..

In vitro and *in vivo* evaluation of the effect of *Aspergillus fumigatus* on epithelial cells.

Project By:

Olga Pitsilioni @00542467

Submitted in Fulfilment of the Requirements for the Award of the Degree of Master of Philosophy (MPhil) Biological Sciences.

School of Science, Engineering, and Environment, SEE

Supervisors: Dr. Sara Namvar, Dr Arijit Mukhopadhyay

April 2024

Final Word Count: 31.896 words

Table of Contents

ACKNOWLEDGEMENTS

I would like to express my appreciation for my supervisor Dr Sara Namvar for giving me the opportunity to do an MPhil in lung physiology research with her, giving me motivation and support through my journey in University of Salford. I gained valuable knowledge and learned many laboratory techniques. She has always been willing to pass me her knowledge and provide valuable feedback in all my steps so far. It was a privilege working with her.

I would also like to thank Dr Arijit Mukhopadhyay for being my co- supervisor during my master's and agreeing to have me on his team, providing valuable knowledge and being a great mentor for me. . I really appreciate all the help and support through my studies as well as the feedback on my work.

Finally, I would like to thank Muna Abubakar the technician of Cockcroft Laboratory 311, for teaching me everything I know in terms of laboratory assays and techniques. It has been a privilege to meet and learn beside you.

ABBREVIATIONS:

Name:	Definition:
ABPA	Allergic Bronchopulmonary Aspergillosis
AHR	Airway Hyperresponsiveness
AIDS	Acquired Immunodeficiency Syndrome
ALI	Air Liquid Interface
AWR	Airway Wall Remodelling
BSA	Bovine Serum Albumin
COPD	Chronic Obstructive Pulmonary Disease
DC	Dendritic Cells
DMEM	Dulbecco's Minimum Essential Media
DMSO	Dimethyl Sulfoxide
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial-To-Mesenchymal Transition
ET1	Endothelin 1
FBS	Foetal Bovine Serum
IA	Invasive Aspergillosis
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
MEM	Minimum Essential Media
PBS	Phosphate Buffer Saline
SAFS	Severe Asthma with Fungal Sensitisation
TEER	Trans Epithelial Electrical Resistance
TGF-β	Transforming growth Factor beta.VEGF Vascular Endothelial Growth Factor

ABSTRACT

Aspergillus fumigatus is a saprophytic fungus and one of the most prevalent airborne pathogens. Asthma is severe and associated with increased mortality in those patients that have fungal sensitisation. Mechanisms of airway wall remodelling and the potential involvement of fibrin deposition, as well as the miRNA biomarkers of airway- *Aspergillus fumigatus* interactions are yet to be identified. The aim of this study was to evaluate the extent of lung pathology, including fibrin deposition in murine models of *Aspergillus fumigatus* sensitisation. Furthermore, this work set out to identify miRNA biomarkers of *Aspergillus fumigatus* induced airway damage and to investigate the effects of co-exposure to fibrin and *Aspergillus fumigatus in vitro.* It was hypothesised that *Aspergillus fumigatus* exposure would be associated with fibrin deposits in the lung. Furthermore, it was hypothesised that fibrin and Aspergillus fumigatus would have a pathological impact on epithelial cells *in vitro.* Lung tissue sections from control mice and models of *Aspergillus fumigatus* sensitisation including those exposed to culture filtrate, culture filtrates plus an Endothelin-1 antagonist and a commercially sourced extract were stained by H&E, Masson's Trichrome, Periodic Acid Schiff and Carstair's staining for fibrin and platelets. The impact of fungal sensitisation and the therapeutic potential of Endothelin-1 antagonism was assessed. *In vitro*, proinflammatory cytokine induction and airway epithelial morphological changes in response to two different strains of *Aspergillus fumigatus* and fibrin were also investigated. Exposure to *Aspergillus fumigatus* caused a significant increase in airway and blood vessel inflammation and remodelling, with no therapeutic benefits of Endothelin-1 antagonism observed. Induction of cytokines, IL-6, IL-8 and Endothelin-1, in response to *Aspergillus fumigatus* exposure was significant in two different cell lines (A549 and 16HBE), and in the co-exposure model of fibrin and *Aspergillus fumigatus*. Finally, *Aspergillus fumigatus* caused a downregulated expression of miRNAs relevant to lung fibrosis including: miR-9-5p, miR-223-5p, miR-155-5p, miR-21-5p miR-320a-3p, miR-130b-3p, miR-223-3p and miR-34a-5p. This work confirms that *Aspergillus fumigatus* and fibrin cause pro-inflammatory cytokine induction *in vitro* and suggest a

possible role of fibrin in airway pathology *in vivo.* . We also observed downregulation of miRNA in response to *Aspergillus fumigatus* exposure ,findings that can give insight for future directions of the research into novel biomarkers in Aspergillosis .

1. INTRODUCTION

1.1 Overview of Lung Physiology and Anatomy

Lung homeostasis and response to injury are functions critical for survival. Lungs are constantly exposed to external factors and are susceptible to damage from microbes, pathogens or toxins. To handle this, they need to obtain ability to clear debris, mount inflammatory and immune responses and facilitate repair after damage avoiding disruption of oxygen absorption. For conduction of these functions, lungs accommodate specialized epithelial cell types in the proximal airway, distal alveoli and mesenchymal compartments that are essential to lung homeostasis and response to injury (Miller & Spence, 2017). Cilia and mucus production together with alveolar macrophages provide the first line of defence against the outside world. Mucus is secreted as a physical defence mechanism triggered by goblet cells and together with the cilia beat clear the majority of unwanted pathogens and allergens (Adivitiya et al., 2021). Respiratory epithelium contains numerous specialized cells that participate in normal function and line both upper and lower respiratory tract (Miller & Spence, 2017).

1.2 Respiratory Epithelium

Epithelium lining the nasal and bronchial mucosa has three distinctive functions in maintaining a healthy state of respiratory mucosa: physical barrier function, innate immune defence function, and mucociliary clearance of inhaled particles (Gohy et al., 2020). These functions aim to prevent pathological inflammation and disease despite the inhalation of infectious agents, pollutants, or allergens. Additionally, epithelial cells have the ability to produce different cytokines and chemokines that attract and activate inflammatory cells that aid in removing or neutralizing foreign molecules (Lambrecht & Hammad, 2012).

1.2.1 Epithelium and its junctional complexes.

The macroscopic appearance of the epithelium along the respiratory tract varies, which each region having a subtly different type of cells to suit the physiological function of that area. The upper respiratory tract is lined with pseudostratified columnar epithelium, whereas the lower is lined with pseudostratified columnar epithelium transitioning into cuboidal cells in bronchioles and a single-layer of squamous cells in the alveoli (Pieter et al., 2015). Alveolar epithelium is responsible for gas exchange and consists of

type 1 and type 2 alveolar epithelial cells (Wang et al., 2018). Pseudostratified epithelial layer of conducting airways is separated from mesenchyme by the basal membrane and consists of basal epithelial cells, club cells, goblet cells and ciliated cells and pulmonary neuroendocrine cells (PNECs) as seen on Figure 1.2.1 (Malte et al., 2022). Basal epithelial cells are progenitor cells and have the ability to differentiate into secretory club cells that can further differentiate into mucus secreting goblet cells or mucus clearing ciliated cells (Dean & Snelgrove, 2018). Club cells have the ability to self-renew and generate ciliated cells to reproduce damaged airway tissue. In patients with asthma and COPD ,reduced numbers of club cells have been reported (Zhu et al., 2019). Mucus-secreting goblet cells are secretory cells containing mucin granules and surfactant proteins. Their main function is to secrete mucin in the internal surface of the airways to trap environmental molecules. The major mucin proteins of the airways are MUC5AC and MUC5B (Bonser & Erle, 2017).

Epithelial cells are tightly connected and polarized owing to the basolateral surfaces expressing cell-cell junctional complexes including desmosomes, adhering and tight junctions(TJs) that collectively impart strength to the integrity of the epithelial layer and regulate solute transport as represented in Figure 1(Calvén et al., 2020). The basolateral membrane is in touch with a basement membrane consisting of extracellular matrix (ECM) proteins such as collagen, laminins and proteoglycans which support structure and are responsible for integrity. Desmosomes have the ability of holding together adjacent cells. In times when the epithelium is under physical stress, epithelial cells couple with adjacent cells using desmosomes. They consist of two cadherin subtypes: desmoglein (Dsg) and desmocollin (Dsc) (Garcia et al., 2018). They also have the ability to mediate cell-cell direct contacts and provide anchorage sites for intermediate filaments important for tissue architecture maintenance (Huber, 2003). Gap junctions are responsible for the connection of cytosol on surrounding cell types. Some of their properties include free diffusion of small molecules between cells. Their permeability can be regulated in response to a variety of physiological stimuli (Ambade et al., 2021).

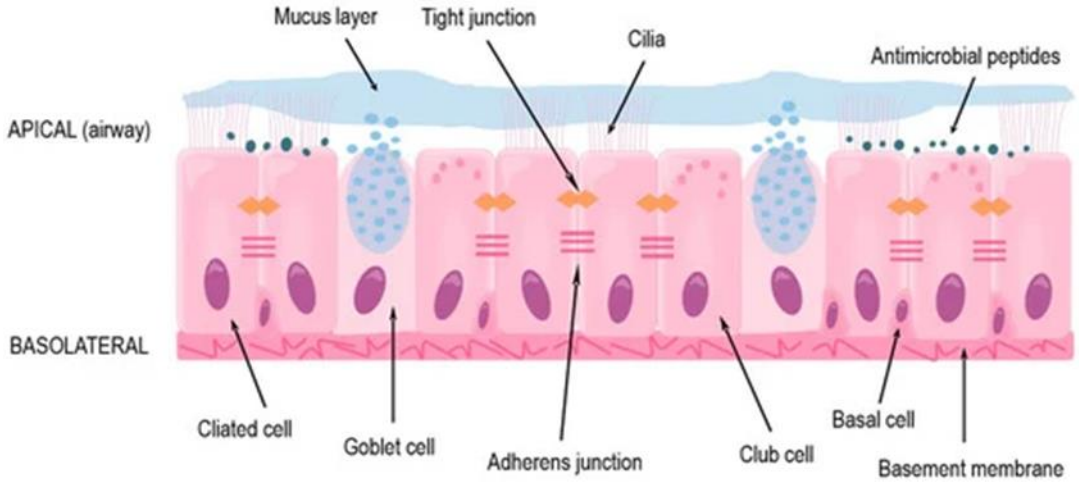

Figure 1.2.1. Schematic representation of the lower respiratory tract airway epithelium. Lower respiratory tract organs like trachea and bronchi are lined with pseudostratified epithelium with a clear apical-basolateral orientation that consists of ciliated cells, goblet cells, club cells, and the underlying basal cells. On the apical side of the airway cilia and tight junctions can be seen, that act as first line of defence mechanism against pathogens and microorganisms.

1.2.2 Epithelium and inflammation.

Respiratory epithelium also plays a very important role in innate and adaptive airway immune responses to external environment. Both responses are induced by release of pro- and/or anti-inflammatory mediators. Airway macrophages, dendritic cells, and innate lymphoid cells act as indicators of physiological and pathological innate immune responses. Whereas T cells, B cells, mast cells, and granulocytes (eosinophils and neutrophils) are orchestrators of physiologic and pathologic adaptive immune responses (Weinstock et al., 2021). Airway macrophages are typically categorized as M1 and M2 macrophages. Macrophages 1 are pro-inflammatory and release cytokines and M2 being anti-inflammatory producing Th2 cytokines (IL-4 and IL-13) (Puttur et al., 2019). Although protecting the lung airway is their main role, macrophages play a crucial role in pathogenesis of both viral, bacterial, and chronic airway diseases like asthma and COPD (Byrne et al., 2015). Airway inflammations is believed to be based on the balance between the macrophage subsets and the adaptive immune responses they elicit (Robbe et al., 2015). Dendritic cells (DCs) are another type of cells actively involved in innate lung defence and adaptive immune responses. They are classified in three main subtypes, two types of myeloid or conventional DCs (type 1

(cDC1) or type 2 (cDC2) and plasmacytoid DCs (pDCs) (Freeman & Curtis, 2017)The cDC1 subtype cells promote the generation of DC precursors and are responsible for antigen presentation to CD8+ T cells. In response to exposure, they stimulate Th1 responses (Fuertes et al., 2011). The cDC2 cells produce proinflammatory chemokines that are responsible for recruitment of effector T helper cells and inflammatory cells including neutrophils (Kim & Lee, 2014). The pDCs cells are dispersed throughout the respiratory system and play an important role in antiviral response, following activation of TLR-7 and contribute to resolution of inflammation by promoting Treg cell differentiation (Condon et al., 2011). However, pDCs are limited in their ability to handle allergen presentation but have the ability control allergic airway responses by promoting apoptosis of Type 2 innate lymphoid cells (Maazi et al., 2018). In response to airway diseases like asthma and COPD, cDC2s have been found to activate both Th2 as well as Th17 immune responses (Voskamp et al., 2020). Lastly, Innate lymphoid cells (ILCs) have been recently identified to participate in innate immune response and their role still remains unclear. There is some evidence suggesting that the majority of all ILCs present in the airway are ILC-2 with ILC-1 and ILC-3 being recruited specifically in response to airway irritants (Barlow & McKenzie, 2019)). Regarding adaptive immune responses, CD4+ T cells or helper T (Th) cells are the primary cells associated. Th cells present the processed antigen to B cells facilitating production of antigen-specific antibodies and are categorized into two main types: Th1 and Th2 cells (Weinstock et al., 2021).Specifically, Th2 cells have been found to play an important role in response to allergic diseases and have been found to remain in the airway submucosa as resident memory to mount a response to subsequent exposure to the specific allergen. Additionally, Th cells are involved in airway epithelial immune response like: Th17 cells, Th22, Th9 and Tregs cells (Zhu et al., 2010). Th17 cells contribute to airway immune homeostasis by controlling epithelial cell response to extracellular pathogens and are known to have an effect in the pathogenesis of asthma (Gurczynski et al., 2018). B cells in the airway are also important effector cells for a healthy immune response by producing antibodies, including Ig M, Ig G, Ig E and Ig A (Polverino et al., 2016)). B cells have been investigated to some extent in regards to asthma. In chronic lung

inflammation, naive B cells migrate to the lung as a result of lung epithelial cells expressing specific chemokines (Habener et al., 2021)Finally, granulocytes (mast cells, basophils, eosinophils and neutrophils) are effector cells for a protective immune response but can also contribute to airway epithelial damage. Mast cells play a key role in the pathophysiology of asthma by producing Ig E, as well as type 2 cytokines in response to cytokines like TSLP and IL-33 secreted by activated airway epithelial cells (Weinstock et al., 2021). Cytokines, such as IL-3, IL-4, and IL-6, have been found to contribute to survival and proliferation of mast cells, while transforming growth factor-β has been observed to have an inhibitory effect. Basophils are similar to mast cells in function and promote Th2 immune response in the airway. Eosinophils have been extensively investigated in the context of allergic airway diseases like asthma and our known to induce inflammation (Holtzman et al., 2014).

1.3 Asthma

1.3.1 Overview and pathophysiology of asthma.

Asthma has been described as a long-term condition affecting the airways of the lungs presenting heterogenicity in aetiology, clinical characteristics, and treatment. In United Kingdom, it has been reported on average that three people die of asthma daily and 5.4 million are currently under treatment (*Asthma and Lung UK*, 2020). Epidemiological studies indicated higher prevalence of asthma diagnosis in women rather than men. It is a fairly common condition that can affect people of any age. Symptoms typically develop early in childhood and include coughing, wheezing, tightness of chest and shortness of breath (Akar-Ghibril et al., 2020). Clinically it is accompanied by airway inflammation, hyperresponsiveness and airflow limitation (Salameh et al., 2023).

There are two main pathological features of the condition: inflammation and Airway Wall Remodelling (AWR). Airway Wall Remodelling is defined as changes in the airway wall structure. Features include extensive epithelial damage, airway smooth muscle hyperplasia and hypertrophy, collagen deposition, subepithelial membrane thickening and fibrosis (Wieczfinska &

Pawliczak, 2023).Asthma is frequently described based on inflammatory status into four main types: eosinophilic, neutrophilic, mixed eosinophilic with neutrophilic and paucigranulocytic (Robinson et al., 2017).

1.3.2 Endotypes and Phenotypes of asthma.

As asthma has been considered an umbrella diagnosis of several diseases, categorization of them into endotypes and phenotypes was essential. According to Global Initiative for Asthma (GINA), the definition of phenotypes focuses on demographic, clinical and/or pathological characteristics and endotypes describe the subset of asthma with distinct molecular mechanisms and treatment response (Borish, 2016). Ever since the understanding of the underlying pathogenesis of asthma, the disease has been categorized into two main endotypes: Th2-high (eosinophilic) and Th2-low (non-eosinophilic). Phenotypes have been categorized as non-atopic or ‘’intrinsic’’ and atopic or ‘’extrinsic’’ asthma (Kuruvilla et al., 2019). Best-researched type of asthma is eosinophilic asthma, which is the most common type and can be categorized into the following phenotypes: early-onset atopic (responsive to steroids), late-onset non-atopic eosinophilic (refractory to steroids), and aspirin-exacerbated respiratory disease (surgical treatment, sensitive to leukotriene modifiers) (McIntyre & Viswanathan, 2023). Non-eosinophilic asthma, also known as T2-low, is a less understood endotype of asthma and is typically defined by asthma signs, such aselevated Ig E levels, the presence of neutrophilic or paucigranulocytic inflammation, and resistance to ICS. Mechanisms underlying the manifestation of T2-low asthma and the maintenance of inflammation are currently unknown, but an association with chronic infection, obesity, smoking, and smooth muscle abnormalities has been proposed (Carr & Kraft, 2016)

1.3.3 Allergic Asthma

Aetiology of bronchial asthma still remains unknown. However, some risk factors, including genetic and environmental conditions have been identified (Mims, 2015). Genetic factors include changes in the expression of several

genes responsible for protein folding in the endoplasmic reticulum, epithelial, and eosinophil dysfunction. Environmental factors include smoking (both active and passive), air pollution from flour dust, animal and plant enzymes, allergens, tree resins, tobacco, polyisocyanate, acids, anhydrides, metals and obesity (Malo & Vandenplas, 2011).Allergic asthma is the most widespread asthma type, developing due to sensitization with environmental allergens, mostly house dust, plant pollen, and fungal spores. After sensitization, asthma symptoms usually develop during second contact with the allergen. The most common mechanism underlying asthma is activation of the Ig E dependent pathways through allergic reactions (Holgate et al., 2015). The main effector of type 1 hypersensitivity underlying the development of asthmatic inflammation is Ig E. Its synthesis occurs in two possible ways: either by direct class-switch recombination from IgM in germinal centres or through a "sequential" switch from IgM to Ig G1 and then from Ig G1 to Ig E outside of germinal centres. The high-affinity receptor of Ig E (FcεRI) is expressed on mast cells and basophils as a tetramer and on monocytes and dendritic cells as a trimer (Wu & Zarrin, 2014). During the step of sensitization in asthma, Ig E leads the allergen on the cell surface through FcεRI, driving the procession of the antigen-IgE complex. Then presented through the complex class II molecules, lowering the threshold for T-cell activation during the allergen challenge. Inflammation is initiated when the antigen contacts Ig E, present on all mast cells and basophiles. After that, cells degranulate, releasing mediators such as histamine, heparin, proteases, and pro-inflammatory cytokines, which are responsible for the chemotaxis of inflammatory cells (Sallmann et al., 2011). Important in the development of asthma also are CD4+ lymphocytes. After recognition of antigen, T helpers type 2 (Th2) secrete pro-inflammatory cytokines, such as IL-4, IL-5, IL-9, and IL-13, which have the ability to stimulate Ig E production and inflammatory cell migration (Johansson, 2014). On the other hand, T helpers type 1 (Th1) inhibit secretion of IL-2 and IFN-γ, activating macrophages and enhancing the cell immune response. T-cell immune response is a process also controlled by IL-1, IL-4, IL-12, and IL-18, cytokines secreted by dendritic cells (McGee & Agrawal, 2009). Innate lymphoid cells (ILCs) are also playing an important role in asthma and post-asthmatic fibrosis development. They are abundantly

present in the tissue of organs performing barrier functions. Specifically, ILC2s have been established as crucial mediators of lung allergy, airway inflammation, and fibrosis. They have been identified to affectthe pathogenesis and clinical course of many respiratory diseases, like, for instance, asthma and cystic fibrosis (Wirtz et al., 2021). Pathophysiological changes in the bronchial asthma lungs can be divided into two patterns: alterations in bronchial epithelium and smooth muscles, signs of asthma exacerbations, and subepithelial fibrosis. All these pathological changes lead to bronchial obstruction, which have been found reversible at the early stages of the disease and irreversible at the later ones (Trejo Bittar et al., 2015). During asthma development, some characteristics observed are hyperplasia and metaplasia of the goblet and epithelial cells of the bronchial epithelium (leading to mucus hyperproduction), thickening of the airways and bronchial obstruction (Gordon et al., 2013).

1.3.4 Asthma and *Aspergillus fumigatus*.

Regarding *Aspergillus fumigatus*, fungi have been recognized to have an association with the formation of allergies for decades. Assessment of respiratory microbiota in the UK population revealed that one of the dominant species present in airways was *Aspergillus fumigatus* (Rick et al., 2020). Another study, indicated that the respiratory fungal load in severe asthmatics on corticosteroids treatment, was found much elevated compared to healthy controls and *Aspergillus fumigatus* was identified as one of the most prominent species (Fraczek et al., 2018). Specifically, the allergen has been well known to be responsible for lung conditions including Severe Asthma with Fungal Sensitization (SAFS). The condition is mainly differentiated from allergic bronchopulmonary aspergillosis (ABPA) by the absence of bronchiectasis and fungal growth in lungs and sensitivity to antifungal treatments (Denning et al., 2006). Mostly SAFS, is Th2 biased.Underlying pathology is characterized by inflammatory cell (predominately eosinophil) recruitment, elevated serum Ig E, peri bronchial and perivascular inflammation, increased airway hyperreactivity, mucus hypersecretion, and airway remodelling (Croisant, 2014). Induction at the epithelial barrier includes

fungal conidia interaction with the airway epithelial barrier, triggering inflammatory signals in response. The major pathogen associated molecular patterns (PAMPs) of fungi include chitin, β-glucans, proteases, glycosidases, and fungal nucleic acids. Fungal components can then be recognized by PRRs on DCs independently or in conjunction and fungal antigen activated DCs trigger both Th2 and Th17 cells (Speakman et al., 2020). Fungal spores and conidia have the ability to produce a variety of proteases during their life cycle. These proteases are recognized and can be pro-inflammatory and lead to tight junction disruption (Sweerus et al., 2017). During SAFS, Th2 cytokines IL-4, IL-5, and IL-13, as well as fungal antigens, stimulate the production of eosinophil chemoattractant, CCL1. Eosinophilia is a common manifestation in SAFS patients and can have an effect in alleviating asthma symptoms (Dhariwal et al., 2021). Eosinophils potentially contribute to fungal asthma pathophysiology by increasing AHR, activating Th2 cells, and inducing airway remodelling. They have the ability to induce AHR by releasing cytokines such as IL-13 and inhibit mast cell and basophil degranulation (Gibson, 2009).

1.4 *Aspergillus fumigatus*

Aspergillus fumigatus is a saprophytic fungus that has been reported in the past as a weak pathogen and plays an important role in carbon dioxide and nitrogen environmental recycling. It accounts as a thermophilic fungus that grows from a range of 37 °C to 50 °C and can survive temperatures as high as 70°C. Over the last ten years, high incidences of fatal and invasive aspergillosis in immunosuppressed patients changed the situation dramatically leading to the fungus being recognized as an airborne pathogen . Regarding its structure, the fungus sporulates abundantly and consists of many conidial heads that further produce airborne conidia. The conidia have a ranging diameter of 2-3 µm, that allows them to be inhaled easily and get attached to the lungs. A human can inhale several hundred conidia per day, but these are typically cleared by the cilia escalator and resident macrophages (Latgé & Chamilos, 2019). Its ability to survive high temperatures and pH and its hydrophobic walls enhance its resistance as a pathogen. The fungi are surrounded by a thick cell wall that mainly comprises

of polysaccharides, protecting it from external aggressions (Latgé et al., 2017).

1.4.1 Aspergillosis

Aspergillus fumigatus is responsible for a spectrum of diseases including Invasive Aspergillosis (IA), Aspergilloma, Allergic Bronchopulmonary Aspergillosis (ABPA) and Severe Asthma with Fungal Sensitization (SAFS) (Hogan & Denning, 2011). Invasive Aspergillosis is responsible for being the main cause of death in 5-25% of patients with acute leukaemia (Patel & Paya, 1997). Also, it has been reported as the main cause of infection in patients diagnosed with cancer, AIDS or chronic granulomatous disease (Sabine Tejpar, 2010). Invasive Aspergillosis can be divided in four different types: acute or chronic pulmonary aspergillosis (which is the most common type), tracheobronchitis and obstructive bronchial disease, acute invasive rhinosinusitis and disseminated disease commonly involving parts of the brain. The specific disease still remains hard to diagnose and biopsy with dissection and collection of mycelia growth in tissue sample is needed (Verweij & Denning, 1997). Allergic bronchopulmonary aspergillosis (ABPA) mostly affects immunocompromised patients diagnosed with asthma or cystic fibrosis. It has been reported as the most severe allergic complication caused by the specific species. Production of thick mucus is observed in these patients so spores inhaled from the allergen cannot be cleared. In normal individuals, Ig G and Ig A antibodies are responsible for the clearance of the spores. However, in ABPA patients, formation of Ig G and Ig E antibodies is observed (Janahi et al., 2017). Aspergilloma is the disease where the growth of a fungal ball in a pre-existing lung cavity or clump (created by another lung condition) is occurring. Possible diseases responsible for the growth of aspergilloma are tuberculosis, coccidioidomycosis, cystic fibrosis, histoplasmosis lung abscess or cancer and sarcoidosis. Treatment of the disease involves surgical removal of the fungal ball or antifungals (Chabi et al., 2015).

Severe Asthma with Fungal Sensitization indicates there is a strong link between *Aspergillus fumigatus* and pathogenesis of asthma and can be

characterised as the continuous fungal sensitization of a severely asthmatic patient. It is often steroid resistant, remains difficult to treat and its underlying pathophysiology remains unclear. SAFS is mainly differentiated from ABPA by the absence of bronchiectasis and fungal growth in lungs and sensitivity to antifungal treatments (discussed further later) (Tiwary & Samarasinghe, 2021).

1.4.2 The interaction of *Aspergillus fumigatus* with the epithelium.

To understand the underlying pathophysiology of disease caused by *Aspergillus fumigatus*, it is very important to comprehend the interaction of this pathogen with the epithelial cells, the first line of defence. Swollen Aspergillus fumigatus conidia bind ECM components, initiating spore recognition via pattern recognition receptors (PRRs). These, include Toll-like receptors (TLR), C-type lectin receptors (CLR) and nucleotide oligomerisation domain-like receptors (NLR) which are able to recognize fungal pathogen-associated molecular patterns (PAMPs). Epithelial cells are also responsible for uptake of fungal spores, which facilitates intracellular killing. The procedure depends upon actin, Dectin-1 a PRR, cofilin (an essential actin regulatory protein) and Phospholipase D (PLD) (regulator of critical cellular processes). *Aspergillus fumigatus* recognition by IL-1 R and TLR also leads to MyD88 intracellular signal transduction, which contributes to the expression of NF-KB chemokines by airway epithelial cells. The airway epithelium can also be activated in response to *Aspergillus fumigatus* secreted proteases and toxins which are produced during hyphal growth. Secretion of proteases and toxins during fungal growth has been shown to cause disruptions in the epithelial barrier function and to promote the progression of *Aspergillus fumigatus* diseases (Crossen et al., 2022). Previous experiments using *Aspergillus fumigatus* hyphae showed induction in the expression of IL-6, IL-8 by bronchial epithelial cells *in vitro* (Earle et al., 2023).

1.4.3 *Aspergillus fumigatus* in innate and immune response.

Understanding of *Aspergillus fumigatus* interactions with neutrophils and macrophages is essential to establish host interactions and immune response. Firstly, neutrophils play a crucial role in the primary host defence against the allergen. They are heavily involved in the distribution of fungal hyphae as long as phagocytosis of conidia, a fact confirmed by the elevated susceptibility of neutropenic individuals to IA (Earle et al., 2023). Existing *in vivo* study in murine model has identified the severity of neutropenia and its correlation with the fungal burden and mortality (Snarr et al., 2020). The likelihood of neutrophils recruitment in response to *Aspergillus fumigatus* being driven by damage and activation of lung epithelium has been proposed (Earle et al., 2023). Secondly, macrophages have an essential role in prevention of the fungal lung disease, participating in creation of 90% of leukocytes found in the airway regularly. Post exposure to *Aspergillus fumigatus*, a healthy host induces polarization of M1 macrophages (Strizova et al., 2023). This type of macrophages is linked with increased antifungal activity and phagocytosis of conidia playing an important role in prevention of the disease development in the host (Kosmidis & Denning, 2015). However, it has been well established that macrophages, demonstrate reduced ability to destroy conidia in immunocompromised individuals, increasing host's susceptibility to infection (Nizet & Johnson, 2009). Macrophages, as professional phagocytes, have the ability to engulf *Aspergillus fumigatus* conidia in an actin dependent manner. Conidia can be detected as early as 30 minutes after internalization (Ibrahim-Granet et al., 2003). Additionally, macrophages participate as important modulators of the immune response secreting inflammatory cytokines such as TNF, IL-6, IL-8 and IL-12. These are crucial for the recruitment of other immune cells such as neutrophils, dendritic cells and initiation of the T helper (Th) immune response (Heinekamp et al., 2015). For the effective clearance of the pathogens, induction of inflammation by these cytokines is extremely important. However, dysregulation of it can have serious effect on the host (Arango Duque & Descoteaux, 2014). As a conclusion, it is currently proposed that even though macrophages are essential for prevention of lung disease, their regulation, is important to prevent serious effects on the host (Earle et al., 2023).

Another important aspect to consider is the link between innate and adaptive responses, driven by dendritic cells (DCs) that act as an important component of antifungal immunity to *Aspergillus fumigatus* (Earle et al., 2023). There are different types of DCs: conventional DCs (cDCs) and plasmacytoid DCs (pDCs), as well as DCs which originate from circulating monocytes (mo-DCs) during inflammation. Each of these types has differing functionalities. Dendritic cells in the lung tissue, mainly cDCs, are continually sampling the airways which enables them to acquire and process inhaled *Aspergillus fumigatus* conidia and activate fungal-specific T cell responses (Bozza et al., 2002). Regulatory responses triggered by DCs in response to *Aspergillus fumigatus* infection, is a field poorly understood. However, existing theoretical approaches propose cDC1s to prevent excessive Th17 response after *Aspergillus fumigatus* infection (Zelante et al., 2015).

1.4.4 *Aspergillus fumigatus* proteases and toxins drive pro-inflammatory responses.

Aspergillus fumigatus produces proteases in response to acidic pH of medium or when grown in liquid culture with proteins such as collagen or elastin. It is reported that after inhalation *Aspergillus fumigatus* initially contacts the mucus layer of the airway epithelium of the host and while it is growing damage to the barrier is observed, exposing the connective tissue and its substrates like collagen and elastin (Farnell et al., 2012). Recent study has revealed that proteases play an essential role in airway wall remodelling and inflammation. In a murine model, mice were intranasally dosed over the course of 5 weeks with culture filtrate from wild type (WT), Asp f 5 or Asp f 13 from *Aspergillus fumigatus* (protease allergens ,important for initiation and progression of allergic asthma) . For the identification of results, Th2 response and airway inflammation by ELISA and cell counts were observed and airway remodelling was assessed by histological analysis. Results have concluded that *Aspergillus fumigatus* secreted proteases play an essential role in recruitment of inflammatory cells and airway wall remodelling. However, protease activity in ABPA or SAFS remain unclear and beneficial effects in preventing pathology have been suggested (Namvar et al., 2015b).

Twenty-five allergens of the species *Aspergillus fumigatus* have been identified with only a small percentage of them producing proteases. Amongst the most well studied strains of *Aspergillus fumigatus* are Af293 and Cea10. Strain Af293 was isolated in 1993 from a lung biopsy taken post autopsy from a neutropenic patient that received treatment for rheumatoid arthritis, developing severe sepsis syndrome and strain Cea10 was isolated in the early ’90s from a patient with invasive aspergillosis (Bertuzzi et al., 2020; Girardin et al., 1993). Additionally, Greer, a commercially available allergen extract, has been documented to have induced collagen deposition and inflammation on a murine model (Labram et al., 2019). Proteases cause the induction of cytokines such as IL-8 which is responsible for cell damage. Studies have shown *Aspergillus fumigatus* stimulation improved the expression of IL-8 and in A549 cells (Chen et al., 2015). More recent studies have also reported IL-8 upregulation after treatment with *Aspergillus fumigatus* in human bronchial epithelial cells (Du et al., 2018). Also, proteases secreted by *Aspergillus fumigatus* have been found to induce the transcription of IL-6 in the lung epithelial cells via the activation of the NFkB pathway and stimulate innate immune response (Russo et al., 2022). Existing literature has suggested the induction of Endothelin-1 (a pro-fibrogenic factor) after in vitro treatment with *Aspergillus fumigatus* in epithelial cells and *in vivo* in a murine model (Labram et al., 2019).

Aspergillus fumigatus genome consists of a variety of biosynthetic clusters that provide secondary metabolites toxins. Some of the most well-known toxins produced by the fungus are gliotoxin, fumitremogin A and B, Hexadehydroastechrome, hemolysin, fumagillin and myotilin. Fumagillin has been well studied for its role on endothelial cell proliferation. It has been used as a treatment option in AIDS and obesity (Guruceaga et al., 2021). Gliotoxin inhibits the immune system of the host and is secreted by newly established hyphae of the fungus (Malcolm, 2009) . It plays an important role in activities such as inhibition of superoxide release, migration, microbicidal activity, cytokine release by leukocytes and T lymphocytes mediated cytotoxicity (Kamei & Watanabe, 2005). Its immunosuppressive role relies on the impairment of macrophage phagocytosis, mitogen-activated T cell proliferation, mast cell activation, cytotoxic T-cell response, monocyte

apoptosis, and neutrophil function (Earle et al., 2023). Hexadehydroastechrome has been associated with enhanced virulence and its over expression has been identified to produce an iron-starvation phenotype (Wiemann et al., 2014). Hemolysin has been known to be lytic for erythrocytes in humans, rabbits and sheep and remains inactivated in the presence of serum or blood plasma (Fukuchi, 2001).

1.5 Airway Wall Remodeling (AWR)

1.5.1 Overview of Airway wall remodelling features

Airway wall remodelling (AWR) accounts as one of the pathological elements of asthma and its terminology is used to describe structural changes in the airway of asthmatic subjects (Bergeron et al., 2010). Airway wall remodelling in asthma constitutes cellular and extracellular matrix changes in the large and small airways, epithelial cell apoptosis, airway smooth muscle cell proliferation, thickening of basement membrane, subepithelial fibrosis, goblet cell and submucosal gland enlargement, increased smooth muscle mass, decreased cartilage integrity and increased airway vascularity are included and fibroblast activation (Figure 1.5.1) (Hough et al., 2020). More specifically epithelial alterations include epithelial shedding, loss of ciliated cells, goblet cell hyperplasia, upregulation of growth factor release and overexpression of receptors such as epidermal growth factor receptor (Bergeron et al., 2010). The process by which AWR is driven, still remains unclear and likely to be extremely complex and multifaceted (Joseph & Tatler, 2022). Up to date, *in vivo*, *in silico* and animal studies on asthma, suggest a link between inflammation to and airway wall remodelling. These theoretical models propose inflammation to promote thickening, although thickening results when biomechanical contractile forces and inflammation are modelled simultaneously, suggesting multiple possible pathways (Hill et al., 2018). Findings in young children with wheezing prior to asthma diagnosis, observed remodelling occurring too early in the disease, suggesting that chronic

inflammation might not be the sole driver of airway remodelling (Lezmi et al., 2015). An alternative possibility initially suggested in 2011, demonstrated that the mechanical environment of the asthmatic airways drives remodelling changes . It is known that contraction of airway smooth muscle cells (ASM) and airways causes activation of the pro-remodelling cytokine TGF-beta and downstream remodelling changes (Choi et al., 2018). Additionally, to the previous statement, non-contractile biomechanical forces might contribute (Noble et al., 2014). Within the asthmatic airway wall, ECM proteins can proliferate ASM cells and drive remodelling *in vivo* (C. Zhang et al., 2017). Increased matrix stiffness has the ability to promote Epithelial-to-mesenchymal Transition (EMT), collagen production by fibroblasts and cell proliferation, all features that contribute to airway remodelling (Brown et al., 2013; Humphrey et al., 2014; Shkumatov et al., 2015).

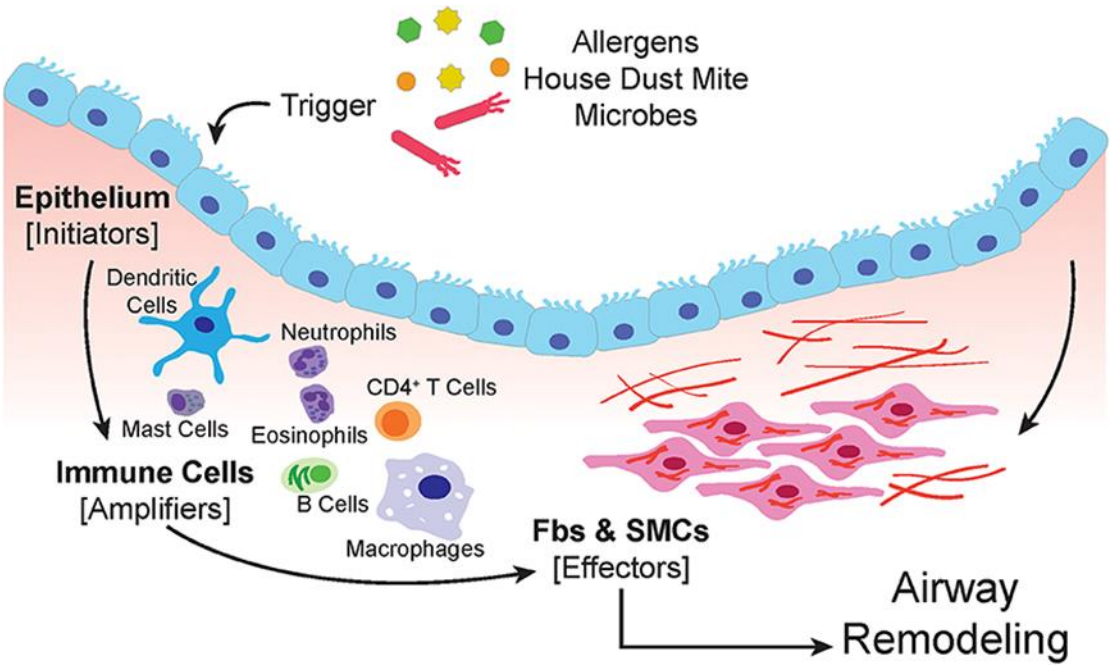

Figure 1.5.1.. Representative image of Airway Wall Remodeling process in asthma. Epithelium after being triggered from microbes and allergens responds by secretion of soluble factors that recruit and activate immune cells. Immune response involves macrophages, dendritic cells, neutrophils, mast cells, eosinophils, and lymphocytes.

1.5.2 Airway Wall Remodelling in Asthma

. Loss of epithelial integrity is observed early in the disease course and results from cellular apoptosis, senescence, and ineffective repair mechanisms (Wu et al., 2013).

Thickening of ASM layer is a common feature of asthmatic airway remodeling. Healthy airway ASM cells, play an important role in maintaining airway tone. However, during disease they have essential role in inflammatory and remodelling procedures by releasing chemokines, pro-inflammatory and/or pro-fibrotic cytokines and ECM proteins that have an effect on asthma pathogenesis(Noble et al., 2014). In asthmatic airways, increased ASM mass appears to result from both increased myocyte size(hypertrophy) and increased myocyte number (hyperplasia),features that are both associated with disease duration and severity (Benayoun et al., 2003). Production of inflammatory cytokines and chemokines and increased ASM proliferation can be promoted from interactions between airway epithelial cells and ASM cells. A paracrine signalling between the two cell types has been suggested (O'Sullivan et al., 2021). Migration of smooth muscle cells has been described as a new feature of AWR, implicating contribution to overall airflow obstruction in asthmatic patients (Joubert et al., 2005). Smooth muscle mass has been correlated with asthma severity (Benayoun et al., 2003).

Goblet cell hyperplasia and submucosal gland hyperplasia are also features mainly evident in fatal asthma incidents. Consequences of these features are increased sputum secretion, airway narrowing (due to sputum secretion) and increased airway wall thickness (Jenkins et al., 2003).

Another feature of AWR in the asthmatic airway, is subepithelial fibrosis occurring in lamina reticularis (below the basement membrane), where ECM proteins like interstitial collagen, fibronectin and proteoglycan assemble (Roche et al., 1989). Subepithelial fibrosis is linked with asthma severity. Patients with moderate or severe asthma presented higher collagen expression compared with those with mild disease (Chakir et al., 2003). Elevated deposition of ECM proteins accounts as a major hallmark of fibrosis regardless of organ or tissue type. The process is primarily controlled by

fibroblasts and myofibroblasts. Number of myofibroblasts in the asthmatic airway presents a link with collagen amount detected in subepithelial region (Joseph & Tatler, 2022). Finally, fibrocytes that have the ability to differentiate into myofibroblasts are increased in asthma and can potentially contribute to subepithelial fibrosis (Wang et al., 2008).

In newly diagnosed asthma patients, increased vascularity of the asthmatic airway is observed. Angiogenesis refers to the process of formation of new blood vessels (Tanaka et al., 2003). Correlation between angiogenesis and lung function in asthma still remains unclear with some studies reporting a link and others concluding to no correlation. However, angiogenesis within the asthmatic airway wall enhances inflammatory cell recruitment causing oedema, which can have an effect on asthma pathogenesis (Salvato, 2001). Vascular Endothelial Growth Factor (VEGF) is an activator of endothelial cell growth and promotes vascular permeability. In asthma, VEGF levels were found in serum and BALF (Lee et al., 2017). Simcock in 2008 proposed that ASM cells isolated from asthmatic subjects have the ability to drive angiogenesis through increased VEGF secretion (Simcock et al., 2008). Some promising data have shown that pharmacological inhibition of VEGF signalling reduces expression of growth factors, improves epithelial barrier function, and reduces markers of airway remodelling (Türkeli et al., 2021; Yuksel et al., 2013; R. Zhang et al., 2017).

1.5.3 *Aspergillus fumigatus* effect on Airway Wall Remodelling

As *Aspergillus fumigatus* has been strongly associated with asthma, understanding of its mode of action in AWR is essential. However, existing literature in the research area still remains limited. Histopathological assessment in lung biopsies from SAFS and ABPA patients indicated signs of extensive airway remodelling, subepithelial fibrosis and smooth muscle hypertrophy (Kurup & Grunig, 2002). Supporting evidence from murine models exposed to *Aspergillus fumigatus* demonstrated that the specific fungi drive airway inflammation, remodelling, and impaired lung function (Desoubeaux & Cray, 2018). *In vivo* models of mice being intranasally dosed

with an unspecified strain of *Aspergillus fumigatus* three times per week for 18 days, revealed airway hyperreactivity, eosinophilia and upregulated IL-4 levels (Urb et al., 2015). In 2014, Buskirk et al, exposed mice by aerosol challenge to strain B-5233 conidia twice a week and revealed peribronchiolar inflammation, subepithelial fibrosis and goblet cell hyperplasia (Buskirk et al., 2014). However, another novel study revealed that exposure to *Aspergillus fumigatus* conidia (Af293 strain isolated from a neutropenic patient embedded in agar beads via an intratracheal route) caused no visible signs of airway inflammation even though hyphal growth was obvious in the airway lumen of the mice exposed (Urb et al., 2015). These findings have suggested a correlation between fungal growth and significant inflammation. More recent studies of mice being intranasally dosed with Aspergillus fumigatus strain Cea10(isolated from IA patients) and with W72310 (isolated from ABPA patients), revealed a persistent presence of WF2310 conidia in lungs on a 2 weeks' time point. These findings were associated with less infiltration of neutrophils and macrophages and reduced phagocytosis of conidia from WF2310 compared to Cea10 (Jones et al., 2021). In conclusion, all these studies have resulted to the statement that animal models of *Aspergillus fumigatus*-induced airway inflammation showed persistent presence of the fungus in in the airways of immunocompetent mice and have the ability to drive pathological features, similar to that observed in SAFS and ABPA patients. However, specific cellular mechanisms and overall understanding of the underlying process still remain unclear.

Fugal-derived proteases produced during germination and growth are believed to be highly involved in AWR. Delivered proteases from *Aspergillus fumigatus* have been found to induce airway epithelial cell disruption by release of pro-inflammatory cytokines and drive mucus production (Farnell et al., 2012). These types of proteases have also been found to upregulate mucin expression of MUC5B and MUC54C genes (Oguma et al., 2011). As mentioned previously, it has been revealed that *Aspergillus fumigatus* secreted proteases (Asp f 5 or Asp f 13 strains) play an essential role in recruitment of inflammatory cells and airway wall remodelling. However, protease activity in ABPA or SAFS remain unclear and beneficial effects in

preventing pathology have been suggested (Namvar et al., 2015b). Another study showed the effect of *Aspergillus fumigatus* Alp1 alkaline protease on mice intranasally challenged three times per week for a 2week-period. Results demonstrated a spontaneous increase in airway resistance and hyperresponsiveness to bronchoconstrictors (Redes et al., 2019)). Lastly, evidence has revealed the role of upregulated levels of Endothelin-1 in driving airway inflammation and remodelling in murine models treated with *Aspergillus fumigatus*. An ET receptor A (ETA) antagonist, BQ-123, was introduced, prior to exposure of mice to *Aspergillus fumigatus*. Results demonstrated for the first time that antagonism of ETA prevents *Aspergillus fumigatus*-induced inflammation and remodelling of the airways (Labram et al., 2019).

1.6 Cytokines Induction

Cytokine induction plays a critical role in lung physiology, participating in both immune responses and homeostasis. Upon exposure to pathogens, lung epithelial cells, secrete a variety of cytokines .Included are interleukins, interferons and tumor necrosis factors (Megha et al., 2021).Cytokines specifically are responsible for recruitment and activation of immune cells (e.g neutrophils and lymphocytes), facilitating pathogen clearance and tissue repair. Dysregulation of cytokine production has been responsible for inducing tissue damage and chronic inflammation , conditions linked with COPD and asthma(Kany et al., 2019).

For the purpose of this study, IL-6 and IL-8 were examined. These two interleukins have been particularly linked with *Aspergillus fumigatus* .Elevated levels of both interleukins have been presented after treatment with the pathogen alongside with decreased pulmonary function (Chaudhary & Marr, 2011; Kousha et al., 2011)

1.7 Interleukin-6 (IL-6)

1.7.1 Features and Signalling Pathways of Interleukin-6

Interleukin 6 (IL-6) is a pro-inflammatory cytokine protein playing a vital role in inflammatory immune response and contributing to host defence. Interleukin-6 is mainly secreted by monocytes, T-cells, fibroblasts, and endothelial cells in humans. In early inflammation, levels are rapidly and strongly upregulated but remain undetectable under physiological conditions (Heinrich et al., 2003). Its expression is controlled by transcriptional and post-transcriptional mechanisms, but continual synthesis has pathological effects on inflammation and autoimmunity (Tanaka et al., 2014). Transcriptional pathway results from IL-6 binding the IL-6 receptor (IL-6R) and glycoprotein 130 (gp130) to Janus Kinase downstream activation (JAKs). Interleukin-6 receptor is only expressed on specific cell types like leukocytes allowing regulation of acute phase inflammation, haematopoiesis, and immune cell proliferation. Trans-signalling from the other hand, results from the binding of a soluble form of SIL-6R to IL-6 in extracellular space and stimulates numerous cell types resulting in physiologic effects (Li et al., 2022). It has been recently established that IL-6 induces the polarization of initial T cells to T helper 17 (Th17) cells and is in turn associated with neutrophil recruitment that can induce airway inflammation in asthma (Lipworth et al., 2020). Levels of IL-6 in peripheral blood of patients with allergic asthma have been identified as significantly increased during the onset of the disease (Li et al., 2022). Additionally, levels of IL-6 have been found elevated through cross-sectional study, proposing that plasma IL-6 can be a potential asthma biomarker (Peters et al., 2020). It has been supported that IL-6 plays an elevated through of asthmatic tissue inflammation by stimulating B and T cells that contribute to the development of Th2. Although pathogenesis and aetiology of lung fibrosis is yet to be understood, studies have demonstrated that IL-6 can cause subepithelial fibrosis, collagen deposition and enhanced accumulation of a-smooth muscle indicating that the cytokine plays a role in airway wall remodelling. It has been indicated that IL-6 plays a crucial role in the inflammatory phase during wound healing. However, if the proliferative phase is uncontrolled, repair can lead to tissue scarring and fibrosis. Studied in transgenic mice that expressed IL-6 gene demonstrated an increase in airway wall thickness in comparison to mice not expressing the gene (Kuhn et al., 2018). Supporting evidence has

suggested that wound repair process has been categorized in three stages: activation of innate immune system and inflammatory cytokine, proliferative phase meaning influx of fibroblasts and differentiation of them to myofibroblasts and wound contraction-remodelling phase meaning type III collagen degradation and type I collagen synthesis. As IL-6 is expressed in fibroblasts it has the ability to regulate their activity. It can participate in their differentiation process to myofibroblasts a process known as FMT characterized by αSMA expression and excessive deposition of ECM (Extra Cellular Matrix) (Li et al., 2022). She et al., in a study in 2021 has indicated that in bleomycin treated mice, M2-like macrophages have been observed to form the IL-6/SIL-6R complex responsible for the IL-6 trans-signalling and promotion of ECM production and cell proliferation. The potentiality of upregulated levels of IL-6 to relate to decreased lung function has been proposed (She et al., 2021).

1.7.2 Role of Interleukin-6 on lung function

According to a study conducted on 16 healthy control and 18 mild-moderate allergic asthmatic individuals, results have indicated significantly elevated IL-6 levels in asthmatic individuals compared to controls leading to the statement that IL-6 has been associated with loss of lung function (Neveu et al., 2010). Additionally, a study on HBECS cells grown under Air Liquid Interface (ALI) suggested that activation of IL-6TS reduced epithelial barrier integrity and caused cell junction disruption. The study included 147 subjects, revealing that asthmatic patients with T2 inflammation independent eosinophilia have been characterized by submucosal inflammation and activation of innate signalling that both act as biomarkers of airway remodelling (Jevnikar et al., 2019).

1.7.3 *Aspergillus fumigatus* effect on Interleukin-6 induction

Regarding *Aspergillus fumigatus*, first observations on A549 cells have indicated elevated IL-6 levels after exposure to fragments of Aspergillus fumigatus mycelium and inactivated spores (Zhang et al., 2005). Chen in

2015 has been the first to analyse transcriptome profiles of A549 cells after exposure to *Aspergillus fumigatus* by the method of RNA sequencing with results concluding on significantly upregulated expression of IL-6 after treatment with the allergen (Chen et al., 2015). Observations on bronchial epithelial cells (BECs) have proposed a highly significant upregulation of IL-6 post exposure to *Aspergillus fumigatus* spores (Labram et al., 2019). Latest findings have observed significant induction of IL-6 after exposure to its Af293 strain conidia in both submerged 16hbe cells and primary epithelial cells grown under ALI (Rowley et al., 2021).

1.8 Interleukin 8 (IL-8)

1.8.1 Features and Signalling Pathways of Interleukin-8

Interleukin 8 (IL-8) is a chemoattract cytokine with a distinct target specificity for neutrophils (Brat et al., 2005). It is member of the chemokine family, produced and secreted by a variety of normal and neoplastic human cell types.IL-8 is mainly secreted by activated monocytes and macrophages that play a vital role in migration of neutrophils, basophils and T lymphocytes. In normal tissue, IL-8 levels remain low or even undetectable (Brennan & Zheng, 2007). IL-8 plays a vital role in mitogenesis, inhibition of angiogenesis, inflammation, chemotaxis, neutrophil degranulation, leukocyte activation and calcium homeostasis (Pease & Sabroe, 2002). Interleukin-8 production is controlled by activation of the transcription nuclear factor (NF)-kB and intracellular activation protein (AP-1) which is the target for induced suppression of IL-8 gene expression (Mukaida et al., 1994). Its effects on neutrophils are applied by two different cell surface receptors, initially named as IL-8 receptors type A and B which has recently been identified as CXCR1 and CXCR2. It is known that CXCR1 binds to only IL-8 and granulocyte chemotactic protein (GCP-2) whereas CXCR2 can bind to other CXC chemokines, neutrophil activating peptide-2, epithelial cell-derived neutrophil activating peptide-78 and (GCP)-2 (Figure 1.8.1). Both receptors have been reported to be present on the surface of endothelial cells (Pease & Sabroe, 2002). Increased migration of neutrophils in response to IL-8 is expressed

from CXCR1 induction of CD28 cross linking (Venuprasad et al., 2001). However, signalling down of CD45, modulates both CXCR1 and CXCR2 suggesting modulation of IL-8 receptor expression (Mitchell et al., 1999).

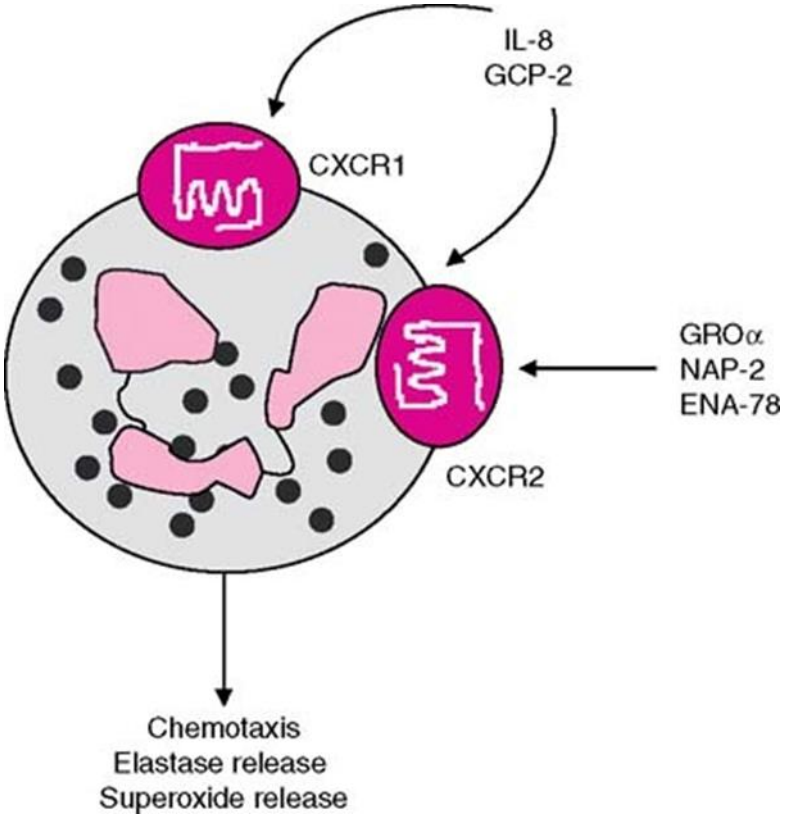

Figure 1.8.1.. Representative image of Interleukin 8 (IL-8) receptors binding. CXCR1 and CXCR2, IL-8 receptors bind IL-8 with high affinity. CXCR1 binds only IL-8 and GCP-2, while CXCR2 is able to bind IL-8 and several other CXC chemokines with similar affinity. ENA-78 = epithelial cell-derived neutrophil activating peptide-78; GCP-2 = granulocyte chemotactic protein-2; GROα = growth-related oncogene-α.

1.8.2 Role of Interleukin-8 on lung function

There is ample evidence of neutrophils recruitment in acute asthma patients. Due to the fact that IL-8 plays a vital role in acute asthma activation, the identification of the correlation between them is very important for the understanding of the underlying pathophysiology of the disease (Wenzel et al., 1997). After the identification of IL-8, its role as an inflammatory factor implicated its participation in the pathogenesis of asthma. Supporting evidence presented the detection of IL-8 in the sera of patients with severe atopic asthma (Jatakanon et al., 1999). Later, elevated IL-8 levels in tracheal aspirates of severe acute asthma patients have been observed in correlation with high levels of neutrophils (Ordoñez et al., 2000). More recent studies have proposed the use of elevated IL-8 levels in serum of asthmatic patients

as a potential biomarker for identification of the disease status and initial treatment (Zhang & Bai, 2017). As airway wall remodelling (AWR) accounts as one of the pathophysiological features of asthma, correlation with IL-8 was inevitable. It has been proven in a mouse model that IL-8 is involved in tissue injury and remodelling (Baek et al., 2013).

1.8.3 *Aspergillus fumigatus* effect on Interleukin-8 induction

Regarding *Aspergillus fumigatus*, it has the ability to induce the production of cytokines like IL-8 by epithelial cells (Borger et al., 1999). More specifically, a study on A549 epithelial cells demonstrated that proteases released from the fungus enhanced the production of IL-8 (Zhang et al., 2005). Another study investigated that the conidia of the allergen are able to induce IL-8 synthesis. Results showed significantly increased IL-8 induction in epithelial cells germinated with hyphae of the specific fungus. Both data indicate that epithelial cells play a role in antifungal immune response with the release of IL-8 which induces activation of neutrophils in response to infection (Balloy et al., 2008). A more recent study has identified upregulated levels of IL-8 6 hours post treatment with *Aspergillus fumigatus* and conidia germination has been observed (Bigot et al., 2020).

1.9 Endothelin 1 (ET-1)

1.9.1 Features and Signalling Pathways of Endothelin-1

Studies over the past twenty years have revealed the critical role of endothelin-1 (ET-1) in neurological function, pulmonary physiology, fluid and electrolyte function, autoimmune disorders and cancer biology (Stow et al., 2011). Importantly, the endothelin system plays a key role in the blood vessel tone. It consists of three ligands (ET-1, ET-2, and ET-3) which are all synthesized by the precursor molecule called preproendothelin. Endothelin-1 is known to be the most abundant of all three and its levels are highly expressed in the lungs (Swigris & Brown, 2010). ET-1 is a peptide consisting

of twenty-one amino acids and is secreted by fibroblasts, endothelial cells, alveolar macrophages, epithelial cells, and polymorphonuclear leukocytes in response to cytokines and growth factors. It is composed of two mediators Endothelin A (ETA) and Endothelin B (ETB). Endothelin A is expressed from vascular smooth muscle cells whereas ETB by endothelial cells, fibroblasts, and smooth muscle cells (Murray et al., 2014). Endothelin A and B are both G-protein coupled receptors (GPCRs) and initiate calcium signalling after their activation. Endothelin (ET) receptor influences the mitogen-activated-protein kinase (MARK)pathway (Houde et al., 2016). Endothelin-1A receptor can follow two different signalling pathways. One is by binding of ET-1 to the receptor and activation of phospholipase C (PLC) that leads to generation of a second messenger inositol triptosphate and diacylglycerol (DAG) which then stimulates release of calcium and protein kinase C activation. The other signalling pathway includes generation of phospholipase D (PLD)-mediated DAG generation, phospholipase A2 (PLA2) -induced arachidonic acid and activation of MAPK referred earlier. These pathways play a crucial role in short-term regulation of vascular smooth muscle tone and long-term control of cell growth in both vasculature and heart. The ET-B receptor from the other hand, is expressed specifically in cells that participate in physiologic processes such as neuronal activities. This leads to the conclusion that ET-B receptor is responsible for the physiologic role of ET-1 (Jenkins et al., 2020).

1.9.2 Endothelin-1 and VEGF

Studies have demonstrated a correlation between pulmonary oedema and high levels of ET-1, but the mechanisms still remain unclear. An upregulation of Vascular Endothelial Growth Factor (VEGF) from ET-1 expression has been suggested (Comellas & Briva, 2009). It is found to be involved in normal wound healing and evidence supports that it is a molecule participating in fibrogenesis. Endothelin-1 can reduce resistance to apoptosis in lung fibroblasts and by working with other molecules such as Transforming Growth Factor-beta (TGF-β) can induce cellular transformation or replication (Swigris & Brown, 2010). However, ET-1 has the ability to enhance expression of adhesion molecules on vascular endothelial cells and stimulate the

aggregation of polymorphonuclear neutrophils (PMNs) contributing to inflammation and endothelial dysfunction (Kowalczyk et al., 2015). Significantly high levels of ET-1 in cells and tissues are observed in a variety of fibrotic diseases. Fibrosis accounts as the result of a response to injury. Endothelin-1 has been observed to differentiate in various types of myofibroblasts and undergo EMT (Epithelial-to-Mesenchymal Transition) Elevated levels of ET-1 expression have been found in patients suffering from pulmonary oedema and acute respiratory distress syndrome. Upregulated levels of ET-1 have been also detected in bronchoalveolar lavage fluid and serum from patients with IPF and increased expression of ET-1 has been detected in small pulmonary blood vessels and macrophages (Abraham, 2008). Evidence has supported that ET-1 plays a vital role in excessive scarring and fibrosis in the lung (Rodríguez-Pascual et al., 2014). Samples collected from patients resulted in high levels of ET-1 in serum and bronchoalveolar lavage samples indicating epithelial dysfunction (Alejandro P. Comellas, 2009). A study which involved murine models exposed to intranasal ovalbumin (OVA) indicated that ET-1 acts as a bronchoconstrictor in asthmatic patients (Gregory et al., 2013). Samples examined by normal individuals in comparison with patients diagnosed with asthma and Chronic Obstructive Pulmonary Disease (COPD) indicated that ET-1 levels were highly upregulated during the night in the patients diagnosed with the diseases (Weil et al., 2011). Both conclusions indicate that ET-1 is a molecule implicated in the pathophysiology of both lung conditions. Although evidence from studies concludes a strong association between ET-1 and pulmonary fibrosis, clinical trials have failed in demonstrating the specific role of the molecule in the pathogenesis of fibrosis (Rodríguez-Pascual et al., 2014).

1.9.3 Role of *Aspergillus fumigatus* on Endothelin-1 induction

Regarding *Aspergillus fumigatus*, literature on its correlation with Endothelin-1 productions still remains unclear and limited. An *in vitro* and *in vivo* study showed elevated ET-1 levels on primary epithelial cells in response to both strains of the allergen (Af293 and Cea10) and BQI23 endothelin-1 antagonist

and BALF from mice exposed reported elevated ET-1 levels respectively . A recent *in vitro* study on Human primary bronchial epithelial cells, has also observed upregulated levels of ET-1 after treatment with the allergen (Labram et al., 2019).

1.10 Fibrin/Fibrinogen

1.10.1 Features and role of fibrinogen

Fibrinogen is a 340-kDa glycoprotein, present in human blood plasma and is essential for homeostasis, wound healing, inflammation, angiogenesis and other biological functions. It is a soluble macromolecule but forms an insoluble clot on conversion to fibrin by the action of thrombin, a cascade activated by vessel wall injury, blood cells or foreign surface. A stable clot is necessary for prevention of blood loss and promotion of wound healing. Fibrinolysis is the term used for dissolvement of fibrin clots by enzymatic reactions. Before fibrinogen is secreted from hepatocytes into the bloodstream, it needs to undergo several steps of assembly of the polypeptide chains (Redman & Xia, 2001). Fibrinogen is now confirmed to play a key role in the acute phase response caused by tissue injury. This procedure can be divided into two phases. First phase is characterized by thrombin cleavage of fibrinogen integrated with an acute inflammatory response that includes tissue damage, stopping loss of blood and prevention of microbial infection. Second phase is characterized by plasmin dissolution of fibrin and other matrix proteins, combined with inflammatory cells functioning to repair and remodel tissue damage. In normal conditions, the acute phase response follows the predicted time course with minimal risk of either complications during the convalescence of tissue injury or failed tissue repair. A dysregulated acute

phase response of inflammation can be detrimental to tissue repair, homeostasis and surviving an injury. This leads to the conclusion that fibrin deposition and degradation following an injury, need to occur in a coordinated manner to prevent side effects like bleeding and infection, control inflammation, and subsequently promote tissue repair (Luyendyk et al., 2019). Elevated blood content of fibrinogen is considered to be high risk factor for cardiovascular diseases and development of diseases such as hypertension, diabetes and stroke which involve inflammatory processes (Chae et al., 2001; Danesh et al., 2005; Lee et al., 1993). Although liver is the primary source of plasma fibrinogen, lung epithelial cells secrete small amounts of fibrinogen in a polarized manner from their basolateral face (Haidaris, 1997).

1.10.2 Role of fibrin on lung physiology

It has been proposed that lung epithelium secretes fibrinogen and incorporates it into ECM under pathological conditions having an effect on lung fibrosis (Lawrence & Simpson-Haidaris, 2004). It has been proved that increased microvascular permeability is a marker of inflammation. Impairment of endothelial cell (EC) integrity is well known to lead significant tissue damage and inflammatory responses (Mehta & Malik, 2006). Pathologically high concentration of fibrinogen has been indicated to impair EC layer integrity by affecting the endothelial cell tight junctions and increase paracellular transport (Patibandla et al., 2009). Endothelial cells can potentially be involved directly in fibrogenesis through endothelial-mesenchymal transition (EndMT) where they develop a contractile, α-smooth muscle actin–expressing phenotype (May et al., 2023). Fibrinogen binds to its endothelial receptor, ICAM-1, induces vasoconstriction and leads to ICAM-1-induced activation of ERK formation of F-actin. Formation of F-actin can potentially cause stiffening of the cells and opening of IEJs, therefore facilitating an increase in EC layer permeability (Tyagi et al., 2008). These results suggest that there may be a link to decreased ZO protein expression and translocation of the ZO proteins from the actin cytoskeleton to the cytosol (Sen et al., 2009).

1.10.3 Correlation of fibrin with Endothelin-1

Fibrinogen has been established to develop vasoconstriction through production of endothelin-1(ET-1) (Lominadze et al., 2005). It binds to ECs through ICAM-1 causing constriction of arterioles in rat. This vasoconstriction is mediated by ETA receptor activity, suggesting a direct link between fibrinogen binding to ICAM-1 on the surface of ECs and production of ET-1 from the ECs. It is now well established that ‘’Big’’ ET-1, an inactive form of ET, is stored in Weibel-Palade bodies (WPBs). Upon induction, regulated production of ET-1, through release of ‘’Big’’ ET-1 and its conversion to ET-1, occurs during exocytosis of WPBs. Therefore, there is a suggestion that fibrinogen induced vasoconstriction may occur by inducing WPB exocytosis. These results have suggested that fibrinogen is involved in production of ET-1 and release of vWF from WPBs of the ECs. It has also been observed that fibrinogen decreased TEER in the EC layer in a dose-dependent fashion was not affected by the presence of an endothelial ET-1 receptor (ETB receptor) blocker. Although fibrinogen and ET-1 are capable of affecting EC layer integrity, the results suggests that fibrinogen has an effect independent of ET-1 (Sen et al., 2009). A key feature of fibrogenesis is abnormal persistence of the myofibroblast phenotype in healing tissue, which leads to high levels of matrix constituents (particularly collagen), ECM contraction, and ultimately, scar formation (Schmitt-Gräff et al., 1994). Endothelin -1 is a molecule that can induce resistance to apoptosis in lung fibroblasts (a process carried out via the pro-survival PI3/AKT signalling pathway), can enhance the expression of the profibrotic moieties, fibronectin and connective tissue growth factor in certain tissues and can act synergistically with other molecules including transforming growth factor-beta (TGF- β), basic fibroblast growth factor, and platelet-derived growth factor to induce cellular transformation or replication (Rodríguez-Pascual et al., 2003).

Regarding ET-1, supporting evidence suggests a link with Vascular Endothelial Growth Factor (VEGF). Vascular Endothelial Growth Factor accounts as a key player in angiogenesis, by increasing the permeability of abnormal blood vessels, resulting in vessel dilation and oedema, which are

features that contribute to airway narrowing (Savin et al., 2023). It is produced and secreted by different cells, including endothelial cells (ECs), podocytes, macrophages, and fibroblasts. It has been suggested, that when produced and secreted by tumour cells, VEGF targets ECs to promote tumour angiogenesis (Lankhorst et al., 2016). It has been established that VEGF stimulates the expression of preproET-1 mRNA as well as the secretion of ET-1. This is mediated by the VEGFR2. Usually If VEGF stimulates the production of ET-1 by ECs, a decrease in ET-1 levels is expected. In the present study, the opposite statement is observed, indicating that it is more likely that activation of ECs underlies the increase in ET-1 in response to VEGF deprivation (Matsuura et al., 1998). Anti-angiogenesis targeting VEGF, is associated with upregulated levels of ET-1. This feature is most likely a consequence of activation of ECs and not a consequence of VEGF deprivation (Salani et al., 2000).

1.11 miRNA biomarkers in respiratory disease

1.11.1 Overview of miRNA function.

In multicellular organisms, maintenance of homeostasis for each cell as well as group of cells, different tissues and organs is very important. Different types of cells and tissues need to relay nutrition states, stress, infection, damage, and signalling pathways to ensure functioning of the organism as a whole. This is accomplished by three well-known biomolecules, DNA, RNA, and proteins. It is well established that DNA is more stable than RNA. However, RNA is the only form that has the ability to store and transmit genetic information and perform regulatory and catalytic functions by itself. Types of RNA can be divided into two broad categories: Coding RNA and non-coding RNA. Coding RNA or else known as messenger RNA (mRNA) is translated into proteins by ribosomes and accounts for only 5% of the total RNA. Non-coding RNA does not translate to proteins but performs other important functions in the cell and accounts for 95% of the total RNA in a human cell. Extracellular RNAs are involved in cell-cell communication and can act as biomarkers. The main RNA sub-types that are found in the

extracellular space – both in vesicles and in vesicle free forms are: mRNA, miRNA, siRNA, lncRNA, piRNA, circRNA, tRNA (Mukhopadhyay et al., 2020).

1.11.2 miRNAs in lung homeostasis

MicroRNAs (miRNAs) have been classified as small endogenous non-coding RNAs with approximate length of 17-25 amino nucleotides that have the ability to regulate mRNA by leading to its degradation and also to adjust the protein levels (Wang et al., 2016). Mostly, they interact with the 3' UTR of target mRNAs to suppress expression and have been shown to activate gene expression under certain conditions (Ha & Kim, 2014; Vasudevan, 2012). They have been shown to be involved in lung development and tissue homeostatic processes like immune response and metabolism. However, miRNA dysregulation has been reported in several pulmonary diseases such as cancer, IPF, COPD, asthma, and Bronchopulmonary dysplasia (BPD). Therefore, miRNAs can potentially serve as biomarkers for the development or progression of a pulmonary disease (Brown et al., 2014). They have firstly been suggested as potential biomarkers in 2008 by Lawrie et al for the examination of diffuse large B-cell lymphoma in the serum of patients (Lawrie et al., 2008). Up to date, research of miRNAs as biomarkers is still in early stages and findings lack reproducibility of results (Condrat et al., 2020).

1.11.3 miRNAs expression in Pathophysiologic conditions

More specifically in asthma, miRNAs are believed to play a pivotal role in the pathogenesis of the disease. Pathology of asthma is manifested by a production of Ig E and consistent recruitment of leukocytes, in particular eosinophils, together with Th2 cells and mast cells (Greene & Gaughan, 2013). Studies so far have identified patterns of upregulated miRNAs like miR-126, miR-145a, miR-21, miR-221/222, miR-106a, and miR-155 and some downregulated like miR-20b, and miR-133a. Targeting microRNAs may represent a potential therapeutic strategy in the treatment of asthma, however mechanisms of delivery still remain unclear (Schembri et al., 2009). Also, contributions of miRNAs in the pathogenesis of IPF has started to

emerge with research on the field to remain limited. Up to date, miR-155 and miR-21 have been identified to play a significant role in the development and progression of lung fibrosis (Pattarayan et al., 2018).

1.11.4 miRNAs expression investigated in response to *Aspergillus fumigatus*.

In the present project, as our research mainly focused on the effects of *Aspergillus fumigatus* on lung fibrosis, we have conducted a literature review and identified the following 8 miRNAs (pro and anti- inflammatory) to play a potential role in lung fibrosis: miR-320a-3p, miR-130b-3p, miR-155, miR-21, miR-223-5p, miR-223-3p, miR-9-5p and miR-34a-5p. We have focused on examining mainly miRNAs that have a potential role on lung fibrosis or diseases that are strongly linked with the allergen like IPF. However, the literature in the area still remains limited and not well-documented. Firstly, miR-320a-3p was found to have a potential role in the pathogenesis of silica-induced pulmonary fibrosis. In a murine model of 6 mice involved, a significant downregulation of miR-320a-3p has been observed, results that confirm the downregulation also proposed from IPF patients from an IPF database. However, the experiment was performed in lung fibroblasts examining the correlation of the results with AlkB homolog 5 (ALKBH5), suggested to play an important role in miRNA regulation and fibrosis formation (Sun et al., 2022). After, miR-130b-3p expression wasassessed in correlation with IPF. Lungs from 4 IPF patients and 3 lungs from normal individuals have been examined on Affymetrix miRNA microarrays. Results from previous studies have revealed, downregulated expression of miR-130b-3p, suggesting it plays an important role in the epithelial-mesenchymal crosstalk (S. Li et al., 2016). Then, the role of miR-155 was examined, a microRNA involved in asthma pathogenesis and consistently found upregulated in fibrotic disorders, suggested to be involved in the activation of the innate and adaptive immune systems. Various studies in different fields investigating lung, cardiovascular, liver and skin Patho physiologies have established a role of miR-155 in fibrosis as it mediates TGF-β1 signalling to drive collagen synthesis (Eissa & Artlett, 2019). Another miRNA involved in the pathogenesis of asthma that has been more studied than others is miR-21. In a mice model it has been

suggested that miR-21 is up-regulated in IPF patients and only a small amount of it is expressed in normal lung tissue of mice. However, after bleomycin stimulation, miR-21 levels have been upregulated, promoting accumulation of fibroblasts (Liu et al., 2010). Existing literature has suggested that miR-21 can act as a potential biomarker for the early diagnosis of IPF (H. Li et al., 2016). The only so far known miRNA to have been examined in response to *Aspergillus fumigatus* has been found to be miR-223. In a study of Eosinophilic esophagitis (EoE) induced mice and biopsy samples, levels of miR-223 have been found upregulated and inversely correlated with IGF1R (insulin-like growth factor receptor 1). However, the impact miR-223 on eosinophilic inflammation of the oesophagus was also assessed, with results demonstrating a reduction of the T2 cytokines IL-5 and IL-13 (Collison et al., 2020). A broad *in vivo* and *in vitro* study on miR-9-5p, has suggested that in the mouse model of bleomycin-induced lung fibrosis, miR-9-5p has been found to dramatically reduce fibrogenesis and inhibition of it prevents its anti-fibrotic effect. Also, in lung specimens from IPF patients, high levels of miR-9-5p were found (Fierro-Fernández et al., 2015). Finally, miR-34a-5p expression has been examined in the lungs of patients with idiopathic pulmonary fibrosis and in mice with experimental pulmonary fibrosis. Results introduced an up-regulated pattern in both human and mouse lung myofibroblasts and suggested that mice with miR-34a ablation developed more severe pulmonary fibrosis than wild-type animals after fibrotic lung injury. Both results have led to the conclusion of a potential role of miR-34a in driving lung fibroblasts senescence (Cui et al., 2017).

1.12 Overview of model systems on respiratory research

1.12.1 Air Liquid Interface (ALI)

Elevated respiratory diseases reports have created the need for research of the pathophysiology and therapeutic measures available. The formation of a model system for the understanding of toxicology, basic functions and modes

of action was essential. Up to date, research mainly focused on animal models, 2D, and 3D cell cultures (Johansen et al., 2020). Firstly, animal models have been used as a research method for the identification of possible biomarkers, drug toxicity studies and understanding of the pathophysiology of diseases. Although research on animal models has identified some very promising findings, limitations apply on the accurate representation of human pathological and physiological characteristics as well as the ethical concerns risen (Shrestha et al., 2020). Secondly, 2D cell culture model has been the most well-known and used method due to its low-cost manufacturing and straightforward handling. However, implications and limitations apply on production and accuracy of results. Set up of the model in a static condition, comprising of only one cell line, indicates inability to represent the complexity of respiratory epithelium and excessive production of toxic waste (Wu & Yu, 2020). Finally, 3D cell culture model is a method recently established and widely used for its ability to overcome 2D models limitations. Amongst the models Air Liquid Interface (ALI) and ECM scaffold are included (Kapetanović, 2011). Scaffolds comprise of ECM gels made of collagen, fibronectin, alginate, gelatine, laminin, and elastin providing an environment with physiological similarities (Lee & Sung, 2018) . Air Liquid Interface cultures are set up on trans well inserts suitable for toxicological and immunological studies. Although this model appears to be the most suitable research approach, limitations on organoid development being hard to control and reproduce still apply (Figure 1.12.1) (Francis et al., 2022).

Cell culture models still remain the most suitable approach for identification of the underlying pathogenesis of a respiratory disease. Primary human epithelial cells and bronchial epithelial cells have been the ideal cell types for enabling study of molecular characteristics. For in vitro experiments, submerged cell cultures of primary cells have been widely used. Recently, ALI model has been identified as the most important cell culture technique in the respiratory field. Cells differentiate on a pseudostratified form including ciliated cells.(Aydin et al., 2021).

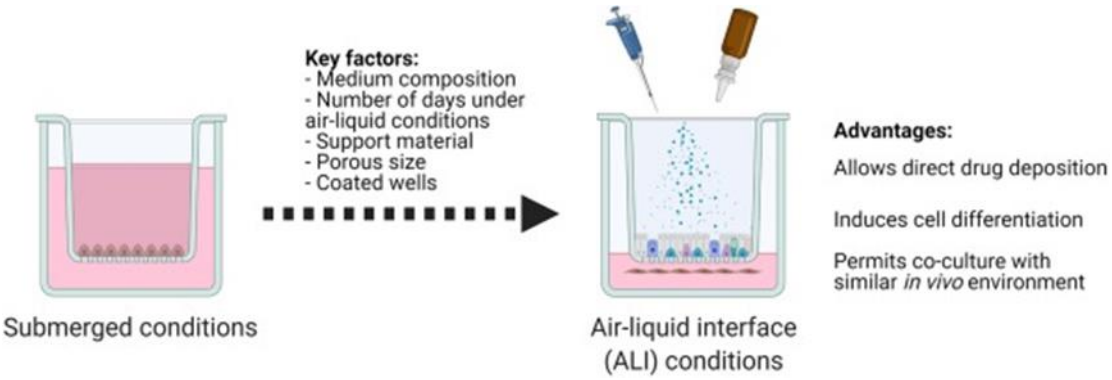

Figure 1.12.1.. Submerged and A.L.I conditions representation.
On the representation of an A.L.I model, specific key factors need to be taken into consideration for the accuracy of results. As a model, it possesses many advantages compared to other procedures conducted.

1.12.2 Air Liquid Interface Technique and limitations.

Air Liquid Interface is a new in vitro structure introduced, allowing in-depth research of barrier properties and metabolic functions (Cao et al., 2021). It is a method, creating a stable *in vitro* 3D human airway model mimicking the respiratory tract epithelia. By the use of this model, physiological and pathological pathways of the respiratory tract, interactions between respiratory epithelial cells, effects of pathogens or allergens on them can be investigated. In the set-up of this model, basal side of cells is in contact with culture media and the apical side is in contact with air. This way, morphological and functional differentiation can occur, allowing the formation of pseudostratified epithelium (Lacroix et al., 2018). As a method it has the ability to induce cell differentiation, promote tight junction formation and so can be used for permeability and molecular signalling pathways investigation (Silva et al., 2023). This system has many potentials and allows for excessive research on mechanistic studies focused on function of epithelial cells as drug permeation barriers or investigating host and pathogen interactions (Wang et al., 2018).

Primary human bronchial epithelial (HBE) culture has been confirmed as a reference model for research on cystic fibrosis pathogenesis. However, it has been identified that HBE cells grown under ALI conditions for 24 to 33 days possess the creation of a fully differentiated human bronchial epithelium consisting of basal, serous, clara, goblet, and ciliated cells. More specifically, the 16HBE14o cell line has been observed to present differentiation

characteristics like formation of tight junctions, ion transport and apical microvilli and cilia when placed in collagen coated filters under ALI conditions. Although the specific cell line accounts as the most ideal for respiratory research, limitations apply regarding its insufficient production of mucus (Silva et al., 2023). As proposed by He et al, 16HBE cells are ideal lung models for investigation of inhalation exposure and ALI culture for weeks as they act as a good indicator of barrier integrity and permeability (He et al., 2021). According to existing literature, the method used for barrier integrity and permeability measurement is Trans Epithelial Resistance (TEER) and values higher than 1.000 Ω x cm2 are classified as tight, between 300-1.000 Ω x cm2 as intermediate and below 300 Ω x cm2 as leaky. Regarding treatment with *Aspergillus fumigatus*, literature still remains limited and unclear. Most of our understanding of pulmonary lung infections comes from animal models which are believed to not fully recapitulate the human airway. Specifically focusing on human airway epithelial studies, literature is very limited indicating a critical gap in knowledge obtained so far (Crossen et al., 2022). To date, results from a study revealed evidence of transcriptional responses by 16HBE cells after treatment with *Aspergillus fumigatus* 6 hours post exposure (Toor et al., 2018).

It is clear that *Aspergillus fumigatus* can be pathological to immunocompromised patients and those who become sensitised to this organism. However, the mechanisms by which Aspergillus fumigatus may drive airway wall remodelling and associated miRNA biomarkers of this process remain unclear. The overall objectives of this MPhil were :

1. To assess induction of IL-6,IL-8 and ET-1 in both alveolar and bronchial epithelial cells *in vitro*, in response to *Aspergillus fumigatus* and fibrin.

2 To assess *in vivo* the impact of *Aspergillus fumigatus* filtrates and extracts on inflammation, collagen mucus secretion and fibrin deposition.

3.To assess expression of miRNAs *in vitro*, in bronchial cells after exposure to *Aspergillus fumigatus* filtrates.

2.MATERIALS & METHODS:

2.1 Human Cell Culture (16HBE14o and A549)

Human bronchial epithelial cells (16HBE14o) were grown using MEM media (Sigma Aldrich,M2279-500) containing 10% FBS (Foetal Bovine Serum, Labtech, FCS-SA/500), 1% Penicillin/Streptomycin (Thermo Fisher Scientific, 15070063), 1% L-glutamine (Sigma Aldrich, G7513). All experiments were conducted with cells between passage 15-45. Cells were propagated in T75 cm^2 flasks and incubated in 37°C temperature in 5% CO_2. For the purpose of experiments, confluent monolayers were detached using trypsin-EDTA (Sigma Aldrich, T4049) and seeded at $12x10^4$ /cm in multi-well plates. The plates were incubated and observed regularly and when 80-85% confluency was reached, the cells were washed with PBS (Thermo Fisher Scientific ,12899712), serum starved for 24 hours and treated with two strains of *Aspergillus fumigatus*, Af293 or Cea10 at 10 µg/ml for 24 hours. Cell culture supernatants were collected for the performance of IL-6, IL-8 sandwich ELISA and QUANTIKINE ET-1 ELISA. The same procedure was followed for human alveolar epithelial cells (A549) but the growing medium used was RPMI (Lonza, 11625220), also supplemented with10% FBS (Foetal Bovine Serum, Labtech, FCS-SA/500), 1% Penicillin/Streptomycin (Thermo Fisher Scientific, 15070063) and 1% L-glutamine (Sigma Aldrich, G7513). All experiments were conducted with cells between passage 8-33.

2.2 *Aspergillus fumigatus* culture

For the experiments conducted, two strains of *Aspergillus fumigatus* were used: Af293 and Cea10. Culture filtrates were generated at the University of Manchester. In brief, conidia were propagated on SAB agar for 72hr and detached using PBS-tween-20 and filtered through lens cloth. To prepare each culture filtrate, conical flasks containing 500ml of sterile Vogel minimal medium were supplemented with 1X106 conidia and incubated for 72hr at 37C at 320rpm (Farnell et al., 2012). Following filtration through J-cloth to remove hyphae and fungal mass, filtrates were 0.2um sterile filtered and aliquoted in 10ml tubes and stored at -80°C. To preserve protein and protease

integrity, filtrates were freeze dried using the appropriate equipment for 36 hours. They were then reconstituted with PBS and aliquoted in 15 ml centrifuge tubes. Freeze-dried filtrates were stored at -80°C and reconstituted in 10ml PBS and a BCA protein assay conducted.

2.3 BCA Protein Assay

The protein concentration of both culture filtrates was calculated using the Thermo Fisher Scientific Pierce BCA protein assay (Thermo Fisher Scientific, 23225) according to manufacturer protocol. In brief, a Bovine Serum Albumin (BSA, Sigma Aldrich, A9418) standard curve was made in PBS with concentrations ranging from 0-1.5mg/ml. The mixture reagent required was created by the addition of 6 ml of Reagent A in 120 µl of Reagent B provided in the protein assay kit. In a 96 -well microplate, 25µl of standard or unknown sample were applied to the wells of a 96 well plate, followed by 200µl of reagent mixture. The plate was sealed with parafilm and incubated for a further 30 minutes in 37°C and 5% CO_2. After the incubation period, the plate was read in a microplate reader at 562 nm absorbance. For the analysis of the results, excel graph in logarithmic scale was used.

2.4 Animal Models

In order to assess the impact of *Aspergillus fumigatus* on airway wall remodelling, inflammation and the possibility of blood vessel leakage and fibrin deposition, tissue sections from a tissue bank at the University of Salford were selected for histological analysis. Mouse tissue from murine inhalational models of respiratory disease previously developed at the UoM under appropriate project licenses were included (Namvar et al., 2015b).
Tissue sections were selected from C57 BL6J male mice repeatedly exposed to saline/ control media (PBS), Af293 *Aspergillus fumigatus* culture filtrate or this culture filtrate plus the Endothelin-1 receptor antagonist BQ123(as it was intranasally administered it is labelled as BQIN). Culture filtrates were developed by growing Af293 in vogals minimal medium for 48hrs after which secreted *Aspergillus fumigatus* products were filtered, sterilised and frozen.(Labram et al., 2019). Finally, one group of mice were exposed to a

commercially available Greer extract. In all cases, five mice were exposed to 50 µl control media, culture filtrate or extract twice per week for four weeks, followed by a ninth final dose 24hr before euthanasia as previously described (Namvar et al., 2015a).

2.5 Histology

2.5.1 Haematoxylin and Eosin(H&E) Staining

For identification of tissue structures and assessment of inflammation, haematoxylin and eosin were used. Slides were dewaxed twice in histoclear for 5 minutes, then incubated in 100% ethanol for 5 minutes and then 95% and 70% ethanol for 2 minutes each. At this point slides were briefly washed in distilled water and then dipped in Harris Haematoxylin for 18 minutes and placed under running water for approximately 5 minutes. Next, slides were dunked 2-3 times in acid alcohol (1% HCL in 70% ethanol), dipped in Scott's Tap water (1g Sodium Hydrogen Carbonate and 10g magnesium sulphate in 500ml of water) for 1 minute and placed again under running tap water for a further 5 minutes. Following a 3 min incubation in Eosin Y and a 2 min rinse in running tap water slides were once again dehydrated in alcohols and exposed to histoclear before cover slipping using DPX.

2.5.2 Masson's Trichrome (M.T)

Trichrome staining is used to visualize connective tissues, particularly collagen, in tissue sections. In a standard Masson's Trichrome procedure, collagen is stained blue, nuclei are stained dark brown, muscle tissue is stained red, and cytoplasm is stained pink. Slides were dewaxed in histoclear and hydrated through incubation in decreasing alcohol concentrations as described above. Once hydrated through to distilled water, slides were dipped in Mayers Haematoxylin for 5 minutes, placed in running tap water for 1 minute, stained in Celestine Blue solution for 5 minutes and placed in running tap water for 1 more minute. At this stage, slides were stained with 1% Ponceau fuchsin in 1% acetic acid for 2 minutes, washed in double distilled water, placed in 1% PMA until collagen decolorized and washed again in

double distilled water. Further, they were counterstained in 0.5% soluble blue in 2.5% acetic acid for 45 seconds. Finally, slides have been washed in double distilled water for a minute, placed in 10% ethanol for 30 seconds and washed twice in histoclear for 1 minute each. The slides were mounted in DPX.

2.5.3 Periodic Acid Schiff (PAS)

Periodic acid-Schiff (PAS) is a staining method used to detect polysaccharides, manifested as glycogen, glycoproteins, glycolipids, and mucin in tissues (Abreu Velez, 2016). Firstly, slides were fixed in 10% formalin and the 0.5% periodic acid solution has been made by adding 0.5 g of Periodic acid in distilled water. The slides have been initially deparaffinised and hydrated to water. Then, they have been oxidised in 0.5%periodic acid solution for 5 minutes and rinsed in distilled water. Slides have been placed into Schiff reagent for 15 minutes before being washed in lukewarm tap water for 5 minutes. Finally, slides have been counterstained with Mayer's haematoxylin for 1 minute, washed in tap water for 5 minutes and dehydrated. The slides were mounted in DPX.

2.5.4 Carstairs' Staining for Fibrin

Carstairs' is a staining method used for identification of fibrin, platelets, collagen, muscle, and red blood cells on tissues. Firstly, slides were fixed using Formalin-Saline for less than 48 hours. Slides have been hydrated in distilled water and mordanted in 5% Ferric Ammonium Sulphate for 5 minutes. They were then rinsed in running tap water and stained in Mayer's haematoxylin for 5 minutes. After being rinsed in running tap water they were stained in Picric Acid Orange G Solution for a further 30 minutes and rinsed once in distilled water. They were stained in Ponceau Fuchsin Solution for 5 minutes, rinsed in distilled water and differentiated in 1 % Phosphotungstic acid until the background appears pale pink. Additionally, they were rinsed in distilled water, stained in Ainline Blue Solution for 1 hour and rinsed again in several changes of distilled water. Finally, the slides were dehydrated, cleared in Xylene, and mounted in DPX. For the identification of the results, fibrin

should appear orange to orange red, collagen should appear bright blue and red blood cells red, green, or yellow.

2.5.5 Imaging and Analysis of H&E, M.T, PAS and Carstair's Results

Images of the histology sections were taken on a Leica microscope connected to a Samsung Galaxy Tab 8 tablet with the Leica Software installed. For the purpose of analysis of airways and blood vessels inflammation, subepithelial collagen deposition, mucus secreting goblet cells and fibrin deposition, images of airways and blood vessels of an appropriate size were taken at a x10 magnification.

For the H&E staining, an arbitrary scoring system ranging from 0-3 was designed similar to the one presented on the Journal of Cystic Fibrosis for the histological evaluation of lung inflammation (Chappe, 2020). The images were blind scored from two different individuals not affiliated with the research project. To begin, scorers received some basic training on identification of inflammation and calibrating the scores they assigned to particular levels of inflammation (Figure 2.5.5.1).

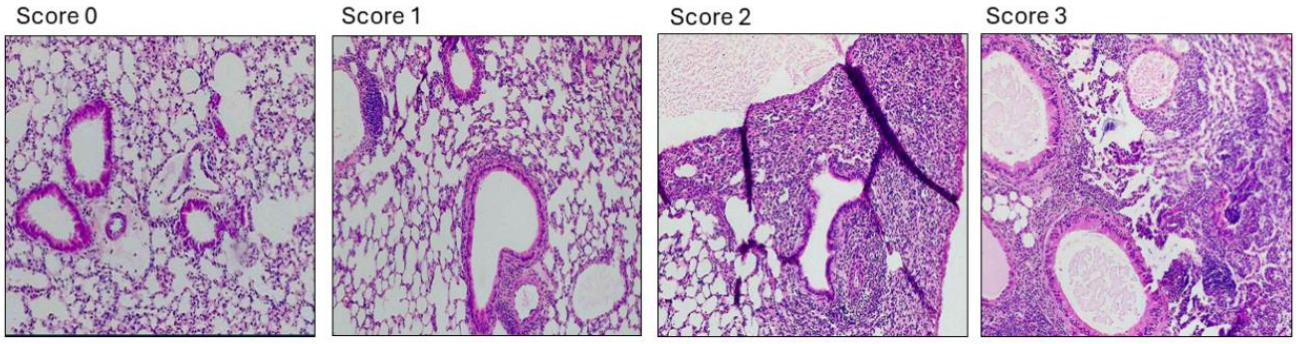

Figure 2.5.5.1. Histological scoring system for the evaluation of lung inflammation. Presence of mild infiltration was scored as 0, moderate was scored as 1, severe infiltration was scored as 2 and lastly diffusion in combination with very severe peri bronchial infiltration was scored as 3.

For the analysis of sub-epithelial collagen deposition, two approaches were adopted. The first involved manually measuring the thickness of 10 points around the perimeter of the airway starting at the point where the epithelium ends to the point

where the collagen staining stopped. Representative image on how the manual measurement was conducted through FIJI can be found below.

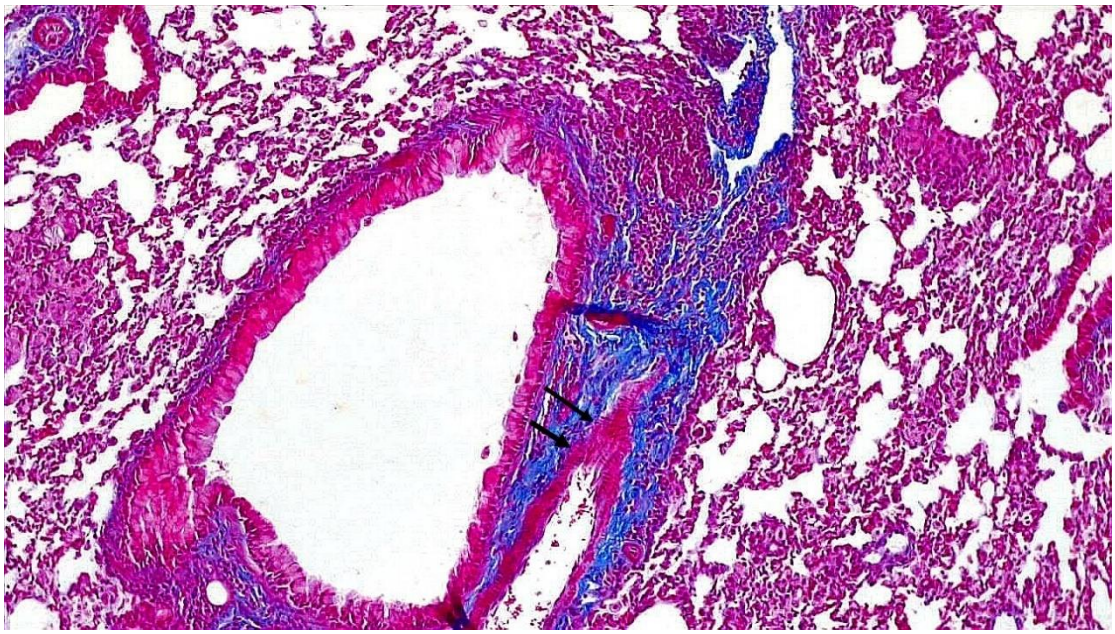

Figure 2.5.5.2. Histological manual measurements for the evaluation of lung inflammation. Manual measurements of ten different areas that inflammation was present around the airway have been measured using the FIJI software and results were collated in excel. **Arrows on the picture indicate the measurement points.**

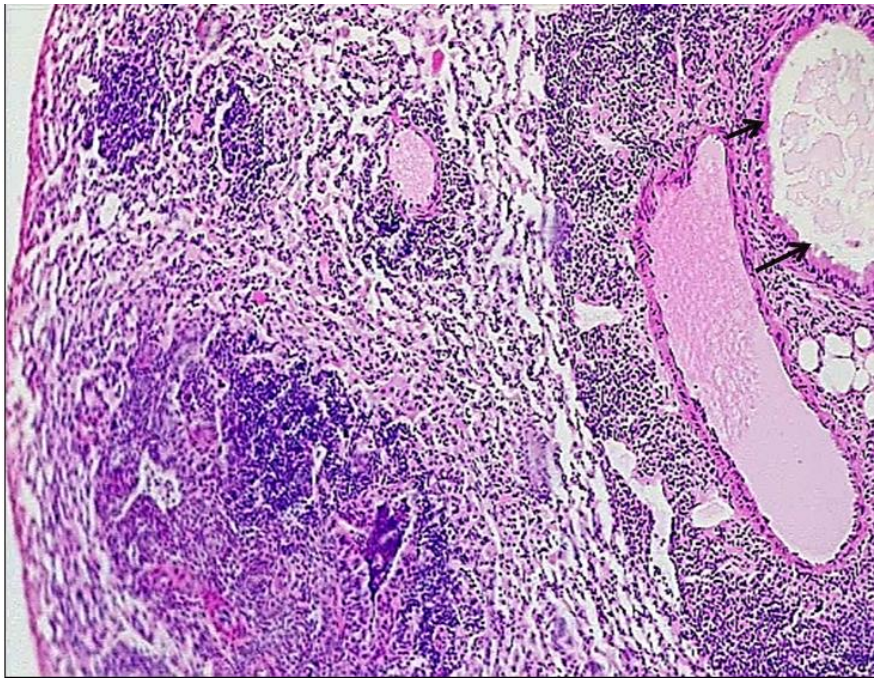

Figure 2.5.5.3. Histological manual measurements for the evaluation of lung inflammation. Manual measurements of ten different areas that inflammation was present around the blood vessels have been measured using the FIJI software and results were collated in excel. Arrows on the picture indicate the measurement points.

The second approach involved use of the colour de-convolution tool in Image J(FIJI) for both the Masson's Trichrome and PAS staining. This tool separates out the histology image into separate channels, one of which represents the stain of interest. The appropriate channels from the colour deconvolution were made binary and the total area for the airway or blood vessels showing positive staining were manually selected. Next the percentage area with positive stain was determined by the Image J Software (National Institute of Health, Maryland, USA). For Carstair's staining, analysis required interpretation of staining according to published guidance (Delta Microscopies,1965). This staining was specifically used to identify instances of fibrin deposition and blood vessel leakage.

Table 1: Stain Results for Carstair's Method for Fibrin and Platelets.

Fixation Time:	**48 hours or more:**	**Less than 48 hours**
Fibrin	Bright Red	Orange to Orange Red
Platelets	Gray Blue or Navy	Light Gray
Collagen	Bright Blue	Bright Blue
Muscle	Red	Red
Red Blood Cells	Clear Yellow	Red, Green or Yellow

2.6 Co-exposure of 16HBEs to fibrin and *Aspergillus fumigatus*

In order to assess the impact of fibrin on *in vitro* cultures an artificial clot was produced. Human fibrinogen (Sigma-Aldrich, F4883) was dissolved in serum-free M199 at a concentration of 3.3 mg/ml media and incubated at 37 °C in a water bath for 3 hours. The final solution was filtered through a 0.45µm filter unit (Millex®-HA, Merck Millipore Ltd, Co. Cork, Ireland) into a 50ml falcon tube and then placed on ice prior to addition of human thrombin (Sigma - Aldrich, T4393) at a final concentration of 0.3 U/ml, before immediately pipetting 0.5 ml to the appropriate wells of 24 well plate already containing confluent monolayers of 16HBE cells. In some cases, fibrin exposed 16HBE cells were also exposed to Af293 or Cea10 culture filtrates, each at 10 µg/ml and comparisons drawn to control cells. Phase contrast images of control cells and those exposed to fibrin, *Aspergillus fumigatus* culture filtrates or those exposed to fibrin and culture filtrates in combination were collected at 0,

24 and 48 hours. Cell culture supernatants were collected at the 48-hour time point for the assessment of IL-6, IL-8 and ET-1 by ELISA.

2.7 IL-6 and IL-8 ELISA

The induction of IL-6 was measured using Duo Set® ELISA Development System following the manufacturers protocol. (R&D Systems). In brief, capture antibody was diluted in coating buffer and 100 µl dispensed onto each well of a 96 -well microplate which was sealed with parafilm and incubated overnight at room temperature. Following 3 wash steps using 400 µl of wash, the plate was incubated with 300 µl of reagent diluent for an hour. The wash step was repeated and 100 µl of the standard or sample added to the appropriate well of the 96 well plate, which was then incubated for a further two hours. Following a repeat of the wash steps, wells were then incubated at room temperature for 2 hours in the presence of 100ul of detection antibody. Following further wash steps, incubation with streptavidin-horseradish peroxidase solution for 20 minutes in the dark and further wash steps, each well received 100 µl of substrate solution (mixture of equal parts of reagent A (H_2O_2) with reagent B (Tetramethylbenzidine), incubated for 20 minutes avoiding contact with direct light. Finally, 50 µl of the stop solution were added in all wells and the results were interpreted using at a microplate reader at 450 nm absorbance using the Magellan software**.** Induction of IL-8 was measured following the same principles. Only difference on the procedure was the preparation of buffers like wash buffer (PBS and 0.05% Tween 20), reagent diluent (100 µg of BSA and 5 µl of Tween in 100 ml of PBS), block buffer (1 g of BSA in 100 ml of PBS).

2.8 QUANTIKINE ELISA Endothelin 1 (ET-1)

The induction of ET-1 was measured using Duo Set® ELISA Development System following the manufacturers protocol. (R&D Systems). In brief, 100 µl of the assay diluent RD1-105 and 100 µl of the standard curve were dispensed and the samples were placed in the plate which was covered with adhesive strip and incubated in an orbital shaker (0.12 orbit) set at 500 ± 50

rpm for 1 hour at room temperature. Following 4 wash steps using 400 µl of wash, the plate was incubated with 200 µl of Endothelin -1 conjugate for 3 hours in the orbital shaker. The wash step was repeated and 100 µl of the standard or sample added to the appropriate well of the 96 well plate, which was then incubated for a further two hours. Following further wash steps, 200 µl of substrate solution (mixture of equal parts of reagent A (H_2O_2) with reagent B (Tetramethylbenzidine) were dispensed and plate was incubated for 30 minutes avoiding contact with direct light. Finally, 50 µl of the stop solution were added in all wells and the results interpreted using at a microplate reader at 450 nm absorbance using the Magellan software.

2.9 Analysis of ELISA

The mean values of the blanks were subtracted from the mean absorbance value of each optical density of the standards and of the samples. A standard curve was produced, and unknown concentrations were interpolated from the standard curve using log-log scale in GraphPad Prism 10 and nonlinear regression analysis was performed.

2.10 Molecular Biology

2.10.1 Tissue Culture preparation

Human bronchial epithelial cells (16HBE) treated with control media and media containing 10 µg/ml of Aspergillus fumigatus strains Cea10 and Af293 for 24 hours. Cell monolayers were washed twice with sterile PBS, harvested using trypsin-EDTA and collected in sterile PBS in Eppendorf tubes stored at -80°C in RNAlater.

2.10.2 RNA extraction

RNA was extracted using the mirVana miRNA Isolation kit (Invitrogen). Firstly, PBS wash was removed from the collected cell layers and 600 µl of the Lysis/Binding solution was added. The samples were then vortexed vigorously

to ensure complete lysis of the cells. For total RNA and small RNAs purification, 60 µl of miRNA Homogenate Additive were added, vortexed to ensure solutions are mixed well and placed into wet ice for 10 minutes. At this stage, 600µl of Acid-Phenol Chloroform were added inside the fume hood and liquid were vortexed for 30-60 seconds to mix. At this step, it was important to ensure that Acid-Phenol Chloroform was withdrawn from the bottom phase of the bottle as the upper phase consists of an aqueous buffer. Samples were then centrifuged at 10.000 x g for 5 minutes at room temperature and upper phase was collected and transferred to a fresh tube. For Total RNA Isolation, 675 µl of 100% ethanol were added to the aqueous phase. For each sample, a filter cartridge was placed into a collection tube. The mixture was pipetted to the filter and centrifuged for 15 seconds at 10.000 x g, so mixture can be passed through the filter. After, the flow through was discarded and 700 µl of wash solution 1 was applied to the filter and centrifuged for 10 seconds and the new flow through was discarded again. The previous step was repeated twice with wash 2/3 this time and the assembly was spun for 1 minute to remove residual fluid from the tube. Finally, the filter cartridge has been transferred to a fresh collection tube and 100 µl of pre-heated (95°C) Elution Solution was applied to the centre of the filter and spun for 30 seconds at 15.000 x g so the RNA can be recovered. The RNA concentration was determined using the ND-1000 Spectrophotometer (Nanodrop, Thermoscientific Massachusetts, USA). To blank the instrument, RNAse free water was used before sample was loaded and RNA concentration determined. The tubes containing the eluate were stored at -20°C until cDNA extraction. Results from Nanodrop measurement can be found in the Appendix (Section 5).

2.10.3 Reverse Transcription (RT)

Reverse transcription (RT) was performed using the QuantiMir RT Kit (System Biosciences, USA). The procedure included three major steps: 1. PolyA Tail, 2. Annealing Anchor dT Adaptor and 3. Synthesis of cDNAs. Each reaction was set up containing the components according to Table 2. Thermal Cycler was set up according to Table 3. Once the reaction had completed the cDNA was diluted 1:100 with RNAse free water and stored in the -80°C medical freezer.

Table 2. Components of Reverse Transcription (RT) reaction.

Component:	Volume in 10 µl of reaction (10 µl):
Total RNA (10 pg-10µg)	5
5X PolyA Buffer	2
25mM $MnCl_2$	1
5mM ATP	1.5
Oligo dT Adaptor	0.5
PolyA Polymerase	0.5
5X RT Buffer	4
dNTP mix	2
0.1M DTT	1.5
RNAse free Water	1.5
Reverse Transcriptase 1	

Table 3. Thermal Cycler program for Reverse Transcription (RT).

Step:	Temperature (°C):	Time (minutes):
Incubation	37	30
Oligo dT Adaptor addition:	60	5
cDNAs Synthesis	42	60
RT Inactivation	95	10

2.10.4 Real time PCR (RT-PCR)

For qRT-PCR, 8 different micro-RNAs have been selected to be tested, proposed by previously existing literature. The microRNAs selected were: miR-320a-3p, miR-130b-3p, miR-144-3p, miR-133a-3p, miR-223-5p, miR-223-3p, miR-9-5p and miR-34a-5p. These reactions were set up as below in Table 4 in 0.2 ml PCR tubes. To these reactions either 5 µl of cDNA or RNase free water for the no template control (NTC) was added.

Table 4: Real -Time PCR Components.

Component:	Volume(µl):
2X SYBR Green qPCR Mastermix buffer	15
User-designed Forward Primer (10 µM)	1
Universal Reverse Primer (10 µM)	0.5
Diluted QuantiMir cDNA	1
RNase free water	12.5
Final Volume	30

Table 5: qPCR Cycling and Data accumulation conditions.

Cycles:	Temperature(°C):	Time(minutes):
1	50	2
1	95	10

40	95	15 sec
40	60	1

Table 6: miRNA Primers for detection.

Oligo Name:	Sequence 5'->3':	Length:
1. miR-320a-3p	AAAAGCTGGGTTGAGAGGGCGA	22
2. miR-130b-3p	CAGTGCAATGATGAAAGGGCAT	22
3. miR-155-5p	TTAATGCTAATCGUGATAGGGGTT	22
4. miR-21-5p	TAGCTTATCAGACTGATGTTGA	22
5. miR-223-5p	CGTGTATTTGACAAGCTGAGTT	22
6. miR-223-3p	TGTCAGTTTGTCAAATACCCCA	22
7. miR-9-5p	TCTTTGGTTATCTAGCTGTATGA	23
8. miR-34a-5p	TGGCAGTGTCTTAGCTGGTTGT	22

The cycling conditions were followed as in Table 5. The data from each different primer was analysed using the Rotor Gene Q software. The sequences for the primers used can be found in the Appendix section.

2.10.5 RT-PCR Analysis

Data obtained from Real Time PCR were analysed according to the ΔΔCT method. Firstly, ΔCT was calculated by subtracting the CT of the reference gene(U6) from the CT of the gene of interest. Then ΔΔCT was calculated by subtracting the average of the control ΔCT from each experimental ΔCT. Relative fold expression was calculated by 2 (-ΔΔCT). For the statistical analysis of ΔΔCT results, GraphPad Prism 10 was used.

2.11 Air Liquid Interface (ALI) Methodology

16HBE were propagated as described earlier (section of human cell culture). A twelve-well plate with cell inserts of 4.0 μm pore size (Millipore, PTHT06H48) was filled with rat tail collagen type -1 (Corning, CLS354236) reconstituted in PBS at a working concentration of 0.03 mg/ml and incubated for 2 hours. After the incubation period, using a cell density of $6x10^4$ the plate was seeded with cells in the apical chamber and 10 ml of MEM media placed in the basolateral chamber. The plate was incubated in 37°C and 5% CO_2 for three days. After the cells reached the desired confluency, media was removed from the apical chamber to initiate the differentiation process. The

plate was incubated for a further twenty-one days. The apical chamber was washed with PBS and the media (MEM) was changed in the basolateral chamber, twice a week. After the differentiation process, Trans Epithelial Electrical Resistance was measured over the 4^{th}, 8^{th}, 11^{th}, 15^{th}, 18^{th}, 22^{nd}, 25^{th}, 28^{th} and 30^{th} day.

Trans Epithelial Electrical Resistance (TEER) is a widely known and used technique indicating the permeability and integrity of the monolayer formed by the epithelial cells. It is measured using the Millicell® ERS-2 Voltohmmeter, calibrated in 1000 Ω which consists of two electrodes that get connected to the cell inserts. One is placed in the upper and is connected to the apical compartment and the other one is placed to the lower part connected to the basolateral compartment.

Additionally to this technique, immunofluorescence and live dead staining of the ALI model was performed. Results have not been accurate to be included on the thesis but methodology can be found in Appendix(Section 6).

2.12 Statistical Analysis

For the statistical analysis of the results obtained from the experiments, data were collated in Excel for Microsoft Windows 365 version 2106 and the statistical analysis was performed using GraphPad Prism 12. ELISAs and ALI have been repeated four times to ensure results accuracy, whereas qPCR has been performed once due to insufficient time. Post-hoc tests and one- and two-way ANOVA tests were conducted for analysis of the significance of the results. Significance was defined as $P < 0.05$.

3.RESULTS

3.1 *Aspergillus fumigatus* culture filtrates and extract cause airway and blood vessel inflammation.

To assess the impact of exposure to *Aspergillus fumigatus* filtrates, Endothelin-1 receptor antagonism and commercially sourced extracts, tissue sections from historic mouse studies were obtained and stained by H&E. Sections have been issued from the tissue bank of University of Salford. Next, ten images from five slides per treatment group were collected for subsequent airway and blood vessel inflammation scoring.

Inflammation for both blood vessels and airways has been quantified using a scoring system of 0-3. Initial statistical analysis by one way ANOVA revealed a significant effect of treatment group ($P < 0.0001$) on average airway inflammation score. Compared to controls, post hoc tests revealed that significant inflammation was evident around the airways of animals exposed to Af293 ($P < 0.0007$) with a similar level of inflammation observed in those animals exposed to Af293+BQIN ($P < 0.0001$), suggesting that Endothelin-1 receptor antagonism had not diminished airway inflammation. Compared to control animals, Greer extract caused a significant induction of inflammation

(*P <0.0007),* but this was no greater to that seen in response to in-house generated filtrates (Figure 3.1.1).

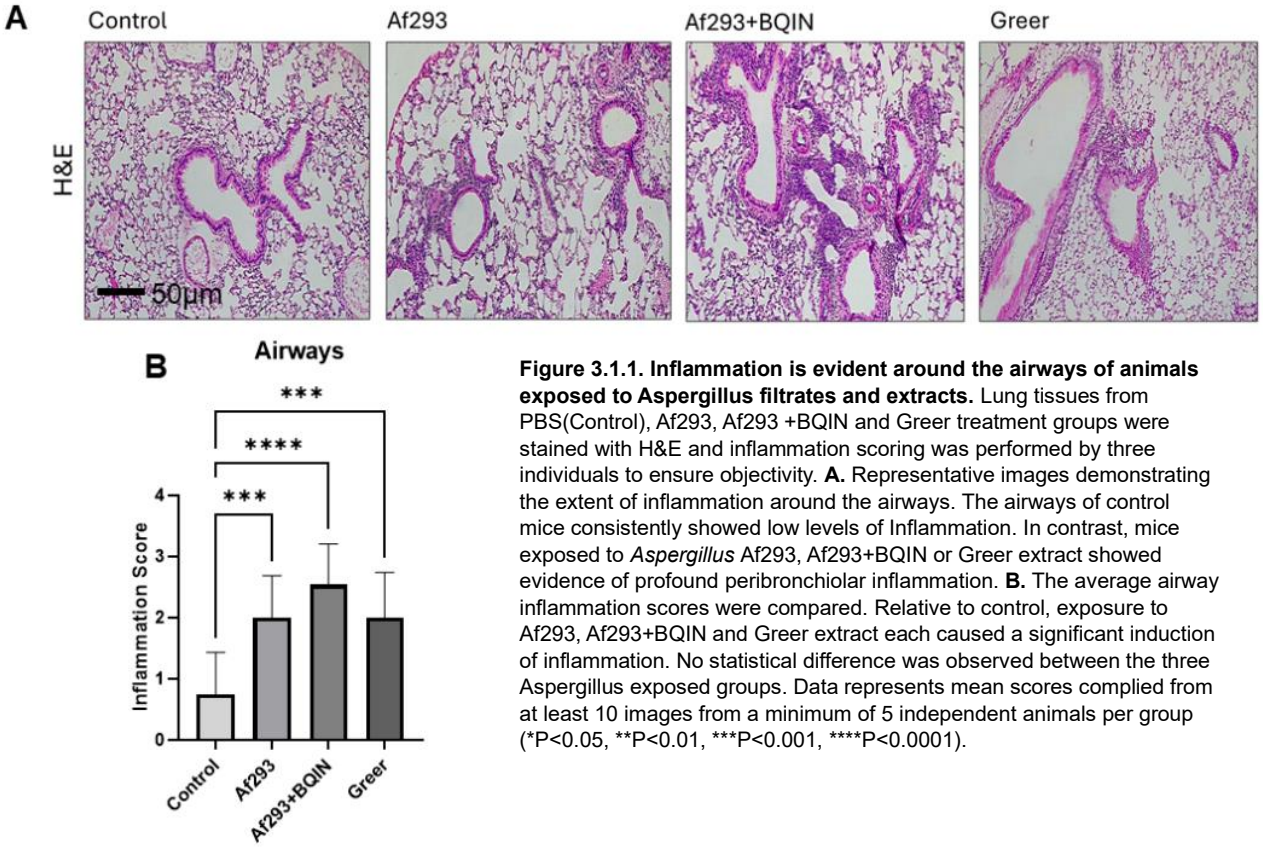

Figure 3.1.1. Inflammation is evident around the airways of animals exposed to Aspergillus filtrates and extracts. Lung tissues from PBS(Control), Af293, Af293 +BQIN and Greer treatment groups were stained with H&E and inflammation scoring was performed by three individuals to ensure objectivity. **A.** Representative images demonstrating the extent of inflammation around the airways. The airways of control mice consistently showed low levels of Inflammation. In contrast, mice exposed to *Aspergillus* Af293, Af293+BQIN or Greer extract showed evidence of profound peribronchiolar inflammation. **B.** The average airway inflammation scores were compared. Relative to control, exposure to Af293, Af293+BQIN and Greer extract each caused a significant induction of inflammation. No statistical difference was observed between the three Aspergillus exposed groups. Data represents mean scores complied from at least 10 images from a minimum of 5 independent animals per group (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

In parallel, we examined the effect of in-house filtrates relative to commercially sourced *Aspergillus fumigatus* extracts on blood vessels. Statistical analysis by one way ANOVA revealed a significant effect of treatment group (*P < 0.0001*) on average inflammation score. Compared to controls, post hoc tests revealed that significant inflammation was evident around the airways of animals exposed to Af293 (*P<0.0104*) with an upregulated level of inflammation observed in those animals exposed to Af293+BQIN (*P<0.0003*), suggesting that Endothelin-1 receptor antagonism had not diminished blood vessel inflammation. Compared to control animals, Greer extract caused a significant induction of inflammation (*P <0.0373*), but this was no greater to that seen in response to Af293+ BQIN (Figure 3.1.2).

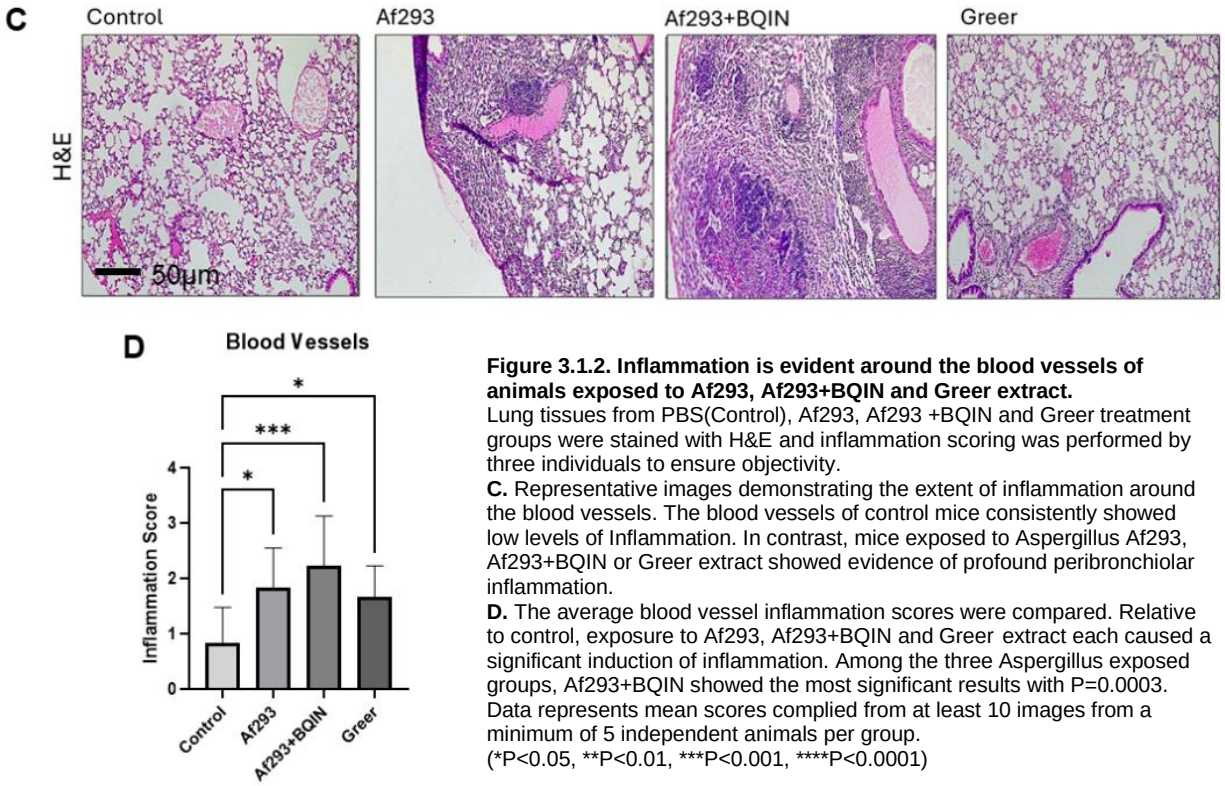

Figure 3.1.2. Inflammation is evident around the blood vessels of animals exposed to Af293, Af293+BQIN and Greer extract.
Lung tissues from PBS(Control), Af293, Af293 +BQIN and Greer treatment groups were stained with H&E and inflammation scoring was performed by three individuals to ensure objectivity.
C. Representative images demonstrating the extent of inflammation around the blood vessels. The blood vessels of control mice consistently showed low levels of Inflammation. In contrast, mice exposed to Aspergillus Af293, Af293+BQIN or Greer extract showed evidence of profound peribronchiolar inflammation.
D. The average blood vessel inflammation scores were compared. Relative to control, exposure to Af293, Af293+BQIN and Greer extract each caused a significant induction of inflammation. Among the three Aspergillus exposed groups, Af293+BQIN showed the most significant results with P=0.0003. Data represents mean scores complied from at least 10 images from a minimum of 5 independent animals per group.
(*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

3.2 *Aspergillus fumigatus* culture filtrates and extract cause airway and blood vessel collagen deposition.

As previous publications have established that *Aspergillus fumigatus* culture filtrates can inhibit collagen deposition and this is abrogated by Endothelin-1 receptor antagonism, we have examined extensively the impact of in-house filtrates relative to commercially sourced *Aspergillus fumigatus* extracts on both airways and blood vessels. Tissue sections from historic mouse studies were obtained and stained by M.T. Next, images were collected for investigation and manual measurements of the collagen deposition were taken through FIJI.

Statistical analysis by one way ANOVA revealed a significant effect of treatment group ($P < 0.0001$) on average manual measurements of collagen deposition. Compared to controls, post hoc tests revealed that significant collagen deposition was evident around the airways of animals exposed to Af293 ($P<0.0001$) with a similar level of inflammation observed in those animals exposed to Af293+BQIN ($P < 0.0001$), suggesting that Endothelin-1

receptor antagonism had not diminished collagen deposition. Compared to control animals, Greer extract caused a significant induction of collagen deposition (*P<0.0001),* but this was similar to the results observed in response to in-house generated filtrates (Figure 3.2.1).

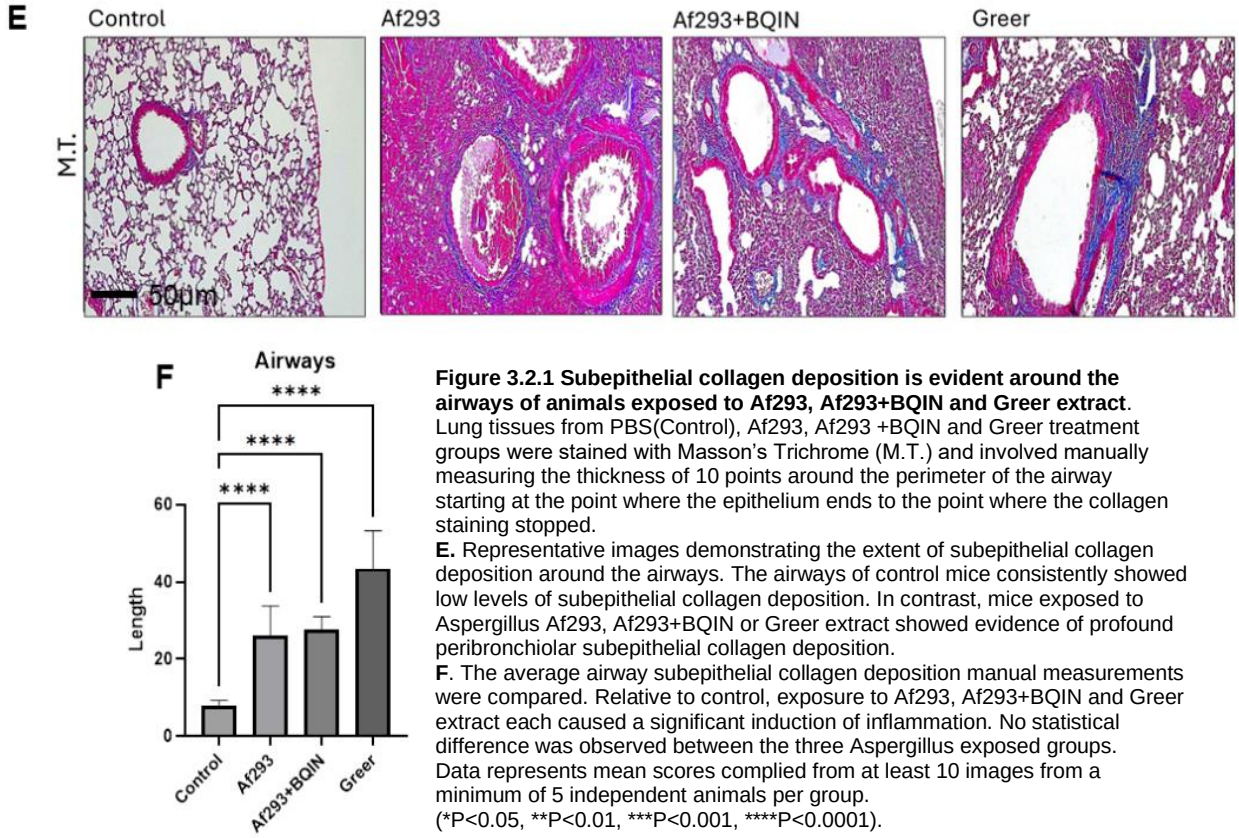

Figure 3.2.1 Subepithelial collagen deposition is evident around the airways of animals exposed to Af293, Af293+BQIN and Greer extract. Lung tissues from PBS(Control), Af293, Af293 +BQIN and Greer treatment groups were stained with Masson's Trichrome (M.T.) and involved manually measuring the thickness of 10 points around the perimeter of the airway starting at the point where the epithelium ends to the point where the collagen staining stopped.
E. Representative images demonstrating the extent of subepithelial collagen deposition around the airways. The airways of control mice consistently showed low levels of subepithelial collagen deposition. In contrast, mice exposed to Aspergillus Af293, Af293+BQIN or Greer extract showed evidence of profound peribronchiolar subepithelial collagen deposition.
F. The average airway subepithelial collagen deposition manual measurements were compared. Relative to control, exposure to Af293, Af293+BQIN and Greer extract each caused a significant induction of inflammation. No statistical difference was observed between the three Aspergillus exposed groups. Data represents mean scores complied from at least 10 images from a minimum of 5 independent animals per group.
(*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Statistical analysis by one way ANOVA for the blood vessels, revealed similar results to those observed on airways. A significant effect of treatment group (*P < 0.0001*) on average manual measurements of collagen deposition was indicated. Compared to controls, post hoc tests revealed that significant collagen deposition was evident around the blood vessels of animals exposed to AF293 (*P<0.0001*) with a similar level of inflammation observed in those animals exposed to AF293+BQIN (*P < 0.0001*), suggesting that Endothelin-1

receptor antagonism had not diminished collagen deposition. Compared to control animals, Greer extract caused a significant induction of collagen deposition (*P<0.0001),* but this was no greater to that seen in response to in-house generated filtrates (Figure 3.2.2).

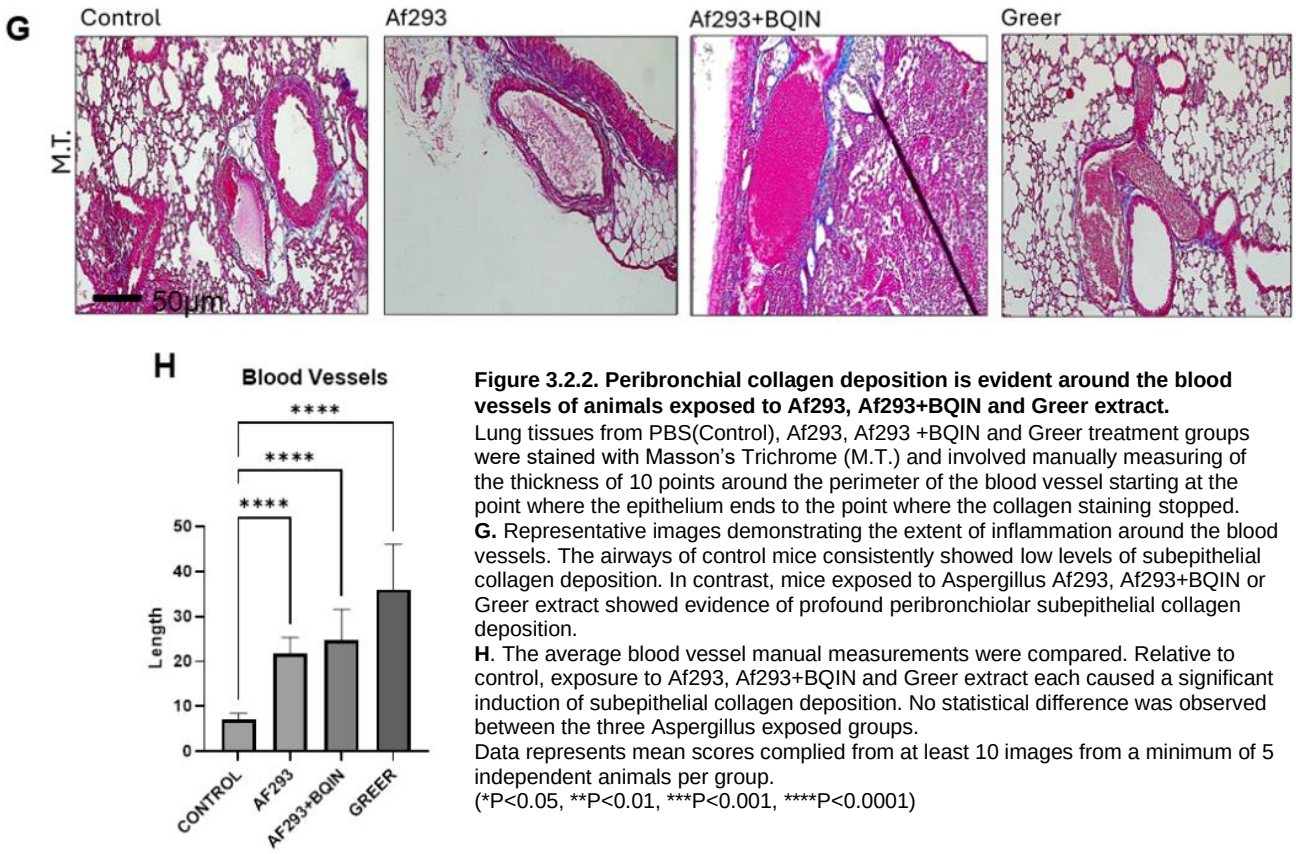

Figure 3.2.2. Peribronchial collagen deposition is evident around the blood vessels of animals exposed to Af293, Af293+BQIN and Greer extract.
Lung tissues from PBS(Control), Af293, Af293 +BQIN and Greer treatment groups were stained with Masson's Trichrome (M.T.) and involved manually measuring of the thickness of 10 points around the perimeter of the blood vessel starting at the point where the epithelium ends to the point where the collagen staining stopped.
G. Representative images demonstrating the extent of inflammation around the blood vessels. The airways of control mice consistently showed low levels of subepithelial collagen deposition. In contrast, mice exposed to Aspergillus Af293, Af293+BQIN or Greer extract showed evidence of profound peribronchiolar subepithelial collagen deposition.
H. The average blood vessel manual measurements were compared. Relative to control, exposure to Af293, Af293+BQIN and Greer extract each caused a significant induction of subepithelial collagen deposition. No statistical difference was observed between the three Aspergillus exposed groups.
Data represents mean scores complied from at least 10 images from a minimum of 5 independent animals per group.
(*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

3.3 *Aspergillus fumigatus* culture filtrates and extract cause airway mucus secretion through goblet cells hyperplasia.

As we have previously investigated the effect of in-house filtrates relative to commercially sourced *Aspergillus fumigatus* extracts regarding airway and blood vessel inflammation and collagen deposition, we thought to investigate the effect on goblet cell hyperplasia, another feature of airway wall remodelling. To assess the impact of exposure to *Aspergillus fumigatus* filtrates, Endothelin-1 receptor antagonism and commercially sourced extracts, tissue sections from historic mouse studies were obtained and

stained by PAS. Next, images were collected for investigation and manual measurements of goblet cell hyperplasia were taken through FIJI Software. Statistical analysis by one way ANOVA revealed a significant effect of treatment group (*P < 0.0001*) on average manual measurements of mucus secreting goblet cells. Compared to controls, post hoc tests revealed that significant goblet cell presence was evident around the airways of animals exposed to Af293 (*P<0.0001*) with a similar level of presence observed in those animals exposed to Af293+BQIN (*P < 0.0001*), suggesting that Endothelin-1 receptor antagonism had not diminished goblet cell hyperplasia. Compared to control animals, Greer extract caused a significant induction of goblet cells hyperplasia (*P<0.0001),* but this was no greater to that seen in response to in-house generated filtrates (Figure 3.3.1).

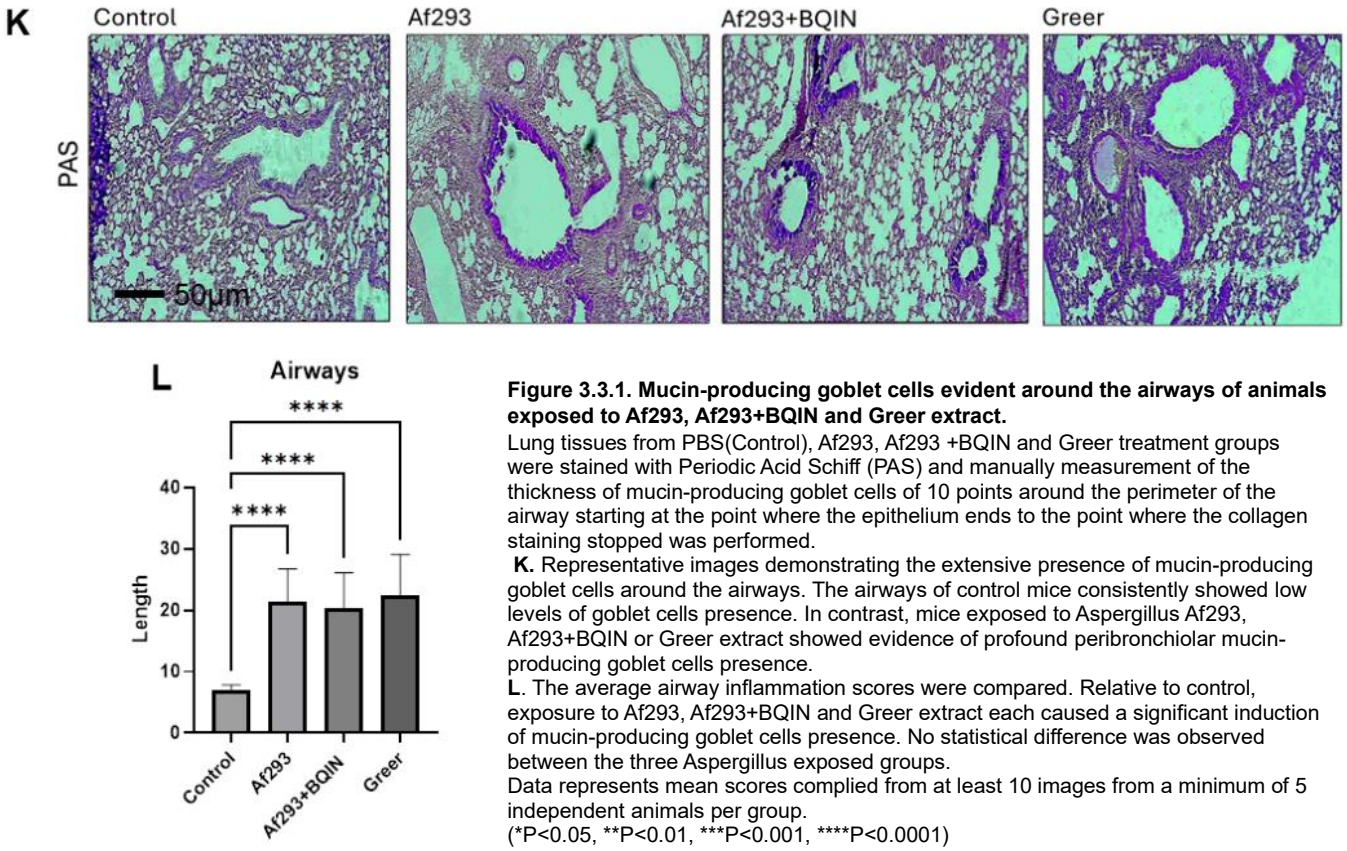

Figure 3.3.1. Mucin-producing goblet cells evident around the airways of animals exposed to Af293, Af293+BQIN and Greer extract.
Lung tissues from PBS(Control), Af293, Af293 +BQIN and Greer treatment groups were stained with Periodic Acid Schiff (PAS) and manually measurement of the thickness of mucin-producing goblet cells of 10 points around the perimeter of the airway starting at the point where the epithelium ends to the point where the collagen staining stopped was performed.
K. Representative images demonstrating the extensive presence of mucin-producing goblet cells around the airways. The airways of control mice consistently showed low levels of goblet cells presence. In contrast, mice exposed to Aspergillus Af293, Af293+BQIN or Greer extract showed evidence of profound peribronchiolar mucin-producing goblet cells presence.
L. The average airway inflammation scores were compared. Relative to control, exposure to Af293, Af293+BQIN and Greer extract each caused a significant induction of mucin-producing goblet cells presence. No statistical difference was observed between the three Aspergillus exposed groups.
Data represents mean scores complied from at least 10 images from a minimum of 5 independent animals per group.
(*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

3.4 *Aspergillus fumigatus* induced *in vitro* epithelial cytokine induction.

Existing literature has observed previously that *Aspergillus fumigatus* conidia and spores can inhibit production of pro-inflammatory cytokines like IL-6, IL-8 and ET-1 in epithelial cells. Originally studies focused on cytokine induction produced by epithelial cells in response to spore inhalation from the allergen. The focus of experiments on the cell lines 16HBE and A549 arose from the fact that they both have distinctive characteristics and represent both bronchial and alveolar epithelium's structure and function. Use of these cell lines allows comprehensive understanding around lung epithelium response to *Aspergillus fumigatus* and underlying mechanisms (Cozens et al.,1994). More recent studies have attempted to assess the effect of allergen strains on cytokine induction. Up to date, IL-6 and IL-8 induction after treatment with inhalation and strain B5233 and there is no evidence of ET-1 being examined in response to the allergen on the specific cell line. Regarding 16HBE, IL-6, IL-8 and ET-1 induction have been quantified in response to *Aspergillus fumigatus* conidia and spores of Af293 strain but not in culture filtrates of Af293 or Cea10.

To assess the impact of exposure to *Aspergillus fumigatus* filtrates on the induction of proinflammatory cytokines IL-6 and 8 as well as the profibrotic growth factor Endothelin-1, experiments were conducted in both 16HBE and A549 cells. Submerged cultures were exposed to culture filtrates derived from Af293 and Cea10.Statistical analysis by one way ANOVA revealed a significant effect of treatment group. Compared to controls, 16HBE showed significant ET-1 upregulation in response to Af293 ($P<0.0001$) with significantly lower levels observed in Cea10 ($P < 0.0124$). On A549 cells post hoc tests revealed significant ET-1 upregulation in response to Af293 ($P<0.0001$). However, in response to Cea10 ($P <0.9990$) results revealed no significant upregulation.

Relative to controls, 16HBEs showed significant IL-6 upregulation in response to Af293 ($P<0.0001$) and similarly to Cea10. On A549 cells, post hoc tests revealed significant IL-6 upregulation in response to Af293 ($P<0.0001$) with a slightly lower level of induction after Cea10 treatment($P<0.0011$). Finally,

compared to controls, 16HBE cells showed significant IL-8 upregulation in response to Af293 (P<0.0001) with a lower level of induction observed in Cea10 (P <0.0011). Similarly, on A549 cells, post hoc tests revealed significant IL-8 upregulation in response to Af293 (P<0.0001) with a lower level of induction observed in Cea10 (P<0.0013) (Figure 3.4.1).

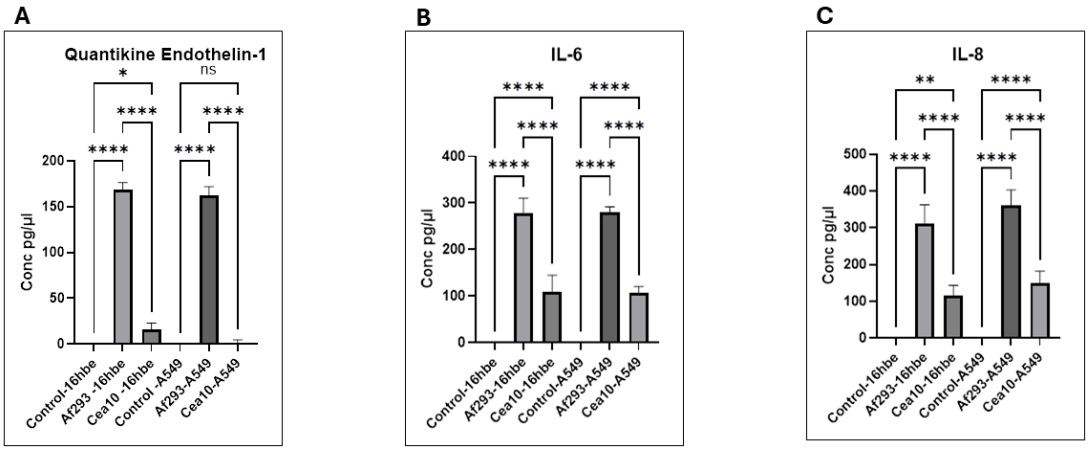

Figure 3.4.1. Upregulation of ET-1, IL-6 and IL-8 in response to *Aspergillus fumigatus* filtrates: Af293 and Cea10 in transformed cell lines.
A549 and 16HBE cells were exposed to control media and media containing 10µg of Af293 and Cea10 derived culture filtrates for 48 hours and cell culture supernatants assessed for presence of ET-1, IL-6 and IL-8. **A.** Representative graph of ET-1 induction after treatment with *Aspergillus fumigatus* strains Af293 and Cea10 from both 16HBE and A549 cells, as assessed by ELISA. **B.** Representative graph of IL-6 induction after treatment with *Aspergillus fumigatus* strains Af293 and Cea10 from both 16HBE and A549 cells, as assessed by ELISA**. C.** Representative graph of IL-8 induction after treatment with *Aspergillus fumigatus* strains Af293 and Cea10 from both 16HBE and A549 cells, as assessed by ELISA. Data represents mean ± SEM, (n=4, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

3.5 The impact of co-exposure to *Aspergillus fumigatus* and fibrin on ET-1, IL-6 and IL-8 induction by human bronchial epithelial cells.

Previous work has observed the correlation between *Aspergillus fumigatus* strain Af293 and key profibrogenic growth factors like TGFβ1, TGFβ2, periostin and Endothelin-1. Results have indicated their upregulated induction by primary airway epithelial cells. It is now well observed that upregulation of VEGF and Endothelin-1 in the asthmatic airway may mediate vascular leak and trigger plasma proteins deposition such as fibrin. That being the case we have decided to set up a model of co exposure of fibrin and *Aspergillus*

fumigatus tested on bronchial epithelial cells, to assess induction of pro-inflammatory cytokines like IL-6, IL-8 and ET-1.

To assess the impact of exposure to *Aspergillus fumigatus* filtrates on profibrotic growth factors, 16HBE cells have been treated with two different strains: Af293 and Cea10 and a co-exposure model of the filtrates and fibrin has been set us. Next, samples were collected, and IL-6, IL-8 and QUANTIKINE ET-1 ELISA were conducted.

Initial statistical analysis by one way ANOVA revealed a significant effect of treatment group. Compared to controls, post hoc tests revealed significant ET-1 upregulation in response to Af293 (*P<0.0001*) with a similar level of presence observed in Cea10 (*P < 0.0001*), in response to Fibrin ,expression of ET-1 reached significance of (*P=0.0003*), in response to Fibrin and Af293 significance of (*P=0.0001*) and in response to Fibrin and Cea10, expression of ET-1 reached significance of *(P =0.0012*). Compared to controls, post hoc tests revealed significant IL-6 upregulation in response to Af293 (*P <=0.0079*) with a similar level of presence observed in Cea10 (*P =0.0027*), in response to Fibrin ,expression of IL-6 reached significance of (*P=0.0038*), in response to Fibrin and Af293 significance of(*P=0.0062)* and in response to Fibrin and Cea10, expression of IL-6 reached significance of (*P =0.0002*). Finally, compared to controls, post hoc tests revealed significant IL-8 upregulation in response to Af293 (*P<0.0001*) with a similar level of presence observed in Cea10 (*P < 0.0001*), in response to Fibrin ,expression of IL-8 reached significance of (*P<0.0001*), in response to Fibrin and Af293 significance of (*P=0.0001*) and in response to Fibrin and Cea10,expression of IL-8 reached significance of (*P =0.0001*) Results indicate that there was no difference between both strains Af293 and Cea10 *(*Figure 3.5.1).

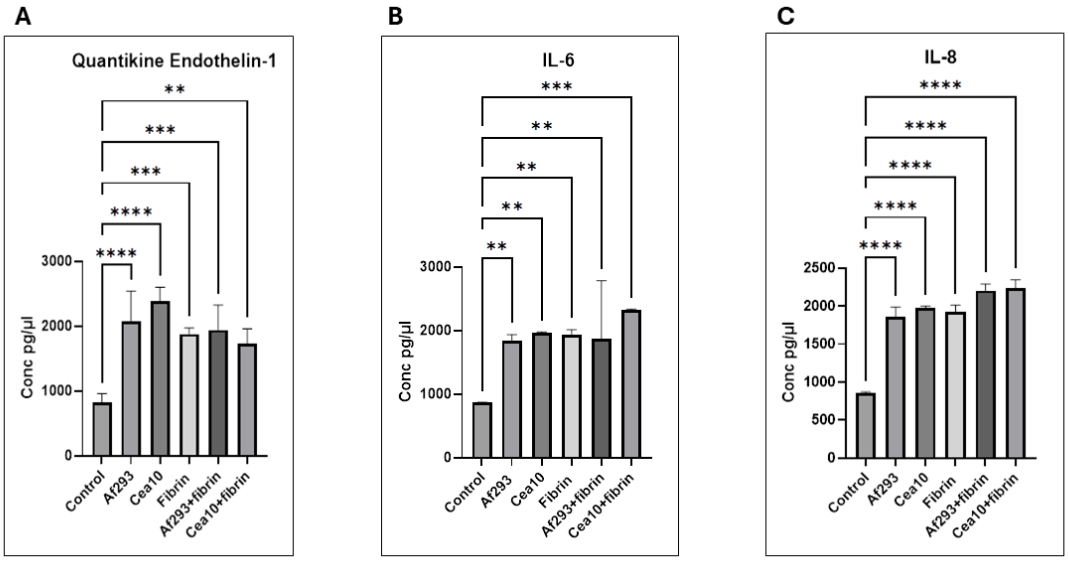

Figure 3.5.1. Upregulation of ET-1, IL-6 and IL-8 in response to co-exposure of fibrin with *Aspergillus fumigatus* filtrates: Af293 and Cea10 in bronchial epithelial cells.
16HBE cells were exposed to control media and media containing 10µg of Af293 and Cea10 derived culture filtrates for 48 hours and cell culture supernatants assessed for presence of ET-1, IL-6 and IL-8. **A.** Representative graph of ET-1 induction after treatment with *Aspergillus fumigatus* strains Af293 and Cea10, fibrin and co exposure model from 16HBE, as assessed by ELISA. **B.** Representative graph of IL-6 induction after treatment with *Aspergillus fumigatus* strains Af293 and Cea10, fibrin and co exposure model from 16HBE cells, as assessed by ELISA**. C.** Representative graph of IL-8 induction after treatment with *Aspergillus fumigatus* strains Af293 and Cea10, fibrin and co exposure model from 16HBE cells, as assessed by ELISA. Data represents mean ± SEM, (n=4, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

3.6 The impact of *Aspergillus fumigatus* strains on cell detachment and bronchial epithelial cell morphology.

As mentioned,16HBE cells were treated with either *Aspergillus fumigatus* Af293 and Cea10 strains, in the presence of absence of fibrin. Images of cultures were collected at 0,24 and 48 hours post treatment to characterize cell morphology. Measurements of cell length and total area covered in the wells, an indicator of cell detachment and/or death were quantified using image J. Initial macroscopic observations revealed a significant effect of treatments on cell morphology and detachment (Figure 3.6.1).

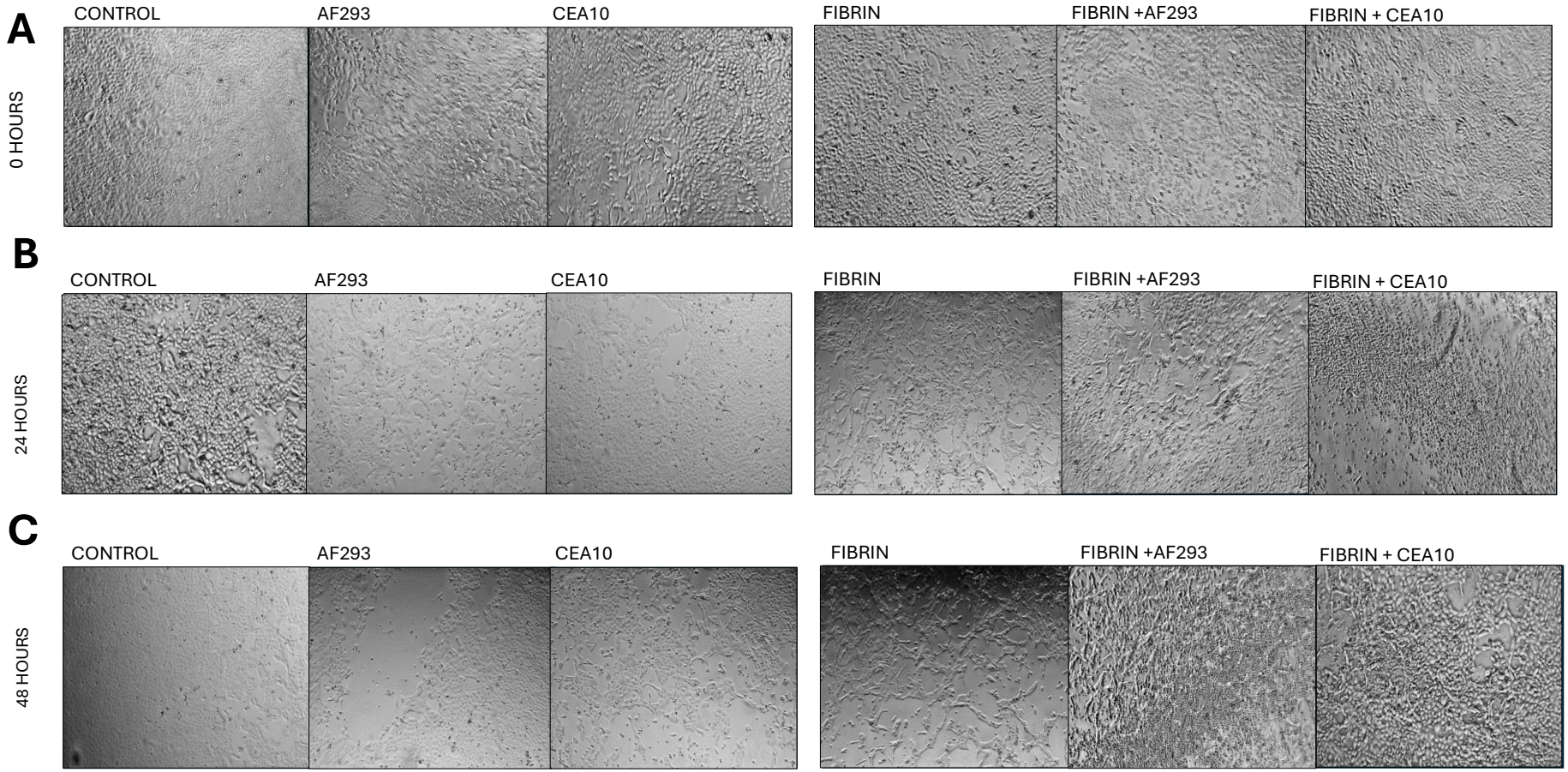

Figure 3.6.1. Evidence of cell viability being reduced and stretching of the cells after exposure to Af293, Cea10 and fibrin co exposure model, revealing a proposed tense of the epithelial cells undergoing EMT. **A.** Representative microscope images of 16HBE cells at 0 hours of treatment with *Aspergillus fumigatus* strains Af293 and Cea10, fibrin and co exposure model. **B.** Representative microscope images of 16HBE cells at 24 hours post treatment with *Aspergillus fumigatus* strains Af293 and Cea10, fibrin and co exposure model**. C.** Representative microscope images of 16HBE cells at 48 hours post treatment with *Aspergillus fumigatus* strains Af293 and Cea10, fibrin and co exposure model.

16HBE cells were exposed to control media and media containing 10μg of the strains Af293 and Cea10 as well as the fibrin co exposure model that has been prepared. Cells were treated and incubated for 48 hours. Pictures were collected at three different timelines: 0,24 and 48 hours post treatment. At 0 hours as displayed in picture A, a monolayer of the human bronchial epithelial cells can be seen in all different treatment groups images indicating normal growth of cells. However, at 24 hours as observed in picture B, the control image only remains the same. Microscopic images for all other treatment groups indicate cell stress as cells can be clearly seen floating on the surface of the wells creating empty spaces in the monolayer and specifically on the fibrin image, morphology of cells appears to be changed. At 48 hours, the same pattern can be observed with control image retaining its full functionality and rest treatment groups presenting clusters of dead cells, monolayer disruption and morphological changes as cells appear stretched indicating the possibility of an epithelial-to-mesenchymal transition pattern.

Initial microscopic inspection at 0,24 and 48 hours post treatment, suggested that compared to controls, Af293, Cea10, Fibrin, Af293+Fibrin and Cea10+Fibrin exposed cells displayed evidence of cell diameter being upregulated (diameter significantly enlarged) and showed evidence of cell death as cells at 24 and 48 hours post treatment have been found detached from the wells and floating.

Statistical analysis confirmed the initial findings. Regarding the cell diameter, compared to controls, it was significantly lengthened after exposure to Af293(*P<0.0001),* Cea10 *(P<0.0001),* Af293+Fibrin(*P=0.0003*) and Cea10+Fibrin(*P<0.0001*). However, treatment with Fibrin (*P=0.8517*) did not show any significant effect on monolayer formation and cell viability. These results can indicate the possibility of bronchial cells undergoing Epithelial-to-Mesenchymal-Transition (EMT) after exposure to *Aspergillus fumigatus* proteases. Although there is supporting evidence to indicate the significance of results, this might have been an outcome due to cells reactions to stress conditions (Figure 3.6.2).

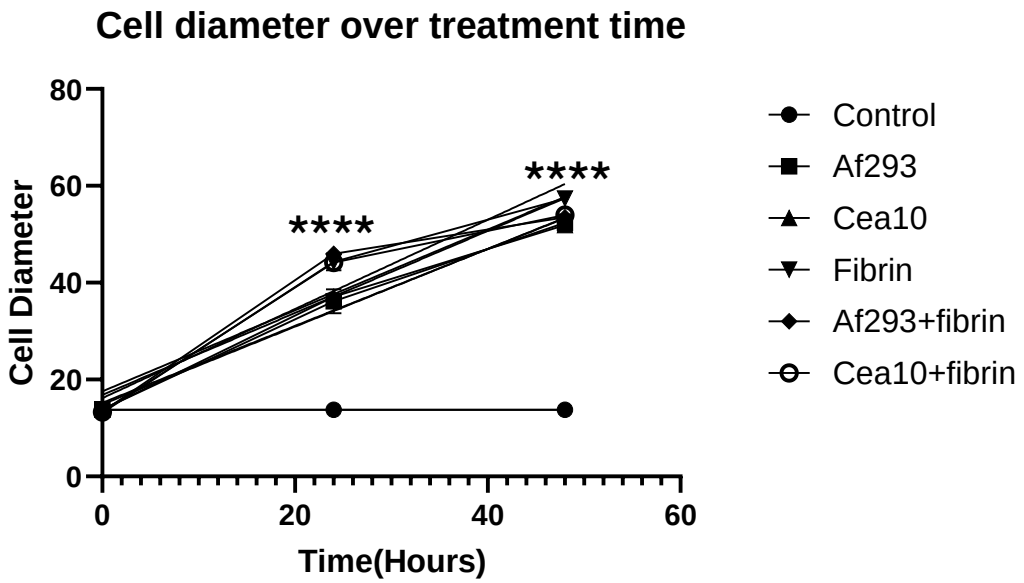

Figure 3.6.2. Diameter of cells increased in response to AF293 and CEA10 with or without Fibrin's presence in 16HBE at 24 and 48 hours post treatment.

16HBE cells were exposed to control media and media containing 10µg of the strains Af293 and Cea10 as well as the fibrin clot that has been prepared and a combination of both isolates and fibrin accordingly. Cells were treated and incubated for 48 hours. Microscopic images have been collected at all three different time points for observation. Using the appropriate tool on image J Software, manual measurements on the cells length (µm) have been conducted. Statistical analysis using one way ANOVA and post-hoc tests was carried out for the significance of results. The representative graph above indicated the transition of cells length at all three different timeframes for all six treatment groups except control. It can be clearly seen that with progression of treatment time there was also observed increased of the cell diameter in all treatment groups except control that has remained the same through all three timeslots.

(n=4, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

Regarding the cell death, compared to controls, it was significantly upregulated after exposure to Af293 (P<0.0001), Cea10 (P<0.0001), Fibrin(P<0.0001), Af293 +Fibrin (P=0.0003) and Cea10+Fibrin (P<0.0001) These results can indicate the possibility of bronchial cells dying and floating on the surface after exposure to *Aspergillus fumigatus* proteases and fibrin. (Figure 3.6.3).

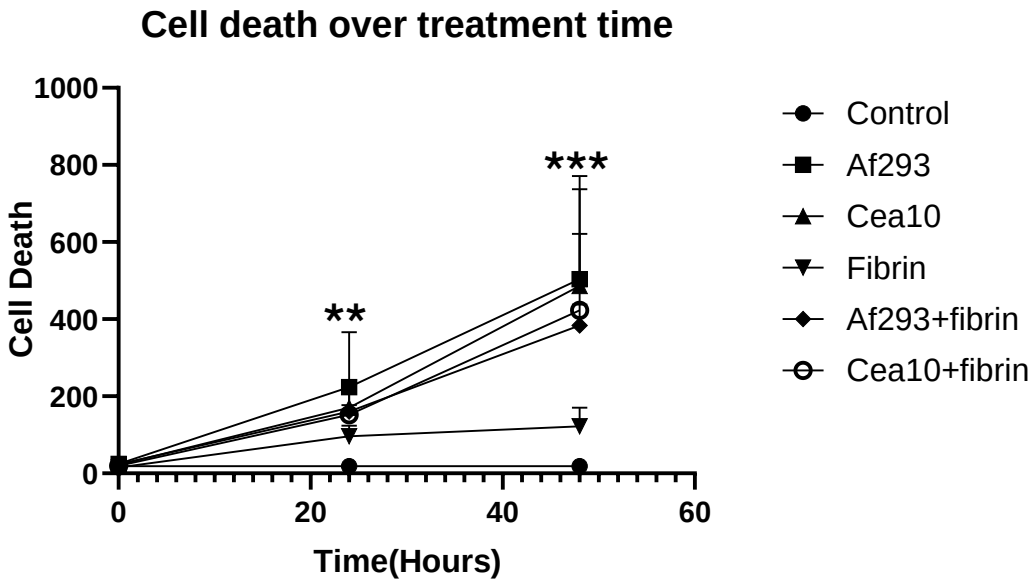

Figure 3.6.3. Death of cells increased in response to AF293 and CEA10 with or without Fibrin's presence in 16HBE at 24 and 48 hours post treatment.
16HBE cells were exposed to control media and media containing 10µg of the strains Af293 and Cea10 as well as the fibrin clot that has been prepared and a combination of both isolates and fibrin accordingly. Cells were treated and incubated for 48 hours. Microscopic images have been collected at all three different time points for observation. Using the appropriate tool on image J Software, manual measurements on the monolayer surface that was free of cells were conducted. Statistical analysis using one way ANOVA and post-hoc tests was carried out for the significance of results. The representative graph above indicated the transition of cells monolayers at all three different timeframes for all six treatment groups except control. It can be clearly seen that with progression of treatment time there was also observed increased of the cell death in all treatment groups except control that has remained similar through all three timeslots.
(n=4, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

3.7 *Aspergillus fumigatus* culture filtrates and extract cause airway and blood vessels fibrin deposition.

Evidence suggests that ET-1 upregulation in the asthmatic airway can promote deposition of plasma proteins like fibrin. Dysregulation of the fibrinolytic pathway in patients with severe asthma has been found to trigger fibrin deposition. High concentrations of Fibrin(ogen) deposition have been identified to impair EC layer integrity by affecting endothelial tight junction proteins and confirm the so far understanding of it playing a key role in the acute phase response caused by tissue injury. A recent study has indicated that fungal proteases may generate fibrin cleavage products which play an important role in driving airway inflammation and remodelling and also promoting vascular leakiness.

We examined tissue mouse model lung sections stained by Carstair's staining to identify possible evidence of fibrin deposition around the airway and

parenchyma as well as blood vessel leakages in response to *Aspergillus fumigatus* exposure.

Initial statistical analysis by one way ANOVA revealed a significant effect of treatment group ($P < 0.0001$) on average inflammation score. Compared to controls, post hoc tests revealed that significant fibrin deposition was evident around the airways and blood vessels of animals exposed to AF293 ($P < 0.0122$) with an elevated level of deposition observed in those animals exposed to AF293+BQIN ($P < 0.0005$), suggesting that Endothelin-1 receptor antagonism had not diminished airway inflammation. Compared to control animals, Greer extract caused a significant induction of inflammation ($P < 0.0002$), and this was greater to that seen in response to in-house generated filtrates (Figure 3.7.1).

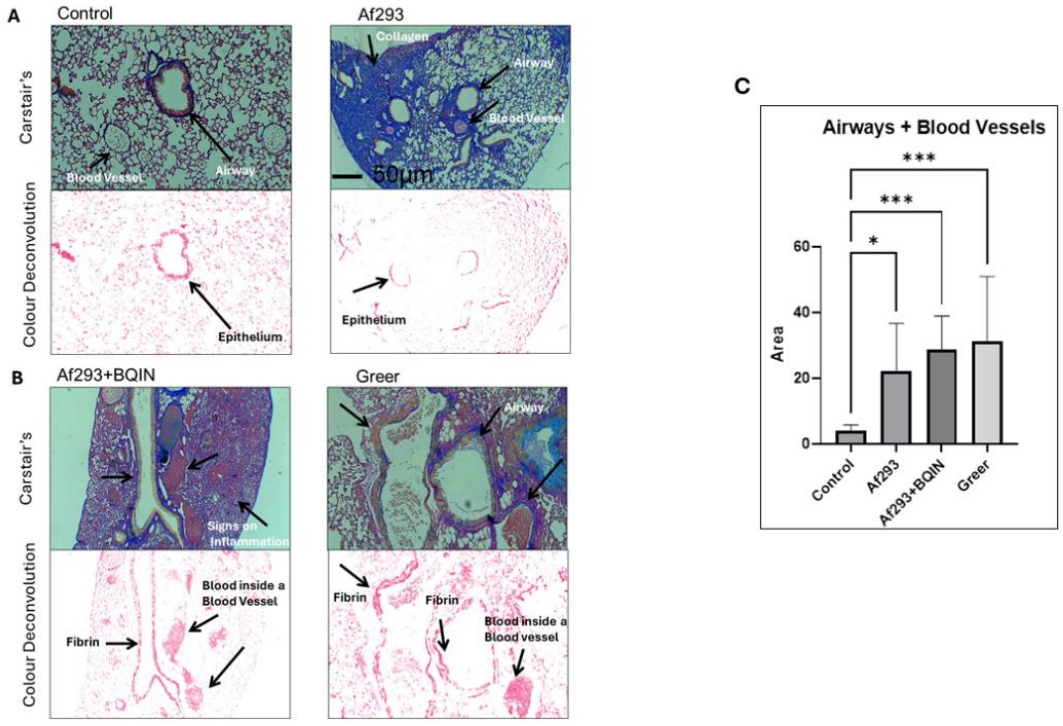

Figure 3.7.1. Fibrin deposition signs around the airways and blood vessels of animals exposed to Af293, Af293+BQIN and Greer extract.
Lung tissues from PBS(Control), Af293, Af293 +BQIN and Greer treatment groups were stained with Carstair's staining for fibrin and platelets and colour deconvolution tool on Image J was used for identification of characteristics around the airways and blood vessels. **A.** Representative images demonstrating characteristics of airways and blood vessels for control and Af293 treatment groups. No signs of fibrin deposition have been indicated**. B.** Representative images demonstrating characteristics of airways and blood vessels for Af293+BQIN and Greer treatment groups. Black arrows demonstrate evident fibrin deposition. **C.** Graph demonstrating the one-way ANOVA and post-hoc tests statistical analysis of colour deconvolution findings indicating that compared to controls, Af293+BQIN and Greer extract caused the most significant results.
Data represents mean scores complied from at least 10 images from a minimum of 5 independent animals per group.

(*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

3.8 The assessment of selected miRNAs by bronchial epithelial cells exposed to *Aspergillus fumigatus*.

Micro(mi) RNAs interfere with pro-inflammatory and pro-fibrotic factors. Existing literature on miRNAs expression in response to lung fibrosis and treatment with *Aspergillus fumigatus* still remains limited. We have decided to examine a variety of 8 different miRNAs, after treatment of human bronchial epithelial cells (16hbe) with two different strains of the allergen Af293 and Cea10 at 10 μg concentration.

Initial statistical analysis by two-way ANOVA revealed no significant effect of treatment group compared to control for miR-9-5p, miR-223-5p, miR-155-5p and miR-21-5p. However, compared to controls, post hoc tests revealed significant miR-320a-3p downregulation in response to Af293 ($P<0.0001$) with a similar level of presence observed in Cea10 ($P < 0.0001$). For miR-130b-3p, compared to controls, post hoc tests revealed significant downregulation in response to Af293 ($P<0.0001$) with a similar level of presence observed in Cea10 ($P < 0.0001$). For miR-223-3p, compared to controls, post hoc tests revealed significant downregulation in response to Af293 ($P<0.0039$) with a decreased level of presence observed in Cea10 ($P < 0.104$). Finally, for miR-34a-5p, compared to controls, post hoc tests revealed significant downregulation in response to Af293 ($P<0.0001$) with a similar level of presence observed in Cea10 ($P < 0.0001$) (Figure 3.8.1).

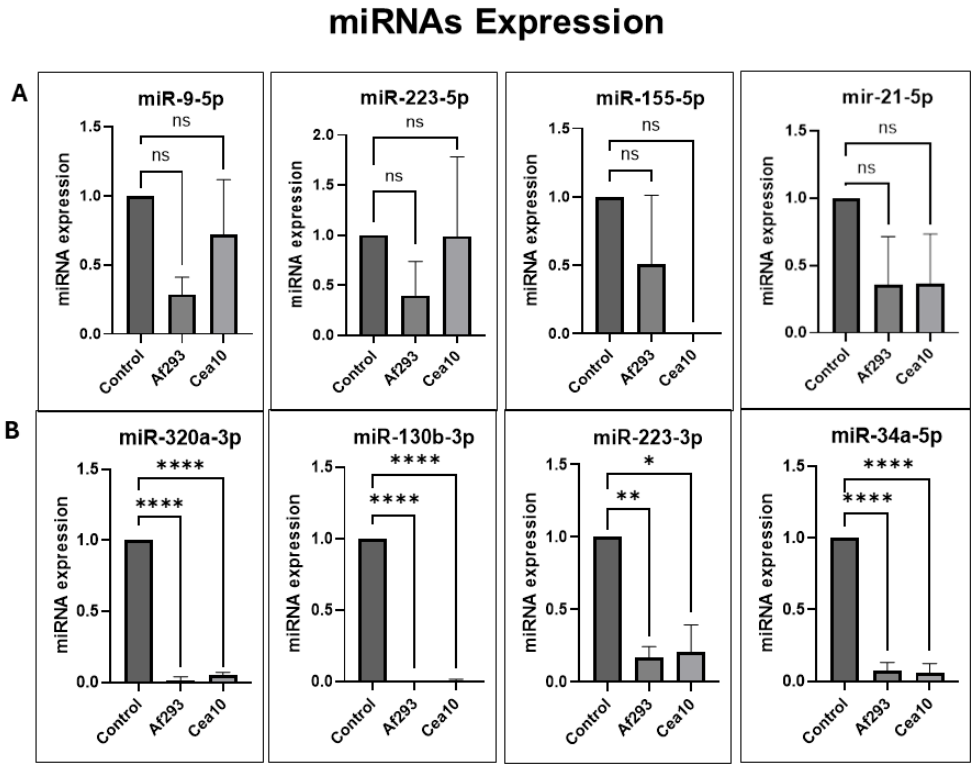

Figure 3.8.1. Downregulated expression of miR-9-5p, miR-223-5p, miR-155-5p, miR-21-5p, miR-320a-3p, miR-130b-3p, miR-223-3p and miR-34a-5p after treatment with both *Aspergillus fumigatus* strains.
16HBE cells were exposed to control media and media containing 10µg of the strains Af293 and Cea10. Cells were treated and incubated for 48 hours. RT-PCR was conducted in two runs and U6 has been used as a reference gene. Statistical tests like two-way ANOVA and post hoc tests were carried out for the data analysis of the results.
B. Representative images of miRNA expression of miR-320a-3p, miR-130b-3p, miR-223-3p and miR-34a-5p that revealed downregulated expression after treatment with both *Aspergillus fumigatus* strains Af293 and Cea10 and results revealed statistical significance compared to controls.

(n=2, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). For the results to be significant P <0.0001.

For the observation of the results ΔΔCT method has been used.

3.9 Trans Epithelial Electrical Resistance (TEER) of Air Liquid Interface (ALI) cultures declines in response to *Aspergillus fumigatus*.

Human airway epithelial cell lines, such as 16HBE, are widely used for the establishment of an Air Liquid Interface (ALI) since they have the ability to form polarized monolayers with tight junctions. We set out to develop a 3D airway model for the assessment of fibrotic responses to Aspergillus and fibrin. To this end, we began by culturing epithelial cells at ALI.

Once hanging insert cultures were setup, TEER was measured on day 0, 4, 8, 11, 15, 18, 22, 25, 28 and 30 to assess developments in membrane polarity. At the 30-day time point, some cells were treated with *Aspergillus fumigatus* AF293 strain for 24 hours and changes in TEER assessed. Results showed

that TEER gradually increased during the differentiation process reaching values of 1.000 Ω. However, after introduction of Aspergillus fumigatus on day 30, values decline to an average of 0.0331 (P value). These results suggest that ALI culture of bronchial epithelial cells is susceptible to a rapid loss of epithelial barrier function after exposure to *Aspergillus fumigatus* (Figure 3.9.1).

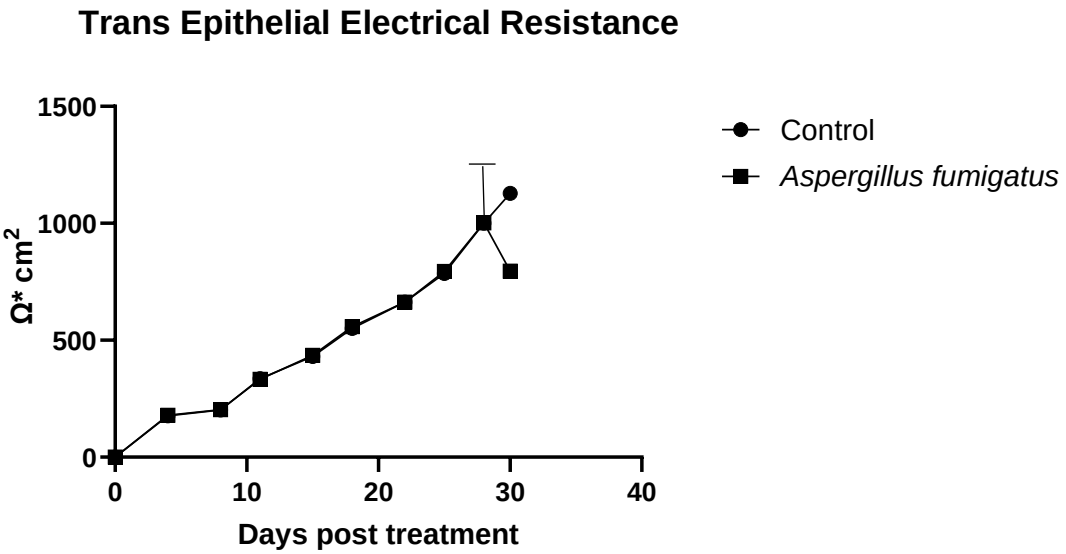

Figure 3.9.1. Trans Epithelial Electrical Resistance (TEER) of 16hbe cells decreased after treatment with Aspergillus fumigatus strain AF293 on Air Liquid Interface (ALI).
Seeded at 6x 10^4 cells per, TEER, was measured as described in the Materials and Methods section beginning on day 4 post-seeding. Data shown are mean ± standard error of mean. The data are composites of multiple time courses, n=4 cell layers for days 4, 8,11,15,18,22,25,28 and 30. At day 30 *Aspergillus fumigatus* strain AF293 at 10 µg concentration has been introduced for 24 hours TEER measurements indicated a decrease of values after treatment with the allergen indicating loss of barrier function and integrity.

4.DISCUSSION

In the current study we observed a significant induction of airway and blood vessel inflammation and subepithelial fibrosis in mice exposed to *Aspergillus fumigatus* filtrates and extracts like Af293, Af293+BQ-123(ET-1 Antagonist) and Greer. We also observed evidence of mucus secretion and goblet cell hyperplasia in airways after exposure to *Aspergillus fumigatus* filtrates and extracts relative to control animals. Surprisingly, there was no protective effect of the ET-1 antagonist BQ123 on these pathological features. Interestingly Aspergillus fumigatus exposure was associated with evidence of fibrin deposition and BV leakage. *In vitro, Aspergillus fumigatus* caused an induction of pro-inflammatory cytokines IL-6, IL-8 and ET-1 in both 16HBE and A549 cell lines. We also explored the impact of an artificial clot on a co-exposure model with the pathogen and found that it also induced cytokines IL-6, IL-8 and ET-1. The co-exposure model also revealed impact of *Aspergillus fumigatus* on cell detachment and cell morphology. We went on to examine the miRNAs expression following exposure to two different strains of *Aspergillus fumigatus*, Af293 and Cea10 and observed that in general miRNAs of interest such as miR-320a-3p, miR-130b-3p, miR-223-3p and miR-34a-5p were significantly downregulated. Progress was also made towards the development of a 3D airway modelling, starting with the growth of 16HBEs at ALI. In these cultures, exposure to *Aspergillus fumigatus* caused a significant decrease in TEER.

Extensive evidence suggests that airway wall remodelling is a significant pathological feature of severe asthma with fungal sensitisation(Holand & Denning, 2011). Findings from CT scans observed bronchial wall thickening, bronchiectasis, mucus plugging, decreased lung attenuation and gas trapping (Patyk,2020). Several animal models have shown that *Aspergillus fumigatus* can drive AWR. More specifically, Porter et al., exposed mice intranasally to *Aspergillus fumigatus* conidia for 18 days an observed airway hyperreactivity and eosinophilia suggesting adherent conidia driving inflammatory responses (Porter, 2011). Another aerosol exposed murine model, suggested peribronchiolar inflammation and evidence of Th2

sensitisation, subepithelial fibrosis and goblet cell hyperplasia (Buskirk, 2014).

Endothelin-1 has previously been shown to be upregulated in the airways of asthma patients (Zietkowski, 2007). Previous research carried out by Labram et al. in 2019, showed that pre-treatment with BQ-123 reduced the extent of peribronchiolar inflammatory infiltration, airway remodelling and collagen deposition in response to *Aspergillus fumigatus* culture filtrates and extracts (Labram et al.,2019). These results suggested that BQ-123 has the ability to successfully diminish the inflammatory response and subepithelial remodelling induced by *Aspergillus fumigatus* filtrates and extracts. In order to assess the impact of *Aspergillus fumigatus* filtrates and extract on airway inflammation and remodelling, tissue sections retrieved from male mice repeatedly exposed to Af293, Af293+BQIN and Greer extract were selected for histological analysis. Surprisingly, ED-1 antagonism had no significant effect on both airways and blood vessels pathology. The difference of results obtained, may be due to differences in tissue sections, airway sizes and staining protocols. To date, current research on ET-1 proposes the statement that it induces connective tissue growth factor expression in human lung fibroblasts by disrupting HDAC2/Sin3A/MeCP2 corepressor complex (Hua, 2023). Studies have reported that HDAC2 activation could prevent airway remodeling through the suppression of airway inflammation and the modulation of fibroblast activation in COPD and asthma (Lai, 2018). Also, a reduction in the HDAC2 protein level or HDAC2 activity is observed in asthma or COPD patients (Barnes,2009).

In vitro and *in vivo* evidence suggests that ET-1 is able to induce fibrosis and plays an essential role in pathophysiology of lung conditions such as asthma (Rodríguez-Pascual et al., 2014). As mentioned previously, ET-1 binds to receptors ET_A and ET_B on different cell types like endothelial cells,fibroblasts and smooth muscle cells, triggering a cascade of signalling events. These lead to fibroblast activation, differentiation into myofibroblasts and collagen deposition, main processes in fibrosis. Recent studies have shed light to the signalling pathway involved in ET-1 induced fibrosis. Endothelin-1 has been shown to activate MAPK/ERK and P13K/AKT pathways in lung fibroblasts

leading to increased expression of profibrotic genes like collagen type I and III, and collective tissue growth factor (CTGF) (Kawano et al.,2020). Related to asthma, ET-1 has been associated with its pathogenesis in the context of airway remodelling. The peptide, has the ability to induce fibroblast proliferation, collagen synthesis and also contributes to smooth muscle cell hypertrophy and deposition of ECM components. Bagnato et al., in 2019 demonstrated that upregulated levels of ET-1 found in broncoalveolar lavage fluid (BALF) of asthmatic patients correlated with airway fibrosis and reduced lung function (Bagnato et al.,2019). It has been suggested that ET-1 could serve as a potential biomarker of airway remodelling in asthma and can be a target for future therapeutic intervention. Also, it has been well documented that both fibrin and endothelin-1 mediate fibroblast activation and collagen deposition (Shi-Wen, 2004). In addition to ET-1, fibrin has been implicated in lung fibrosis. Fibrin deposition in lung intersistium has been chracterised as a common feature of fibrotic lung diseases. Evidence suggests that ET-1 and fibrin act synergistically to promote fibroblast activationan and collagen synthesis. Studies have indicated that ET-1 and fibrinogen (precursor of fibrin) interaction can enhance profibrotic effects of ET-1 . Endothelin-1 specifically has been shown to increase expression of tissue factor, enhance fibrin formation and deposition (Kumar et al.,2022).

Role of ET-1 in fibrosis and lung diseases has introduced its potential role in a therapeutic target. Research on Endothelin receptor Antagonists (ERAs) has shown promising results regarding pulmonary arterial hypertension, a condition characterised by vascular remodelling and fibrosis. Most recent clinical trials has explored the potential role of ERAs on IPF patients,with results remaining unclear. Although some studies have shown reports of reducing lung fibrosis and improving lung function, other studies have reported no immediate effect (King et al., 2019). Results combined can propose that it might be of benefit not only targeting ET-1 signalling pathways but a combination target of TGF-beta or coagulase cascade.

A key aim of this research project was to develop *in vitro* models that could be used to unpick the mechanism of airway wall remodelling. Towards this work, we began with simple submerged cultures of two different airway epithelial

cell lines which were exposed to *Aspergillus fumigatus*. We observed upregulation of IL-6, IL-8 and ET-1 in both cell lines after 24 hours of treatment. Interleukin-6 levels have been found elevated in asthmatic patients indicating loss of lung function (Neveu et al., 2010). Similarly, IL-8 levels have been found elevated in serum of asthmatic patients proposing use of IL-8 as a potential biomarker in asthma (Zhang & Bai, 2017). Our findings support those of Rowley et al, 2021 indicating that 16HBE cells exposed to Af293 conidia showed upregulated levels of IL-6 and IL-8 compared to controls at 24 hours of treatment (Rowley et al.,2021). Similarly, Chen et al. in 2015 observed IL-6 and IL-8 upregulated induction on A549 after infection with *Aspergillus fumigatus* (Chen et al.,2015). Elevated levels of ET-1 after treatment with *Aspergillus fumigatus* on bronchial epithelial cells, confirming our findings have been also observed from Labram et al. in 2019 (Labram et al.,2019)

Intriguingly, we observed evidence of blood vessel leakage and fibrin deposition in the lungs of mice exposed to Aspergillus *fumigatus.* We hypothesised that the induction of ET-1 may feed into fibrin deposition in response to *Aspergillus fumigatus* exposure. As mentioned previously, ET-1 is a potent vasoconstrictor playing a critical role in vascular homeostasis and pathology and is produced mainly by endothelial cells lining the blood vessels (Kedzierski & Yanagisawa, 2018). It has been reported that under certain conditions like hypertension, vascular smooth muscle cells (VSMCs) can produce ET-1 (ET-1 produced by VSMCs can promote vascular remodelling and fibrosis (Dhaun et al.,2022). An *in vivo* study by Li et al., investigated the effects of ET-1 on vascular permeability. Mice were intravenously administered ET-1 and vascular leakage was investigated. Results demonstrated that ET-1 induced significant vascular leakage (Li et al., 2020). Evidence of ET-1 inducing vascular leakage is linked with endothelial barrier disruption (Kanda et al., 2021). It is known that ET-1 can induce expression of inflammatory cytokines and adhesion molecules compromising the barrier and enhancing permeability. This effect can contribute to pathogenesis of conditions characterised by vascular leakage like acute lung injury or oedema. Fibrinogen has been established to develop vasoconstriction through

production of ET-1 (Lominadze et al., 2005). Pathologically high concentration of fibrinogen has been indicated to impair EC (Endothelial Cells) layer integrity by affecting the endothelial cell tight junctions and increase paracellular transport (Patibandla et al., 2009). In a recent murine model of Transfusion-related acute lung injury, significant fibrin accumulation was observed associated with enhanced coagulation and impaired fibrinolysis (Yu, 2021).

An artificial fibrin clot has been prepared and examined in co-exposure with the allergen. Overall, our findings indicated upregulated levels of IL-6, IL-8 and ET-1, changes on cell detachment and morphology and signs of fibrin deposition on tissue sections retrieved from a variety of animal models. Recent studies have indicated the role of fibrin deposition in the pathogenesis of aspergillosis and more specifically IPA. Fibrin has extensively been detected in lungs of patients with aspergillosis, contributing to tissue damage and impaired pulmonary function. Findings indicate that *Aspergillus fumigatus* can induce a procoagulant state that leads to excessive fibrin formation and deposition (Bruns et al., 2019). Recent evidence suggests that antifibrinolytic therapies might be critical in mitigating fibrin deposition in aspergillosis. This way tissue damage can be reduced and improve outcomes of IPA patients (Sheppard et al.,2016). To date, the literature on the topic remains limited

After, our study focused on the molecular biology. Dysregulation of miRNAs has been reportedly linked with severe lung diseases such as asthma and can potentially serve as biomarkers for the development or progression of a pulmonary disease (Brown et al. 2014). For the assessment of the miRNAs expression involved in pulmonary and lung fibrosis as well as eosinophilia and IPF , 8 different miRNAs have been selected and tested using the RT-PCR method. These specific miRNAs have been targeted due to the fact that they are involved in pathological conditions linked with asthma and *Aspergillus fumigatus.* Results have been analysed using two-way ANOVA as there were no previously identified expected results. The literature regarding miRNAs expression on epithelial cells after treatment with *Aspergillus fumigatus* remains limited, indicated a gap of knowledge on the field. We have chosen to examine the specific miRNAs as they have been documented in pulmonary and lung fibrosis. Results revealed that miR-9-5p, miR-223-5p, miR-155-5p

and miR-21-5p have been found downregulated with no significant effect compared to controls and miR-320a-3p, miR-130b-3p, miR-223-3p and miR-34a-5p have been found downregulated as well but with results significant to control. Regarding miR-9-5p our results contrast the ones proposed by Fierro-Fernández et al. that observed upregulated levels of miR-9-5p (Fierro-Fernández et al., 2015). However, contrast of results can be due to the fact that this experiment was conducted in vivo including a mouse model of bleomycin-induced lung fibrosis. Furthermore, miR-223 has been the only so far known miRNA to have been examined in response to *Aspergillus fumigatus*. Our results have indicated that miR-223-5p and miR-223-3p have been both found downregulated but only miR-223-3p revealed significant results compared to controls. Findings from Collison et al, revealed upregulated miR-223 levels on Eosinophilic esophagitis (EoE) induced mice and biopsy samples (Collison et al., 2020). Differentiation of results possibly occurred due to the experimental design set up. Another miRNA that we chose to examine regarding its correlation to the pathophysiology of asthma and lung fibrosis is miR-155, that its expression has been consistently found upregulated. Findings from Eissa and Artlett et al, observed upregulated expression of miR-155 on Wild-type and NLRP3-deficient murine fibroblasts (Eissa & Artlett, 2019). Our results differed from the proposed findings as the experiment was set up on human bronchial epithelial cells treated with *Aspergillus fumigatus*. After, expression of miR-21-5p has been found to be upregulated in IPF patients as proposed by Liu et al in 2010, contrasting our initial findings (Liu et al., 2010). This may have been due to the fact that we have assessed the expression *in vitro* whereas Liu findings have been assessed *in vivo.* On the other hand, miR-320a-3p levels have been found downregulated having significant effect compared to controls. These findings were confirmed from a murine model of 6 mice involved with IPF as observed from Sun et al. Similar were the results observed for miR-130b-3p that were confirmed from S.Li et al that examined microarrays of lungs of 4 IPF patients compared to control lungs (S. Li et al., 2016). Finally, miR-34a-5p expression has been found downregulated after treatment with *Aspergillus fumigatus*, results differentiated from the ones proposed from Cui et al. that stated upregulated expression in human and mouse lung myofibroblasts, indicating a

potential role in lung fibroblasts senescence (Cui et al., 2017). Variety of results could, as mentioned before, be due to the differentiated experimental design and techniques used. To date, not known miRNA has been acknowledged as a biomarker on pulmonary diseases. Research is still on early stages and findings lack reproducibility (Condrat et al., 2020).

Lastly, our research was directed towards the setup of a 3D model on epithelial cells. This kind of model has recently been established, overcoming limitations from 2D cell culture models (Francis et al., 2022). Results obtained from our study observed reduced TEER measurements after treatment of the ALI model of 16HBE cells, with *Aspergillus fumigatus.* Findings from Dunne et al in early 2023, had observed declined TEER measurements after treatment with Af293 in HBEs suggesting disruption of TJs, confirming our initial results (Dunne et al.,2023)

In summary, our in *vivo* and *in vitro* findings on the underlying mechanisms of the pathophysiological effects of treatment with *Aspergillus fumigatus*, provide valuable insights and confirm the existing literature. All these observations have been of great importance investigating in depth the role of *Aspergillus fumigatus* in AWR, cytokine induction and miRNAs expression contributing to the limited available literature. However, limitations still apply on interpretation of results. Firstly, for in vitro experiments except from A549 and 16HBE cells, primary cells have been suggested to be good indicators of respiratory disease and they could have also been assessed in regards to *Aspergillus fumigatus.* Additionally, 2D cell cultures might have been widely used but as proposed by Qi Tal they have implicated a lot of limitations in terms of production of toxic waste and failure of complexity reproducibility of the respiratory epithelium as they usually comprise of only one cell line (Qi Tal, 2019). This was one of the main reasons why we decided to set up an ALI model, which is categorised as a 3D model known to overcome 2D limitations. However, still in 3D models organoid development and lack of control and reproducibility appear to be factors for consideration. Regarding animal models, a lot of research has focused on them, and exciting findings have been announced. To date, ethical considerations are still arising along with

their proposed in ability to accurately reproduce human respiratory physiology and pathophysiological features (Buckley et al., 2018)

Despite, our results providing a better understanding of *Aspergillus fumigatus* mechanisms, excessive research is needed to elucidate the existing gap of knowledge. Regarding miRNAs expression, to our knowledge our experimental design was the first to examine the effect of specific strains of *Aspergillus fumigatus* on bronchial epithelial cells. In general miRNAs have been proposed in different diseases as possible biomarkers. In respiratory physiology they have not been well studied and absence of replication of results fails to propose them as a good indicator of a lung pathophysiology. However, future research can include investigation in depth of possible miRNAs observed in lung fibrosis that can shed light and be used as a precautious method. Additionally, as our project was coming to an end, we attempted the setup of an ALI model of 16HBE cells. Due to limited time, the only results we could present included decreased TEER measurements after treatment with *Aspergillus fumigatus.* However, we have attempted some immunofluorescence and live dead staining to examine the effect in depth. Also, we have initiated some work on MRC-5 fibroblasts by OCT embedding so we can represent the complexity and diversity of the respiratory epithelium (protocols followed are included in the appendix section).

That being said, some future directions can include repetition of the attempted immunohistochemistry protocols or set up of an ALI with co-exposure of different cell lines for the accurate representation of lung physiology.These models can be expanded to include immune cells and and other lung cell types, to enhance understanding of pathogen’s impact on the respiratory system and enhance development of disease models. Regarding the observed downregulation of the selected miRNAs, future research can aim on stabilising *in vitro* models to characterise functional implications of these models. Finally, given the observed fibrin deposition and blood vessel leakage, future research should examine coagulation and vascular permeability pathways activated by *Aspergillus fumigatus*.

In conclusion, our study demonstrated that exposure to *Aspergillus fumigatus* culture filtrates and extracts (Af293, Af293+BQIN and Greer) significantly induced inflammation, subepithelial fibrosis, collagen and fibrin deposition and mucus secretion.Of note, ET-1 antagonist (BQIN) did not present a potent effect against the pathogen. *Aspergillus fumigatus* exposure also led to blood vessel leakage. *In vitro* experiments revealed that Aspergillus fumigatus induced production of cytokines like IL-6, IL-8 and ET-1 in both 16HBE and A549 cell lines. Significant downregulation of miRNA expression following treatment with Af293 and Cea10 has been observed. Finally, development of a 3D airway model using 16HBEs grown at ALI indicated that Aspergillus fumigatus plays a critical role in fungal-induced airway pathology highlighting potential therapeutic targets.

5.APPENDIX

Table 7. RNA extraction results using Nanodrop.

TREATMENT GROUP:	A260:	A260/A280 RATIO:	CONCENTRATION (ng/µl):
CONTROL (1)	1.545	2.04	61.8
CONTROL (2)	0.804	2.00	32.2
CONTROL (3)	0.309	1.98	12.3
AF293(1)	1.644	1.86	65.7
AF293(2)	2.475	2.00	99.0
AF293(3)	3.090	2.04	123.6
CEA10(1)	2.635	2.04	105.4
CEA10(2)	2.611	2.00	104.5
CEA10(3)	2.045	2.03	81.8

5.1 MRC-5(FIBROBLASTS) Cell Culture

MRC-5 are fibroblasts isolated from the lung tissue derived from a White, male, 14-week-old embryo by J.P. Jacobs in 1966.). The cell culture was initiated from frozen stock in passage number 8 and was grown under sterile conditions. Cells were grown using MEM media containing 10 % FBS (Foetal Bovine Serum), 1% Penicillin/Streptomycin and 1% L-glutamine. Cells were propagated in T75 cm^2 flasks and incubated in 37 ^{0}C temperature in 5 % CO_2.

5.2 Introducing Fibroblasts to Matrigel

Fibroblasts were grown under sterile conditions in T75 flasks. For the purpose of the experiment, cells were trypsinised and a cell count was performed of 10^4 cells. Matrigel was used and placed in dry ice so the appropriate temperature can be obtained. Using a sterile pipette 100 μl of the gel was applied in some wells of a twenty-four-well plate and the plate was further incubated for two hours. Using a petri dish, two cell inserts (0.4 μm pore size) were turned upside down and 100 μl of the gel were applied. Cell inserts were incubated for a further two hours. When Matrigel was steady in both versions, cells were inserted in the gel and MEM media with all the appropriate supplements was applied. The plate was incubated for a further 72 hours. For the tissue processing, cells were fixed using PFA and were placed in the fridge overnight. The following day the process involved washing of the tissue with 70%, 90% and 100% ethanol and finally histoclear. Using a scalpel the tissue was removed from the cell inserts and was inserted in paraffin overnight. Next day wax embedding was conducted so the tissue can be collected and microtomed so further histological analysis can be performed.

5.3 Immunofluorescence

For the characterisation of the Air Liquid Interface (ALI) model immunofluorescence staining was performed. Firstly, media was removed from the plate and cells were washed with PBS and fixed with ice cold 4%PFA for twenty minutes. Then, the PFA was removed, cells were washed with PBS three times and the block (3%BSA) was applied for thirty minutes at room temperature. After, the block was removed and 100 μl of the antibody was applied for 1 hour. The antibodies used were ZO-1 monoclonal antibody (rabbit), alpha-tubulin (mouse) and mucin(mouse). Cells were again washed three times with PBS and the secondary antibody was applied at 1:400 dilution for forty-five minutes at room temperature. Secondary antibody for ZO-1 was donkey anti-rabbit, for alpha tubulin and mucin was goat anti-mouse. Cells were finally washed three times with PBS and DAPI has been applied for 5 minutes so nuclei can be stained. The plate was then read under the specific microscope.

5.4 Live/Dead Staining

To determine the efficacy of the fibroblasts introduced to Matrigel under the confocal microscope, Live/Dead staining (04511Cellstain double staining kit) was performed. The cells were firstly inserted in the Matrigel that was placed inside 0.4μm pore size trans well inserts in a 24-well plate. First step was the preparation of the assay solution which included the mixture of 10 μl of the solution A with 5 μl of the solution B in 5ml of PBS. The cells were washed several times with PBS so residual esterase activity can be removed. Then,100 μl of the assay solution were added to the cells which were incubated at 37 ^{0}C for fifteen minutes. Fluorescence was detected at 490 nm.

5.5 OCT EMBEDDING

Fibroblasts were grown under sterile conditions in T75 flasks. For the purpose of the experiment, cells were trypsinised and a cell count was performed of 10^4 cells. Matrigel was used and placed in dry ice so the appropriate temperature can be obtained. Using a sterile pipette 100 μl of the gel was applied in some wells of a twenty-four-well plate and the plate was further incubated for two hours. Using a petri dish, two cell inserts (0.4 μm pore size) were turned upside down and 100 μl of the gel were applied. Cell inserts were incubated for a further two hours. When Matrigel was steady in both versions, cells were inserted in the gel and MEM media with all the appropriate supplements was applied. The plate was incubated for a further 72 hours. For the OCT embedding, cells were fixed using 4% PFA, gently to avoid removal of gel, for thirty minutes at room temperature. PFA was removed, gel was washed with PBS and 30% sucrose was introduced in the gel and was incubated at room temperature overnight. Next day, 30% sucrose was replaced with 50/50 30% sucrose/OCT solution and was incubated for a further 4 hours. At this point a tray with dry ice and foil was prepared. Solution was removed from gel and a cryomold was filled up to ½ with OCT solution. Using a scalper gel was removed from cell inserts and placed into the cryomold which was then filled with OCT and placed into the ice tray. When OCT has completely frozen, the sample was microtomed in the cryostat and tissue was placed in polylysine charged slides that were further stained with H&E staining.

REFERENCES: