UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Investigating the role of the gut-lung axis in chronic respiratory diseases: chronic obstructive pulmonary disease (COPD) and severe steroid resistant asthma (SSRA)

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Doctor of Philosophy

Under the supervision of Prof. Phil Hansbro,

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, *Piyush Jha*, declare that this thesis is submitted in fulfilment of the requirements for the award of *Doctor of Philosophy*, in the *School of Life Science/Faculty of Science* at the University of Technology Sydney.

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

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Preface – Contribution by others

In order to provide a cohesive set of experimental data the following sections contain data contributed by others.

Chapter 3 and Chapter 4 – Dr. Sj Shen and Dr. Annalicia Vaughan

Appendix Figure 25 and 26: Dr. Annalicia Vaughan

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List of Abbreviations used in the thesis

AAD: Allergic Airway Disease

AHR: Airway Hyper-responsiveness

BALB/c: an albino, laboratory-bred strain of the house mouse

BALF: bronchoalveolar lavage fluid

CFU: Colony Forming Unit

Cmu: *Chlamydia muridarum*

CRDs*:* Chronic respiratory diseases

COPD: Chronic Obstructive Pulmonary Disease

C57BL/6: C57 black 6; common inbred strain of laboratory mouse

DC: Dendritic cells

DEX: Dexamethasone

FMT: Faecal microbial transfer

FVC: Forced vital capacity

FEV0.1: Forced expiratory volume in 0.11 second

GAPDH: Glyceraldehyde 3-phsophate dehydrogenase

GBD: Global burden of disease

i.n. intranasal

i.p. intraperitoneal

ICS: inhaled corticoids

IgE: Immunoglobulin E

IL-1β: Interleukin -1 beta

IL-5: Interleukin -5

LABA: long-acting beta2 agonists

LAMA: long-acting muscarinic receptor antagonists

LRT: Lower Respiratory Tract

LTRA: leukotriene receptor antagonists

Mch: methylcholine

MDC: Macrophage-derived chemokine

MMP9: Matrix metalloproteinase-9

o.g. oral gavage

OCS: oral corticosteroids

OVA: Ovalbumin

PBS: phosphate buffered saline

qRT-PCR: Quantitative real-time polymerase chain reaction

rCDI: recurrent *Clostridium difficile* infection

SABA: short-acting beta2 agonists

SAMA: short-acting muscarinic receptor antagonists

SCFAs: short chain fatty acids

SPG: Sucrose Phosphate Glutamate Buffer

SSRA: Severe steroid resistant asthma

SSRAAD: Severe steroid resistant allergic airway disease

TARC: Thymus- and activation-regulated chemokine

TSLP: Thymic stromal lymphopoietin

TNF-α: Tumour necrosis factor alpha

URT: Upper Respiratory Tract

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Abstract

Chronic respiratory diseases (CRDs) including chronic obstructive pulmonary diseases (COPD) and severe asthma are very common and both do not effectively respond to corticosteroids therapy. The compositional changes in gut microbial diversity or abundance has been well documented in CRDs. However, the precise effect of modulation of gut microbiota and its effect on CRDs are to be explored further to establish a strong link for therapeutic pathways. In this thesis, I investigated modulation of gut microbiota by faecal microbial transfer (FMT) through oral gavage in experimental COPD and SSRAAD mice models. This thesis concluded, FMT with COPD microbiome in 6 weeks smoke exposure predisposes to COPD (Chapter 3), and in 8 weeks smoke exposure aggravates COPD features (Chapter 4). In SSRAAD, gut microbiome plays a role in airway neutrophilic inflammation (Chapter 5).

Chapter 1 – Literature review

1.1 Overview of chronic respiratory diseases

Chronic respiratory diseases (CRDs) are one of the most frequent non-communicable diseases globally. This is mainly attributable by the noxious environmental, occupational and inhalational behavioural exposures, such as exposure to cigarette smoke and biomass burning [1]. The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) report of 2017 estimated around 545 million people on the planet suffer from a CRDs. CRDs have increased by 39.8% since 1990 and accounted for 3.9 million deaths in 2017, with an increase of 18% since 1990. The most prevalent among the CRDs are the chronic obstructive pulmonary disease (COPD) and asthma (3.9% and 3.6% global prevalence, respectively). COPD and asthma are the top contributors to CRD-related deaths worldwide [2]. Current pharmaceutical treatments from COPD and asthma include bronchodilators and/or corticosteroids. Though there has been substantial progress in the long-term management of most of the CRDs, the immune-pathophysiology of these CRDs remains poorly understood, especially the mechanism behind the development of steroid resistance. Hence, there is an urgent need for novel therapeutics agents to treat disease, particularly for patients with poor response to treatment.

1.2 Chronic Obstructive Pulmonary Disease (COPD)

COPD is characterized based on persistent respiratory symptoms and partially irreversible airflow limitation [3]. COPD is now the 3rd leading cause of death with approximately 300 million cases reported globally and 3.2 million COPD-related deaths in 2017 [1]. COPD is expected to increase in the coming years due to the increasing exposure with environmental risk factors and an aging population [4]. Common risk factors include tobacco smoking, air pollution, smoke from biomass fuels and the genetic susceptibility [5, 6]. Repeated exposure to these environmental inhalants leads to persistent, chronic airway inflammation, resulting in

gradual parenchymal tissue destruction (emphysema) and small airway fibrosis. These underlying pathological changes in COPD causes gas trapping and progressive airflow limitation. COPD symptoms includes shortness of breath, chronic coughing, production of sputum, wheezing, and in some patient's chest tightness and fatigue is also observed. COPD is diagnosed via spirometry testing and is based on the severity of airflow obstruction. There are four progressive stages (I-IV) of COPD what are determined by the severity of airflow obstruction [3]. Similar to asthma, the focus of COPD management is directed towards relaxation of smooth muscles and reduction of airway inflammation.

The foremost management of stable COPD is typically directed to non-pharmacological means such as cessation of smoking, increase in pulmonary rehabilitation, life-style changes and eventually pharmacological therapy [3]. The pharmological arsenal for COPD management is very similar to asthma, comprising of the use of one or a combination of short acting beta-2 agonist (SABA), long acting beta-2 agonist (LABA), short acting muscarinic antagonist (SAMA), long acting muscarinic antagonist (LAMA) or inhaled corticosteroids (ICS), as well as methylxanthines (theophylline), phoshodiesterase-4 (PDE4) inhibitors, mucolytic and, antibiotics [7]. The bronchodilators acts through by relaxing the smooth muscle of the airways and reducing lung hyperinflation. ICS reduced airway inflammation but is ineffective at reducing symptoms when used alone. The use of ICS/LABA combination therapy is often typically beneficial in patients with higher burden of symptom and also high risk of exacerbation [8].

Acute exacerbations of COPD (AECOPD) is described as the sudden worsening of airway function and respiratory symptoms in patients with established COPD [9]. Oral corticosteroids are mostly used in treating the acute exacerbations during emergency department visits and hospitalised patients and has proven to reduce the rate of treatment failure, relapse, improving lung function and breathlessness [10]. AECOPD are generally managed with systematic

steroids and antibiotics along with the inhaled therapy. Though steroids are highly effective in asthma, but are not as effective in suppressing inflammation in COPD. This could be because of the difference in the nature of the inflammation in asthma and COPD, as COPD is neutrophilic and so do the severe steroid resistant asthmatic. Corticosteroids do not supress neutrophilic inflammation. In fact, it prolongs the survival of neutrophil *in vitro* by inhibiting the apoptotic pathways involved [11]. Steroid resistance in COPD is also understood to be associated with the cytokines released by alveolar macrophages that are resistant to corticosteroids [12]. This resistance can partly be explained as an inhibitory impact of cigarette smoking as well as of oxidative stress on histone deacetylases (HDACs) [13], which has been observed in SSRA as well as COPD [14]. Overall, current therapies are only partially effective, hence there is a clear need for novel therapies with an alternate mode of action to improve the treatment of COPD as well as Severe Steroid Resistant Asthma (SSRA) [15].

1.3 Asthma and severe asthma

Asthma is characterized by clinical features with variable respiratory symptoms, expiratory airflow limitation with chronic airway inflammation affecting around 1-18% of the global population. Symptoms of asthma include shortness of breath, wheezing, with tightness of chest and productive cough. These symptoms are inconsistent and vary with time and intensity imposing variable expiratory airflow limitation [16]. The most common trigger for asthma symptoms include allergens, irritant exposure, and exercise, changes in weather as well as viral and/or bacterial infection in the respiratory airways. Once triggered, these asthmatic symptoms and airflow limitation mostly resolve itself over time or in response to pharmaceutical intervention [16].

The symptoms of asthma are a result of two key features: bronchoconstriction and inflammation of the airways. These symptoms can be managed by three types of medications:

controller medications (e.g. fluticasone propionate), reliever medication (e.g. salbutamol) and/or newer biologic therapies (e.g. anti-IgE monoclonal antibody). Controller medication work to reduce airway inflammation that includes ICS, leukotriene receptor antagonists (LTRAs), LABAs, LAMAs. Reliever medications work to resolve bronchoconstriction and opening the airways by delivering either rapid-acting inhaled beta2-agonist and inhaled anticholinergics. A newer form of asthma medications include biologic agents which target specific immune signallers in order to control the immune response in asthma. These agents include anti-IgE and anti-IL-5 monoclonal antibodies [16, 17].

The current therapeutic recommendation for the maintenance of persistent asthma and its exacerbation depends on the severity of diseases and frequency of exacerbation depending on the individual needs of the patient. ICS alone or in combination with LABAs is the first choice of maintenance therapy, with LTRA, SAMA/LAMA and SABA as alternative add-on therapies. Between 3-10% of asthmatic have persistent, uncontrolled symptoms despite treatment with high dose of ICS-LABA [16] or other combinations. In this instance, an oral corticosteroid (OCS) may be prescribed. The socio-economic burden of severe steroid resistant asthma (SSRA) is estimated to be more than 50% of direct and indirect healthcare costs associated with asthma [18].

The current understanding of the development of asthma is constantly evolving with recent research identifying different asthmatic phenotypes such as allergic asthma, eosinophilic asthma and non-eosinophilic (steroid resistant) asthma. Severe steroid resistant asthma (SSRA) is defined as failure to improvement of lung function of more than 15% even after treatment with high dose of prednisolone (30-40 mg daily) for two weeks [16]. It is hypothesised that SSRA may be cause by genetic abnormalities in glucocorticoid receptors (GR-α and GR-β) leading to reduced GR binding capacity and also its receptor density, increased expression of inflammatory cytokines (IL-2, IL-4, IL-13), increased expression of an alternatively spliced

GR-β, impaired nuclear localization of GR in response to high dose of corticosteroids (probably with the increased activation of p38 MAP kinase), abnormalities in histone acetylation pattern (in some patients defect in specific acetylation of lysine 5 of histone-4), defective secretion of IL-10 from macrophages and circulating monocytes, cigarette smoking (consequences of oxidative stress) and latent infection with virus [19, 20]. There are limited therapeutic option for SSRA patients, therefore there is an urgent need of more effective anti-inflammatory agents or an alternate approach to inhibit the inflammatory pathway of asthma.

1.4 Patho-immunology of lung in asthma and COPD

Both COPD and asthma can be basically characterized by variable airflow obstruction and inflammation of the airways. However, in asthma, airflow limitation is reversible with pharmaceutical intervention, whereas airflow limitation is only partially reversible in COPD. Further, the cause of airflow limitation are distinctly different with marked differences in the inflammatory pattern due to the underlying disease pathology [21, 22]. Overall, the inflammatory cell pattern in COPD and asthma differs and these differences lead to very distinct immunopathology of each disease [23]. It is evident that the immune mechanisms driving inflammatory processes of COPD and asthma are different as these are mediated by different immune cell types, particularly the different subsets of T-cells.

Immunopathology in COPD

In COPD, inflammation is observed in the large and small airways as well as the lung parenchyma [24, 25]. In the majority of COPD cases, airway inflammation is predominantly neutrophilic. Neutrophilic inflammation produces CXC-chemokines, such as CXC-chemokine ligand 1 (CXCL1; or GROα) and CXCL8 (or IL8), that acts on CXCR2 [26, 27]. Further, there is a correlation between the level of neutrophilic infiltration and disease severity [28]. The macrophage number in the lungs of patient with asthma and COPD are generally increased, but

in very high proportions in COPD. These macrophages are derivative of the circulating monocytes, those migrating in response to chemoattractants such as CCL2 (or MCP1) acting through CCR2, and CXCL1 acting through CXCR2 receptor. There are strong evidence that the migrated macrophages orchestrate the inflammatory direction in COPD by releasing certain chemokines that further attracts neutrophils, monocytes and T cells and also the releasing proteases such as MMP9. T cell population plays a vital role in disease pathology. CD4+ T cells found in the respiratory airways in COPD are mainly TH1 cells. In few patients with more severe form of disease there is significant increase in B cell numbers [29, 30].

Immunopathology of asthma

In inflammation in asthma, is mainly localised to the larger airways and to some extent to small airways in more severe form of disease. The lung parenchyma remains free of pathology. It is now been well documented that inflammation in allergic asthma is eosinophilic whereas SSRA is predominantly neutrophilic. The eosinophilic inflammation secretes chemotactic factors like CC-chemokine ligand 11 (CCL11; or eotaxin-1) and other related CC-chemokines are mostly through airway epithelium. The functional role of eosinophils in pathogenesis of asthma yet to be fully understood but current evidence suggests a role in subepithelial fibrosis [31, 32].

T-cell populations play a critical role in the immunopathology of asthma. The number of CD4+ T cells are increased which are predominantly T helper 2 (TH2) cells. TH2 cells mostly secretes cytokines such as IL-4, IL-5, IL-9 and IL-13. Among them, IL-4 and Il-13 provoke IgE production by B cells and IL-5 coordinates differentiation of eosinophils in the bone marrow. IL-9 is responsible for attracting and directing the differentiation of mast cells. Collectively, this all indicates that TH2 cells plays central role in allergic inflammation of airways of asthmatic patients [33]. It is now evident that in some COPD phenotypes, even the TH2 cells are found to be increased in the lavage fluid, further in patients with more severe form of asthma

TH1 cells are found to be activated. This makes the distinction between the TH-cell patterns in the two closely related diseases [34]. This also correlates with eosinophilic asthma being predominantly TH2 type and steroid responsive and the neutrophilic severe asthma and COPD which are predominantly neutrophilic and dominated by TH1 type.

Other cells involved in the severe asthma include mast cells, B cells and dendritic cells. Mast cells play a crucial role in asthma by releasing several bronchoconstrictors, including histamine from the pre-formed granules and lipid mediators such as leukotriene C4, leukotriene D4, leukotriene E4, and prostaglandin D2. These are secreted after the activation of mast cells. It is hypothesised these mediators play a pivotal role in bronchoconstriction. Mast cells are also associated with release of cytokines related with allergic inflammation, such as interleukin-4 (IL-4), IL-5 and IL-13. However, we do not known if mast cells has any contributory role in bronchoconstriction in COPD [35].

B cells is known to play a pivotal role in allergic form of asthma through releasing allergen-specific IgE and the TH2-cell type provoked cytokines like IL-4 and IL-13 induces B cells to undergo immunological class switching to further produce IgE. Dendritic cells (DCs) plays an important role in regulation of TH2 cells in asthma and presents the processed peptides of the inhaled allergens to TH2 cells [36]. The thymic stromal lymphopoietin (TSLP) cytokine secreted by epithelial cells and mast cells in large amounts is believed to play a critical role in maturation of myeloid DCs and also the recruitment of TH2 cells in the respiratory airways through inducing the release of CCL17 (or TARC) and CCL22 (or MDC), these binds to CCR4 which is selectively expressed by TH2 cells [37, 38]. The role of DCs in COPD is still not clear, though cigarette smoking is linked to an increase in mature DCs [39]. A recent study found a decrease in DCs in the in respiratory tract of COPD patients who smoke when compared to smokers without any airway obstructive disease. This leads to the assumption that DCs do not have a major role in COPD immuno-pathogenesis [40-43].

1.5 Risk factors for COPD and Asthma

1.5.1 Genetic factor

The various environmental risk factors are considered important in disease progression, however; both COPD and asthma has been well known to be a heritable diseases with genetic components playing vital role in disease development of obstructive airway diseases. Many of the genetic variants have been identified from genome-wide association studies in asthma and COPD [44]. The identification and recognition of genetic variants are crucial in understanding the underlying mechanisms of disease development [44]. With the remarkable advances in genome-wide association studies, many of the genetic risk factors associated with COPD and asthma has been reported. In COPD, genetic loci of more than 58 genes (eg. CHRNA3/CHRNA5/IREB2, RIN3, MMP12, TGFB2, HHIP FAM13A, CYP2A6, MTCL1, SFTPD, SNRPF, PPT2, and AGER) have been so far associated with COPD pathogenesis [45-50]. Similarly in asthma, loci of more than 108 genes (eg., IL2RB, HLA-DQ, IL18R1, IL33, SMAD3, ORMDL3/GSDMB) has been claimed to be associated with the disease [44-48]. There are some of the genes that have been identified to be implicated in both asthma and COPD include *ADRB2*, *GSTM1*, *GSTP1*, *IL13*, *TGFB1*, *TNF*, *ADAM33*, *CCL5*, and *IL17F* [49].

1.5.2 Viral and bacterial infections

Acute exacerbation (AE) of both COPD and asthma is recognized to be mostly triggered by either viral or bacterial infections. These infectious exacerbations are associated with rapid decline in lung function and increased morbidity and mortality [50]. The role of infections is complicated in COPD as both chronic bacterial infections and viral infections, which is associated with worsening of airway inflammation and airflow obstruction [51, 52]. The half of burden reported from bacterial causes that includes non-typeable *Haemophilus influenza*

(NTHi), *Moraxella catarrhalis, Streptococcus pneumonia,* or *Pseudomonas aeruginosa,* and other half by viral infections such as influenza virus, human rhinovirus (HRV), respiratory syncytial virus (RSV) and coronavirus to name a few [53-57].

Asthma is mostly associated with respiratory viral infections and also found responsible for more severe acute disease [54, 55]. Several respiratory viruses are documented to induce asthma exacerbation like respiratory syncytial virus (RSV), human rhinovirus (HRV), metapneumovirus and influenza virus among others [54, 56-58]. Further, there is strong evidence linking asthma exacerbation and bacterial infections. The bacteria mostly reported includes *Mycoplasma pneumonia, Chlamydophila pneumonia, Streptococcus pneumonia* and *Coxiella burnetti* reported to be related with asthma attacks [59-61]. Co-infections with both virus and bacteria are found to be in 18% of the asthma exacerbation [62].

The advancement of culture-independent techniques has indicated that there is persistence of virus and colonization of lower airways with resident bacterial microbiota in stable COPD patients, this indicates a possible role of lung microbiota in disease pathogenesis and prognosis [63-66].

1.5.3 Cigarette Smoking

Cigarette smoking is the frequent and dominant risk factor for COPD development and is linked with accelerated decline in FEV1, progressive worsening of symptom and higher rate of mortality when compared to non-smoker [67-70]. In asthmatics, cigarette smoking is associated with accelerated decline in lung function, worsening severity, reduced response to corticosteroids and poor asthma control and frequent hospital admission and increased morbidity and mortality rates when compared to non-smoker asthmatics [71-74]. The exact mechanism underlying the adverse effects in asthmatics and COPD in active smokers are not

known, but thought to be because of the increased airway inflammation and reduction in corticosteroid response [75]. One of the study reports decreased in number of CD83+ve mature dendritic cells and B lymphocytes in smoker with asthma when compared to asthmatics who never smoked [76]. In yet another study the total IgE levels were found higher in smokers versus non-smokers with asthma. This indicates that smoking might play a pivotal role in IgE secretion from B cells, though the underlying mechanism is far from clear [77, 78]. In COPD, inhaled corticosteroids are no longer recommended as first line therapy, but are used as add-on therapies in severe exacerbations. Research shows a reduction in overall COPD exacerbation rate with inhaled corticosteroids, yet this does not improve the overall mortality but rather increases chances of adverse effects such as pneumonia [79-84]. The therapeutic efficacy of oral corticosteroids treatment in chronic asthma is seen to be impaired in active smokers [79]. The general overview suggests that cigarette smoke exposure in both COPD and asthma is strongly associated with the development of relative resistance to corticosteroid therapy.

Cigarette smoking is considered to be one of the most important risk factor associated with gradual decline of lung function, increased mortality and worsening of the symptoms in both COPD and asthma. Interestingly, cigarette smoking alters the inflammatory mechanisms in asthma that is similar to that found in COPD with increased CD8 cells and neutrophils. Furthermore, cigarette smoking is been seen to be associated with poor inhaled corticosteroid response. Some of the main mechanism proposed for this altered response to inhaled corticosteroid includes reduction of the histone deacetylase 2 (HDAC2) enzyme system, overexpression of GR-β, activation of p38 MAPK pathway and increased production of some of the pro-inflammatory cytokines (such as IL-2, IL-4, IL-8, TNFα and NF-kβ [85, 86].

1.5.4 Dysbiosis of Gut and Lung Microbiota

Human body surfaces such as skin, intestines, respiratory and urogenital tracts are all colonized by microorganisms, including bacteria, viruses, protozoans, fungi and archaea, and gut being

colonized extensively and most densely [87]. The recent advancement of culture-independent methods based on 16S rRNA genetic sequencing and metagenomic sequencing have provided insight into varying microbial populations throughout the human body [87, 88]. In this thesis, I have exclusively focused on bacterial populations. The gastrointestinal (GI) tract of humans harbours about 10^{14} bacteria, of more than 1000 different species [87]. The composition of gut microbiota is constantly and continuously shaped by various factors such as diet, mode of delivery, feeding practices and drugs [87, 88].

Healthy Gut Microbiota

The gut microbial phyla that are dominant includes Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia, in which only two phyla Firmicutes and Bacteroidetes accounts for 90% of all gut microbiota in average healthy individuals [87]. The phylum Firmicutes represents more than 200 different genera which includes *Lactobacillus, Bacillus, Clostridium, Enterococcus,* and *Ruminicoccus*. The genera *Clostridium* alone makes 95% of the total Firmicutes phylum. In the phylum Bacteroidetes, the predominant genera are *Bacteroides* and *Prevotella*. Similarly, in the Actinobacteria phylum *Bifidobacterium* are the most represented genus, though proportionally less abundant in comparison to other phylum groups [88].

Healthy Lung Microbiota

Previously, before the development of culture-independent molecular techniques, the lungs were considered sterile [88, 89]. It is now known that healthy lungs are colonized by diverse bacterial communities. The microbial biomass in the lungs is much lower than in the gut, with 10^3 to 10^5 bacteria per gram of tissue in the lungs versus 10^{11} to 10^{12} bacteria per gram of tissue in the gut [90, 91]. The lung microbial community is regulated by the process of microbial

migration, elimination of microbes and the relative reproduction rates of the colonies in the airways [92].

The healthy lung microbiome is mainly formed of four phylum Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria [93-95]. The most abundant genera in a healthy lungs includes *Prevotella, Streptococcus, Veillonella, Neisseria, Haemophilus* and *Fusobacterium* [96]. The human respiratory tract (70 m^2 surface area) is inhabited by the microbiota in varying quantities and qualities, the upper and lower respiratory tract (URT and LRT) have distinct bacterial compositions [97, 98]. The healthy microbiome in the URT comprises of genera *Staphylococcus, Propionibacterium, Dolosigranulum, Corynebacterium, Moraxella, Streptococcus, Haemophilus, Rothia, Veillonella, Prevotella,* and *Leptotrichia* [99-101]. Similarly, the healthy microbiome of the LRT is mainly composed of six dominant bacterial phyla: Firmicutes, Bacteroides, Proteobacteria, Fusobacteria, Acidobacteria, and Actinobacteria [102-104].

Extensive recent research in the field has enhanced our understanding of how various microbial populations interact with the human body particularly in the context of human health and diseases [105-107]. It is now widely understood that a rich and diverse microbiota, particularly in the lungs, plays an important role in the maintaining the human health, and any impairment or imbalance in the composition of the human microbiota leading to dysbiosis and causing disease [108]. The associations between altered gut microbiota and disease has been widely reported, while role of gut microbiome and is effect on remote organs and immune function has also been demonstrated in many of the recent studies [109, 110]. The difference in the composition and function of gut microbiota has been associated with varying chronic diseases ranging from inflammatory gastrointestinal diseases, metabolic conditions, autoimmune, neurological, cardiovascular and respiratory disorders [111]. Several reports states that

dysbiosis or the alteration in gut microbiome composition in chronic respiratory diseases including COPD and asthma [112-114].

Gut Microbiota in COPD

In COPD, an alteration of different sets of gut microbiota has been documented. On the family level, a relatively decreased abundance of *Fusobacteriaceae, Prevotellaceae,* and enriched level of *Bacteroidaceae* and *Prevotella* have been identified [113]. 16 S rRNA gene sequencing shows COPD patients shows increased abundance of genera *Streptococcus, Rothia, Romboutsia* and *Intestinibacter* whereas the *Bacteroides, Roseburia* and *Lachnospira* from the family *Lachnospiraceae* and some of the unnamed genera of *Ruminococcacea* are decreased. *Streptococcus* species are identified as key organism in COPD group as compared to healthy group. Multiple members of the family *Lachnospiracease* such as *Streptococcus sp000187445*, *Streptococcus vestibularis* has been found to be correlated with reduced lung function [114].

Lung Microbiota in COPD

In clinically stable COPD, many of the recent reports suggests towards a rich lung microbiome that is distinctly different than seen in healthy controls. In these people with stable COPD, the common phyla identified are Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes, and the most dominant genera includes with *Pseudomonas, Streptococcus, Prevotella* and *Haemophilus* [115-117]. It has been well documented that during COPD exacerbation there are distinct changes in the lung microbiota, specifically raised abundance of Proteobacteria and decreased abundance in Firmicutes [118-119]. Existing literature suggests the relative increase in *Haemophilus influenza, Moraxella catarrhalis* and colonization of *Pseudomonas aeruginosa* and decrease in *Streptococcus pneumonia* species [120-122]. The difference in the upper airway microbiome in smokers and non-smokers includes a decrease in the relative abundance of *Porphyromonas, Neisseria,* and *Gemella* and higher a relative abundance of *Megasphaera*

species, *Streptococcus, Veillonella, Atopobium* species, and *Actinomyces* [123, 124]. Further, the lung microbiome has bene correlated with disease severity. In GOLD I (mild) patients, *Streptococcus, Streptococcus salivarius* and *Streptococcus thermophilus* are found to be the most highly abundant, whereas in GOLD II and IV (moderate and severe) patients *Streptococcus pseudopneumoniae* and *Streptococcus pneumoniae* are dominant species, respectively [125].

Gut Microbiota in Asthma

Research has shown that infants who are at high risk of developing asthma have significantly reduced relative abundance of the genera *Lachnospira, Veillonella, Faecalibacterium* (phylum Firmicutes), and *Rothia* (phylum Actinobacteria) [126]. Taxonomical classification of the gut bacteria of children with asthma have shown significant reduction in abundance of genera *Faecalibacterium* and *Roseburia*, while enhancement of genera *Enterococcus* and *Clostridium* (phylum Firmicutes) when compared to healthy controls [127]. In yet another study, it was found that *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* bacterial species were found to be decreased in the asthmatic patients than that compared to healthy controls [128]. A similar consistent finding was reported by Fujimura et al., in 2016, in which they stated that infants with relatively reduced abundance of *Akkermansia, Bifidobacterium,* and *Faecalibacterium* and raised relative abundance of *Candida* and *Rhodotorula* in the gut [129]. Furthermore, reduction in abundance of *Akkermansia municipila* has been found to be linked with severe form of asthma in adults [130].

Lung Microbiota in Asthma

Several studies have characterised the lung microbiome in asthma patients and have shown to have increased relative abundance of the Proteobacteria phylum [131 -133]. The alteration of lung microbiome at the genus level corresponds with increase in the proportion of

Haemophilus, *Neisseria*, *Moraxella*, *Pseudomonas, Klebsiella* species [131, 134]. One of the most recent studies have reported that Firmicutes, particularly the *Streptococci* are increased in severe asthmatics as compared to controls [135]. However, yet another study reported that the *Gammaproteobacteria*, particularly the *Enterobacteriaceae* and *Pseudomonadaceae* species are more often found in the severe asthma compared to non-severe asthma group [136].

1.6 Targeting the gut-lung axis to treat CRDs

The growing body of evidence suggests there is bidirectional crosstalk between the gut and lungs, in which gut microbiota influences immune-pathophysiology in the lungs. This cross-talk between the gut microbiota and lungs is termed as the "gut-lung axis," This axis is thought to facilitates endotoxins, microbial metabolites, cytokines, and hormonal regulation into the bloodstream connecting the gut and lungs [137 – 139]. This cross-talk between gut and lung is bidirectional and the disease in lung have an effects on gut and vice versa [140-142]. Although the underlying mechanisms regulating the gut-lung axis is yet poorly understood, the alterations in gut microbial species and metabolites have strongly been linked with changes in immune response, inflammation and development of CRDs. The gut microbiota is now known to be producing some of the metabolites through fermentation of conventionally non-digestible dietary fibres by hosts, and among all the most prevalent being the short chain fatty acids (SCFAs). These SCFAs have potent anti-inflammatory effects, which reduces the chemotaxis and adherence in immune cells and promotes the release of anti-inflammatory cytokines [143, 144].

The patho-immunological regulation through gut-lung axis in CRDs is an emerging field of interest to researcher. The growing body of evidence suggests towards possible prophylactic and therapeutic approach through interventions to modulate the gut microbiota and its products. These could include either faecal microbial transfer (FMT), supplementation with probiotics

(live microorganisms having potentially beneficial health effects), supplementation with prebiotics (nondigestible dietary fibre), and supplementation with gut microbial metabolites (such as short chain fatty acids) [145-148].

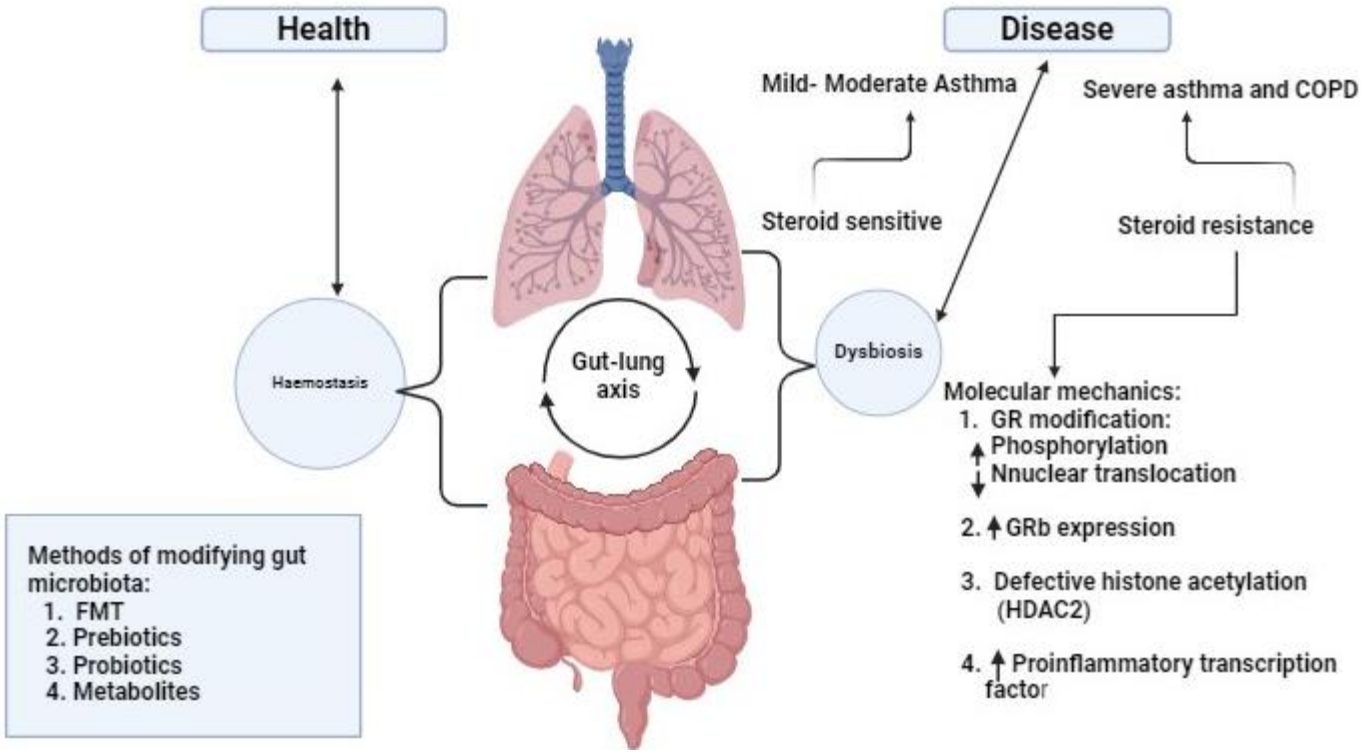

Figure 1: Schematic diagram showing the mechanism of steroid resistance and factors modifying gut microbiota

1.7 FMT as a therapeutic intervention in COPD and severe asthma

FMT is a way to reshape the composition of the gut microbiota, in which faecal material is transferred from healthy donor to a diseased recipient. The ultimate goal of FMT is to restore the richness and diversity of intestinal microbial population. This is an attractive strategy to restore healthy immunomodulation along the gut-lung axis, which is often hindered in COPD and severe asthma. FMT has been successfully established as therapy in recurrent *Clostridium difficile* infection (rCDI) associated diarrhoea and has been recommended in several international guidelines as an effective therapy option for recurrent rCDI [149, 150]. The rate

of cure is promisingly high up in recurrent or refractory CDI to approximately 90% and is much higher to the recommended anti-microbial therapy which has success rates of just 20-30% [151, 152]. FMT is now been considered for many other inflammatory, autoimmune and metabolic disorders including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), Crohn's disease, rheumatic arthritis, type I diabetes, cancer, neuropsychiatric conditions, cardiovascular and other systemic disorders [153]. The stool preparations can be administered either through frozen capsule, enema, colonoscopy, duodenal infusion or oral gavage in animals [154]. Several clinical case reports and animal models in severe multiple sclerosis, autism, multi-drug resistant organism infections, multiple organ dysfunction in critical patients and melanoma have shown probable beneficial therapeutic effects in these disease condition [155-159]. One prominent positive aspect observed from recent clinical trials is that there is no any known absolute contraindication for FMT [160].

The emerging evidence from experimental studies and several clinical trials with probiotics is promising with asthma [161]. It is assumed that FMT would be more effective therapeutic choice then even the probiotics since the faecal infusion overcomes the intrinsic quantative gap and are more durable in altering the recipient's gut microbiota [162]. A recent study which looked at the effect of FMT as well as a high fibre diet showed that both interventions together attenuated CS-exposure-induced emphysema [163]. The research to-date endorses a more directed vigorous research to explore and establish FMT as a noble therapeutic option in chronic inflammatory respiratory diseases asthma and COPD.

1.8 Study rationale

In general, FMT is a way to reshape or restore the altered gut microbiota (dysbiotic state) to a more homeostatic or healthy state. Similarly, in concept when FMT is done from dysbiotic or

diseased faecal matter into a healthy mice, this should aggravate the disease and increase the severity.

FMT is an attractive strategy to restore healthy immune cross-talk along the gut-lung axis and provides an exciting opportunity to develop novel treatments for COPD. The studies described hereafter were designed to investigate the role of gut microbiota in pre-disposition and increase in severity of COPD and its therapeutic role in SSRAAD.

1.9 Hypothesis

In this study, we hypothesised that transplantation (FMT) of the CS-induced COPD microbiome contributes to the development and in increasing the severity of COPD, after 6 to 8-week chronic CS exposure. It is also our hypothesis that the gut microbiome has a critical role in the development and severity of Severe Steroid Resistant Allergic Airway Disease (SSRAAD). Treatment with FMT from a healthy donor will ameliorate disease and treatment with FMT from a diseased donor will exacerbate disease.

1.10 Aim

To test this hypothesis, I have performed the following studies for my PhD:

1. To assess whether a smoke-induced COPD microbiome can predispose to disease.
2. To interrogate the role of the COPD gut microbiome in the development of COPD and aggravation of severity.
3. To investigate the relationship between the relative abundance of dominant bacterial strains in the COPD gut microbiome and the hallmark features of COPD (chronic inflammation, airway remodelling, alveolar destruction and airflow obstruction) in order to extrapolate potential mechanisms of action.

4. To investigate FMT as a potential therapy for SSRAAD and to further understand the role of the gut microbiome in the development of SSRAAD.

Chapter 2: Methods

In this chapter, I have described the material, methods and statistical analysis that has been applied throughout my PhD study to generate the data for this thesis. Here, I have briefly outlined the experimental details (Sections 2.1 – 2.7), for each experimental model. The specialised experimental techniques and the statistical analyses that has been specifically used in specific chapters has been outlined in concerned chapters as appropriate.

2.1 Animal details

2.1.1 Ethics statement

This study was performed with strict guidance and recommendation in the Australian code of practice for animals care and only for the scientific purposes issued by the National Health and Medical Research Council of Australia. In this thesis, I have performed three experimental cigarette smoke (CS)-induced COPD (CS-COPD) mouse models and one model of severe steroid resistant allergic airway diseases (SSRAAD). The ethics for all these models were approved by the Animal Welfare Committee of Centenary Institute and Sydney Local Health District (SLDH) (CS-COPD: Ethics Approval no./ Protocol No. 2020/023, 2019/029, 2020/002 and SSRAAD: Protocol No 2019/029A).

2.1.2 Mice, acclimatisation, and housing conditions

For the CS-COPD models, 5-8 week-old C57BL/6, adult, female mice were sourced from Australian BioResources. For the SSRAAD experimental model, 4-6 week old Balb/c, female mice were sourced from Australian BioResources. The female mice

were preferred over male mice as they are less susceptible to microbial infections, have higher innate immune response and are more stable in terms of behaviour. As per the ethical requirement and Centenary Institute policy, the mice arriving at Animal Facility were kept for one week without any procedural intervention, termed as acclimatisation period. The experimental mice are rearranged in the different groups to ensure an even distribution of baseline body weight. The mice were distributed in planned manner in individually ventilated cages, 4 mice per cage, which were kept 12 hour's dark and light cycling conditions. The water and food were made available for the mice *ad libitum*.

2.1.3 Microbiome normalisation

To ensure comparability between the groups, the gut microbiome was normalised between each mice to reduce cage effects and variability. To achieve normalisation, a combination of bedding swaps and cohousing was utilized. During the bedding swap, half of all the dirty bedding is mixed with clean beddings and then redistributing this new mix of dirty/clean bedding to all the cages. For co-housing, the mice in each cages are redistributed by planned rotation in various cages. This was conducted two times a week for four weeks prior to the start of the models in experimental COPD and SSRAAD mice models.

2.2 Established murine model in our laboratory

2.2.1 Cigarette smoke exposure and established COPD smoke models

In this current experimental model, mice were exposed with reference cigarettes (3R4F; University of Kentucky, Lexington) twice per day, five times per week, for up to twelve weeks using a custom-designed and purpose-built nose-only and direct flow inhalation with smoke-exposure system (CH Technologies, Nj) housed in a specifically designed

fume and laminar flow hood. Experimental research cigarettes were used in the experiment and exposure was done for 75 minutes in one run. All experiments were assessed and approved by our institutional animal ethics committee [164-167]. The clinical characteristic features after 8 weeks of smoke exposure in wild type mice mimic with established clinical COPD, the symptoms include chronic inflammation, mucus hypersecretion, airway remodelling, emphysema and reduction of the lung function [167]. The continuing smoke exposure even after 8 weeks further worsen the symptoms and increases the disease severity.

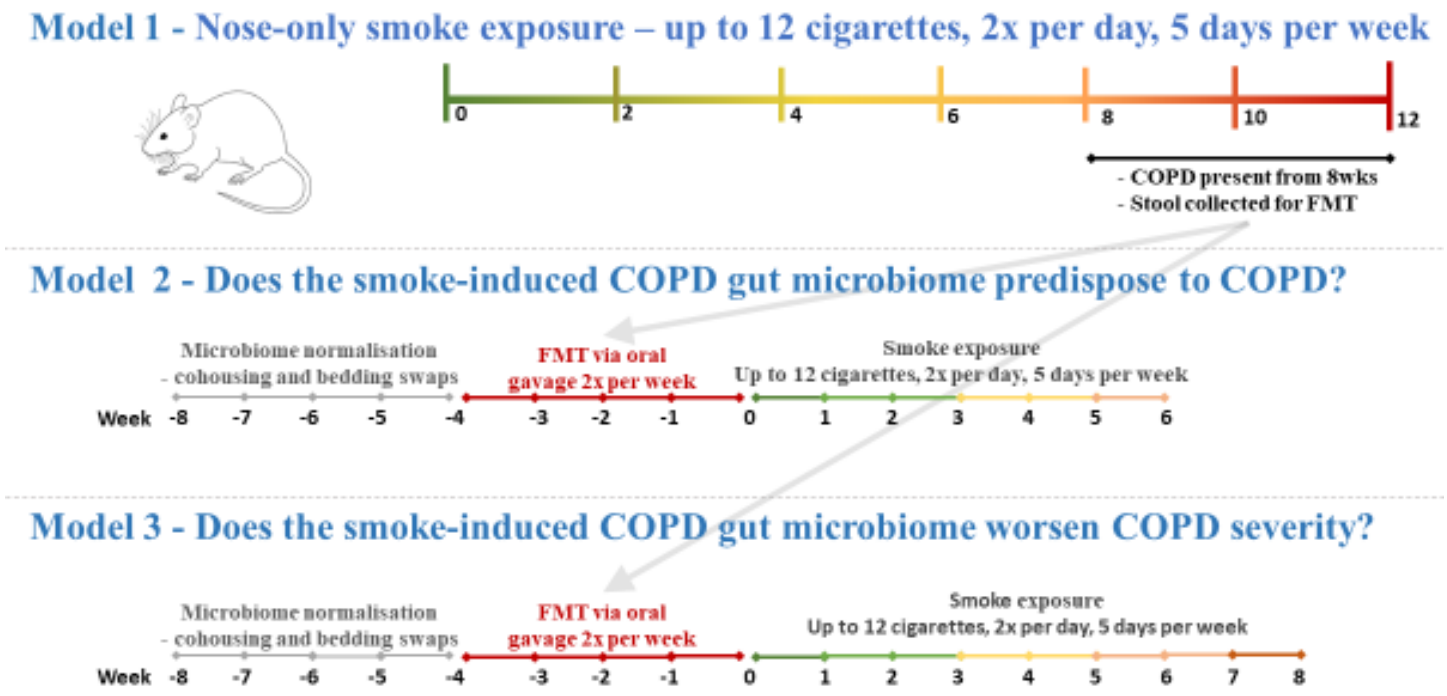

Figure 2: Novel mouse model of cigarette smoke-induced COPD mouse model.

The mouse in Model 1 are smoke exposed with nose-only with 12 cigarettes, twice per day, 5 times per week, for up to 12 weeks. The clinical characteristics of COPD is only seen after 8 weeks of smoke exposure an then after it is further worsening of COPD. The subsequent models (Model 2 and Model 3) are transplanted with FMT for 4 weeks and then smoke exposed for 6 weeks in Model 2 and 8 weeks in Model 3. In treatment models (Model 2 and Model3) mice underwent microbial normalization before start of FMT through oral gavage.

2.2.2 Murine model of established SSRAAD

In the SSRAAD mouse model, the mice are sensitized to ovalbumin (Ovalbumin; 50µl) with the adjuvant alum by intraperitoneal (i.p.) injection on d0 and allergic airway disease (AAD) is induced by intranasal (i.n.) challenge with ova (Ova; 10µg) on d12-13 followed by subsequent rechallenge (Ova; 10µg) on d33-34. The mice were inoculated with *Chlamydia muridanum* (Cmu; 100 inclusion-forming units, 27µg) or sucrose phosphate glutamate buffer (SPG) at d14 via i.n. Dexamethasone (2 mg/kg; 50µL phosphate buffered saline (PBS)) was given to mice via i.n. on d32- 34. On day 35, lung function and airway hyperresponsiveness was measured. Then the mice were euthanized and lung tissue, bronchoalveolar lavage fluid (BALF), faeces, caecum content and gut tissues were collected for further analysis [168, 169]. In this model mice were done with microbial normalization before the start of the actual allergy induction.

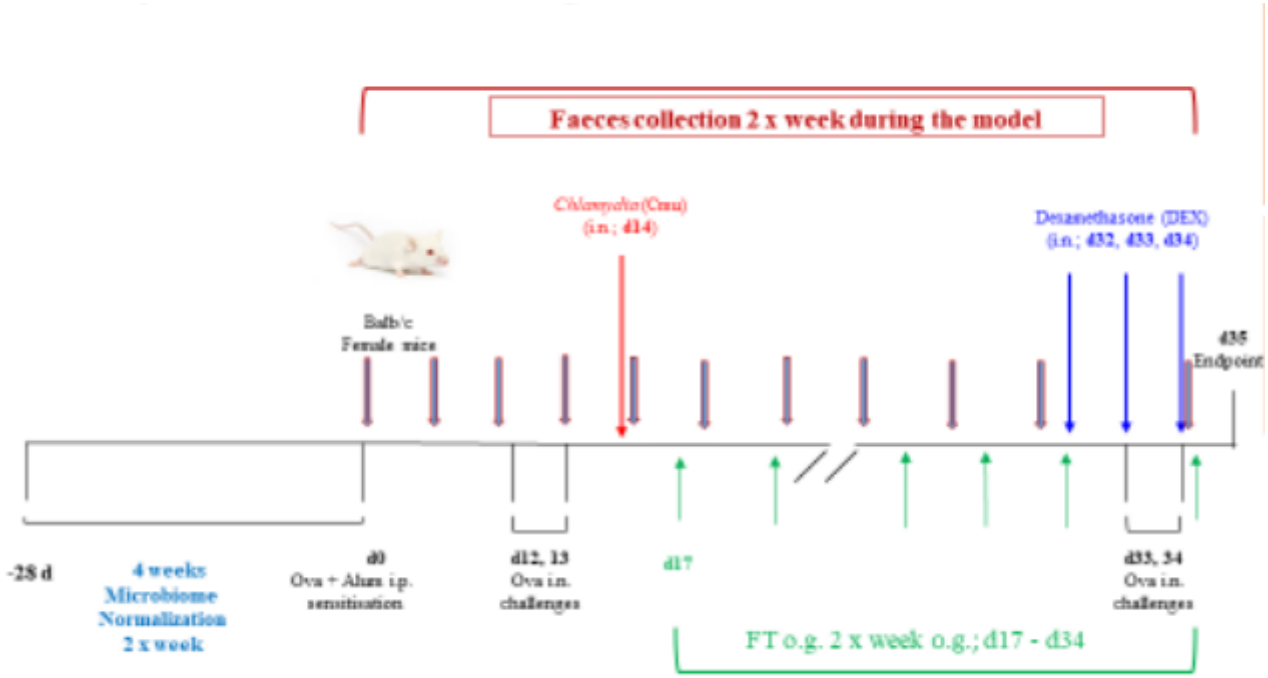

Figure 3: Novel mice models of infection-induced, severe steroid-resistant, allergic airways disease (SSRAAD).

The mice were sensitised with ovalbumin (Ova) through the intraperitoneal (IP) route (d0) and AAD was induced through intranasal (IN) Ova challenge (d12–13) followed by rechallenge with Ova (IN) (d33–34). The infected groups were inoculated with intranasal 100 inclusion-forming units of Chlamydia muridarum in between the two sets of Ova challenges (Cmu; d14) and 3 doses of Dexamethasone (DEX; d32-34). The uninfected controls received phosphate buffered saline (PBS), and non-allergic controls were sham-sensitised with Sal. Microbial normalization was performed for initial 4 weeks (d-28-0) before Ova sensitization. FMT through oral gavage was done twice a week for 2 weeks (d17-34). Faecal samples were collected for metagenomic analysis at different time points (d0, d4, d8, d12, d15, d18, d24, d28, d32, d34, d35).

2.3 FMT methods and techniques

2.3.1 Faecal suspension preparation and oral gavage FMT

Faecal pellets were collected and immediately transferred into an anaerobic chamber (Whitley DG250 Anaerobic Workstation). Under anaerobic conditions, the faecal pellets were homogenised and suspended in 100mg/mL phosphate buffered saline (PBS). The faecal/PBS mixture were centrifuged for 1 minute and the aqueous supernatant was collected and transferred into Eppendorf tubes. For mice in the treatment groups, 200µl of supernatant was administered via oral gavage twice a week in order to transplant the faecal samples in the recipient mouse. Specific timeline for each model is detailed in subsequent chapters (Chapter 3-5).

2.3.2 Faecal sample collection for metagenomics analysis

Approximately 100 mg (2-3 pellets) of faecal pellets were collected in a sterile 1.5 ml of Eppendorf tubes that were and stored at -80°C until further investigation. These samples were collected over the time course of the experimental model in order to monitor the changes in the gut microbiome over the course of the experiment. The details of time point of faecal samples collected is included in the subsequent chapters (Chapter 3-5).

2.4 Endpoint Day Assessment

2.4.1 Endpoint sample collection and assessments

On each endpoint day, lung function was measured using plethysomometry in order to assess the presence and severity of disease. Following lung function, the mice were euthanized and samples collected (tissue, blood, stool, and BALF) to assess the pathophysiology of disease. A detailed description of lung function testing, sample collection and sample storage are described below.

2.4.2 Lung function

Airflow obstruction in COPD

Lung function for COPD is measured using the Scireq flexiVent FX1 system (Montreal, Canada) and the forced oscillation and forced manoeuvre techniques were employed [170]. The presence of airflow obstruction in this experimental model of COPD was defined at a significant decrease in FEV1% predicted, FEV1/FVC and/or an increase in total lung capacity, which is indicative of emphysema. Several additional lung function parameters such as inspiratory capacity, area, static compliance and vital capacity were also measured.

Airway hyperresponsiveness in SSRAAD

The key lung function parameter used to assess the presence of SSRAAD is airway hyperresponsiveness (AHR) was evaluated using the whole body plethysmography in anaesthetised mice. The ketamine: xylazine (100mg/kg: 10mg/kg in 200µL of PBS; Troy Laboratories, Australia) was used as anaesthetic with tracheas been cannulated (tracheostomy with ligation) [171-174]. The FlexiVent apparatus (FX1 System) was used to assess airway resistance (Rn; tidal volume of 8mL/kg at a respiratory rate of

450 breaths/min) in response to increasing challenging doses of nebulised methacholine (up to 10mg/kg; Sigma-Aldrich, Australia) [173]. The assessments was repeated for three times per dose of saline/methacholine and the average percentage change in Rn to methacholine challenge was compared with the nebulised saline in order to measure the AHR in mice in the experiment.

2.4.3 Sample collection

Plasma collection

Blood was collected by cardiac puncture into the ethyl-enediamine-tetra-acetic acid (EDTA)-coated vacutainers (Becton Dickson, USA) and were stored on ice until further processing. The blood was then processed by centrifugation at 1200xg for 10 min at room temperature. The plasma was then collected by the pipette and kept in the 1.5 ml epindroff tubes and stored at -80°C for further analysis at a later date.

Bronchoalveolar lavage fluid (BALF) collection

The right lung was tied off at the right bronchi to prevent loss of the immune cell population within the tissue during BALF collection. BALF was then collected from the large lobe of the left lung by washing the lung two times with 500µL phosphate buffered saline (PBS; 0.13M NaCl, 0.002M potassium chloride, pH 7.4). Both 500µL washes were collected into Eppendorf tubes and centrifuged at 130xg for 5 min at 4°C to separate the cellular matter and the supernatant. The collected supernatant are stored at -80°C for assessment at a later date. The cells were centrifuged and aspirated before resuspension in 160µL of PBS. The total number of cells in the BALF was counted with haemocytometer and dead cell were excluded via trypan blue staining. The remaining volume was used for assessment of differential immune cell counts. The BALF and other tissue were collected from the same mice.

Lung tissue collection and storage

The lung lobes are perfused with 0.9% saline in order to remove red blood cells, and then inflated and fixed with 1ml volume of 10% neutral buffered formalin (Lonza Australia Pty Ltd), by injection. The left lung was tied at the left bronchi, excised at the trachea and stored in formalin for next 48 hours and then transferred to 10% ethanol in PBS that can be stored for longer duration. The right lobes were removed and kept separately in three separate tubes, snap frozen with liquid nitrogen and were stored at -80°C for further extraction of RNA and molecular analysis at a later date.

2.5 Airway inflammation

2.5.1 Differential immune cell counts

After storage of the BALF supernatant and total cell counts, the remaining cells were transferred into a cytospin column loaded with a filter and microscope slide and spun at 500g for 7 min. The various inflammatory cellular components were assessed by differential cell counting under light microscopy (40x magnification). The protocol was followed as established in our lab (Hansbro lab, Centenary Institute).

2.5.2 RNA extraction

The single right lung lobe collected from mice were transferred in TRIzol (1mL; Invitrogen) and were thoroughly homogenised using bead homogenisation (2 x 3min 50 oscilations/sec; [TissueLyser II] Qiagen, USA). The homogenates are transferred in fresh 1.5 ml tubes and centrifuged (12,000 x g for 10 mins; 3ºC). The clear homogenate are again transferred to another fresh tubes and supplemented with 250 µl of Chloroform (Pulse-vortexed; 5 seconds), incubated at room temperature (10 mins) and centrifuged (12,000 x g; 15 mins; 3ºC). The aqueous phase is again transferred to fresh tubes and RNA precipitated (with 500 µl of ice-cold Isopropyl Alcohol), then pulse-

vortexed and incubated (at room temperature; 10 mins). Now again pulse-vortex and centrifuge (12,000 x g; 10 mins). Remove supernatant and wash RNA pellet (1 ml of 75% Ethanol). Pulse-vortex to dislodge pellet and centrifuge (8000 x g; 5 mins; 3ºC). Washing with 75% Ethanol and pulse-vortex repeated as in previous step. Removal of ethanol and kept for air-drying on ice (15 mins). The RNA pellet were resuspended with nuclease-free water (30 µl and quantified using Nanodrop bioanalyser. The procedure is as per the established protocol in our lab (Hansbro lab, Centenary Institute).

2.5.3 Reverse transcription

The extraction of RNA (1000 ng) was done by treating the lung lobe with DNAse 1 for 15 min at room temperature (Sigma Aldrich, Australia) to remove contaminated DNA. The DNAse activity was then terminated with the supplementation of stop solution 1 µL (Sigma Aldrich, Australia) before removal of secondary RNA structures by heating (10 min, 65°C). The procedure was followed as per the recommended protocol.

2.5.4 Quantitative real-time quantitative PCR (qRT-PCR)

The qPCR was carried out with cDNA (2 µL) combined with SYBR Green (6.25µL; Biorad), nuclease free water and both the forward and reverse primers (10µM each) that makes the final volume of 12.5µL. Gene expression was normalised using housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in lung tissue. Gene expression is presented as relative to the healthy control group.

2.6 Histology

2.6.1 Preparation of lung tissue for histology

The lung tissue is formalin-fixed, ethanol-stored, embedded with paraffin, sectioned and stained. The lungs were washed with ethanol (3 x 100%), chloroform (2x) and paraffin wax (3x) before embedding with blocks of paraffin serially sectioned (5µM thick) and mounted on slides. The slides were stained and counterstained with haematoxylin and eosin.

2.6.2 Alveolar destruction

The lung tissue were haematoxylin/eosin stained on the slides then were assessed for alveolar destruction by measuring the mean linear intercept (MLI) technique. The mean linear intercept is a measure of morphometric change based on serial measurements of the lung using test lines. MLI is the most direct interpretation of the mean free distance between gas exchange surfaces in the acinar airway complex [9]. The random images were taken at 40x magnification (Image Pro Plus 7.0 software, Media Cybernetics, Inc.). The average intercept of the lung section were calculated as per the established protocol.

2.6.3 Collagen deposition

The collagen deposition in the small airways were quantified by using sirus red/fast green section stained as described previously [10].

2.6.4 Epithelial thickness

The airway epithelial (µm2) of the small airways was measured in a minimum of four (basement membrane [BM] perimeter <1,000µm) per section [9-12]. Data were

quantified and normalized to BM perimeter (μm) using ImageJ software (Version 1.50, NIH).

2.7 Statistical analysis

All data in this thesis are expressed as mean ± standard error of the mean (SEM). The statistical analysis of various data generated were compared between the various groups were made using unpaired T-tests or a nonparametric equivalent where appropriate. For airway inflammation, comparisons between groups were made using one-way ANOVA and an appropriate post-hoc test. For lung function, two-way ANOVA was used to compare the data between the multiple doses and groups with an appropriate post-hoc test. GraphPad Prism Software version 8.2.1 (San Diego, California) was used to perform analysis. The data shown in this thesis are representative of individual mice (n=6-8).

Chapter 3: Investigating the role of gut microbiome in COPD development.

3.1. Background

COPD is generally characterised by the presence of persistent airflow limitation, neutrophilic inflammation, macrophagic accumulation, and the production of certain cytokines, chemokines and proteases involving the airway and lung parenchyma [12-14, 139, 145]; and is now the third leading cause of death globally. The focus of COPD management is directed towards relaxation of smooth muscles and reduction of inflammation, typically through non-pharmacological and pharmacological means.

Though steroids are highly effective in asthma, but are not to that extent in suppressing inflammation in COPD. This could be explained by the difference in the nature of the inflammation in asthma and COPD, as COPD is neutrophilic and so do the severe steroid resistant asthmatic. Indeed, corticosteroids do not supress neutrophilic inflammation in fact it prolongs neutrophilic survival in vitro by inhibiting apoptotic pathways [11]. The resistant of COPD to steroids is also be explained with its association with the cytokines released by alveolar macrophages that are basically responsible for resistant to the anti-inflammatory effects of corticosteroids [12].

The overall magnitude of clinical benefits of the various pharmacological options are somewhat limiting and disappointed and for this reason it is essential to focus on alternative therapeutic approach such as investigating and intervening the gut-lung axis in COPD. Recent microbiome studies have established dysbiosis or the alteration in gut microbiome composition in chronic respiratory diseases including COPD [126]. In COPD patients an alteration of different sets of gut microbiota has been documented. On the family level, a relatively decreased abundance of Fusobacteriaceae, Prevotellaceae, and enriched level of Bacteroidaceae and Prevotella have been identified [113]. The faecal microbiome 16 S rRNA gene sequencing of COPD patients shows increased abundance of genera *Streptococcus, Rothia, Romboutsia* and *Intestinibacter* whereas the *Bacteroides, Lachnospira* and *Roseburia* belonging to family *Lachnospiraceae* and several unnamed genera of *Ruminococcacea* are found to be decreased. *Streptococcus* species are identified as key organism in COPD group as compared to healthy group. Multiple members of the family Lachnospiracease such as *Streptococcus* sp000187445, *Streptococcus vestibularis* has been found to be correlating with reduced lung function [114]. Bowerman and colleagues looked into the difference between the gut microbiota of COPD patients and healthy controls, and came up with the conclusion that *Streptococcus, Rothia, Romboustia, Interstinibacter*, and *Escherichia* are increased in

abundance in COPD, while *Bacteroides, Roseburia,* and *Lachnospira* were found reduced [114]. One of the study compared the duodenal microbiome in smokers and people who never smoked, and reported that smokers had more Firmicutes, particularly *Streptococcus* spp., and *Veillonella* spp., and less *Prevotella* and *Neisseria* spp [174, 175]. There has been many ongoing efforts to understand the composition and changes in the gut microbiota in COPD, however; we are still away from the complete understanding of the role of gut microbiota in COPD and also either it is the cause or effect is still to be established.

3.2. Study rationale

The evolving concept indicates towards dysbiosis of gut microbiome in COPD, however only little is known if gut microbiome is the cause or effect of the disease. In this study, we designed the experiment in such a way that FMT was done from healthy and smoke exposed COPD groups to either healthy or COPD group. Through this experimental model we intend to show the causality of gut microbiome in COPD progression. In this study, the experiment were designed in such a way that FMT is done from the faeces of the donor group (Model 1) to the recipient group (Model 2). In this experiment we investigated and evaluated the effects of FMT from a donor mice with COPD when given to healthy mice prior to smoke exposure and predisposition and prognosis to COPD.

FMT helps us to evaluate the gradual changes in taxonomy of the gut microbiota of the different groups. We expect that the FMT from healthy (air-exposed) group of mice when given to the diseases (smoke-exposed) group will change the gut microbiota of the diseases group to a more healthy state or the haemostatic state. Similar, the FMT from diseased (smoke-exposed) group of mice when given to the healthy group of mice will change the gut microbiota to a more diseased state or the dysbiotic state. Through this experiment we also want to assess whether if the faecal transplant from the diseased (smoke-exposed) group of mice to the diseased

(smoke-exposed) group aggravates the disease and also when FMT is done from the diseased (smoke-exposed) group of mice to a healthy (air microbiome) and does it predisposes to COPD.

3.3. Hypothesis

It was predicted that the cigarette smoke-induced COPD microbiome will accelerate the development of experimental COPD.

3.4. Aim

The aim of this current experiment was to investigate to role of the COPD microbiome on the development on COPD. This was achieved by addressing three key objectives:

1. To assess whether a smoke-induced COPD microbiome can predispose to disease.
2. To assess the relationship between the relative abundance of dominant bacterial strains in the COPD gut microbiome and the hallmark features of COPD (chronic inflammation, airway remodelling, alveolar destruction and airflow obstruction) in order to extrapolate potential mechanisms of action.

3.5. Methods

3.5.1. Study design

The study design for this chapter is summarised in Figure 1 (Chapter 2; Section 2.2.1). Briefly, the mice received a FMT from either healthy mice or cigarette smoke-induced COPD mice. After 4 weeks for FMT treatments, the mice were chronically exposed to cigarette smoke or air exposure for 6 weeks. To achieve this, we ran two cigarette smoke models in parallel: a donor model (Model 1) and a recipient model (Model 2). The donor model (Model 1) was a standard 12 week model of experimental COPD which has been shown to have the hallmark feature of COPD at 8 week and severe disease at 12 weeks [167]. The hallmark characteristics features of COPD is established

after 8 weeks of smoke exposure to the mice. From 8-12 weeks of the donor model (Model 1), the recipient model received the FMT prior to smoke exposure. The recipient model (Model 2) are then exposed to smoke for 6 weeks. This model where recipient mice exposed to 6 weeks of smoke after 4 weeks of FMT provides us an opportunity to evaluate if the FMT from the diseases mice or COPD microbiome is able to or not push or predispose the 6 weeks smoke exposed mice towards an established COPD phenotype. At endpoint, the major hallmarks of COPD were assessed including lung airway inflammation, airway remodelling, alveolar destruction and lung function. The parameters we looked includes the total and differential count (TC and DC) in BALF, pro-inflammatory cytokines such as IL-1β, TNF-α, Casp1, Nalp3, total collagen deposition, the genes related to collagen such as Col5a3, Eln, Smad2 and Fn. Also, remodelling marker, alveolar diameter and Mmp12 were assessed. The lung function parameters we looked into are FEV0.1, FVC, FEV0.1/FVC and TLC.

3.5.2. Ethics statement

The ethics for this study was approved by the Animal Welfare Committee of Centenary Institute and Sydney Local Health District (SLDH) (Ethics Approval no./Protocol No - 2020/023, 2019/029, 2020/002). All the experimental procedure involved in this PhD thesis followed the strict recommendation by the Australian code of practice for the use of animals for experimental purposes with issued by the National Health and Medical Research Council of Australia.

3.5.3. Mice acclimatisation, housing conditions and microbiome normalisation

Details of the handling of mice prior to smoke exposure (acclimatisation, housing and microbiome normalisation) can be found in Section 2.1.2 and 2.1.3 of Chapter 2.

3.5.4. FMT and cigarette-smoke model

After microbiome normalisation, female C57BL/6 (B6) mice received oral gavage FMT twice weekly for 4 weeks as per Figure 1 and Table 1. The mice then underwent chronic smoke exposure to 6 weeks as per the parameters defined in Section 2.2.1 (Figure 1) in Chapter 2.

Table 1: *Table showing the experimental group, source of FMT, type of exposure (air or smoke) and duration of exposure, (n=8).*

Experimental group	Sample size	FMT source	Exposure type	Duration of exposure
Healthy Microbiome – Air exposed	8	Healthy mice	Air	6
COPD Microbiome – Air exposed	8	COPD mice	Air	6
Healthy Microbiome – CS exposed	8	Healthy mice	CS	6
COPD Microbiome – CS exposed	8	COPD mice	CS	6

3.5.5 Faecal sample collection during the model and assessment

We collected the faecal samples on -4 weeks (after microbial normalization), 0 weeks (after 4 weeks of FMT) and at 6 weeks (after CS exposure). These samples were processed and stored for microbial molecular analysis at a later date. Detailed can be found in Section 2.3.2 of Chapter 2.

3.5.6 FMT preparation and administration

Faecal pellets are collected from all the mice in the group (n=8). The pellets are immediately kept inside the anaerobic phosphate buffer saline (PBS); 100mg/ml. and processed for suspension preparation. The details can be found in Section 2.3.1 of Chapter 2.

3.5.7 FMT through oral gavage in CS-COPD mice model

In this mice model FMT from Model 1 healthy group (air exposed) mice is transplanted to either healthy (air-exposed) or diseased (smoke-exposed) group of mice in Model 2. Also, the faecal matter from diseased group (smoke-exposed) from Model 1 is been transplanted in both healthy (air-exposed) and diseased (smoke-exposed) group of mice. The details of stool sample collection and processing of FMT can be found in Section 2.3.1 and 2.3.1 of Chapter 2.

3.5.8 Endpoint sample collection and assessments

On the day of endpoint, the recipient model (Model 2) mice were anaesthized with an i.p. injection of xylazine and ketamine (up to 25 mg/kg xylazine + up to 400 mg/kg ketamine) diluted with sterile PBS. The tracheal access through cannula is fixed through surgery. The mice are then run on the FlexiVent to evaluate the lung function of each individual mouse. Blood is collected by cardiac puncture, two lung BALF is collected by pushing 1 ml of Haenks buffer. Right lung lobes are tied and lung collected for RNA and protein analysis. The left lobes are fixed with formaline by pushing 500 µl of formaline collected in a formaline tube. Faeces, are collected in epitubes and snap freezed immediately and kept in -80º C till further analysis.

3.5.9 Lung function

Lung function for COPD was measured using the Scireq flexiVent FX1 system (Montreal, Canada) and the forced oscillation and forced manoeuvre techniques were employed [170]. The various lung function parameters were assessed including FVC, FEV0.1, FEV0.1/FVC and TLC. The details are enclosed in Section 2.4.2 of Chapter 2.

3.5.10 Airway inflammation.

As the total leucocyte counts (TC) and differential leucocyte counts (DC) are the most established clinical indicators of inflammation. The bronchoalveolar lavage fluid (BALF) is collected on the endpoint and total leucocyte counts (TC) is done using light microscopy. The BALF fluid is stained with MayGrunwald Giemsa stain and differential leucocyte counts (DC) performed under light microscopy. The details can be found in Section 2.5.2 of Chapter 2.

3.5.11 RNA extraction

The single right lobe of the mice are snap frozen, to be used latter for extraction of RNA using TRIzole (1mL; Invitrogen) and processed as per the protocol and the final extract was resuspended in nuclease free water. The concentration and quality of RNA was measured using a Nanodrop (Spectrophotometer ND-1000). The details of the protocol can be found in Section 2.5.2 of Chapter 2.

3.5.12 mRNA reverse transcription and quantitative real-time polymerase chain reaction (qPCR)

The extracted RNA from the lung tissue is then processed to transcribe to complementary DNA (cDNA) and further qPCR with the standard protocol. The details are enclosed in Section 2.5.3 and Section 2.5.4 of Chapter 2.

3.5.13 Statistical analysis

The statistical analysis was basically done by comparisons between groups using unpaired t-Tests or a nonparametric equivalent as appropriate. The details can be found in Section 2.7 of Chapter 2.

3.6. Results

3.6.1. The effect of a COPD gut microbiome on airway inflammation after 6 weeks smoke exposure

Chronic airway inflammation is leading drivers of COPD development and progression. In order to understand the effect of the COPD gut microbiome on CS-induced airway inflammation, the levels of immune cell counts BALF (Fig. 3 a-d) and gene expression of some of the proinflammatory cytokines in lung tissue (Fig. 4 a-d) were assessed.

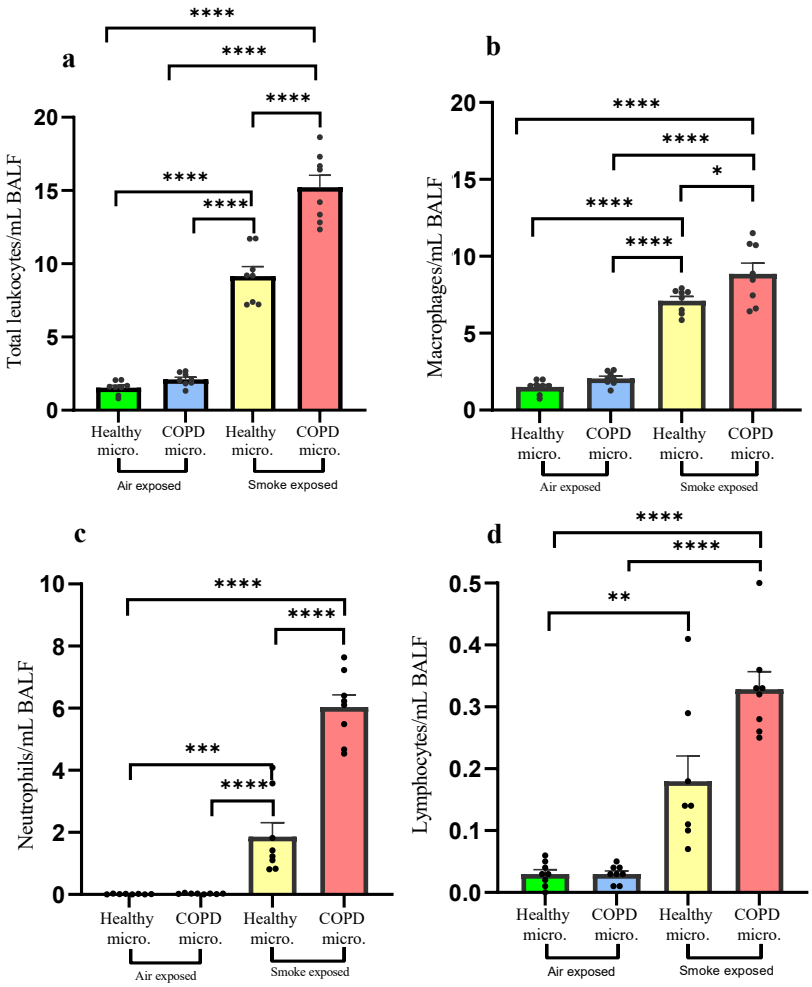

Figure 4: The level of immune cells in BALF after 6 weeks of CS exposure.

*Nose-only exposure of the lungs of BALB/c mice to cigarette smoke induces the hallmark features of human COPD. Relative to air exposed mice, smoke-exposed mice had has increased number of (a) Total leukocytes, (b) Macrophages, (c) Neutrophils, and (d) Lymphocytes numbers were determined (n=8). Results are presented as mean ±SEM. Statistically significant differences are shown between groups as *p<0.05, **P<0.01 and ***P<0.001.*

Figure 3 shows the total number of total leukocytes (a), macrophages (b), neutrophils (c) and lymphocytes (d) in BALF from air and smoke exposed mice after 6 weeks, with either transplanted with healthy or COPD microbiome. In the smoke-exposed groups, the mice which were transplanted with a COPD microbiome showed significantly higher total leukocytes, macrophages and neutrophils after 6 weeks of smoke exposure (P<0.0001, P=0.0235 and P<0.0001, respectively). The difference in lymphocyte levels was comparable but not significant (P=0.0014) (Fig. 3 a-d). In contrast, we noted no significant changes in gene expression of proinflammatory genes (Casp1, IL-1β, TNF-α and Nalp3) when comparing between the smoke-exposed groups with a healthy versus COPD microbiome (Fig. 4 a-d). There were no significant changes in immune cell counts for the air exposed mice, regardless of gut microbiome profile.

The gene expression of some of the common proinflammatory cytokines such as Interleukin-1β (IL-1β), Tumour-necrosis factor alpha (TNF-α), Caspase 1 (Casp1) and Nalp3 were assessed to compare this with the inflammatory cells of BALF. The effects of FMT on the inflammatory cytokines in the lung tissue, was assessed by measuring the gene expression of proinflammatory cytokines (Fig. 4 a-d).

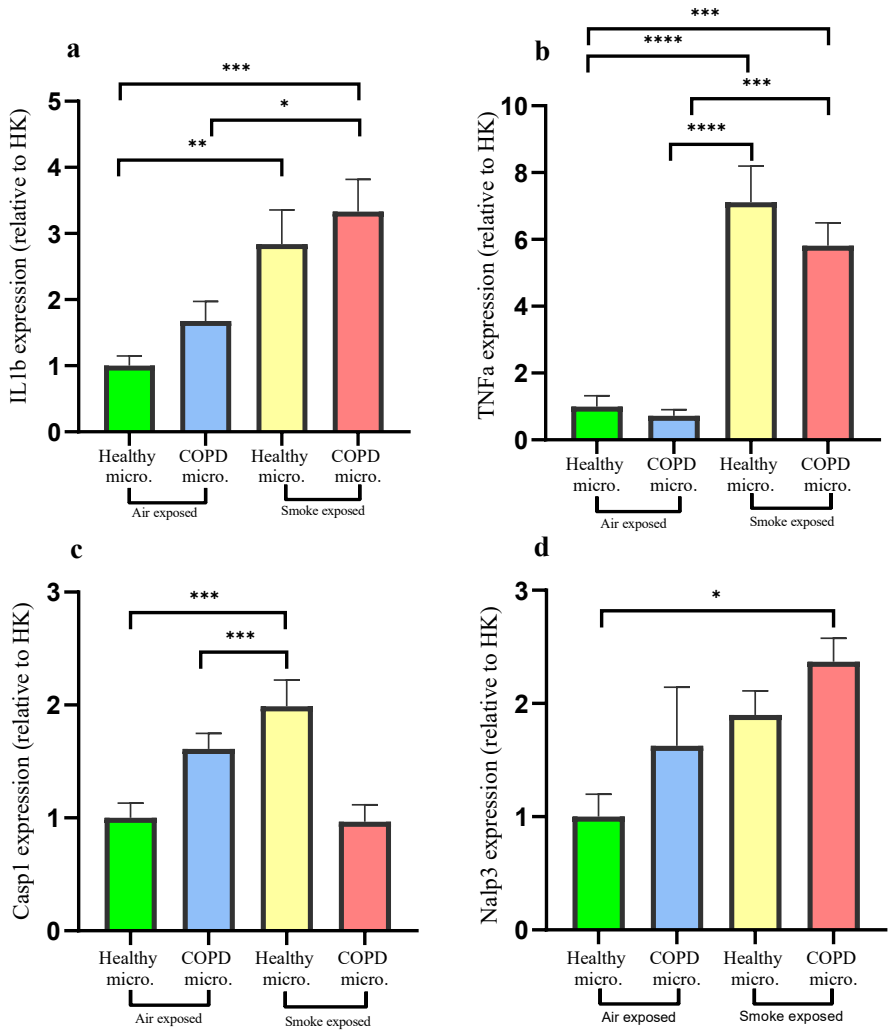

Figure 5: Relative mRNA expression of proinflammatory genes in lung tissue.

*(a) Interleukin-1β (IL-1β), (b) Tumour-necrosis factor alpha (TNF-α), (c) Caspase 1 (Casp1) and (d) Nalp3 Results are presented as mean ±SEM, n=8 for each group. Statistically significant differences are shown between groups as *p<0.05, **P<0.01 and ***P<0.001.*

Figure 4 shows the expression of Interleukin 1β (a), Tumour-necrosis factor α (TNF-α) (b), Caspase 1 (Casp1) (c) and Nalp3 (d) in lung tissue from air and smoke exposed mice after 6 weeks, with or without a transplanted COPD microbiome. Mice exposed to 6 weeks of smoke exposed mice transplanted with COPD gut microbiome had significantly higher IL-1β and comparably higher TNF-α (P=0.0214 and P= 0.0002, respectively). The mice groups with healthy microbiome transplanted with COPD microbiome had significantly higher IL1 β, TNF-

α, Casp1 and no difference in Nalp3 (P = 0.0074, P < 0.0001, P = 0.0004 and P = 0.2778, respectively), when compared to the air-exposed control group.

3.6.2. The effect of a COPD gut microbiome on airway remodelling after 6 week CS exposure

The extra cellular matrix (ECM) in the lung are the basic building blocks of the lung. The disturbance in the ECM leads to important consequences such as lung tissue remodelling, which affects in all compartments including fibrosis of airway and emphysema in COPD. The ECM compartments includes the basement membrane, lamina propria and alveolar interstitium, and ECM is considered to connect the alveoli and blood vessels, forming the lung parenchyma. We assessed collagen deposition and gene expression of important proteins that play a vital role in lung tissue remodelling in COPD. This study showed no difference in collagen deposition around the airways between experimental groups after 6-week exposure to CS or air (Fig. 5 a, b).

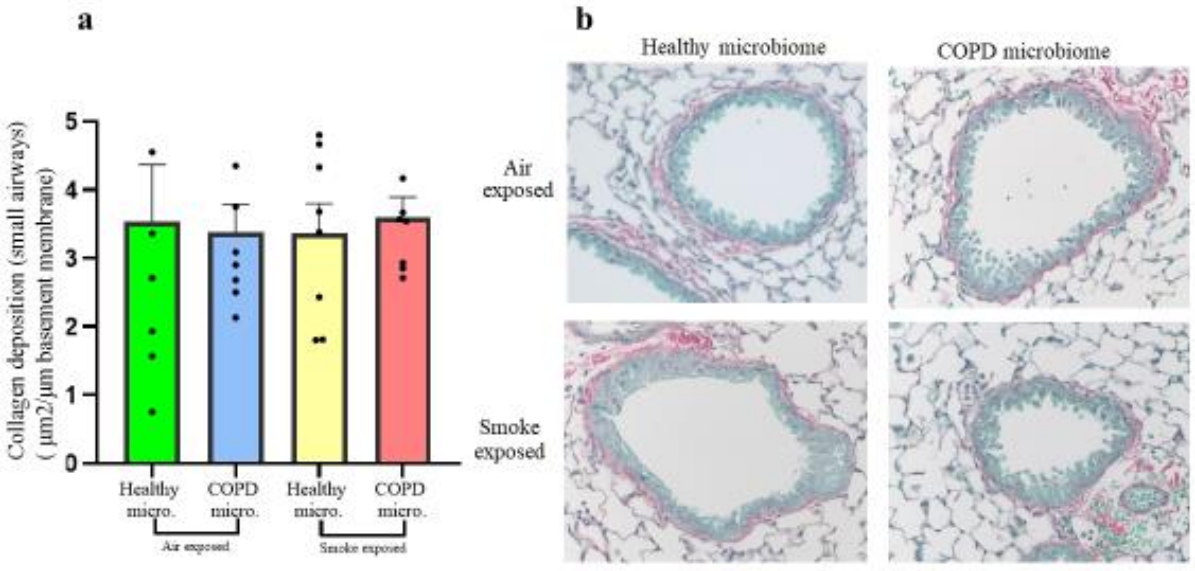

Figure 6: Collagen deposition after 6 weeks smoke exposure.

*(a) Area of collagen around small airways (b) The representative images (40x magnification; Scale bar = 50μm). The data presented as mean ± SEM; n=8. Statistically significant differences are shown between groups as *p<0.05, **P<0.01 and ***P<0.001.*

I found significant increase in the expression of Col5a3 and fibronectin within the CS-exposed groups, comparing between the smoke exposed mice with either healthy verses COPD gut microbiome (P= 0.0015 and P < 0.0001, respectively) (Fig. 6 d).

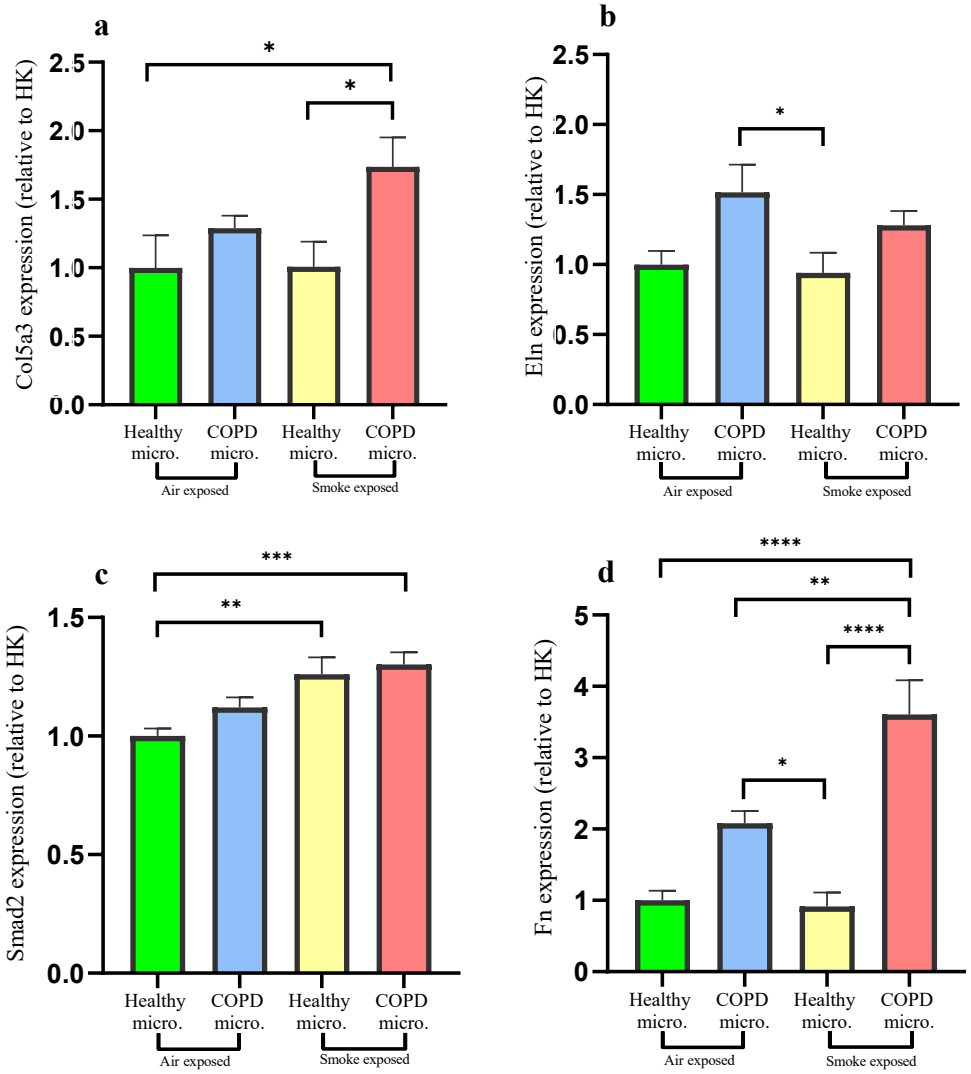

Figure 7: Gene expression of remodelling biomarkers.

*Figure showing gene expression levels of remodelling genes Col5a3 (a), Eln (b), Smad2 (c) and Fn (d) from lung tissue after FMT for either healthy (air exposed) mice or diseased (smoke exposed) mice. Data presented as mean ± SEM; n=8. * = p<0.01.*

3.6.3 The effect of a COPD gut microbiome on epithelial thickness after 6 weeks CS-exposure

The airway epithelial cells does play a crucial role in the development and pathogenesis of COPD. The epithelial cells in the process becomes dysregulated, and inflammatory response causes excess mucus production and hypersecretion, resulting in increased mucus production, airway obstruction, and tissue remodelling from several downstream events. This study show no significant different in epithelial thickness between experimental groups (Fig. 7 a, b).

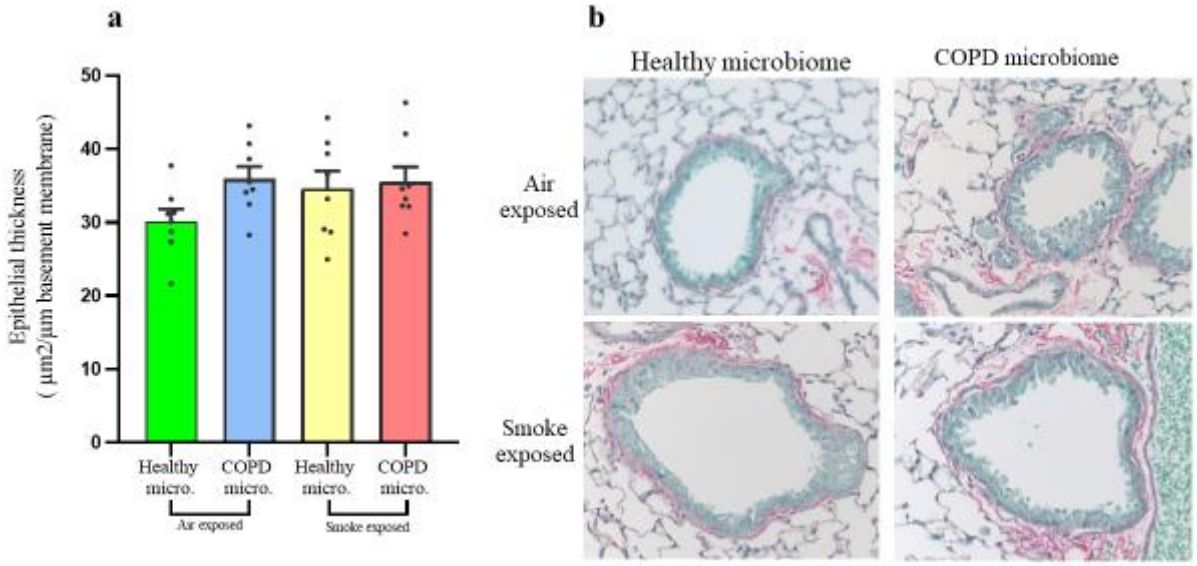

Figure 8: Changes in epithelial thickness

*The figure showing the effects of FMT on (a) Epithelial thickness around the small airways was quantified using Sirus Red and Fast Green-stained lung sections. (b) Representative images (40x magnification; Scale bar = 50µm). Data presented as mean ± SEM; n=8. * = p<0.01.*

3.6.4 The effect of a COPD gut microbiome on alveolar destruction after 6-week CS exposure

One of the hallmark features of COPD is alveolar destruction. This is driven by chronic inflammation which can lead to an imbalance of proteases such as neutrophil elastase and matrix metalopeptidases (MMP) such as MMP12. Using histological analysis, we have measured the diameter of the alveoli. An increase in alveolar diameter is indicative of alveolar destruction. Figure 8 shows that only the CS-exposed group with a COPD microbiome has a significantly higher alveolar diameters compared to the air-exposed healthy group ($P = 0.0103$) (Fig. 8 a, b). The macrophage elastase Mmp-12, is basically secreted from alveolar macrophages in COPD patients, is known to mediate the lung injury and emphysema development. I noted no significant difference in the gene expression of Mmp12 gene by qPCR (Fig. 8 c). Also, there was no noted significant difference in Mmp12 expression between smoke exposed groups with a healthy micbriome versus a COPD microbiome.

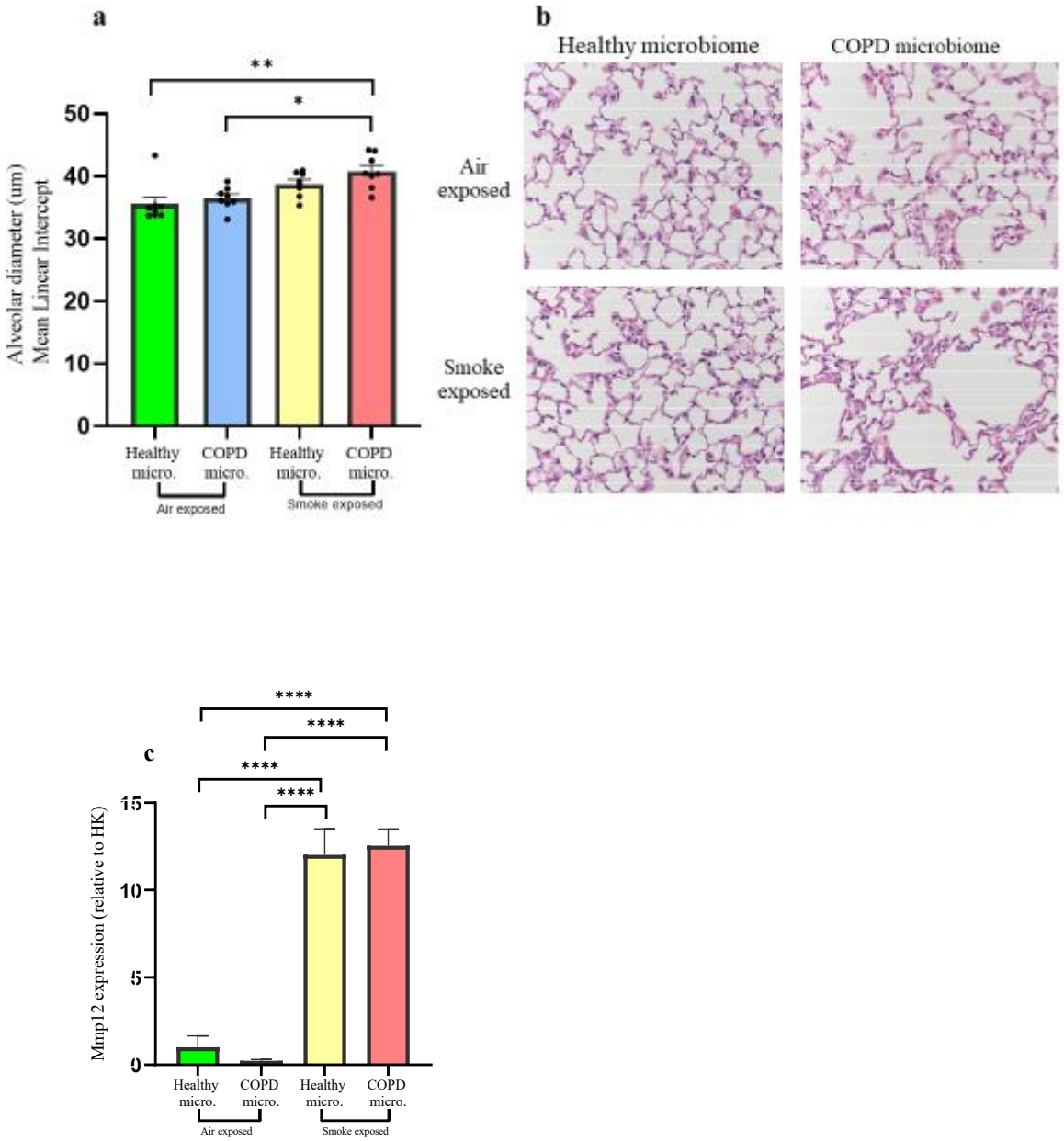

Figure 9: Indicators of emphysema

*The alveolar diameter was measured by mean linear intercept (a). Representative images (40x magnification; Scale bar = 50µm) (b). Mmp12 lung gene expression results obtained by qPCR in the in transplanted mice with either healthy (Healthy microbiome) or diseased (COPD microbiome) group (c). The data are expressed as mean per group. Gene expression data are displayed as fold-changes compared to the Air/Air control group. * p Data presented as mean ± SEM; n=8. * = p<0.01.*

3.6.5 The effect of a COPD microbiome on lung function after 6-weeks of CS-exposure

Lung function is the ultimate hallmark of COPD as it measures the degree of airflow obstruction. Lung function was measured using plethysomometry. Both smoke groups showed significantly reduced $FEV_{0.1}$ and $FEV_{0.1}$/FVC compared to the healthy air exposed group. Only the CS-exposed mice with a COPD microbiome showing significant increase in TLC compared to the healthy air exposed mice (Fig. 9 a-d).

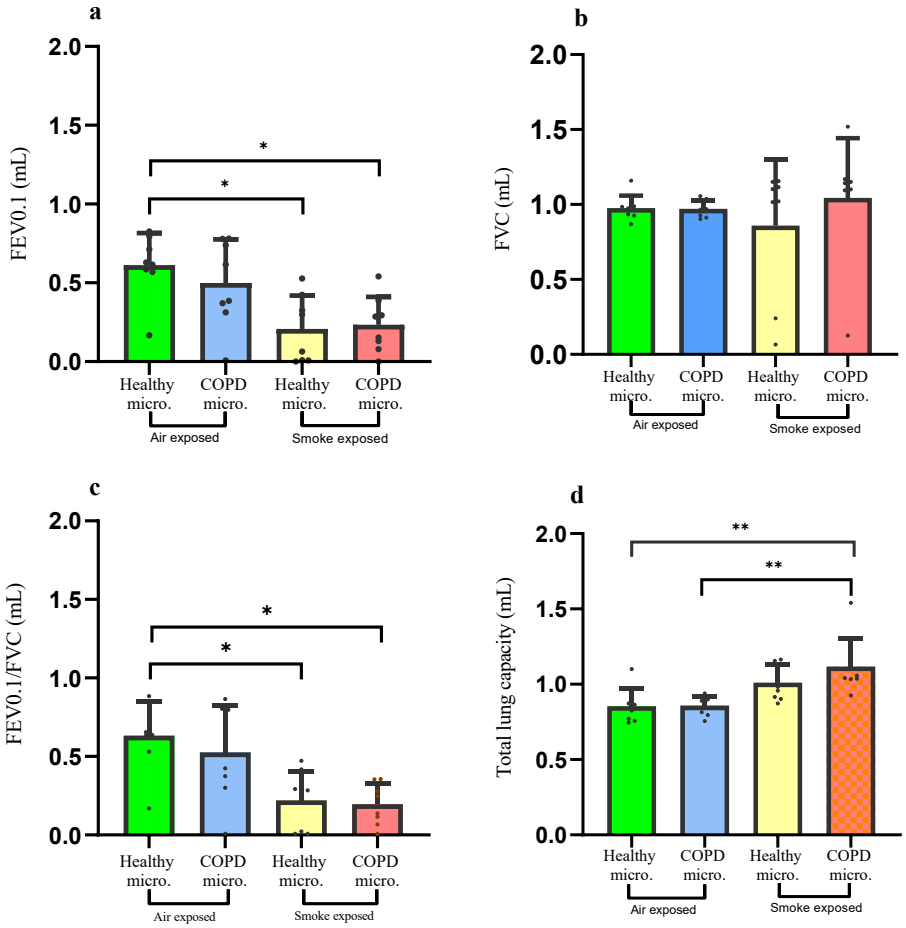

Figure 10: Changes in lung function

*Figure showing the effects of FMT on Lung function of the C57BL/6 female mice when transplanted with either healthy or diseased/COPD microbiome. Lung function was assessed using a Flexivent apparatus. The data presented as mean ± SEM; n=8. * = p<0.0001*

3.7 Discussion

In this present study, I investigated the effects of FMT when given prophylactically from the diseased group of mice (smoke exposed for 12 weeks) to the mice groups further exposed with 6 weeks of CS [164-167]. This showed that FMT from diseased group of mice (COPD microbiome) predisposes to COPD features even with shorter exposure to CS.

3.7.1. Main results

COPD is a chronic inflammatory condition and generally having high infiltration of leukocytes in the lung and airways. These leukocytes, if in excess and when poorly controlled, tend to damage the lung that leads to the pathophysiology associated with the disease [175]. The long-term exposure to inhaled pollutants such as cigarette smoke (CS), leads to the chronic inflammation in the airways due to the activation of the structural and inflammatory cells in the lungs including epithelial cells and alveolar macrophages. These activated cells releases the chemotactic mediators which then recruit additional inflammatory cells such as neutrophils, monocytes, lymphocytes, and CD8+ T cells in the lung. This results in chronic inflammation which leads to structural changes, airway obstruction and respiratory symptoms [176].

In this study we observed significantly higher number of total leukocyte counts, macrophages and neutrophils and noticeably higher lymphocytes in the BALF fluid of the mice group with a transplanted COPD gut microbiome when compared to mice with a healthy gut microbiome (Figure 3 a-d). We also noted that the healthy microbiome when treated with smoke exposed faeces have significantly raised total leucocyte count, macrophages, neutrophils and lymphocytes as compared to the healthy controls (Healthy microbiome exposed with air) (Figure 3 a-d). Overall, in this present study the comparable difference can be seen in the inflammatory cells between the groups with most notable changes observed between the two smoke exposed groups with a healthy versus COPD gut microbiome. This indicates towards

the predisposition of lung airway to inflammation in the mice group when treated with the faces of mice already been in COPD state; between 8 – 12 weeks of smoke exposure.

3.7.2. The COPD gut microbiome exacerbates inflammasome activation in the lungs

Inflammation is a complex biological process in response to the pathogenic and harmful stimuli involving the immune system in which a cascade of vicious cycle initiates. In COPD, due to the inflammatory responses the eosinophils and neutrophils migrate from the circulating bloodstream into lung tissues and upon activation, they release some of the pro-inflammatory cytokines which further contribute in the recruitment of monocytes and T cells. The two prominent pro-inflammatory cytokines considered important in COPD are interleukin 1-beta (IL-1β) and tumour necrosis factor-alpha (TNF-α). The elevated levels of IL-1β and TNF-α have been found in COPD and are mostly responsible for amplifying inflammation through the activation of cellular nuclear factor kappa-light chain-enhancer of activated B (NF-kB), activator protein 1 (AP-1), and also few of the other transcription factors [177]. IL-1β are also secreted by monocytes that are involved in initiation and flaring of inflammation [178]. IL-1β and TNF-α, are both potent activator of alveolar macrophages in COPD patients responsible for alveolar septa and fibrosis of the airway walls [179].

The caspase-1 (Casp1)/Interleukin-1 converting enzyme (ICE) is considered as an evolutionarily enzyme that is known to proteolytically cleaves the other proteins, such as the precursors of the some of the inflammatory cytokines and most prominent among them is IL1-β [180, 181]. It is now identified that the activation of infammasomes is responsible for the pathogenesis of COPD. One of the most investigated inflammasome is the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) or

Napl3 infammasome which are activated by different agonists [182]. NLRP3/Nalp3 is reported to be an activator of Casp1 [183].

In this present study we assessed the change in gene expression of IL-1β, TNF-α, Casp1 and Nalp3 in the lung tissue and looked at the relationship with proinflammatory cytokines in order to determine the effects on inflammasome activation, believed to play important role in COPD pathogenesis and prognosis. This study showed a trend for increasing expression of IL-1β, TNF-α and Nalp3 with no difference in Casp1 in the CS-exposed transplanted with COPD microbiome as compared to control groups; Healthy microbiome or COPD microbiome treated either air exposed faecal matter (Figure 4 a-d). Though there was no difference noted between the COPD microbiome and healthy microbiome treated with smoke exposed faecal matter. This finding suggests that the FMT from COPD microbiome when exposed to CS for even 6 weeks is enough to activate pro-inflammatory cytokines in lung tissue. This further indicates that FMT with COPD microbiome does pushes mice towards COPD prognosis.

3.7.3 The COPD gut microbiome accelerates lung remodelling gene expression

The major components of extracellular matrix (ECM) of lung includes collagen, elastic fibres, proteoglycans, fibronectin and tenascin [184, 185]. Some of the previous literature have reported a decrease in elastin [186, 187] and proteoglycans [188] and an increase in total collagen content in the alveoli of COPD patients [189]. This study showed no change in collagen deposition between the groups (Figure 5 a-b). This could be explained partially as the mice were only smoked for 6 weeks following FMT for 4 weeks. In our experimental model, collagen deposition is not evident until 8 weeks of smoke exposure.

Col5a3 gene encodes collagen alpha-3(V) chain which is a basic protein in humans [190, 191]. It is believed that the more collagen deposition is related with increase in Col5a3 expression in

the lung tissue. Similarly, *Eln* gene encodes for Elastin protein in humans. Elastin is considered a key element of the extracellular matrix [192, 193]. It is highly elastic and are basic component of the connective tissue allowing the various other tissues to resuming their shape after stretching or contracting. Deletions and mutations in this Eln gene is associated with various disease conditions including emphysema caused by α_1-antitrypsin deficiency [193-195]. The Mothers against decapentaplegic homolog 2 which is more commonly known as SMAD family member 2 or SMAD2 protein encoded by the *Smad2* gene. SMAD proteins act through as a signal transducers and transcriptional modulators that influence multiple signalling pathways [196]. Fibronectin is an important player in morpho-regulation during the lung development. In adult life, this proteins is altered after any local tissue injury and inflammation that regulates cell adhesion, migration and differentiation [197]. Fibronectin is found to be higher in COPD patients, mostly in the small airway level. Also, fibronectins induces matrix metalloproteinase expression and activity [198], contributing to the perpetuation of local tissue injury. Despite the lack of elevated collagen deposition, the expression of Col5a3, Smad2 and Fibronectin (Fn), (Fig. 6a, c, d) is found to be significantly raised in the CS-exposed microbiome transplanted with COPD microbiome compared to air-exposed microbiome transplanted with COPD microbiome mice faeces. However, we did not find any difference in expression of Elastin (Eln) (Fig. 6 b). This finding shows that FMT with COPD microbiome and CS exposure for even only 6 weeks is sufficient to accelerate Col5a3, Smad2 and Fn, Further, supporting our hypothesis that FMT with COPD microbiome is able to push the pathology forward towards COPD phenotype.

3.7.4 The COPD gut microbiome induces lung damage and promoter proteins

Emphysema is a common phenotype of COPD where the air sacs (alveoli) are damaged and oxygen exchange capacity of the lung is reduced. The epithelial thickness of the alveolar sac

in COPD patients and mice models have been found increased. The air sac walls become loose and overall surface area increases and alveolar diameter increases [199]. Matrix metalloproteinase 12 (Mmp-12), commonly known as matrix metallopeptidase 12 and also as macrophage metalloelastase in mice which are mainly produced by macrophages, in the lower airspaces under normal conditions and is essential for the development of emphysema in cigarette smoke exposed mice [200-207].

The results of this study show no difference in the epithelial thickness (Fig. 7 a, b), however the alveolar diameter (Fig. 8 a, b) from the lung tissue shows significant increase between the smoke exposed COPD microbiome group and the air controls. The Mmp12 gene expressed increased in both the smoke exposed groups (Fig. 8 c). With this findings we can state that after 6 weeks of smoke exposure and FMT from diseased mice group does not effects the epithelial thickness however it does increase the alveolar diameter and increased expression of Mmp12 gene. This suggests that there is alveolar destruction with FMT COPD microbiome and just 6 weeks of CS exposure, though no changes seen in epithelial thickness, indicating that emphysema is not yet well established at this stage however swinging towards COPD phenotype.

3.7.5 The COPD gut microbiome reduces lung function capacity of mice

COPD is diagnosed using spirometry which measure the amount and/or speed in which a person can inhale and exhale air and can therefore assess for airflow obstruction in the lungs. This is based on three key parameter of lung function: forced expiratory volume in one second (FEV1), forced vital capacity (FVC), and the FEV0.1/FVC ratio. An FEV1/FVC ratio of <0.70 after bronchodilator is typically considered diagnostic of COPD. Further, lung tissue is destroyed in emphysema, leading to loss of elastic recoil which allows the lungs to be re-stretches to abnormally large volumes, that resulting in an increased TLC [208].

In this study, we assessed the lung function parameters of the mice in groups, there were few differences noted between the different groups. We see a significant decrease in FEV0.1 and FEV0.1/FVC in smoke exposed COPD microbiome group as compared to air control (Fig. 9 a, c). The TLC which in inversely related to FEV0.1 and FEV0.1/FVC is elevated (Fig. 9 d) which is very much consistent with the existing literature [208]. We did not note any change in FVC between the groups (Fig. 9 b).

Though we note some changes in the lung function parameters there is no overall obvious difference between the groups which indicates that FMT with CS mice faeces and further exposure with CS for 6 weeks is not enough to induce significant decline in lung function parameters.

Table 2: ***Summary table showing the experimental groups and various parameters investigated in this chapter (n=8). Groups in this chapter are: HM-AE (Healthy Microbiome – Air Exposed); COPDM-AE (COPD Microbiome – Air Exposed); HM-CSE (Healthy Microbiome – Cigarette Smoke Exposed); COPDM-CSE (COPD Microbiome - Cigarette Smoke Exposed).***

Experimental group/Findings	HM-AE (Column I)	COPDM-AE (Column II)	HM-CSE (Column III)	COPDM-CSE (Column IV)
TLC	-	-	↑	↑
Macrophages	-	-	↑	↑
Neutrophils	-	-	↑	↑
Lymphocytes	-	-	↑	↑
IL1b	-	-	↑	↑
TNF-α	-	-	↑	↑
Casp1	-	-	↑	-
Nalp3	-	-	-	↑
Collagen deposition	-	-	-	-
Col5a3	-	-	-	↑
Eln	-	-	-	-
Smad2	-	-	↑	↑
Fn	-	-	-	↑
Epithelial thickness	-	-	-	-
Alveolar diameter	-	-	-	↑
Mmp12	-	-	↑	↑
FEV0.1	-	-	↓	↓
FVC	-	-	-	-

3.7.6 Characteristics of the COPD gut microbiome which might contribute to disease pathology

In COPD patients have dysbiosis of the gut microbiome. On the family level, a relatively decreased abundance of *Fusobacteriaceae, Prevotellaceae,* and enriched level of *Bacteroidaceae* and *Prevotella* have been identified [113]. The faecal microbiome 16 S rRNA gene sequencing of COPD patients shows increased abundance of genera *Streptococcus, Rothia, Romboutsia* and *Intestinibacter* whereas the *Bacteroides, Roseburia* and *Lachnospira* from the family *Lachnospiraceae* and several unnamed genera of *Ruminococcacea* are decreased. *Streptococcus* species are identified as key organism in COPD group as compared to healthy group. Multiple members of the family *Lachnospiracease* such as *Streptococcus sp000187445*, *Streptococcus vestibularis* has been found to be correlating with reduced lung function [114].

In clinically stable COPD, many of the recent research has reported a rich lung microbiome that is distinctly different than seen in healthy controls. In these stable COPD patients, the common phyla identified are Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes, and the most dominant genera includes with *Pseudomonas, Streptococcus, Prevotella* and *Haemophilus* [115 -118].

In order to assess the effectiveness of the FMT and the changes in gut microbiome over the course of the smoke exposure, microbial DNA extracted from stool was sent for metagenomic sequencings. Unfortunately, at the time of submission for this thesis, the bioinformatics on this sequencing was not complete. To address this limitation, quantitative PCR was used to assess the abundance of specific bacterial targets that are known to be affected by cigarette smoke exposure. The abundance of *segmented filamentous bacteria (SFB), Bacteroidetes, Lactobacillus, Prevotellaceae,* and *Bifidobacterium* were assessed after normalisation, after

FMT and after 6 weeks CS exposure. *The abundance of segmented filamentous bacteria (SFB), Bacteroidetes, Lactobacillus,* and *Bifidobacterium* was significantly reduced after 6-weeks of smoke exposure in the smoke-exposed group with a transplanted COPD gut microbiome (P=0.0007, P=0.0069, P=0.0009 and P=0.025, respectively) but not the Prevotellaceae (Appendix - Fig. 26 a-e). This is consistent with the existing literature which indicates towards reduced commensal bacterial in COPD [113-115].

3.7.7 Limitation

This study has several limitations. Firstly, the microbiome of the FMT was not assessed so we cannot confirm components and composition of this suspension preparation. However, our method of FMT preparation is a commonly used method used in the literature and we are confident with its effectiveness of transplantation. Secondly, the FMT is done only twice a week. It is possible that this was not sufficient to transplant the microbiome. However, there is a risk that increased oral gavage can have an unintended adverse effect and twice per week standard practice for murine model. Thirdly, the faecal samples was been collected at different time points of the model for metagenomic sequencing. Though the experimental analysis has been done and currently this is with our bioinformatics team for further analysis. However, we could not finalise the bioinformatics data at the time of final thesis submission.

3.7.8 Conclusions

In this chapter, I showed that the COPD gut microbiome predisposes to disease in a 6 week model of CS-exposure. Overall, this study showed that the COPD gut microbiome contributed to the predisposition towards COPD. It accelerates the local and systemic inflammation and worsen the alveolar destruction and collagen deposition. The pro-inflammatory cytokine, inflammasome, inflammation signalling molecules are increased with the worsening disease condition in the smoke exposed COPD microbiome group as compared to air controls. These

findings indicates towards the potential for targeted therapies to restore a healthy gut microbiome may be beneficial in preventing disease progression in COPD.

Chapter 4: Investigating the role of gut microbiome in COPD severity

4.1 Background

There is increasing interest of the scientific community to explore this ever-expanding field, and now researchers are investigating the role of gut microbiota in influencing the distal organs through the gut-lung axis. There are many studies in murine model as well as in human trials showing therapeutic effects of FMT. This study is the continuation of the previous chapter (Chapter 3) where we investigated the role of gut microbiome in COPD development where we smoked the mice for 6 weeks and transplanted with COPD microbiome and noted some signification differences among the groups. I found that the COPD gut microbiome is able to predispose the mice towards COPD and accelerates many of the local and systemic inflammation featuring COPD. In this present study, we investigated the effects of a COPD gut microbiota from the mice group smoked for 12 weeks on the severity of an experimental model of 8 weeks CS-induced COPD.

4.2 Study rationale

The current literature suggests dysbiosis of gut microbiome in COPD. However, not much is known either if the diseased COPD microbiome has any role to play in the causation and severity of the disease. Through this experimental model we intend to show the causality of

gut microbiome in COPD severity. In this study, the experiment were designed in such a way that FMT is done from the faeces of the donor group (Model 1) to the recipient group (Model 3). The rational for this study is to access that the COPD microbiome when transplanted to the mice with smoke exposure enhances disease process or increases severity.

4.3 Hypothesis

It is hypothesised that transplantation of COPD microbiome prior to CS exposure will increase severity of disease after 8-week chronic CS exposure.

4.4 Aim

The aim of this study is to interrogate the role of the COPD gut microbiome in the development of COPD.

4.5 Methods

4.5.1 Study design

This study is the continuation of the previous chapter (Chapter 3) with additional component of extended 2 more weeks smoke exposure in recipient model (Model 4), i.e a total of 8 weeks of smoke exposure.

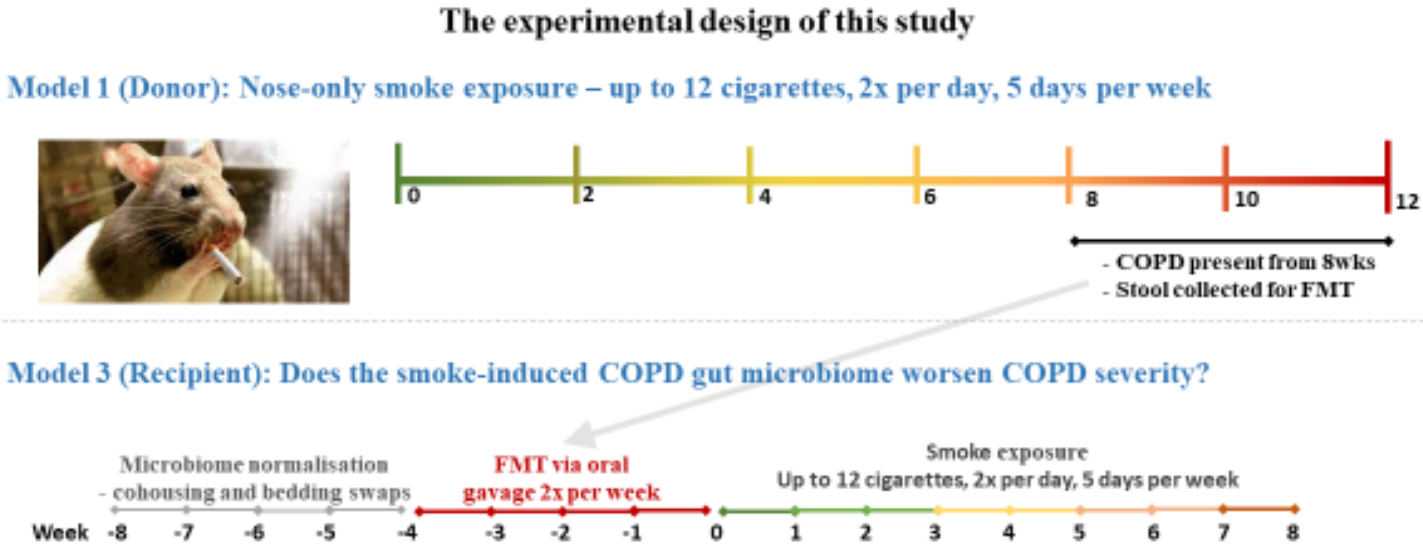

Figure 11: Figure showing the two model – Model 1 and Model 3.

Model 1 is the donor group of mice and model 3 is the recipient group of mice. The mice in Model 1 were either air or smoke exposed for 12 weeks. The FMT was done from the fresh faecal samples collected from model 1 between 8 to 12 weeks. The Model 3 mice underwent microbial normalization for initial 4 weeks. FMT was fed in the recipient model (Model 3) for 4 weeks; 2 x weekly between the microbial normalization and start of the smoke exposure (for further 8 weeks).

4.5.2 Ethics statement

The ethics for this study was approved by the Animal Welfare Committee of Centenary Institute and Sydney Local Health District (SLDH) (Ethical Approval no./ Protocol No -2020/023, 2019/029, 2020/002). This study was performed in recommendation and in strict accordance with the Australian code of practice for the care and use of animals for scientific purposes issued by the National Health and Medical Research Council of Australia.

4.5.3 Mice, acclimatisation and housing conditions

Female c57BL/6 mice, 4-6 weeks of age are being used in this CS exposed-COPD experimental model for both the donor and recipient models. The details included in Section 2.1.2 of Chapter 2.

4.5.4 Microbiome normalization

The microbial normalization was done in order to ensure all the experimental animals in the cohort have similar gut microbiome at the start of the experimental procedure. The details of the procedure is included in Section 2.1.3 of Chapter 3.

4.5.5 Murine model of established CS exposed COPD

The female C57BL/6 (B6) mice were used in this study. In each experiment, 12 mice were simultaneously exposed to reference cigarette (3R4F) twice per day, 5 times per week, for 1–12 weeks. The mice were exposure for 75 minutes. All experiments were approved by our institutional animal ethics committee.

4.5.6 Mice groups and state of disease

In this experiment two models were run parallels – Model 1 and Model 3. The Model 1 includes two groups of mice (n=8) one exposed to room air and the other exposed to cigarette smoke for 12 weeks. The faeces were collected from this model (Model 1) between week 8 and 12 twice a week and these faeces were given to the mice in Model 3. The mice from Model 3 were normalized for microbiome for initial four weeks and then FMT through oral gavage was conducted and done for four weeks before the start of smoke exposure for further 8 weeks. Mice were smoked twice/day, 5 times/week, for 1-12 weeks.

Table 3: ***Description of experimental groups, Table showing the mice groups in this experimental model (Model 3; n=8); 3 groups, 8 x 3 = 24 total mice in this experiment.***

Experimental group	Sample size (n)	FMT source	Exposure type	Duration of exposure (wks)
Healthy Microbiome – Air exposed	8	Healthy mice	Air	8
Healthy Microbiome – CS exposed	8	Healthy mice	CS	8
COPD Microbiome – CS exposed	8	COPD mice	CS	8

4.5.7 Faecal sample collection during the model and assessment

The freshly defecated faecal pellets were collected for gut microbiome molecular analysis and investigation. The faecal samples were collected on day 28 (after microbial normalization) and after 4 weeks of FMT and then after twice a week during the 8 weeks of smoking period.

4.5.8 Faecal suspension preparation and administration

Faecal pellets are collected from all the mice in Model 1 and processed and prepared for faecal suspension for FMT in the mice in the Model 3. The details are included in Section 2.3.1 of Chapter 2.

4.5.9 Endpoint day assessment

On the day of endpoint the mice are anaesthized with an i.p. injection of xylazine and ketamine (up to 25 mg/kg xylazine + up to 400 mg/kg ketamine) diluted with sterile PBS. The tracheal access through cannula is fixed through surgery. The mice are then run on the flexiVent to evaluate the lung function of each individual mouse. Blood is collected by cardiac puncture, two lung BALF is collected by pushing 1 ml of Haenks buffer. Right lung lobes are tied and lung collected for RNA and protein analysis. The left lobes are fixed with formaline by pushing 500 µl of formaline collected in a formaline tube.

4.5.10 Lung function

Lung function for COPD was measured using the Scireq flexiVent FX1 system (Montreal, Canada) and the forced oscillation and forced manoeuvre techniques were employed [170]. The various lung function parameters were assessed including FVC, FEV0.1, FEV0.1/FVC and TLC were measured. The details are enclosed in Section 2.4.2 of Chapter 2.

4.5.11 Airway inflammation

The bronchoalveolar lavage fluid (BALF) is collected on the endpoint and total leucocyte counts (TC) is done using light microscopy. The BALF fluid is stained with MayGrunwald Giemsa stain and differential leucocyte counts (DC) performed under light microscopy.

4.5.12 RNA extraction

RNA extraction was done from the snap frozen lung lobe in accordance with the established protocol. The details of the procedure is described in Chapter 2 (Section 2.5.1).

4.5.13 Transcription and quantitative polymerase chain reaction (qPCR)

Reverse transcription of mRNA to cDNA was done following the established protocol. Details of the procedure and protocol can be found in previous sections, Section 2.5.2 and Section 2.5.3 of Chapter 2.

4.5.14 Statistical analysis

The details can be found in Section 2.7 of Chapter 2.

4.6 Results

4.6.1 The effects of COPD gut microbiome on airway inflammation after 8 weeks smoke exposure

The effects of FMT on the airways inflammation in the lumen BALF, was assessed by measuring the immune cell infiltration in the lungs (Fig. 11 a-d). Figure 11 shows the number of total leukocytes (a), macrophages (b), neutrophils (c) and lymphocytes (d)

in BALF from air and smoke exposed mice for 8 weeks, with either transplanted air or smoke exposed mice groups. Mouse with smoke exposed with COPD microbiome and air exposed with healthy microbiome shows significant increase in total cell count, macrophages and lymphocytes (P= 0.0063, P=0.0045, P=0.0162, respectively) but no significant change in neutrophils.

The mice groups with smoke exposed with COPD microbiome had significantly higher total leukocyte count, macrophages, neutrophils and lymphocytes ($P < 0.0001$, $P = 0.0002$, $P = 0.0006$ and $P < 0.0001$, respectively), when compared to the air exposed healthy microbiome control group. The COPD microbiome group when transplanted with diseased gut microbiome (smoke exposed) had significantly higher lymphocytes ($P = 0.0018$), when compared to the healthy microbiome transplanted with smoke exposed faeces whereas total leukocyte count, macrophages and neutrophils did not show any change.

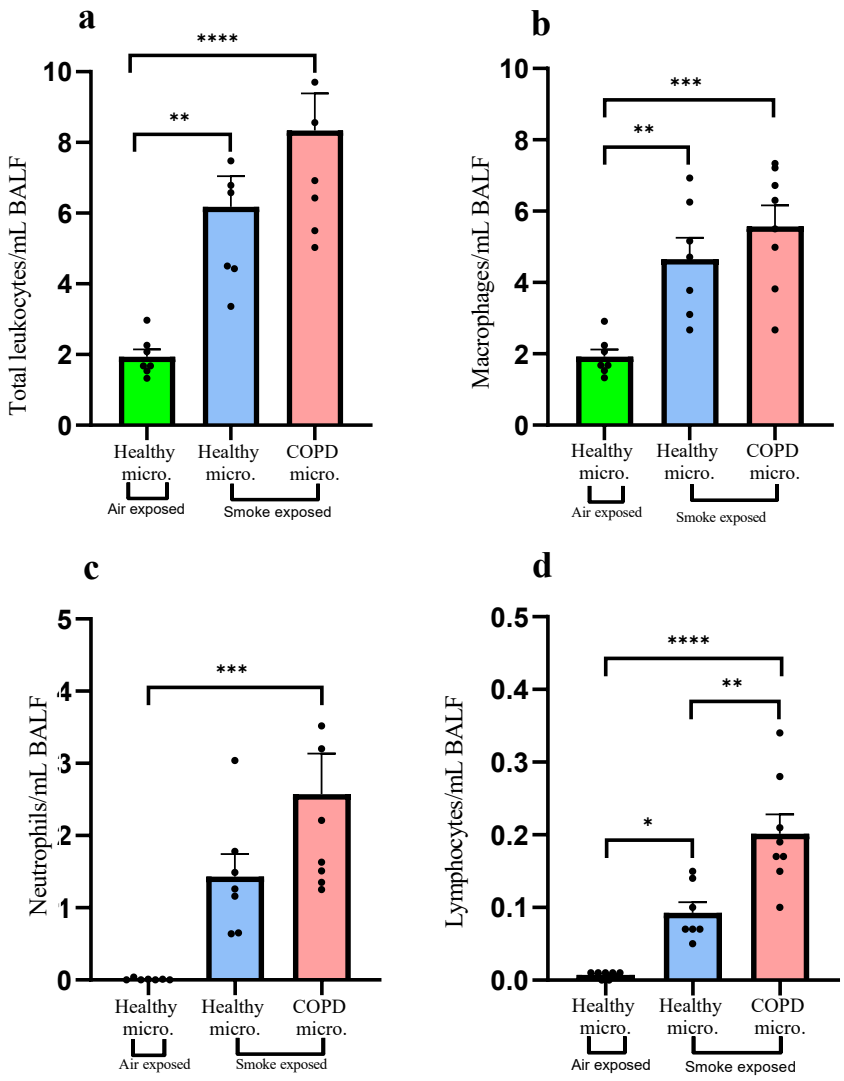

Figure 12: Changes in total leucocytes after 8-week smoke exposure.

*The figure showing the effects of FMT on airway inflammatory cells in the healthy (healthy microbiome) and diseased group (COPD microbiome) when given FMT through oral gavage from either healthy (air exposed) or diseased group (smoke exposed). (a) Total leukocytes, (b) Macrophages, (c) Neutrophils, and (d) Lymphocytes numbers were determined (n=8). Results are presented as mean ±SEM. Significant differences are shown between groups as *p<0.0001.*

The gene expression of some of the common proinflammatory cytokines such as IL-1β, TNF-α, Casp1 and Nalp3 were assessed to correlate this with the inflammatory cells of BALF. The effects of FMT on the inflammatory cytokines in the lung tissue, was assessed by measuring the gene expression of proinflammatory cytokines (Fig. 12 a-d).

Figure 12 shows the expression of IL1-β (a), TNF-α (b), Casp1 (c) and Nalp3 (d) in lung tissue from healthy microbiome and COPD microbiome treated with FMT through oral gavage from the air or smoke exposed mice.

Mouse with air exposed healthy microbiome and transplanted with smoke-exposed faeces shows significant increase in TNF-α ($P = 0.0089$) but does not show any difference in either IL-1β, Casp1 or Nalp3 as compared to the healthy microbiome group of mice when transplanted with faeces from air-exposed group of mice, controls. This correlates with the findings for immune cells in airway lumen BALF particularly total leukocyte counts, macrophages and lymphocytes.

The mice groups with COPD microbiome with transplanted gut microbiome from smoke exposed mice had significantly higher IL1 β, TNF-α and Casp1 ($P=0.0002$, $P<0.0001$, and $P=0.0400$, respectively) and no difference in Nalp3, when compared to the healthy microbiome group of mice with transplanted air-exposed control group faeces. The COPD microbiome group when transplanted with smoke exposed mice gut microbiome had significantly higher IL-1β and TNF-α ($P = 0.0123$ and $P = 0.0152$, respectively), and no significant difference in Casp1 and Nalp3 gene expression, when compared to the healthy microbiome treated with smoke-exposed faeces.

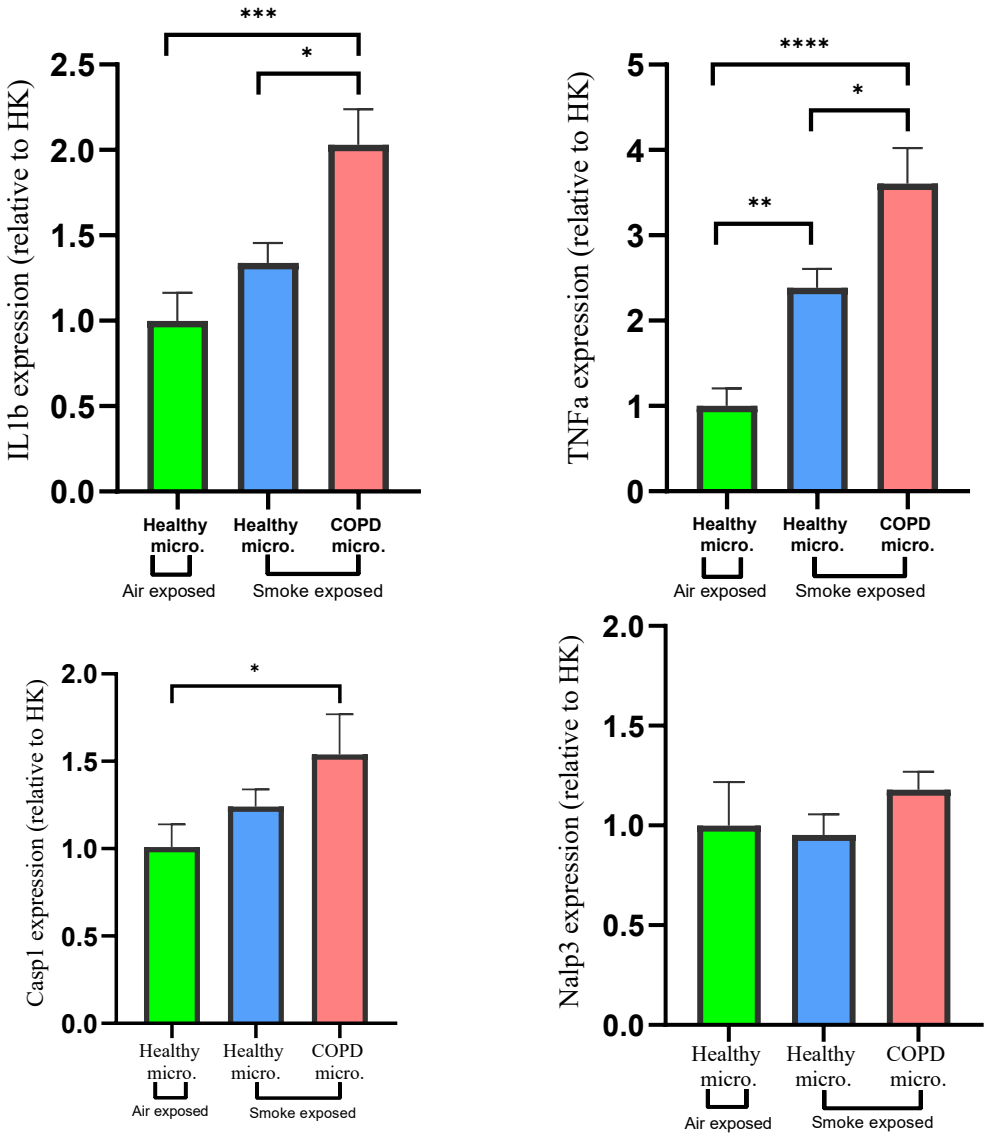

Figure 13: Changes in inflammatory biomarkers after 8 weeks of smoke exposure.

*The figure showing the effects of FMT on inflammatory cytokines in the lungs of healthy group (healthy microbiome) and diseased group (COPD microbiome) when given FMT through oral gavage of either healthy (air exposed) or diseased group (smoke exposed). (a) Interleukin-1β (IL-1β), (b) Tumour-necrosis factor alpha (TNF-α), (c) Caspase 1 (Casp1) and (d) Nalp3 were determined (n=8). The result has been presented as mean ±SEM. Significant differences are shown between groups as *p<0.0001.*

4.6.2 The effects of a COPD gut microbiome on airway remodelling after 8 weeks of CS exposure

After 4 weeks of FMT and 8 weeks of either air or smoke exposure, there was significant difference in collagen deposition in and around small airways in the COPD microbiome transplanted with smoke exposed faeces (P = 0.0066) when compared to healthy microbiome group of mice transplanted with air-exposed faeces (Fig. 13 a, b). Similarly, the COPD microbiome treated with smoke-exposed faeces has significantly higher (P = 0.0413) collagen deposition then compared to healthy microbiome treated with faeces from smoke-exposed group of mice.

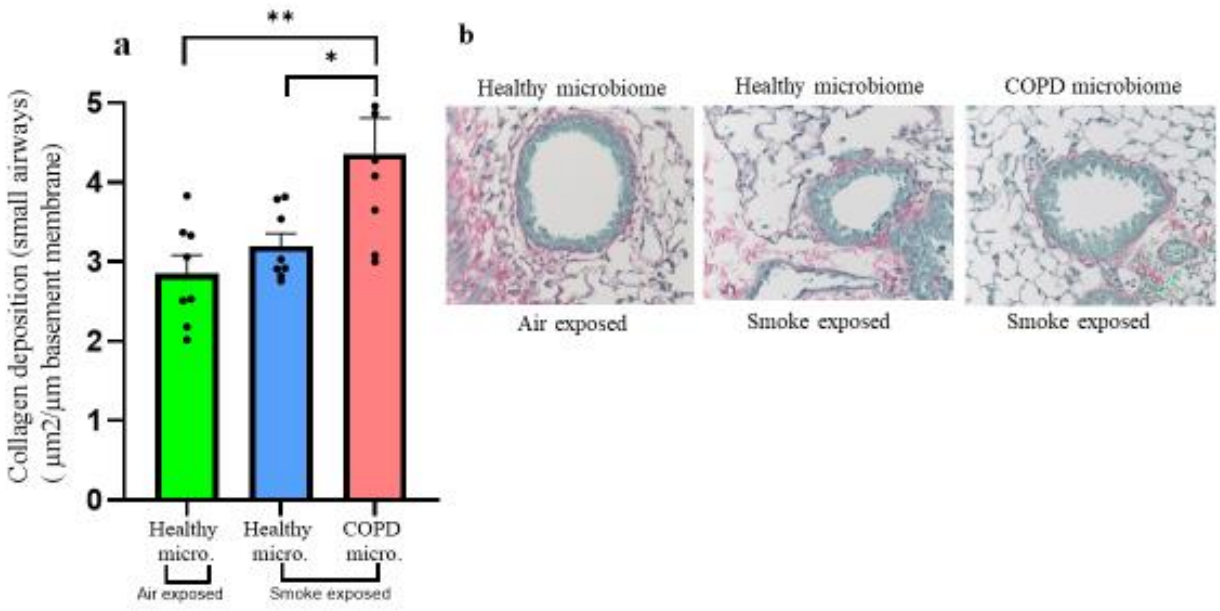

Figure 14: Change in collagen deposition after 8 weeks of smoke exposure

*The figure showing the effects of FMT on (a) Area of collagen around small airways was quantified in Sirus Red/Fast Green-stained lung sections. (b) Representative images (40x magnification; Scale bar = 50µm). Data presented as mean ± SEM; n=8. * = p<0.01.*

The extra cellular matrix (ECM) in the lung are an important building block of the lung. The disturbance in the ECM leads to important consequences such as lung tissue remodelling, affecting the airway wall fibrosis and emphysema of the lung in COPD. The ECM compartments include the basement membrane, lamina propria of the airways and alveolar interstitium, and ECM is responsible to connect the alveoli and blood vessels, forming the lung parenchyma. We assessed the gene expression of some of the important proteins that plays vital role in lung tissue remodelling in COPD. This was to assess the effects on gut-lung axis after FMT and transplanted microbiome from either air-exposed or smoke-exposed mice.

The gene expression of the common genes involved in lung tissue remodelling such as the collagen fragment Col5a3, elastin (Eln), SMAD group of transcription factor (Smad2) that regulates Transforming growth factor-beta1 (TGF) signals, and Fibronectin (Fn) was assessed (Fig. 14 a-d). Mouse with healthy microbiome with transplanted gut microbiome from smoke-exposed has significantly lower gene expression of Col5a3 ($P < 0.0001$), then compared with healthy microbiome treated with air-exposed mice faeces controls. There was no change in Eln, Smad2 or Fn. The COPD microbiome group when transplanted with diseased gut microbiome (smoke exposed) also had significantly reduced expression of Col5a3 ($P<0.0001$), to that compared with healthy microbiome transplanted with air-exposed mice faeces. There were no difference noted in either Eln, Smad2 or Fn when compared between these two groups.

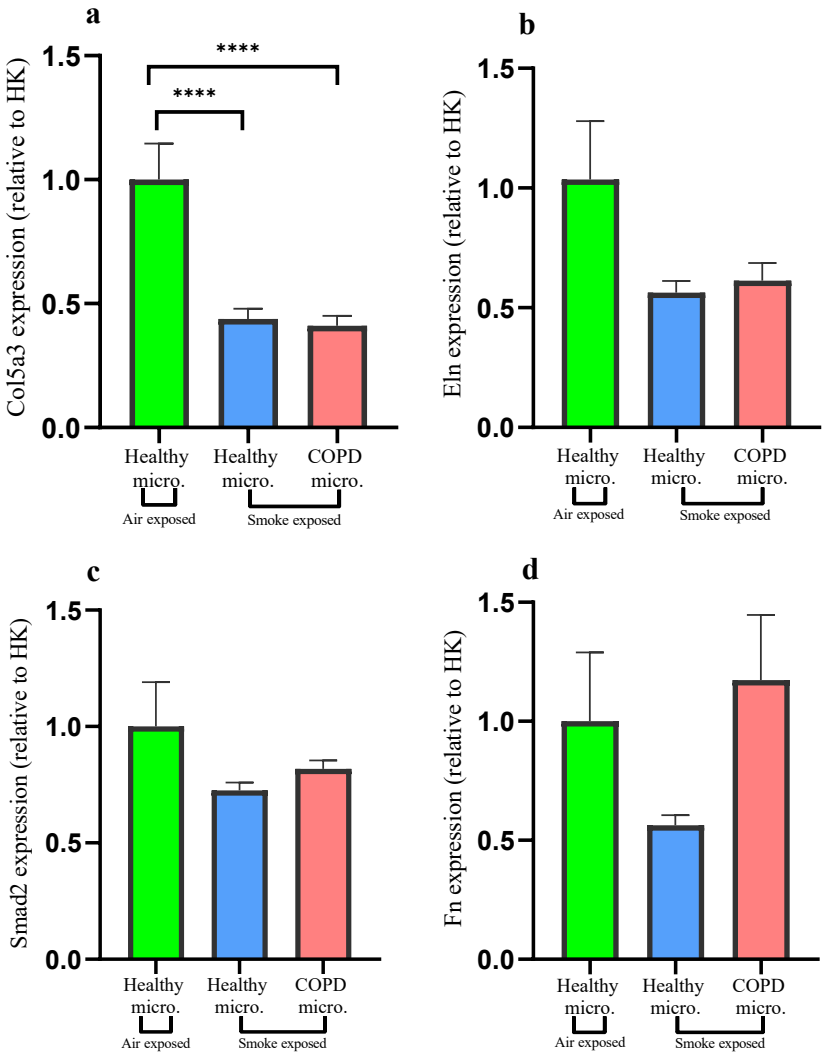

Figure 15: Changes in biomarkers for airway remodelling

*Figure showing gene expression levels of remodelling genes Col5a3 (a), Eln (b), Smad2 (c) and Fn (d) from lung tissue of healthy or COPD microbiome, after FMT through oral gavage for either healthy (air exposed) mice or diseased (smoke exposed) mice. Data presented as mean ± SEM; n=8. * = p<0.01.*

4.6.3 The effects of a COPD gut microbiome on epithelial thickness after 8 weeks of CS-exposure

The airway epithelial cells is known to play a major role in the development and pathogenesis of COPD. The airway epithelial when becomes dysregulated, and the inflammatory response causes increased mucus production and hypersecretion,

resulting in mucostasis, airway obstruction, and tissue remodelling through the various downstream events.

The effects of FMT through oral gavage was assessed for epithelial thickness of the lung alveoli. After 4 weeks of FMT and 8 weeks of either air or smoke exposure, there were significantly increased epithelial thickness around the small airways in the healthy microbiome group transplanted with smoke-exposed mice (P = 0.0009) when compared to the healthy microbiome transplanted with air-exposed mice faeces. Similarly, the COPD microbiome transplanted with smoke-exposed mice faeces had significantly raised epithelial thickness of the small airways (P = 0.0009) compared to the healthy microbiome transplanted with air-exposed mice faeces. Here we noted no significant difference observed between COPD microbiome and healthy microbiome both transplanted with smoke-exposed mice group faecal samples. (Fig. 15 a, b).

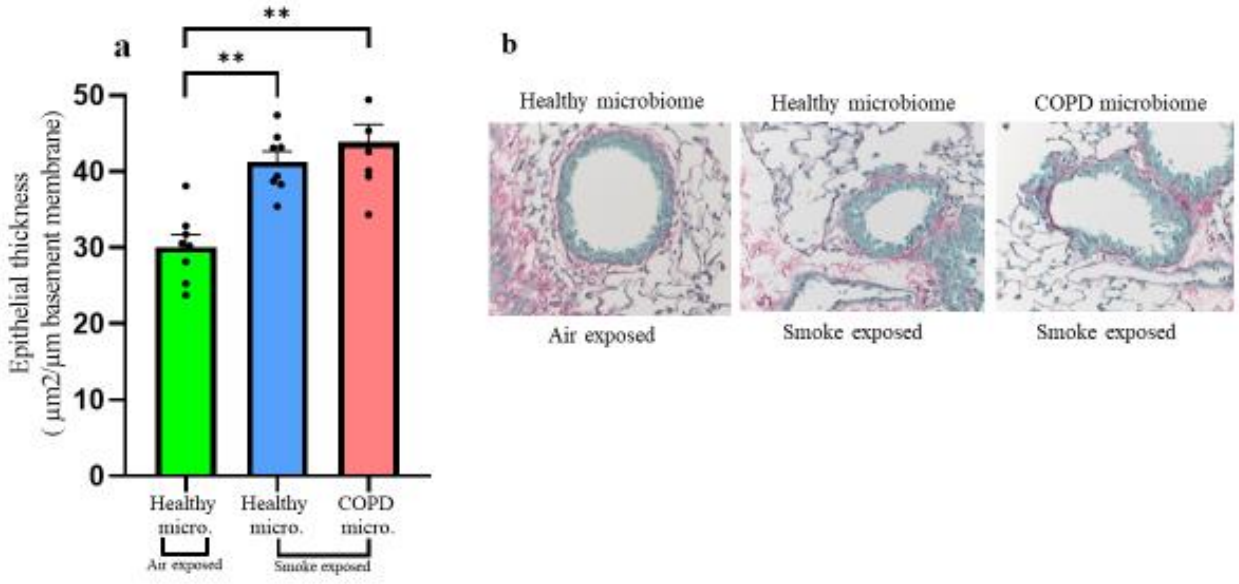

Figure 16: Changes in epithelial thickness after 8 weeks of smoke exposure

*The figure showing the effects of FMT on (a) Epithelial thickness around small airways was quantified in Sirus Red/Fast Green-stained lung sections. (b) Representative images (40x magnification; Scale bar = 50µm). The data presented here is mean ± SEM; n=8. * = p<0.01.*

4.6.4 The effects of a COPD gut microbiome on alveolar destruction after 8 weeks of CS-exposure

The histopathological analysis was done in formalin-fixed, paraffin embedded lung tissue sections (Fig. 16 a, b). The alveolar diameter is found to be increased in COPD patients. The alveolar diameter was determined by mean linear intercept (MLI).

The healthy microbiome group when transplanted with diseased gut microbiome (smoke- exposed) had significantly higher alveolar diameter (P = 0.0152), when compared to the healthy microbiome transplanted with air-exposed mice faecal samples. Similarly, the mice group with COPD microbiome when transplanted with smoke-exposed faecal samples have significantly higher alveolar diameter (P <0.0001) then that compared to healthy microbiome group of mice transplanted with air-exposed mice faeces, control group. Furthermore, the COPD microbiome mice treated with smoke-exposed mice faecal samples has significantly increased alveolar diameter (P = 0.0270), compared to the healthy microbiome transplanted with smoke-exposed mice faecal samples. A similar pattern of changes in alveolar diameter in the Model 2 mice group (Chapter 3; section 3.6.6) was also seen with only 6 weeks of smoking after FMT.

The Mmp-12, is prominently secreted from alveolar macrophages in COPD patients and is known to mediate the initiation of lung injury and development of emphysema. We assessed the gene expression of Mmp12 gene by qPCR (Fig. 16 c). Mouse with healthy microbiome with faecal matter transplanted from smoke-exposed mice is significantly raised gene expression of Mmp12 (P = 0.0263) compared to the healthy microbiome treated with air-exposed mice faecal matter; the control group. Also, the COPD microbiome transplanted with faeces from smoke-exposed mice has also significantly increased Mmp-12 (P = 0.0043) in comparison to the air control

group. Here we found no significant difference observed between the healthy microbiome or COPD microbiome, both when transplanted with faecal matter of the smoke-exposed mice groups.

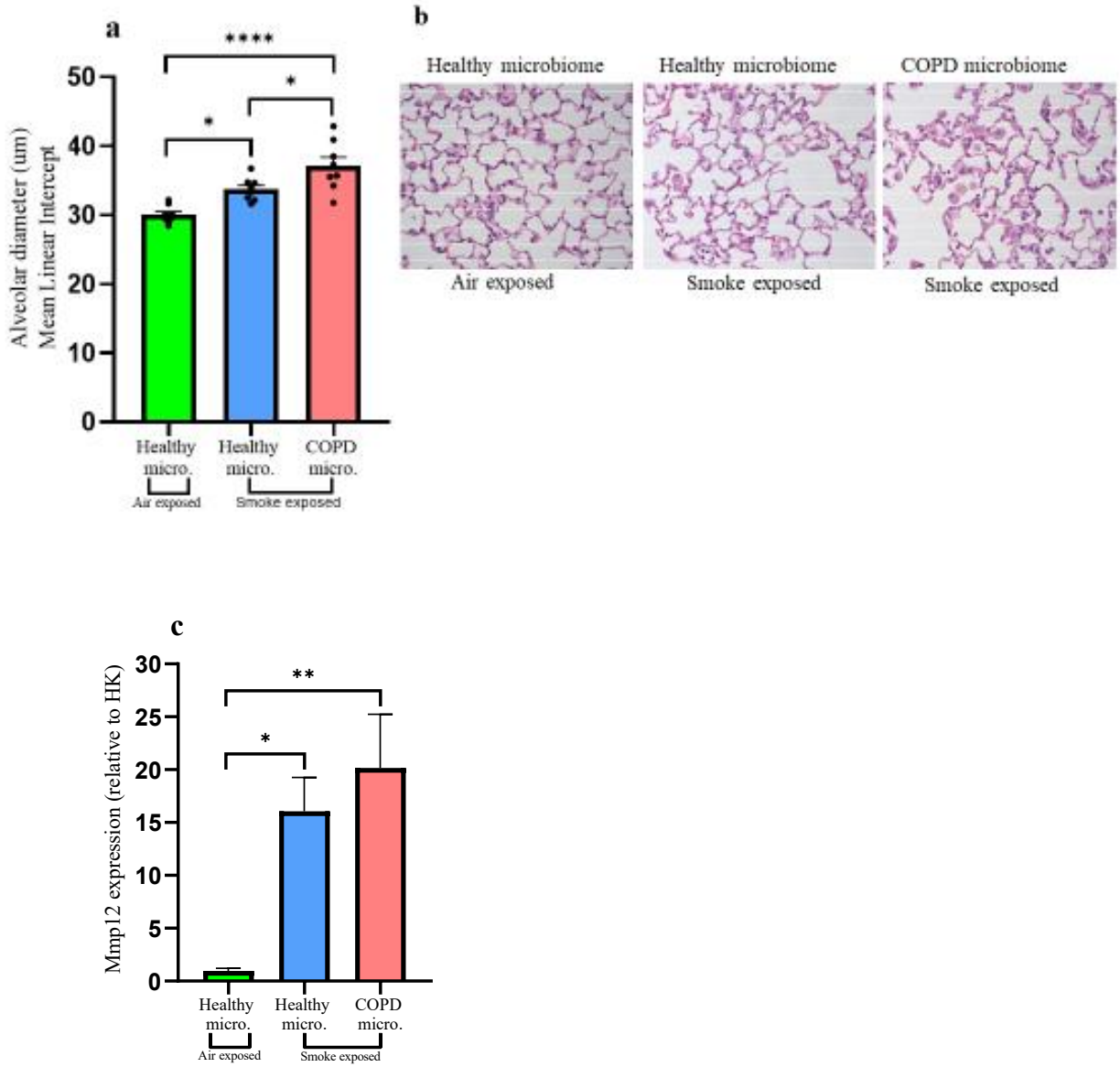

Figure 17: Changes in alveolar diameter and biomarkers for alveolar destruction

The figure showing the effects of FMT on alveolar diameter and Mmp12 gene expression (a) Alveolar diameter was measured by mean linear intercept. (b) Representative images (40x magnification; Scale bar = 50µm). Small airways was quantified in Sirus Red/Fast Green-stained lung sections. (c) Mmp12 lung gene expression results obtained by qPCR in the mice groups with either healthy or COPD microbiome transplanted with mice faeces of either air-exposed or smoke-exposed mice (n=8 per group). Data are expressed as mean per group. Gene expression data are

*displayed as fold-changes compared to the Healthy microbiome/Air exposed control group. * P Data presented as mean ± SEM; n=8. * = p<0.01.*

4.6.5 The effects of a COPD microbiome on lung function after 8 weeks of CS-exposure

The effects of FMT on lung function was assessed in anaesthetised mice using the Flexivent apparatus (ScireqTM) (Fig. 17 a-d). Figure 17 shows the Forced Expiratory Volume in 0.1 seconds (mL), Forced Vital capacity (mL), FEV0.1/FVC ration (mL) and Total Lung Capacity (TLC) from healthy or COPD microbiome after transplantation of faecal matter from either air or smoke exposed mice for 4 weeks and then after 8 weeks of air or smoke exposure.

Mouse with healthy microbiome with a transplanted smoke-exposed mice faeces shows significantly difference in FVC and TLC (P = 0.0019 and P = 0.0016, respectively) when compared with mouse group having healthy microbiome transplanted with air-exposed mice (control group) faecal matter. FEV0.1 and FEV0.1/FVC shows no difference irrespective of groups. Similarly, COPD microbiome treated with smoke-exposed mice faeces also has increased FVC and TCL (P = 0.0008 and P = 0.0008, respectively) compared to control group. FEV0.1 and FEV0.1/FVC were non-significant irrespective of groups.

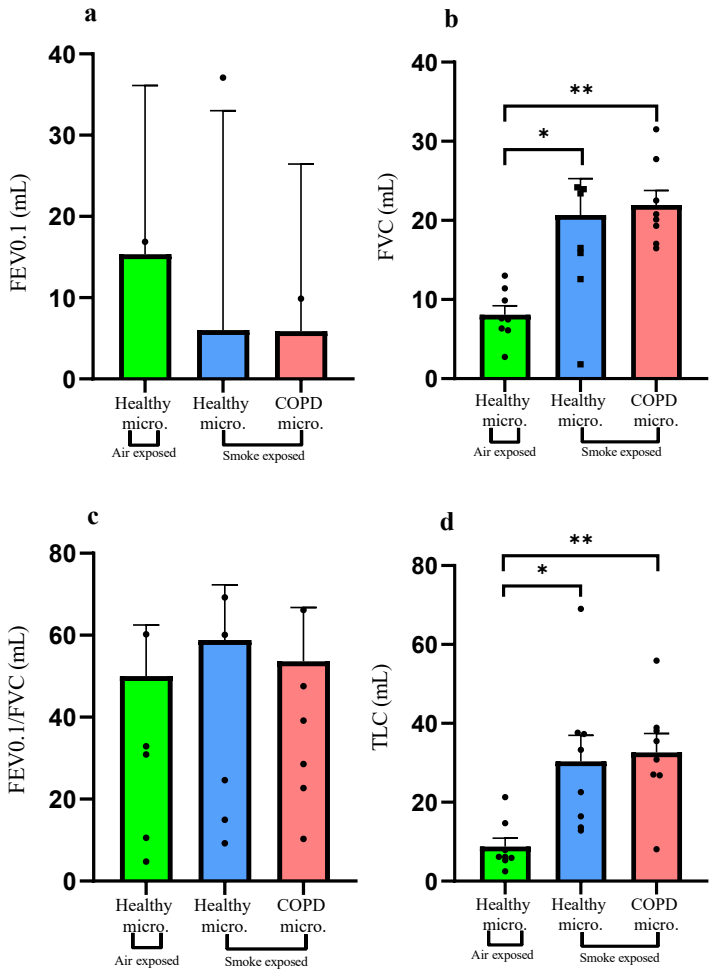

Figure 18: Changes in lung function after 8 weeks of smoke exposure

*Figure showing the effects of FMT on Lung function of the C57BL/6 female mice which are either healthy or COPD microbiome and transplanted with air or smoke exposed mice faeces. Lung function was measured using a Flexivent apparatus. Data presented as mean ± SEM; n=8. * = p<0.0001*

4.6.6 Changes in commensal gut bacterial abundance after FMT and cigarette smoke exposure

In COPD patients an alteration of different sets of gut microbiota has been documented at a large extent. The relative abundance of some of the family such as *Fusobacteriaceae* and *Prevotellaceae* decreases, and *Bacteroidaceae* and *Prevotella* increases and have been documented in literature [113]. In one of the study from our

research group reported, faecal microbiome metagenomic sequencing of COPD patients shows increased abundance of genera *Streptococcus, Rothia, Romboutsia* and *Intestinibacter* whereas the *Bacteroides, Roseburia* and *Lachnospira* from the family *Lachnospiraceae* and several unnamed genera of *Ruminococcacea* are decreased. *Streptococcus* species are identified as key organism in COPD group as compared to healthy group. Multiple members of the family *Lachnospiracease* such as *Streptococcus sp000187445*, *Streptococcus vestibularis* has been found to be correlating with reduced lung function [114].

In order to assess the effectiveness of the FMT and the changes in gut microbiome over the course of the smoke exposure, microbial DNA extracted from tool was sent for metagenomic sequencing. Unfortunately, at the time of submission for this thesis, the bioinformatics on this sequencing was not complete. To address this limitation, quantitative PCR was used to assess the abundance of specific bacterial targets that are known to be affected by cigarette smoke exposure. The abundance of *segmented filamentous bacteria (SFB), Bacteroidetes, Lactobacillus, Prevotellaceae,* and *Bifidobacterium* were assessed after normalisation, after FMT and after 6 weeks CS exposure.

The abundance of *segmented filamentous bacteria (SFB), Bacteroidetes, Lactobacillus,* and *Prevotellaceae* was significantly reduced after 8-weeks of smoke exposure in the smoke-exposed group with a transplanted COPD gut microbiome (P=0.006, P=0.004, P=0.04 and P=0.0012, respectively) (Appendix; Fig. 26). This is consistent with the literature which shows that commensal bacterial are reduced in COPD. This work was performed by Dr Annalicia Vaughan on behalf of Piyush Jha.

4.7 Discussion

In this present study, we investigated the effects of FMT prophylactically from the diseased group of mice (smoke exposed for 12 weeks) to the diseased mice with COPD microbiome (smoke exposed for 8 weeks) and showed that mice with a COPD gut microbiome predisposed to more severe disease.

4.7.1. Main results

In this study we observed noticeably higher number of total leukocyte counts, macrophages, neutrophils and lymphocytes in the BALF fluid of the mice group with a transplanted COPD gut microbiome when compared to mice with a healthy gut microbiome (Fig. 11 a-d). We also noted that the healthy microbiome when treated with smoke exposed faeces have significantly raised total leucocyte count, macrophages, and lymphocytes, though no difference in neutrophils counts, as compared to the controls (Healthy microbiome/air-exposed) (Fig. 11 a-d). We also found that the lymphocytes were significantly increased in the mice group of the COPD microbiome treated with smoke exposed faeces COPD microbiome/Smoke-exposed) (Fig. 11 d), whereas no other difference in the total leukocyte count, macrophages, or the neutrophils. Overall, in this present study the comparable difference can be seen in the inflammatory cells between the groups with most notable changes observed between the two smoke exposed groups with a healthy versus COPD gut microbiome. This indicates towards the worsening of airway inflammation in the lung of the mice group when treated with the faeces of mice already been in COPD state; after 8 weeks of smoke exposure.

4.7.2. The COPD gut microbiome exacerbates inflammasome activation in the lungs

Inflammation is a well-known to be a complex biological response in response to pathogenic and harmful stimuli involving the immune system in which a cascade of vicious cycle initiates. In COPD, due to the inflammatory response, eosinophils and neutrophils migrate from the bloodstream into tissues and upon activation, they release some of the pro-inflammatory cytokines which in turn further contribute in recruitment of monocytes and T cells. The two pro-inflammatory cytokines considered important in COPD are IL-1β and TNF-α. The elevated levels of IL-1β and TNF-α have been found in COPD and are responsible for amplifying inflammation through the activation of cells nuclear factor kappa-light chain-enhancer of activated B (NF-kB), activator protein 1 (AP-1), and other transcription factors [177]. IL-1β are also secreted by monocytes involved in initiation and perpetuation of inflammation [178]. IL-1β and TNF-α, are both potent activator of alveolar macrophages in COPD patients responsible for alveolar septa and fibrosis in airway walls [179].

Casp1/ICE is an evolutionarily enzyme and is responsible to proteolytically cleaves proteins, such as the precursors of the inflammatory cytokines interleukin particularly the 1β [180, 181]. It is now identified that the activation of infammasomes is involved in the pathogenesis of COPD. The most studied inflammasome is the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) or Napl3 infammasome which are activated by different agonists [182]. NLRP3/Nalp3 is reported to be an activator of Casp1 [183].

In this present study we investigated the gene expression of IL-1β, TNF-α, Casp1 and Nalp3 in the lung tissue and correlate the proinflammatory cytokines and

inflammasome, believed to play important role is COPD pathogenesis and prognosis. There is increase in expression of IL-1β, TNF-α, Casp1 (Fig. 11 a-c) with a similar trend of Nalp3 (Fig. 12 d) in between the groups. The highest expression seen in smoke exposed COPD microbiome group, followed by air exposed with healthy microbiome group.

4.7.3 The COPD gut microbiome accelerates lung remodelling gene expression

The major component of lung extracellular matrix (ECM) includes collagen, elastic fibres, proteoglycans, fibronectin and tenascin [184, 185]. Some of the previous studies reported a decrease in elastin [186-188] and proteoglycans [189] and increased total collagen content in the alveoli of COPD patients [190]. Accordingly, this study showed a significantly increase in collagen deposition between the groups of Healthy microbiome/Air-exposed, Healthy microbiome/Smoke-exposed and COPD microbiome/Smoke-exposed having the highest collagen deposition (Fig. 13 a, b).

Col5a3 gene encodes collagen alpha-3(V) chain, a protein that is encoded in humans and believed increase collagen deposition in the lung tissue [191, 192]. Similarly, Eln gene encodes Elastin protein in humans and are the key component of the extracellular matrix [193]. Deletions and mutations in this Eln gene is associated with various disease conditions including emphysema caused by α_1-antitrypsin deficiency [194]. SMAD2 is a protein that in humans is encoded by the *Smad2* gene and are believed to be the signal transducers and transcriptional modulators that mediate multiple signalling pathways [195]. Fibronectin is an important player in morph-regulation during lung development. In adult life, this proteins is altered after any tissue injury and inflammation, regulating cell adhesion, migration and differentiation [196]. Fibronectin

is found to be higher in COPD patients, mainly at the small airway level. Also, the fibronectin induce matrix metalloproteinase expression and activity [197], contributing to the perpetuation of tissue injury.

In this study, we observed that though the collagen deposition in increased in the CS-exposed group with a transplanted COPD gut microbiome group as compared to the other groups (Healthy microbiome/Smoke-exposed) and (Healthy microbiome/Air-exposed) control (Fig. 13 a, b). The expression of Col5a3, the gene that represent a core protein of collagen, is found to be significantly reduced in the COPD microbiome/Smoke-exposed and Healthy microbiome/Smoke-exposed then compared to control (Healthy microbiome/Air-exposed) group (Fig. 14 a). Some of the important gene playing role in extra-cellular matrix remodelling are generally found to be expression high in COPD patients, these includes Elastin (Eln), Smad2 and Fibronectin (Fn). We have a different finding in our study where all these genes in the lung tissue of the mice are trending lower in diseased mice groups (Fig. 14 b-d).

4.7.4 The COPD gut microbiome induces lung damage and promoter proteins

Emphysema is a common phenotype of COPD where the air sacs (alveoli) are damaged and oxygen exchange capacity of the lung is reduced. The epithelial thickness of the alveolar sac in COPD patients and mice models have been found increased. The air sac walls become loose and overall surface area increases and alveolar diameter increases [198].

Mmp-12 is mainly produced by macrophages, in the lower airspaces under normal conditions and is essential for the development of emphysema in mice exposed to cigarette smoke [199-204]. The results of this study is aligned with the literature, we

found that the epithelial thickness (Fig. 15 a, b), alveolar diameter (Fig. 16 a, b) and Mmp12 (Fig. 16 c) gene from the lung tissue both shows significantly increasing trend from control; (healthy microbiome/air-exposed), healthy microbiome/smoke-exposed) and the highest been that of the diseased group transplanted with diseased faces (COPD microbiome/Smoke-exposed) (Fig. 16 a-c).

4.7.5 The COPD gut microbiome reduces lung function capacity of mice

The lung tissue are destroyed in emphysema that leads in loss of elastic recoil which allows the lungs to stretch to abnormally large volumes, resulting in an increased TLC [205].

In this study we assessed the lung function parameters of the mice, there were no overall difference between the groups. The FEV0.1 showed no difference (Fig. 17 a), FVC in the mice group transplanted with faeces of smoke-exposed mice shows significantly increased Healthy microbiome/Smoke-exposed and COPD microbiome/Smoke-exposed compared to the control group of Healthy microbiome/Air-exposed (Fig. 17 b). Though there was no comparable difference in the FEV0.1/FVC ratio (Fig. 17 c). The TLC in the mice group transplanted with smoke-exposed faeces is increased and this is aligned with the current understanding of the COPD pathology. TLC increases in emphysema and this can be seen in our current study where both the group either Healthy microbiome/Smoke-exposed or COPD microbiome/Smoke-exposed have significantly greater TLC compared to controls; Healthy microbiome/Air-exposed (Fig. 17 d). Though we note some changes in the lung function parameters there is no overall obvious difference between the smoke exposed COPD microbiome group and air exposed air microbiome groups which could be conclusive.

Table 4: ***Summary table showing the experimental groups and various parameters investigated in this chapter (n=8). Groups in this chapter are: HM-AE (Healthy Microbiome – Air Exposed); HM-AE (Healthy Microbiome - Air Exposed) and COPD Microbiome (COPD Microbiome – Cigarette Smoke Exposed).***

Experimental group/ Findings	HM-AE (Column I)	HM-AE (Column II)	COPDM-CSE (Column III)
TLC	-	↑	↑
Macrophages	-	↑	↑
Neutrophils	-	↑	↑
Lymphocytes	-	↑	↑
IL1b	-	-	↑
TNF-α	-	↑	↑
Casp1	-	-	↑
Nalp3	-	-	-
Collagen deposition	-	↑	↑
Col5a3	-	↓	↑
Eln	-	-	-
Smad2	-	-	-
Fn	-	-	-
Epithelial thickness	-	↑	↑
Alveolar diameter	-	↑	↑
Mmp12	-	↑	↑
FEV0.1	-	-	-
FVC	-	↑	↑
FEV0.1/FVC	-	-	-
TLC	-	↑	↑

4.7.6 Characteristics of the COPD gut microbiome which might contribute to disease pathology

In order to assess the effectiveness of the FMT and the changes in gut microbiome over the course of the smoke exposure, microbial DNA extracted from tool was sent for metagenomic sequencings. Unfortunately, at the time of submission for this thesis, the bioinformatics on this sequencing was not complete. To address this limitation, quantitative PCR was used to assess the abundance of specific bacterial targets that are known to be affected by cigarette smoke exposure. The abundance of *segmented filamentous bacteria (SFB), Bacteroidetes, Lactobacillus, Prevotellaceae,* and *Bifidobacterium* were assessed after normalisation, after FMT and after 8 weeks CS

exposure. The abundance of *segmented filamentous bacteria (SFB), Bacteroidetes, Lactobacillus,* and *Prevotellaceae* was significantly higher after 8-weeks of smoke exposure in the smoke-exposed group with a transplanted COPD gut microbiome (P=0.006, P=0.004, P=0.04 and P=0.0012, respectively) but not the *Bifidobacterium* (Appendix; Figure 26 a-e). This is consistent with the literature which shows that commensal bacterial are reduced in COPD [113].

4.7.7 Limitation

There are several limitations in this study and are similar to as mentioned in the limitation section of previous chapter (Chapter 3; Section 3.7.7).

4.7.8 Conclusions

In the previous chapter (Chapter 3), I showed that the COPD gut microbiome predisposes to disease in a 6 week model of CS-exposure. To continue this investigation into the role of the gut microbiome in the progression of COPD, I extended the previous 6 week model to 8 weeks to see if the COPD gut microbiome causes more severe disease. The disease is established, may be due to some underlying feedback mechanism. Overall, this study showed that the COPD gut microbiome contributed to the worsening of disease. It accelerates the local and systemic inflammation and worsen the alveolar destruction, epithelial thickness and collagen deposition. The pro-inflammatory cytokine, inflammasome, inflammation signalling molecules are increased with the worsening disease condition between the groups. These findings highlight that the potential for targeted therapies to restore a healthy microbiome may be beneficial in preventing disease progression in COPD.

Chapter 5 – Investigating the role of Faecal Microbial Transfer (FMT) to treat experimental Severe Steroid Resistant Asthma

5.1. Background

Asthma is a very common, chronic respiratory disease affecting more than 340 million people worldwide and more than 400,000 deaths occurring due to asthma every year. Symptoms of asthma include wheezing, shortness of breath, with chest tightness and cough. These symptoms vary over time and intensity imposing variable expiratory airflow limitation [16]. The symptoms and airflow limitation of asthma may resolve spontaneously by itself or in response of recommended medications. Our understanding of the mechanisms driving disease have improved over the recent decades and it is now understood that there are complex asthma phenotypes based on demographic, clinical and pathophysiological characteristics. The pathophysiology of asthma has two components: bronchoconstriction and inflammation of the airway, even in mild asthma. There are three main categories of pharmaceutical therapies which work to address both bronchoconstriction and inflammation. These include controller medications, reliever medication and some specific biological therapies. The controller medication (to achieve control primarily through anti-inflammatory effects) includes ICS, LTRAs, LABAs, LAMAs. The reliever medications available are rapid-acting inhaled beta2-agonist and inhaled anticholinergics. Some add-on specific biological agents are also available as well which includes anti-IgE and anti-IL-5 [16, 17]. The previous assumption that reversal of bronchoconstriction should be targeted first has now can changed to reduction of airway inflammation. The current therapeutic recommendation to achieve and maintain control of persistent asthma and its exacerbation depends on the severity of diseases and frequency of exacerbation in a step-care manner and as per need of individual patients. The socio-economic burden of these uncontrolled asthma or difficult to treat asthma, severe asthma or

corticosteroid-resistant asthma is estimated to be more than 50% of direct and indirect healthcare costs associated with asthma [206]. Corticosteroid-resistant (CR) asthma is basically failure to improve lung function by more than 15% even after treatment with high dose of prednisolone (30-40 mg daily) for 2 weeks.

Many severe asthmatics respond poorly to current medications, hence there is an urgent need for more effective therapies in order to improve the treatment and management of severe asthma. There is a growing body of evidence suggesting that the gut microbiota plays a significant role in health and disease. The human gastrointestinal (GI) tract contains a complex and dynamic microorganism's population referred generally as the gut microbiota. It is now understood that the gut microbiota exerts significant effect on homeostasis and disease [206].

There are now growing evidence that dysbiosis of gut microbiota is associated with many of the chronic inflammatory disease condition. It is understood that the more diverse the abundance of gut microbiota the more healthful. One promising approach could be to investigate and intervene the gut-lung axis of asthmatic and severe asthmatic patients by manipulating the gut microbiota and their fermentation by-product such as microbial metabolites (such as short chain fatty acids). In this present study, we have assessed the role of FMT through oral gavage as therapy in the previously established SSRAAD mice model. We have an established murine model of SSRAAD, where we induce allergy with Ova (egg allergen) and induce infection (*Chlamydia muridanum*) which changes the phenotype to steroid resistant. This SSRAAD model exhibits various groups and different sensitization resulting in different disease phenotypes including healthy group, steroid sensitive group and steroid resistant group. Thorough, these different phenotypes and treatment with FMT from the groups within the model would give us an opportunity to look into different patho-physiological parameters of disease.

5.2. Study rationale

Research has indicated dysbiosis of the gut microbiota in early life with increased risk of asthma development in later life. The children who develop asthma has less diverse gut microbiota [9]. The taxonomic classification of gut microbiota of the children with asthma shows the decreased abundance of the genera *Faecalibacterium* and *Roseburia* whereas increased *Enterococcus* and *Clostridium* in healthy controls [10]. In more severe form of disease, dysbiosis is considered even more pronounced. The gut-lung axis in patho-immunological regulation in lung diseases is an emerging field of interest to researchers [11].

The growing body of evidence suggests that modulation of the gut microbiota and its product may be a possible prophylactic and therapeutic approach to treating severe asthma. These could include either faecal microbial transfer (FMT), supplementation with probiotics (live microorganisms having potentially beneficial health effects), supplementation with prebiotics (nondigestible dietary fibre), and supplementation with microbial metabolites (such as short chain fatty acids) [12 -15]. FMT is a way to reshape the altered gut microbiota, in which faecal material is transferred from healthy donor to another diseased recipient. The principle of FMT is simple and straightforward to restore the diversity of intestinal microbial population. This is an attractive strategy to restore appropriate and healthful cross-talk between the gut and lungs. This immunomodulatory effect of the gut-lung axis provides an exciting opportunity to develop novel treatment for severe asthma patients.

5.3. Hypothesis

It is our hypotheses that the gut microbiome plays a critical role in the development and severity of SSRAAD. Thus, the FMT from a healthy donor to the healthy recipient will have no effect in terms of disease outcome, further FMT from healthy donor to the diseased recipient will

ameliorate disease and treatment with FMT from a diseased donor to healthy recipient will worsen disease.

5.4. Aim

The main aim of this study is to test FMT as a potential therapy for the treatment of SSRAAD. Our secondary aim is to better understand the role of the gut microbiome in the development of SSRAAD.

5.5. Methods

5.5.1. Study design

This study is designed to see if FMT from a healthy donor could ameliorate SSRAAD by improving response to corticosteroid. In a previously established, experimental model of SSRAAD, mice are sensitised to Ova to induce allergic airways disease. This sensitisation is combined with an infection with *Chlamydia muridanum* (Cmu), which induces steroid resistance [206]. The complete details of this model are below in figure 18. Briefly, the SSRAAD is a 35 days model preceded by four weeks of microbial normalization, faecal sample collection at various time course, FMT through oral gavage was done from day 17 to day 32 twice a week and on endpoint day (day 35), lung function analysis was done under anaesthesia and then mice were culled and samples collected and stored at -80ºC. The BALF were processed and total leukocyte count done on the same day and slide stained for differential white blood cell counts. The stool samples were collected fresh and processed in anaerobic conditions and the supernatants was collected and given to the mice through oral gavage needle. The details of the model and procedures has been discussed in detail in the Section 2.2.2 in Chapter 2 (Figure 2).

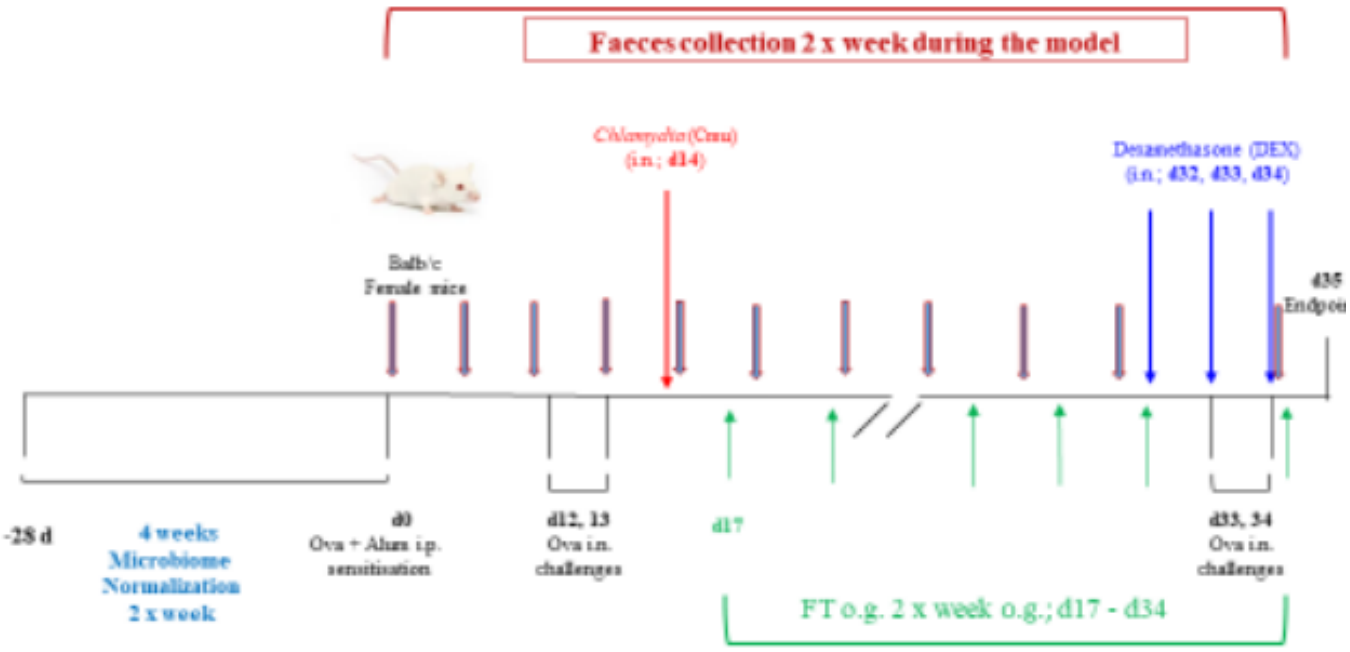

Figure 19: Study design for SSRAAD study.

Figure showing the faecal sample collection and faecal transfer time points. The faecal samples will be collected on day 0, 5, 11, 14, 15, 24, 30, 34 and 35. The faecal transfer starts on day 17 of the model with a frequency of 2/week and the last treatment will be done on day 32.

5.5.2. Ethics statement

The ethics for this study was approved by the Animal Welfare Committee of Centenary Institute and Sydney Local Health District (SLDH) (Protocol No 2019/029A).

5.5.3. Mice, acclimatisation and housing conditions

Female Balb/c mice, 4-6 weeks of age are being used in this SSRAAD experimental model. The details included in Section 2.1.2 of Chapter 2.

5.5.4. Microbiome normalisation

The purpose of microbial normalization is to ensure that all the experimental mice have similar gut microbiome at the start of the model. The details of the procedure is included in Section 2.1.3 of Chapter 2.

5.5.5. Murine model of established SSRAAD

In this model of SSRAAD, female (6-8 weeks old) BALB/c mice, infection-induced, severe steroid resistant allergic airway disease (SSRAAD). The details of the model and procedures has been discussed in detail in the Section 2.2.2 in Chapter 2 (Figure 2).

5.5.6. Mice groups and state of disease

Table 5: ***Table showing treatments in the different groups and the disease state of the following group of mice. (n=8); 11 groups. 8 x 11 = 88 total mice in this experiment.***

Group (n=8)	Disease state	Donor/Recipient/ Control
Control Groups		
1. Sal/SPG (D)*	Healthy	Donor
2. Ova/SPG	Allergic	Control
3. Sal/Cmu	Only Infection	Control
4. Ova/Cmu (D)#	SSRAAD	Donor
5. Ova/SPG/Dex	Allergic+Dex	Control
6. Ova/Cmu/Dex (D)$	SSRAAD+Dex	Donor
Treatment Control		
7. Sal/SPG (R) *	Healthy	Recipient
8. Ova/Cmu/Dex (R) $	SSRAAD+Dex	Recipient
9. Sal/SPG (R) #	Healthy	Recipient
10. Ova/Cmu (R) *	SSRAAD	Recipient
11. Ova/Cmu/Dex (R) *	SSRAAD+Dex	Recipient

5.5.7. Donor and recipient groups for FMT in this experiment

The arrow in the figure show the transfer of faeces from control to the treatment groups. There are 11 groups (n=8) and donor and recipient groups are mentioned. The symbol

*, # and $ corresponds to specific donor and recipient groups in between which the FMT is done.

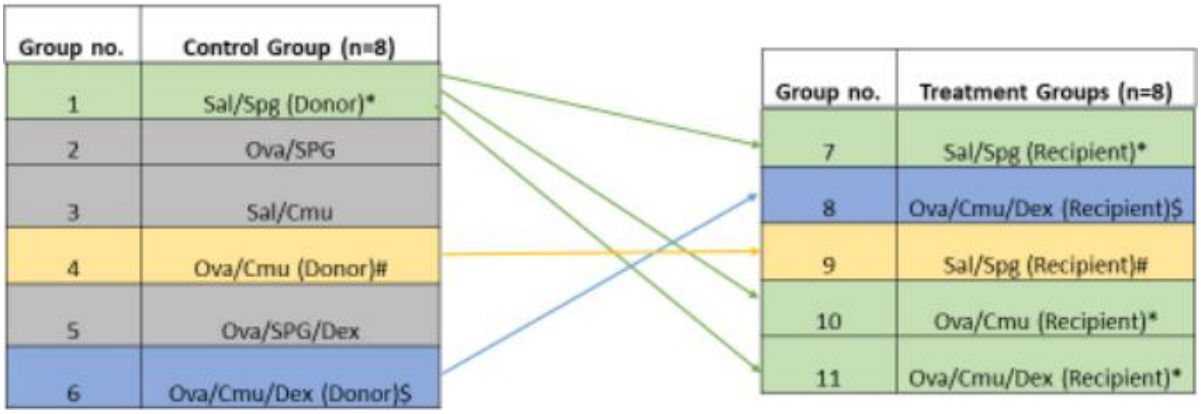

Figure 20: Summary of experimental groups examining the effect of FMT from the healthy donor.

Figure showing the control and the treatment groups. The arrow showing the corresponding faecal transfer from the control to the treatment group. The Sal/SPG which is the healthy group of mice. The faecal transfer done from Sal/SPG (Donor) group to the Sal/SPG (Recipient) group to show that there is no change in the patho-immunological status of the healthy mice when treated with the faeces of the healthy mice. The FMT when done from Sal/SPG or the healthy group to the Ova/Cmu or the Severe Steroid resistant group should change the gut microbiota of the Ova/Cmu to a more healthy state and reverse the dysbiotic state of gut microbiota to a more healthy or haemostatic state which in turn has a beneficial or anti-inflammatory effect on lung patho-immunology and should reverse the disease. Similarly, to ensure that dexamethasone does not have any individual impact when treated with healthy faecal matter we did FMT from Sal/SPG to Ova/Cmu/Dex group. Further, FMT from diseased group Ova/Cmu to healthy group Sal/SPG, we expect this would change the gut microbiota of healthy group to a diseased state. To ensure that the diseased microbiota when given to the diseased mice does not have any beneficial outcome on the lung patho-immunology we are doing FMT from diseased group Ova/Cmu/Dex (Donor) to Ova/Cmu/Dex (Recipient) group.

Table 6: Summary of experimental groups examining the effect of FMT from the diseased donor

Table showing the transfer of FMT from either healthy to healthy, healthy to diseased, and diseased to diseased group of mice, or healthy to healthy, with Dex administration or expected outcome from these faecal transfers.

S.N.	Donor group	Direction of FMT	Recipient group	Expected Outcome
1.	Sal/SPG (D)* (Healthy)	→	Sal/SPG (R)* (Healthy)	No change
2.	Sal/SPG (D)*	→	Ova/Cmu (R)*	Induce health

	(Healthy)		(Diseased or SSRAAD	
3.	Sal/SPG (D)* (Healthy)	→	Ova/Cmu/Dex (R)* (Diseased or SSRAAD with Dex treatment)	Induce health
4.	Ova/Cmu (D) # (Diseased)	→	Sal/SPG (R) # (Healthy)	Induce disease or SSRAAD
5.	Ova/Cmu/Dex (D) $ (Diseased or SSRAAD with Dex treatment)	→	Ova/Cmu/Dex (R) $ (Diseased or SSRAAD with Dex treatment)	No change or may be more severe disease.

5.5.8. Faecal suspension preparation and administration

Faecal pellets are collected from all the mice from donor group and processed and prepared for faecal suspension for FMT in the mice in recipient group. The details are included in Section 2.3.1 of Chapter 2. Faecal pellets are collected from all the mice in the group (n=8).

5.5.9. FMT through oral gavage in SSRAAD mice model

In this mice model faecal matter from healthy group of mice is transferred to diseases mice and from diseased group to healthy group. In order to see the controls for the experiment we will be doing FMT from healthy to healthy groups and from diseased to diseased groups. This will help us to evaluate the gradual changes in taxonomy of the gut microbiota of the different groups. We expect that the FMT from healthy group of mice when given to the diseases group will change the gut microbiota of the diseases group to a more healthy state or the haemostatic state. Similar, the FMT from diseased group of mice when given to the healthy group of mice will change the gut microbiota to a more diseased state or the dysbiotic state. This study will help us understand that if changes in the gut microbiota through FMT has an impact on the disease outcome. Furthermore, we will be doing FMT from healthy to healthy group and diseased to diseases group to ensure that transferring the gut microbiota between the similar patho-immunological states of health or disease doses not impart any change on patho-

physiological outcome. Through this experimental model we want to assess the effects of FMT through oral gavage on the lung and gut microbiome and immune response FT treatment has on patho-immunology in SSRAAD mice model.

5.5.10. Endpoint sample collection and assessments.

On the day of endpoint, the mice are anaesthized with an i.p. injection of xylazine and ketamine (up to 25 mg/kg xylazine + up to 400 mg/kg ketamine) diluted with sterile PBS. The tracheal access through cannula is fixed through surgery. The mice are then run on the FlexiVent to evaluate the lung function of each individual mouse against methacholine resistance. Blood is collected by cardiac puncture, two lung BALF is collected by pushing 1 ml of Hanks buffer. Right lung lobes are tied and lung lobes collected for RNA and protein analysis. The left lobes are fixed with formalin by pushing 500 µl of formalin collected in a formalin tube. Faeces, colon, jejunum, caecum content, kidneys, liver, spleen is collected in Epindroff tubes and snap freezed in liquid nitrogen then transferred to -80º C till further analysis.

5.5.11. Lung function

AHR was measured by anaesthetised, cannulated mice using the Scireq FlexiVent FX1 system (Montreal, Canada). Detail can be found in Section 2.3.2 of Chapter 2.

5.5.12. Airway inflammation.

The total leucocyte counts (TC) and differential leucocyte counts (DC) from bronchoalveolar lavage fluid (BALF), collected on the endpoint is done. The details can be found in Section 2.5.1 of Chapter 2.

5.5.13. Statistical analysis

Comparisons between two groups were made using unpaired T-tests or a nonparametric equivalent where appropriate. The details can be found in Section 2.7 of Chapter 2.

5.6. Results

5.6.1. The effect of steroid in an experimental model of SSRAAD

5.6.1.1 Airway inflammation

The five basic control groups were evaluated for overall phenotype of the model which includes healthy (Sal/SPG), allergic (Ova/SPG), allergic with dexamethasone treatment Ova/SPG/Dex), SSRAAD Ova/Cmu) and SSRAAD with dexamethasone treatment (Ova/Cmu/Dex). In the allergic group (Ova/SPG), the total leukocyte count, eosinophils, macrophages and neutrophils were all significantly higher (P = 0.0010, P = 0.0001, P = 0.0018 and 0.0106, respectively.) (Fig. 24 a-d). This clearly shows the induction of allergy in this experimental model. The allergy suppressive effects of the dexamethasone can be seen in the experimental cohorts. The total leukocyte counts, eosinophils and neutrophils are significantly reduced (P = 0.0165, P = 0.0003, and P = 0.0365 respectively) and a decreasing trend in macrophage counts (figure 24 a-d). The immune cells profile in the SSRAAD (Ova/Cmu) and SSRAAD treated with dexamethasone (Ova/Cmu/Dex) does not show any differences between the groups.

5.6.1.1 Airway hyperresponsiveness

The airway hyperresponsiveness was assessed in anaesthetised mice using the Flexivent apparatus (ScireqTM) (Fig. 24 e). The dose responses to methacholine and airway hyperresponsiveness were measured. However, there were no significant differences between experimental groups (figure 24 e).

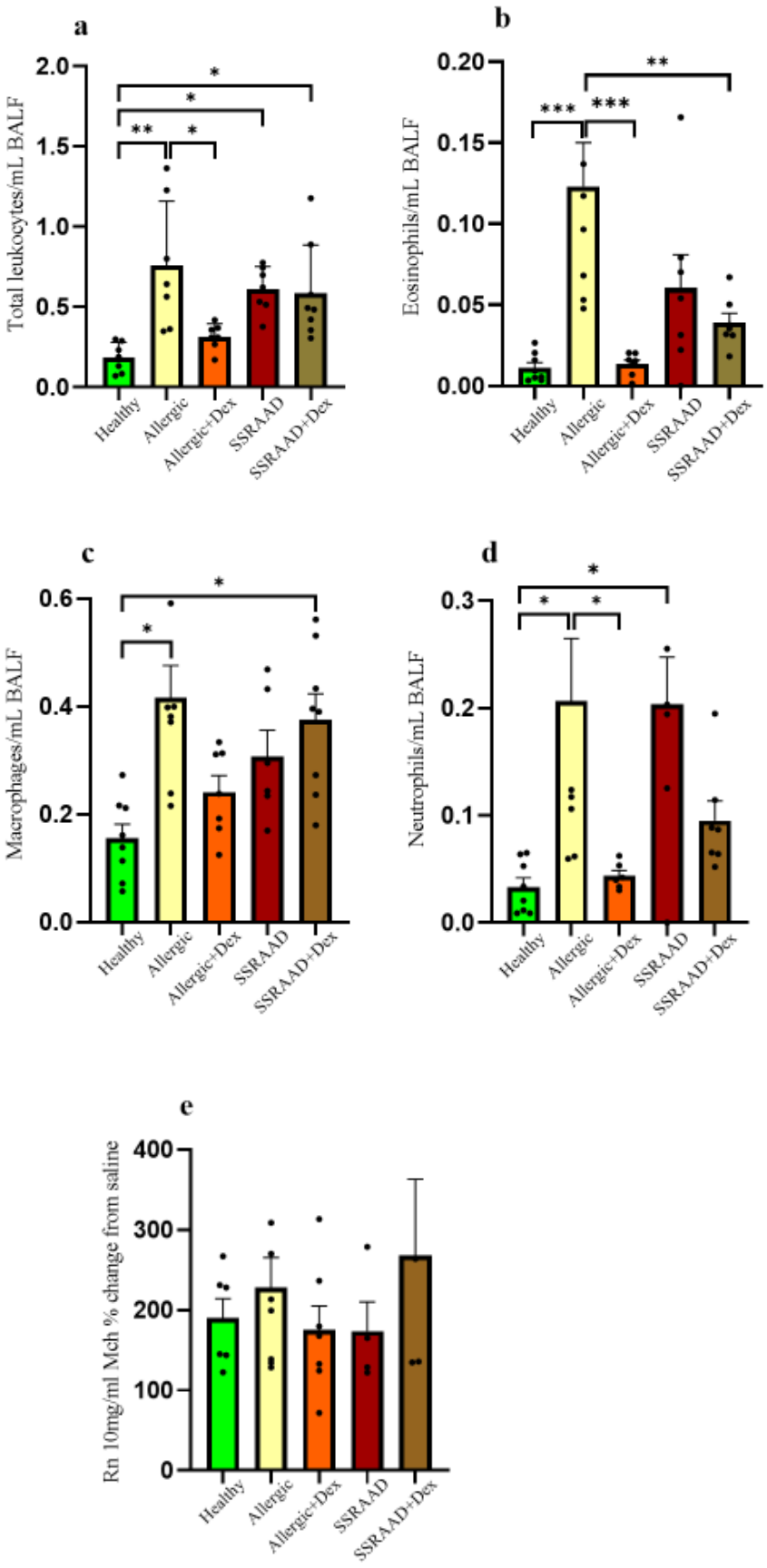

Figure 21: Changes in airway inflammation and AHR in the SSRAAD model.

*The basic model of SSRAAD showing the airway inflammatory cells in the absence and presence of SSRAAD and response to Dex administration. (a) Total leukocytes, (b) Eosinophil, (c) Macrophage, and (d) Neutrophil numbers were determined, (e) SSRAAD was assessed in response to increases doses of Mch, (n=8). Results are presented as mean ±SEM. Significant differences are shown between groups as *p<0.0001.*

5.6.2. The effects of healthy gut microbiome on airway inflammation and airway hyperresponsiveness (AHR) in an experimental model of SSRAAD

In this experimental SSRAAD mice model, we looked for the effects of FMT through oral gavage from healthy gut microbiome. The FMT was carried from healthy (Sal/SPG) to either healthy (Sal/SPG), diseased (SSRAAD; Ova/Cmu) or SSRAAD with Dex administration (Ova/Cmu/Dex) (Sal/SPG → Sal/SPG; Sal/SPG → Ova/Cmu; Sal/SPG → Ova/Cmu/Dex) (figure 25 a-d).The inflammatory immune cells in the BALF fluid does not show any suppression of either total leukocyte count, eosinophils, macrophages and neutrophils in the diseased group; SSRAAD (Ova/Cmu) and SSRAAD treated with dexamethasone (Ova/Cmu/Dex) (figure 25 a-d). There were no overall effects of Dex in the experiment, the only significant difference was seen in eosinophil counts between SSRAAD [Ova/Cmu (R)*] and SSRAAR with Dex treatment [Ova/Cmu/Dex (R)*] (figure 25 b).

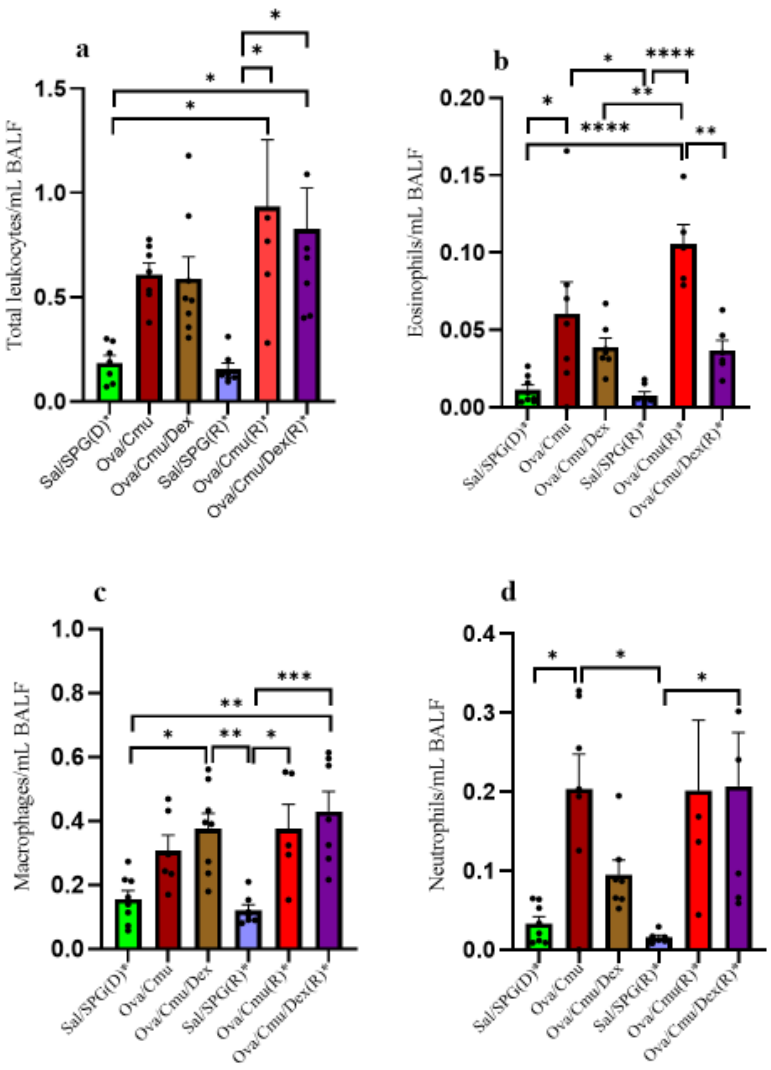

Figure 22: Changes in airway inflammation with an FMT from healthy mice.

*The figure showing the effects of FMT on the airway inflammatory cells in the absence and presence of SSRAAD and response to Dex administration. (a) Total leukocytes, (b) Eosinophil, (c) Macrophage, and (d) Neutrophil numbers were determined (Five experiments, n=8). Results are presented as mean ±SEM. Significant differences are shown between groups as *p<0.0001.*

The AHR was measured in the anaesthetized mouse to look into the effects of healthy gut microbiome by FMT. In accordance with airway inflammation, there was no significant difference between the groups (Sal/SPG → Sal/SPG; Sal/SPG → Ova/Cmu; Sal/SPG → Ova/Cmu/Dex) (table 4).

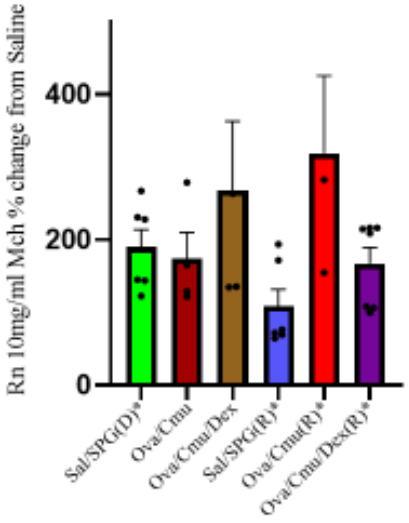

Figure 23: Changes in AHR after FMT from healthy mice.

*Effects of FMT through oral gavage on airways hyperresponsiveness after faecal transfer from healthy group to healthy, diseased (SSRAAD) and SSRAAD with Dex administration. The effect of FMT in respect to AHR in, healthy, diseased (SSRAAD) and SSRAAD with Dex administration was assessed in response to increases doses of Mch (n=8). Results are presented as mean ±SEM. Significant differences are shown between groups as **p<0.0001.*

5.6.3. The effects of diseased gut microbiome on airway inflammation and airway hyperresponsiveness (AHR) in an experimental model of SSRAAD

In this next experiment, the effects of FMT of a diseased gut microbiome was assessed. FMT from a mouse with SSRAAD was transplanted to either healthy (Ova/Cmu → Sal/SPG) or diseased (Ova/Cmu/Dex → Ova/Cmu/Dex) mice to investigate the effects on airway inflammation in BALF fluid and AHR. The FMT from diseased (Ova/Cmu) to healthy (Sal/SPG) was expected to increase the inflammation in the airway BALF. There was no change in either of the BALF immune cells; total leukocyte, eosinophils macrophages or neutrophils, in the FMT treated healthy group [Ova/Cmu (D) # → Sal/SPG (R) #] (Fig. 27 a-d) or diseased group [Ova/Cmu/Dex (D) $ → Ova/Cmu/Dex

(R) $]. With exception, neutrophil count is raised (P = 0.0158) (Fig. 27 d). This shows that the FMT through oral gavage does not influence the airway inflammation and also the FMT seems to have playing no role in modulating the effects of steroids.

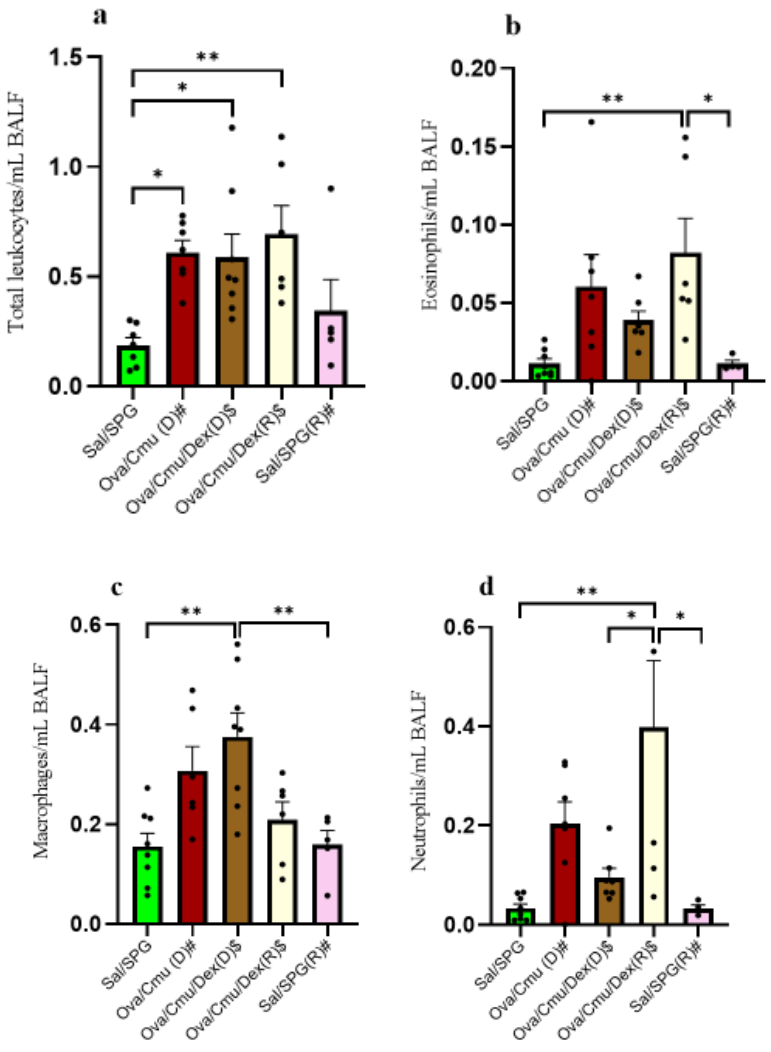

Figure 24: Changes in airway inflammation after FMT from diseases mice

*The figure showing the effects of FMT on the airway inflammatory cells in the absence and presence of SSRAAD and response to Dex administration. (a) Total leukocytes, (b) Eosinophil, (c) Macrophage, and (d) Neutrophil numbers were determined (One experiments, n=5-8). Results are presented as mean ±SEM. Significant differences are shown between groups as *p<0.0001.*

The AHR was measured on the endpoint day to investigate the effects of diseased gut microbiome to healthy (Ova/Cmu → Sal/SPG) and diseased SSRAAD with Dex

treatment (Ova/Cmu/Dex → Ova/Cmu/Dex). In accordance with airway inflammation, there was no significant difference between any of the experimental groups.

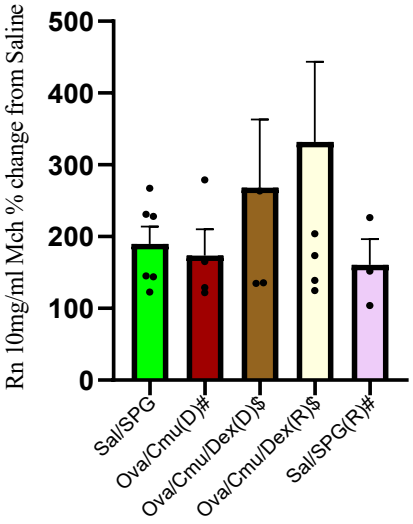

Figure 25: Changes in AHR after FMT from diseased mice.

*Effects of FMT through oral gavage on airways hyperresponsiveness after faecal transfer from diseased group to healthy and from diseased (SSRAAD) to diseased group. The effect of FMT in respect to AHR in, healthy, diseased (SSRAAD) and SSRAAD with Dex administration was assessed in response to increases doses of Mch (n=5-8). Results are presented as mean ±SEM. Significant differences are shown between groups as **p<0.0001.*

5.7. Discussion

Asthma is a chronic disease characterizing with airway inflammation and airway hyperresponsiveness gradually leading to airway remodelling, and the prevalence of asthma is anticipated to reach 400 million by 2025 [211]. The pathogenesis of asthma is yet to be clearly understood, but several factors has been linked in shaping the diseases such as genetic, environmental, infectious, nutritional and recently it has been recognized that the composition of the gut and lung microbiome present also impacts [208, 209]. The “dysbiotic” changes in both the gut and airway microbiome have been linked to respiratory diseases including asthma,

however, the appropriate mechanism and the casual pathway is yet not well characterized [210 -214]. One important tool in this direction to better understand the underlying mechanism and connection between gut microbiome and asthma is the *in vivo* mouse model of allergic airway inflammation, mimicking human conditions as closely as possible [215]. We have an established murine model of SSRAAD, where we induce allergy with Ova (egg allergen) and induce infection (*Chlamydia muridanum*) which changes the phenotype to steroid resistant. This is detailed in Section 2.2.2 of Chapter 2.

In this present study we intended to investigate the effects of FMT through oral gavage in SSRAAD mice model from healthy to diseased and vice versa. To the best of our knowledge there has been no reports on FMT as a treatment for severe asthma.

5.7.1 Main results

The effect of FMT from a healthy donor was limited and the severity of steroid resistance was not significantly different than the healthy control group. However, one interesting observation in this set of FMT (SSRAAD → SSRAAD) was enhanced neutrophilic inflammation and worsened AHR. This supports the hypothesis that the gut microbiome play a role in the development of steroid resistance in severe asthma.

5.7.2 Effects of steroid (Dex) on allergic and SSRAAD group

Corticosteroids (inhaled, oral, or by injection) are anti-inflammatory drugs that are used in asthma to calm the airway inflammation, swelling, and mucus production. These steroids bind to intracellular glucocorticoid receptors that trigger increased expression of anti-inflammatory genes and supress pro-inflammatory gene activation in asthmatics [217]. In majority of cases, inhaled corticosteroids are clinically efficient in improving lung function and preventing exacerbation. However, in about 5-10% of the asthmatic patients, even higher doses of inhaled as well as systemic corticosteroids are not able to control the symptoms [217]. Steroid

resistance in asthma is defined as < 15% improvements in forced expiratory volume in 1s (FEV1), even after 2 weeks of steroid treatment either inhaled or systemic [218, 219].

In this current study, we investigated the effects of steroid (Dex) on allergic and SSRAAD groups. In concept, Dex should supress the inflammation in the allergic group and have very limited or no effect on the SSRAAD group. In this experimental model we noted that Dex is able to significantly reduce inflammatory immune cells including total leukocyte count, eosinophils, neutrophils and a decreasing trend in macrophages count (Fig. 24 a-d). in the allergic groups Though, measuring the AHR, the dose response to methacholine we find no significant difference between the groups, but a decreasing trend is seen in allergic with Dex treatment group compared to allergic group, and an instead increase in SSRAAD with Dex treatment compared with SSRAAD group (Fig. 24 e).

5.7.3 The effects of healthy gut microbiome on SSRAAD on airway inflammation and airway hyperresponsiveness

The gut microbiota, composed of 100 trillion resident bacteria that plays an important role in homeostasis and immunity [220]. The change in composition of gut microbiota results in certain disease conditions and restoration of gut microbial homeostasis is considered a potential therapeutic target in various diseases [221, 222]. Several recent studies have highlighted the relationships between gut microbiota and many of the chronic conditions such as inflammatory bowel syndrome, ulcerative colitis, atopic dermatitis, obesity, diabetes, immunological disorders, neurological diseases and chronic respiratory disorders including asthma [223, 224]. High diversity of gut microbiota is considered to be indicator of healthy state and this imparts homeostatic immune functioning with dominant species [225, 226]. The four most dominant bacterial phyla in the intestinal tract includes Firmicutes, Bacteroidetes, Actinobacteria and Verrucomicrobia [227]. However, there are large interindividual variations in the composition

of gut microbiota which are influenced by factors including age, diet, genetics and any existing disease conditions [228]. Poor microbial diversity is not the only factor attributed to disease. For instance, specific gut bacteria such as *Clostridium* and *Eggerthella lenta* are found to be more abundant in gut of asthmatic patients. Further, the abundance of *Bifidobacterium*, *Akkermansia*, and *Faecalibacterium* decreases and that of *Candida* and *Rhodotorula* increases in child at risk of allergy and asthma [229-231].

The gut microbiota can be modulated with either probiotics, prebiotics, postbiotics or faecal microbiota transplantation (FMT). FMT is promising and emerging medical therapy involving gut microbiota transfer from a healthy donor to a diseased recipient [232, 233]. This causes change in the gut microbiota of the recipient to a more homeostatic state and resulting in improved symptoms and revert disease [234]. The identification of dysbiosis in asthmatic patients has lead the researchers to attempt to modulate the immune response of the lung in order to prevent and treat asthma through modulating the gut microbiota [235].

To the best of our knowledge, there has been no study to evaluate the effects of FMT as therapy in SSRAAD mice model. In this present study, we transplanted faecal matter from the healthy group (Sal/SPG) to healthy (Sal/SPG), diseased SSRAAD group (Ova/Cmu) and diseased SSRAAD with Dex treatment (Ova/Cmu/Dex). Our hypothesis is that the healthy gut microbiota should ameliorate SSRAAD. FMT when done from healthy to healthy (Sal/SPG → Sal/SPG) has no effect, as expected. However, the diseased groups Ova/Cmu and Ova/Cmu/Dex (Sal/SPG → Ova/Cmu; Sal/SPG → Ova/Cmu/Dex) does not have any impact on reduction of inflammation. The only significant reduction is seen in eosinophil counts (Fig. 25 a-d).

The airway hyperresponsiveness (AHR), Rn was measured in responses to increases doses of Mch, in response to stimuli is the cardinal feature of asthma that otherwise in healthy subjects

have little or no effect [236]. AHR is used to diagnose, classify the severity and management of asthma [237-239]. The presence of AHR is also linked with the decline in lung function [240]. In this study, we observed no difference in AHR between the groups irrespective of FMT treatment and steroid treatment (Fig. 26).

5.7.4 The effects of SSRAAD gut microbiome on airway inflammation and airway hyperresponsiveness

In Chapter 3 and Chapter 4 of this thesis, we investigated the effects of diseased (COPD) gut microbiome in predisposition and worsening severity of COPD respectively. Similarly, in this study we evaluated the effects FMT from diseased (SSRAAD; Ova/Cmu) gut microbiota to healthy (Sal/SPG) [Ova/Cmu → Sal/SPG] and from diseased (SSRAAD with Dex treatment; Ova/Cmu/Dex) to diseased (SSRAAD with Dex treatment; Ova/Cmu/Dex) [Ova/Cmu/Dex → Ova/Cmu/Dex] (Tab. 5). The findings of this study shows that FMT from diseased gut microbiota (SSRAAD; Ova/Cmu) to healthy (Sal/SPG) (Ova/Cmu → Sal/SPG) does not increase inflammation or airway hyperresponsiveness (Fig. 27 and 28). Similarly, when FMT is fed to the diseased mice (Ova/Cmu/Dex) to diseased mice (Ova/Cmu/Dex), there was no difference in either total leukocyte count, eosinophils or macrophages. However, one interesting observation from this study is that there is neutrophilic infiltration in the airway lumen of diseased group (Ova/Cmu/Dex). This is an interesting finding and draws attention for further research and investigation.

Table 7: Summary table showing the various groups and findings of FMT in SSRAAD mice model.

Effects of steroid in experimental model of SSRAAD						
Groups/ Findings	Healthy (Sal/SPG)	Allergic (Ova/SPG)	Allergic+Dex (Ova/SPG/Dex)	SSRAAD (Ova/Cmu)	SSRAAD+Dex (Ova/Cmu/Dex)	
TLC	-	↑	-	↑	↑	
Eosinophils	-	↑	-	-	-	
Macrophages	-	↑	-	-	↑	
Neutrophils	-	↑	-	↑	-	
AHR	-	-	-	-	-	
Effects of FMT from healthy gut microbiome						
	Sal/SPG(D)*	Ova/Cmu	Ova/Cmu/Dex	Sal/SPG(R)*	Ova/Cmu(R)*	Ova/Cmu/Dex(R)*
TLC	-	-	-	-	↑	↑
Eosinophils	-	↑	-	-	↑	-
↑	↑	↑	↑	↑	↑	↑
Neutrophils	-	↑	-	-	↑	↑
AHR	-	-	-	-	-	-
Effects of FMT from diseased gut microbiome						
	Sal/SPG	Ova/Cmu(D)#	Ova/Cmu/Dex(D)$	Ova/Cmu/Dex(R)$	Sal/SPG(R)#	
TLC	-	↑	↑	↑	-	
Eosinophils	-	-	-	↑	-	
Macrophages	-	-	↑	-	-	
Neutrophils	-	-	-	↑	-	
AHR	-	-	-	-	-	

5.8. Limitations

There are some limitations with this study. Firstly, the basic SSRAAD model did not produce steroid resistance when measured with AHR. To test an effective treatment against the development of steroid resistance, a sufficient diseases control group is required. This lack of significance may be due to inherent variability and future experiment will increase the sample size for each group. Secondly, the method and techniques used to prepare the faecal suspension is questionable as in this study we used the faecal suspension supernatant and the components and composition of this supernatant is unknown. Thirdly, the model is a short 5 weeks model and the frequency of FMT in this model might not be sufficient therapeutic intervention Future studies will extend the FMT period. Finally, a total of eight mice from various groups died during the model, this decreased the statistical strength of some experimental groups.

5.9. Conclusion

From this study, the gut microbiome plays a role in airway neutrophilic inflammation and steroid resistance. Future studies will address the limitations of this study and continue to disseminate this finding to elucidate the mechanisms in which the gut microbiome can alter the gene signalling in the lungs. It is possible with a longer length of treatment, FMT with a healthy gut microbiome may prove an effective treatment for SSRAAD.

Chapter 6. General Discussion

In this chapter, I have discussed the significance and novelty of my research to date, and the implication of these studies for future research into the mechanism and disease modulating capacity of FMT through oral gavage in steroid resistant chronic respiratory disease including COPD and severe asthma. Overall, I have drawn major associations between the results of these findings with previous studies, and how this may help researchers to investigate further into the use of FMT to understand the underlying mechanisms of CRD in order to prevent and/or treat COPD and severe asthma. I highlight the reliability of murine models in assessing the effects of FMT through oral gavage and its associated changes in smoke-induced COPD and infection induced SSRAAD. Herewith, I draw attention to the key components of the experiments and implications on improving future studies in this field. I also note to point future investigations that can be performed to gain more clear insight into the mechanisms gut microbiota modulation and its remote effects in respiratory conditions such as COPD and severe asthma through various immunological pathways signalling the gut-lung axis and finally the limitation of these studies.

6.1 Significance of Research

The chronic respiratory diseases such as COPD and asthma are primarily the obstructive pulmonary diseases that affects millions of people worldwide and Australia is no exception. In Australia CRDs affects almost one-third (7.4 million; 31%) of Australians, in which 2.7 million (11%) have asthma and 464,000 (4.8%) aged 45 years and over had COPD [241-243]. According to Australian Institute of Health and Welfare (AIHW) 2017 report that the economic burden imposed by these two disease conditions costs about 1.8 billion AUD ($ 997 million for COPD and $ 770 million for asthma) every year [241]. The burden of both the disease

conditions are projected to be increasing in the coming years [243]. Both these disease, COPD and asthma have their characteristic features though have many differences and some similarities. The mainstay of pharmological therapy mainly includes bronchodilators and anti-inflammatory agents (steroids) in both COPD and asthma [244]. The steroids are well effective in controlling symptoms in majority of asthmatic patients, however in some patients (5-10%) with more severe form of asthma or the steroid resistant asthmatics, even the steroids fail to be effective in controlling symptoms [245-249]. This severe and persistent phenotype of asthma is neutrophilic whereas the mild to moderate asthma is eosinophilic [250-252]. On the other hand, in most cases of COPD, steroids are ineffective due to the neutrophilic nature of the chronic inflammation. Neutrophilic inflammation in the airway lumen of COPD and severe asthma are both associated with steroid resistance. This necessitates the research focused on understanding the underlying mechanism of disease and investigating new therapeutic potential for these steroid resistant chronic inflammatory respiratory diseases such as COPD and severe steroid resistant asthma.

Recently, the role of the microbiome and in particular the gut microbiome has drawn attention in health and disease [253]. There is growing body of evidence revealing that the gut microbiota is dysbiotic in various chronic diseases, including COPD and asthma [254, 255]. However, it is still unclear whether this dysbiosis in a particular disease condition is the cause of disease or effect of disease. In allergic asthmatics, the alteration of gut microbiota by antibiotic agent azithromycin shows reduction in airway inflammation [256]. One of our previous studies has revealed that the faecal microbiome of stable COPD patients are distinct from that of healthy controls [114]. Though there are studies linking association of changes in the gut microbiome composition and disease development in the lungs, how these changes impact the gut-lung axis and contribute to the development of COPD and asthma is still poorly understood [257, 258].

Therefore, in this study I have used an established murine model of cigarette smoke-induced experimental COPD and infection-induced experimental SSRAAD to investigate the role of gut microbiome in disease development. Firstly, I investigated into the role of COPD gut microbiome on the development of disease in a 6 weeks model of CS-exposure. COPD gut microbiota increased airway inflammation and worsened lung function, which was supported by reported changes in the histopathology and gene expression in lung in individual sample types as discussed in **Chapter 3.**

Secondly, I assessed the role of COPD gut microbiome in worsening the disease or increasing severity in an 8 weeks model of CS-exposure. FMT of a diseased gut microbiome worsened airway inflammation, histopathology, gene expression and lung function as discussed in **Chapter 4.**

Finally, I examined the effects of gut microbiota as therapy and disease progression in SSRAAD murine model. Both FMT treated, with either health and diseases gut microbiome, had a limited or no effect on disease progression or severity. However, an interesting feature is noted when FMT given from diseased/SSRAAD mice to diseased/SSRAAD mice (Ova/Cmu/Dex; SSRAAD → Ova/Cmu/Dex; SSRAAD), there is neutrophilic influx in the airway lumen, and discussed in **Chapter 5**.

Together, these findings demonstrates that COPD gut microbiota predisposes to disease and further worsen the disease severity. In SSRAAD murine model, FMT not effective in modulating disease progression or severity.

6.2 Modulating the gut-lung axis in COPD with dietary interventions.

The risk factors for COPD development are multi-factorial, with cigarette smoking being the most influential. The other well-established factors includes air pollution (eg., biomass fuel exposure), occupational hazards, infections, genetic susceptibility (eg., deficiency of α1-

antitrypsin) and epigenetic interactions. Diet is recently been recognised as modifiable risk factors in various chronic diseases such as cancer, cardiovascular (CVD) and has been pointed to have role in obstructive lung diseases, including COPD and asthma [259-262]. Over the decades, the food habit of the general population at large have changed significantly, with decreased consumption of fruits, vegetables, whole grains, and fish, and replaced by increased consumption of processed and refined foods. This has been attributed to the increasing prevalence of chronic diseases, including COPD and asthma [263]. Diet has been shown to modulate the effect environmental exposures or the genetic predisposition, but can also have a direct effect, either protective or harmful on the patho-physiology, disease development and outcomes [264-266]. Furthermore, COPD patients are known to have poor nutritional status, particularly in advanced disease which often coincides with loss of weight including muscle mass. This provides basis for nutritional intervention as therapeutic target in COPD [267]. The Western diet is often considered to have deficient in adequate nutrition and has been linked with important features of COPD, includes cough and sputum production, alteration of airflow hyperresponsiveness and systemic inflammation [268].

Many studies have investigated the association of fruits, vegetables and fibre consumption in various chronic conditions [138, 267, 269]. There is strong link of long-term fruit consumption and lower risk of COPD development. Further, there is a strong inverse relationship between high fibre diet consumption and low risk of COPD [269]. Another study reported a positive correlation between high consumption of fruits, vegetables, fish oil and whole cereals with FEV_1 and FVC in COPD patients [270]. The two recent large population-based prospective studies from Sweden in men [277] and women [278], found an inverse and independent correlation between long-term high consumption of fruits (in both men and women) and vegetables (only in men), lowers COPD incidence by 35% in men and 37% in women [277, 278]. One of the clinical trials found a significant improvement in FEV_1 after three years of

dietary counselling which was done bi-annually, particularly focused on benefits of antioxidants rich diet [271]. One systematic review on vitamin D supplementation reported decrease in COPD exacerbation rates [272]. Another trial with cold-pressed black seed oil supplementation found significant reduction in systemic inflammatory markers after twelve weeks of intervention [273]. This all recommends for promotion of diet rich in fruits, vegetables, high fibres and fish oil in individuals with COPD.

The food we eat is digested by the host enzymatic machinery or the gut microbiota [274]. The gut microbiota mainly helps in fermentation of "otherwise" non-digestible substrates such as dietary fibres and endogenous intestinal mucus. This fermentation also helps specialist microbes to grow and produces metabolites such as short chain fatty acids (SCFAs) and various gases [275]. The major SCFAs produced in gut are acetate, propionate, and butyrate. These SCFAs are reported to play important role in modulation of immune system both locally and at distant organs [276].

6.3 Modulating the gut-lung axis in COPD with antibiotics and probiotics

The gut microbiota shares a close symbiotic relationship with the host locally and to other distant organs and helps maintain good health [279]. The composition and diversity of gut microbiota is found altered in many chronic disease conditions including COPD. If this alteration of the gut microbiota is the cause or effect of a certain disease process is yet to be established. However, we now know that in certain diseases conditions as the *C. difficle* associated diarrhoea, the disease is reverted when transplanted with faecal matter from healthy donors. This medical success has directed medical scientist over the world to think of possibility of modulating gut microbiota in other chronic conditions. The modulation of gut microbiota can be approached in four ways: FMT, dietary intervention, antibiotics or

probiotics. The concept of gut microbiota and its impact on various disease processes has been well documented in recent years.

The discovery of antibiotics have revolutionized the treatment of infectious diseases and has contributed in increased life expectancy on a global scale [280]. However, their overuse and misuse in humans and animals have resulted in global crisis of antibiotic resistance [281, 282]. Further, the effects of broad-spectrum antibiotics have been found to have detrimental impact on gut microbiota. The use of broad spectrum antibiotics is to kill the pathogen of concern which also unintentionally eradicates beneficial microbes [283], and reducing the gut microbiota diversity [284] leading to deleterious consequences in health and disease. Many studies have reported the effects of short and long term use of antibiotics in clinical as well as pre-clinical models. In one of the study, a course of clindamycin for 7 days resulted in significant alteration in the gut microbiota bacterial community, a sharp decrease in Bacteroides [285, 286], and enterococcal colonies [287]. This remained at low levels for 2 years post-treatment. Some studies have shown long-lasting on the gut microbiota community composition [288 -290]. In *H. pylori* infection, the use of triple therapy which includes clarithromycin, metronidazole, and omeprazole, alters the fecal taxonomy rapidly but recovers only partially in some cases and the effects persists for over four years [291]. Antibiotic therapies are very common for COPD patients, as bacterial infections are a major cause of acute exacerbations.

Acute exacerbation of COPD is common and plays an important role in disease progression. In one of the study with bronchoscopic samples, the colonization rate of potential pathogenic bacteria was 63%, significantly higher than the healthy controls [292]. There are evidences suggesting the presence of potentially pathogenic bacteria in the lower airway tract and actively involved in exacerbation, accelerated lung function decline and impaired quality of life in clinically stable COPD patients. These potential pathogens include *H. influenzae,*

Streptococcus pneumoniae, M. catarrhalis, Staphylococcus aureus, P. aeruginosa and few members of an *Enterobacteriaceae* family [293, 294]. Prevention of acute exacerbation is a major challenge in the management of stable COPD. There are publication supporting the long-term use of antibiotics in COPD patients that prevents exacerbation [295]. Erythromycin or azithromycin was found to be associated with decrease in exacerbation frequency and prolonged time intervals between the exacerbations. It also significantly reduces the inflammatory markers such as total cells in sputum and the neutrophil elastase [296, 297]. We note that a vicious cycle is going on, antibiotics are used to prevent or treat COPD exacerbation and this antibiotic also destabilizes the entire microbiota community to various extent depending on the spectrum of antibiotics and duration of course. This alteration exists for few weeks to several years until they revert back to state of health. Then the altered gut microbiota contributes to the pathogenesis and prognosis of the COPD further in long term. This strongly suggests the need for prudence and restraint when considering the use of antibiotics in management of COPD.

Changes in the normal gut flora can lead to enhanced inflammatory reactions in distant organs including lungs. Therefore, it is possible that depleted commensal gut bacteria may be replaced by specific live microorganism (probiotics) in order to improve the course of disease. These probiotics are generally found in yogurt, fermented foods and various dietary supplements [298]. Several studies have reported that the lactic acid bacteria (*Bifidobacterium* and *Lactobacterium genera*) have role in suppression of the opportunistic bacterial pathogens and also play a part in immunomodulation and signal transduction [299-301]. A recent in vivo study showed that *Lactobacillus paracasei,* when given orally, can protect against the influenza virus and modulates lung immunity [302]. Another study showed that dietary supplementation of *Lactobacillus rhamnosus* and *Bifidobacterium breve* prevents airway inflammation and damage in lungs. This probiotic treatment in mice attenuated cellularity in BALF, restored

cytokines and chemokines balance that results in reduced alveolar enlargement and collagen deposition [303]. All these findings suggests that treatment with certain specific probiotics in certain disease conditions yields positive outcome, suggesting its potential as therapeutics, though needs to be justified by large-scale randomised clinical trials (RCTs) [304].

6.4 Modulating the gut-lung axis in COPD through FMT intervention

In the recent past there has been growing interest to understand the role of human gut microbiota in various disease conditions and its therapeutic potential by its manipulation. The advancement of metagenomic techniques indicates the more the richness and diversity of bacterial species community of human gut, is an indicator of good health [305-307]. Further, the presence of certain groups of bacteria are found to impart health advantages. These microbes includes *Bacteroides, Bifidobacterium, Clostridium* clusters XIVa/IV, and *Lactobacillus*; are seen to enhance metabolism, immune system, endocrine signalling and cancer resistance [308-310]. The gut microbiota remains relatively resilient over time, however, external factors such as antibiotic consumption, diet change, travel and disease conditions can lead to the alteration of the gut microbiota [311-313]. There is evidence of manipulation of gut microbiota resulting in better health outcome through FMT.

The use of FMT as therapy was been practiced in fourth-century China for various conditions including diarrhoea [316], however, the success story of FMT in treating recurrent or refractory *Clostridium difficile* infection (CDI) is very new [317]. Very recently, Food and Drug Administration (FDA), USA has approved Rebyota (Ferring Pharmaceutics Inc.), in the form of capsule and is recommended in CDI patients over age of 18 years and who have completed antibiotic treatment for recurrent CDI (American Gastroenterological Association, February 2023). Although FMT as therapy in CDI is proven successful, the underlying mechanism involved is not fully understood. The most convincing logic is that the donor microbiota

competes exclusively with the pathogens and outcompeting *C. difficile* for nutrients and creates an unfavourable environment for its growth [318].

There are many research groups exploring the role of the gut microbiota in many other disease conditions including inflammatory bowel disease (IBD), necrotizing enterocolitis, liver disease, colorectal cancer, oesophageal and gastric adenocarcinoma, autism and chronic respiratory diseases [319-326]. Additionally, in functional bowel disorders such as Crohn's disease (CD) and ulcerative colitis (UC), FMT has been shown to improve symptoms, revealing the possibility of gut microbiota manipulation as therapeutics in various disease conditions [327].

FMT as therapy in COPD has evaluated by some research groups. For instance, FMT from fresh faeces of high fibre diet fed mice attenuates emphysema development by suppressing inflammation and apoptosis [328]. The authors demonstrated that alveolar structures were preserved and levels of pro-inflammatory cytokines such as IL-6 and IFN-γ were reduced, indicating decrease in inflammation. A recent clinical trial noted that in respiratory diseases the effects of eight months post-FMT treatment have significantly higher faecal microbial diversity and relative abundance [329].

In this thesis, I have shown the effects of FMT on predisposition of disease (Chapter 3) and worsening the severity of disease (Chapter 4). To the best of our knowledge there are only few studies reporting into the effects of FMT on COPD in murine model of CS-induced COPD. We noted that FMT from the diseased mice (COPD microbiome) has overall effects in increasing the airway inflammation, increased expression of remodelling and infammasome genes, with a trend for worsening lung function. It is likely that the manipulation of gut microbiota by FMT modulates the immune cells into the lungs through the translocation of metabolites such as SCFAs and LPS [330].

When comparing the results from chapter 3 and 4, we find some of the parameters having similar trend and few differences. The inflammatory cells (total leukocyte counts, macrophages, neutrophils and lymphocytes) in the BALF of mouse airway and lung, in both the model have similar increasing trend showing that FMT through oral gavage from the smoke-exposed mice to the COPD microbiome group predisposes to COPD in chapter 3 and worsen COPD severity in model 3, based on inflammatory cells in the BALF (Section 3.6.1 and Section 4.6.1).

In this PhD thesis, I also investigated the expression of some of the most prominent pro-inflammatory cytokines such as IL-1β and TNF-α and important inflammasome genes Casp1 and Nalp3. In both the models (Model 2 and Model 3); we noted that the IL-1β and TNF-α are expressing significantly higher in the healthy or COPD microbiome group which is transplanted with faecal matter of smoke exposed mice (Fig. 4 a, b and Fig. 12 a, b), irrespective of either the mice were exposed to cigarette smoke for 6 weeks (Model 2) and 8 weeks (Model 3). A similar increasing trend is seen in the expression of Casp1 and Nalp3 genes in both the models (Fig. 4 c, d) and Figure 12 c, d). This increase in the pro-inflammatory cytokines and inflammasome gene expression in the lung of the mice could be either in response to direct exposure of the mice with cigarette smoke or it could also the effect of change in the gut microbiome after FMT through the gut-lung axis. This needs to be further explored to understand the underlying mechanism.

I also investigated the histopathological features from the lung tissue in both the models. The collagen deposition in Model 2, we observed no difference between the groups (Chapter 3; Section 3.6.3), whereas in Model 3 we noted significant increase in the collagen deposition in the diseased group transplanted with faecal samples from smoke-exposed mice(Chapter 4; Section 4.6.3). A similar trend is seen with epithelial thickness where no change is seen

between the groups in Model 2 (Chapter 3; Section 3.6.5), however; in Model 3 (Chapter 4; Section 4.6.5) we see significant increase in epithelial thickness in the mice airway.

However, the alveolar diameter of the airway sac in both the model (Model 2 and 3) are significantly higher is the diseased group of mice transplanted with faeces from diseased mice group (i.e. COPD microbiome/Smoke-exposed). This indicates that the alveolar sac are damaged and alveolar diameter increases in size with 6 weeks of smoke exposure (COPD microbiome), transplanted with diseases mice faeces (Smoke-exposed). Though the changes in collagen deposition and epithelial thickness appears only after 8 weeks of smoke-exposure irrespective of treatment with faecal transplantation. The matrix metalloproteinase 12 (Mmp12) is established to be an important component in development in emphysema in COPD patients and is found to be significantly raised in both the models. This is consistent with the findings of other studies in murine model.

To further investigate the downstream mechanism that could be correlate with our histopathological findings, we further looked into the gene expression of some important proteins important in emphysema formation. Col5a3 gene (Collagen alpha-3(V) chain protein) is known to increase with collagen deposition, though in our models we noted an increase in the 6 weeks smoke exposure model (Model 2; Section 3.6.4) though in 8 weeks smoke exposure (Model 3) it shows decreasing trend in the diseased group (Model 3; Section 4.6.4).

The other important component of ECM are the elastin (Eln gene), Fibronectin (Fn) and the signalling pathway gene Smad2 shows a similar pattern as that of the Col5a3, where in 6 weeks smoke exposure model (Model 2) the gene expression of Eln, Smad2 and Fn is decreased and there was no change detected in the 8 weeks smoke exposure model (Model 3).

This could be partly due to the expression and role of these genes in the initial phase of emphysema development or ECM alteration and then becomes silent once the disease is established, may be due to some underlying feedback mechanism. All these findings indicates towards a strong association between gut microbiota manipulation and gut-lung axis modulation in COPD.

6.5 Modifying the gut microbiome in SSRA and potential for therapy

There are several studies linking gut microbiota dysbiosis in early life with increased risk of asthma development later in adult life. One study noted that children who develop asthma at school going age have lower diversity of gut microbiota up to 1 month of age, compared to healthy non-asthmatic children [331]. Another study identified the gut microbiota of the infants who are at risk of developing asthma in the first 100 days of their life, had decreased relative abundance of the genera *Lachnospira*, *Veillonella*, *Faecalibacterium* (phylum Firmicutes), and *Rothia* (phylum Actinobacteria) [332]. There are evidences from both human and animal studies, convincing enough that gut microbiota plays an important role in regulating immune response and development of asthma. The manipulation or the modification of gut microbiota in various disease conditions is pointing towards it as a therapeutic target in asthma as well. The ways to manipulate includes as discussed before, changing diet pattern (prebiotics), FMT from non-diseased donors, introducing specific probiotics, consumption of antibiotics and probiotics. Studies have reported that FMT of faecal flora induces a durable alteration in the gut microbiota of the recipient [333]. Further, the probiotics are only able to colonise temporarily in the gut lumen [334]. In one of the clinical trials, 33 days post-FMT showed that the recipient stool had almost similar gut microbiota as that of the donor. This suggests that the FMT is able to restore a healthy microbiota colony when transplanted from healthy to diseased

subjects. However, there are no much studies reporting FMT as therapy in murine model of asthma and further in severe steroid resistant experimental model. Hence, I planned to look into the effects of FMT in severe steroid resistant allergic airway disease (SSRAAD), in which allergy is induced by Ova and steroid resistance is induced by *C. muridanum*.

In Chapter 5 of this thesis, I evaluated the role of gut microbiota FMT from healthy (Sal/SPG) to diseased/SSRAAD (Ova/Cmu) and vice versa to investigate the role of gut microbiota on steroid resistance and its effects on gut-lung axis modulation. I found that the effect of FMT from a healthy donor was limited, however the severity of steroid resistance was not significantly higher than the healthy control group. One interesting observation in this set of FMT (SSRAAD → SSRAAD) was enhanced neutrophilic inflammation and worsened AHR. This supports the hypothesis that the gut microbiome play a role in the development of steroid resistance in severe asthma. Although, the steroids should supress the inflammation in the allergic group treated with steroid and does not impose any effect on the SSRAAD group. However, in this experimental model we noted that Dex is able to significantly reduce inflammatory immune cells including total leukocyte count, eosinophils, neutrophils and a decreasing trend in macrophages count (Fig. 24 a-d). Though, measuring the AHR, the dose response to methacholine we find no any significant difference between the groups, but a decreasing trend is seen in allergic with Dex treatment group compared to allergic group, and an instead increase in SSRAAD with Dex treatment compared with SSRAAD group (Fig. 24 e).

6.6 Conclusion

The aims of this project were to characterise the changes in the experimental CS-induced COPD and infection-induced SSRAAD mice model and assess the effects of intervention with gut microbiota by FMT through oral gavage. The treatment with FMT and further smoke

exposure significantly altered the inflammatory profile in the airway lumen, lung tissue (histopathological feature and specific gene expression), though only limited alteration in the lung function parameters, in experimental CS-exposed mice model. The COPD gut microbiota transplantation (FMT) through oral gavage is capable of predisposing and worsening the severity of COPD. This provides the basis for future research into gut microbiota and host response in COPD development and progression. The COPD gut microbiome induces inflammation in airway lumen, stimulates the pro-inflammatory cytokines, infammasomes, remodelling genes and alters the histopathogical features in lung tissue. Finally, in the infection-induced SSRAAD experimental mice model the healthy (Ova/SPG) gut microbiota FMT through oral gavage has limited benefits and also the diseased (Ova/Cmu) gut microbiome is not inducing disease. However, one important note from this study is that the FMT from diseased (Ova/Cmu/Dex) to diseased (Ova/Cmu/Dex) increases neutrophilic influx in the airway lumen.

Whilst these studies require further investigation and profiling of immune and metabolic pathways to elucidate the mechanism in detail, as well as validation in the human studies. This study nevertheless provides useful pre-clinical data in regards to the effects of gut microbiota and host response in COPD and SSRAAD pathogenies. I believe these studies provides an important platform from which future studies can be developed, and will ultimately help us to understand the mechanism and also to develop novel preventive and therapeutic strategies in COPD and severe asthma.

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Appendix

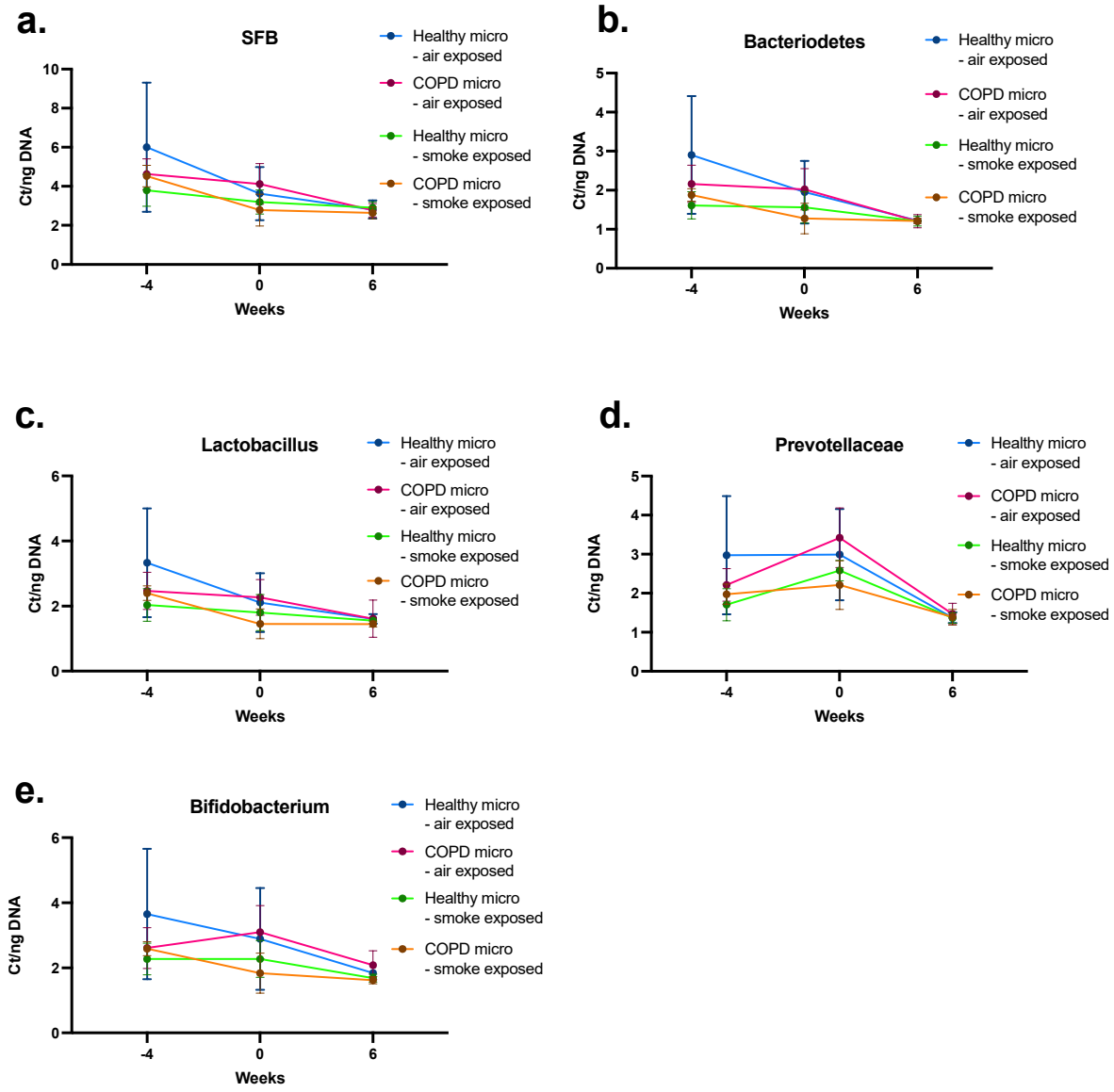

Figure 26: Relative abundance of bacterial targets after normalisation (-4 weeks), after FMT (0 weeks) and after chronic smoke exposure (6 weeks). a) Segmented filamentous bacteria (SFB), b) Bacteroidetes, c) Lactobacillus, d) Prevotellaceae, and e) Bifidobacterium. This work was performed by Dr Annalicia Vaughan on behalf of Piyush Jha.

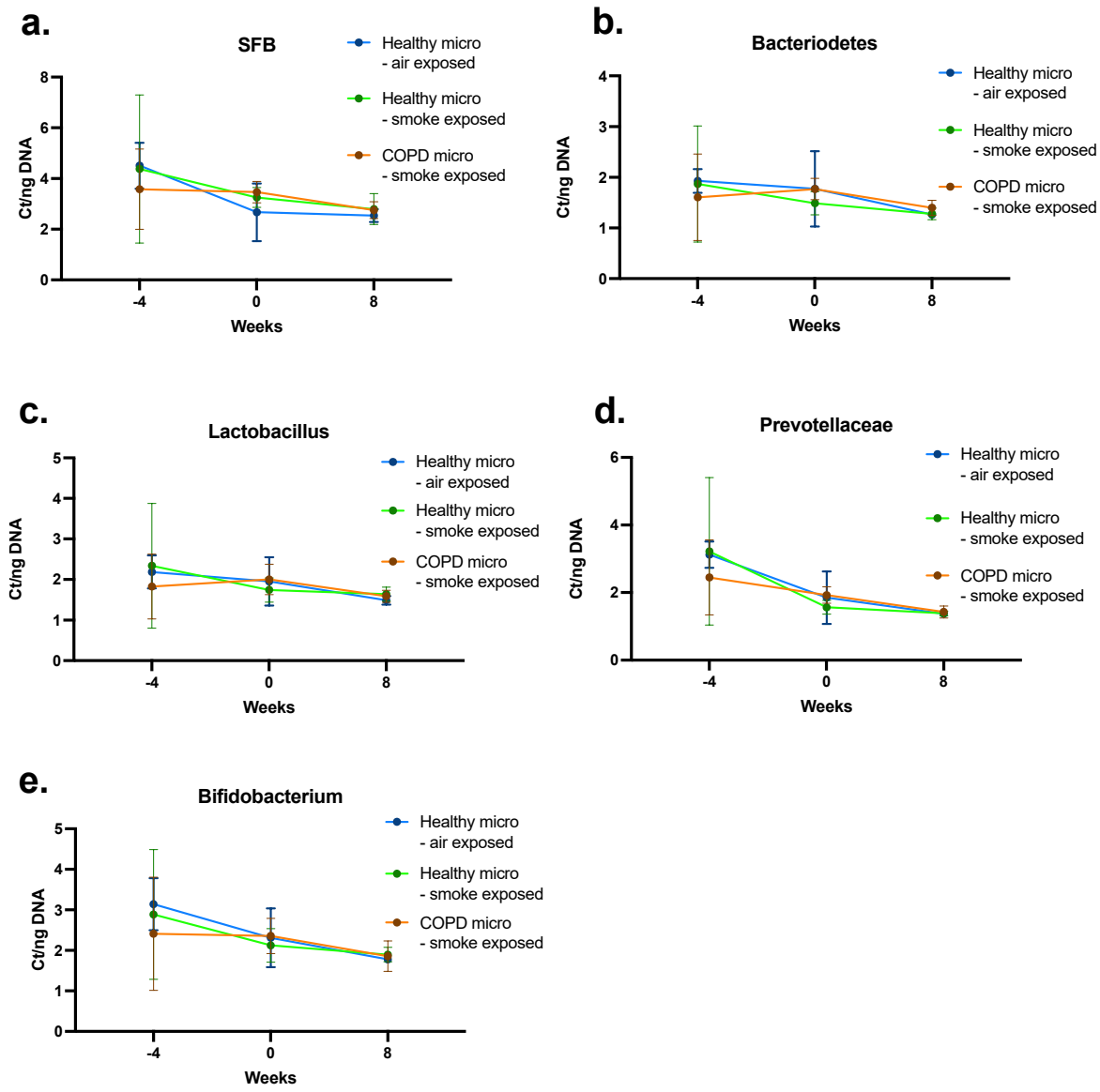

Figure 27: Relative abundance of bacterial targets after normalisation (-4 weeks), after FMT (0 weeks) and after chronic smoke exposure (8 weeks). a) Segmented filamentous bacteria (SFB), b) Bacteroidetes, c) Lactobacillus, d) Prevotellaceae, and e) Bifidobacterium. This work was performed by Dr Annalicia Vaughan on behalf of Piyush Jha.

Table 8: Oligonucleotide sequences used for qPCR analyses in this thesis.

Primer	Nucleotide sequence	Target gene
GAPDH forward	TGCACCACCAACTGCTTAG	GAPDH

GAPDH reverse	GGATGCAGGGATGATGTTC	GAPDH
IL-1βforward	TGGGATCCTCTCCAGCCAAGC	IL-1β
IL-1β reverse	AGCCCTTCATCTTTTGGGGTCCG	IL-1β
TNF-α forward	TCTGTCTACTGAACTTCGGGGTGA	TNF-α
TNF-α reverse	TTGTCTTTGAGATCCATGCCGTT	TNF-α
Casp1 forward	AGGAATTCTGGAGCTTCAATCAG	Casp1
Casp1 reverse	TGGAAATGTGCCATCTTCTTT	Casp1
Nalp3 (Nlrp3) forward	GCTCCAACCATTCTCTGACC	Nalp3
Nalp3 (Nlrp3) reverse	AAGTAAGGCCGGAATTCACC	Nalp3
CollagenV (Col5a3) forward	CAG TGT GAG CAG CAC AGA AAA GT	Col5a3
CollagenV (Col5a3) reverse	CAG GGT GAC CGA GCA TTC A	Col5a3
Elastin (Eln) forward	CTC GAC AGG TGC TGT GGT GCC	Eln
Elastin (Eln) reverse	ACC CCC ACA CCA GGG AAC CG	Eln
Smad2 forward	AATACGGTAGATCAGTGGGACA	Smad2
Smad2 reverse	CAGTTTTCGATTGCCTTGAGC	Smad2
Fibronectin (Fn) forward	TGT GGT TGC CTT GCA CGA T	Fn
Fibronectin (Fn) reverse	GCT ATC CAC TGG GCA GTA AAG C	Fn
MMP12 forward	CCT CGA TGT GGA GTG CCC GA	MMP12
MMP12 reverse	CCT CAC GCT TCA TGT CCG GAG T	MMP12