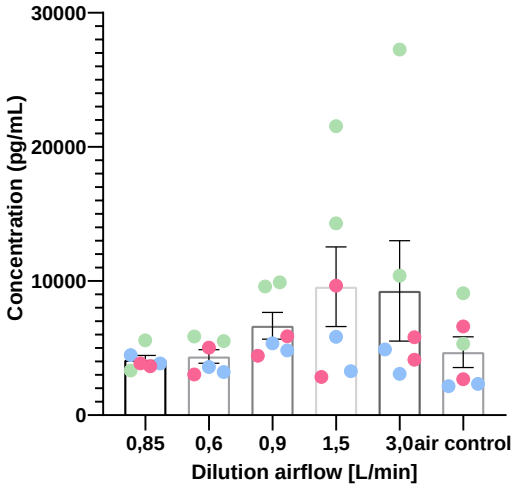
Figure B.1.5: NQO1 gene expression of smoke-exposed SAECs and air control presented as fold change to air control, according to dilution airflow (L/min). Presented as mean ± SEM with $P < 0.05$.

B.2 Immunoassay results

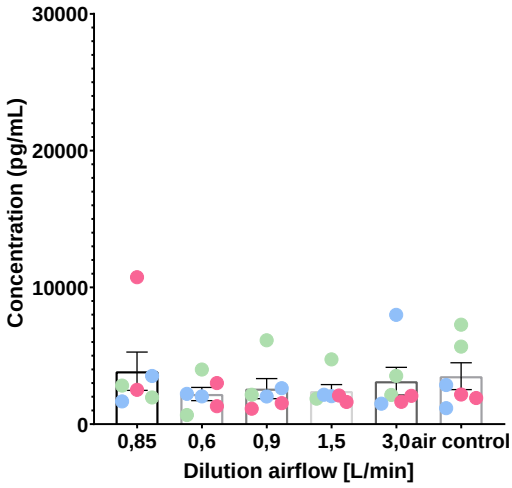
In the following section, inconclusive and/or non-significant results from the cytokine expression analysis is presented.



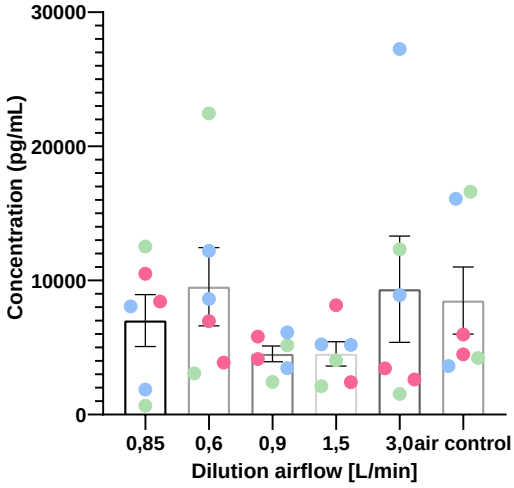
(a) 24 hours post 1 CS exposure.



(b) 48 hours post 1 CS exposure.



(c) 24 hours post 3 CS exposure.



(d) 48 hours post 3 CS exposure.

Figure B.2.1: Levels of cytokine MCP-1 in samples 24h (a) and 48h (b) post one cigarette smoke exposure and 24h (d) and 48h (d) post three cigarette smoke exposures related to air control presented ng/mL. The green points represent donor D387, blue donor D083 and pink donor D841.

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