Investigating IL-36 receptor signalling in COPD:

A novel drug target

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Statement of Originality

I confirm that I have performed all the experiments and analysis reported in this thesis. I confirm that all other sources of information within this thesis have been appropriately referenced.

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Abstract

Chronic pulmonary obstructive disease (COPD) is a chronic inflammatory lung condition and leading cause of mortality and morbidity worldwide. Currently there are no treatments licenced for COPD that slow or reverse disease progression.

Interleukin (IL)-36 receptor signalling promotes neutrophilic inflammation in COPD. There is increased IL-36 receptor agonist (IL-36γ) is released by small airway epithelial cells (SAEC) and causes small airway fibroblasts (SAF) to release neutrophil chemoattractants (CXCL1, CXCL8). Macrophages are a key inflammatory cell in COPD. This thesis shows that IL-36γ directly inhibits COPD macrophage phagocytosis of *H. influenzae*. Meanwhile the effect of IL-36γ on SAF causes healthy and COPD macrophages to be more pro-inflammatory. Respiratory viruses stimulate IL-36γ release from SAECs. Thus, IL-36γ may drive virally induced exacerbations and secondary bacterial infections.

Lung macrophages are a major source of IL-36RA. This thesis shows that COPD monocytes are innately different to healthy monocytes and have reduced IL-36RA mRNA (*IL-36RN)* expression. In healthy cells there is significant upregulation of *IL-36RN* from monocytes to GM-CSF differentiated macrophages, but this is not seen in COPD monocytes. As such *IL-36RN* is almost 2-fold higher in healthy GM-MDM compared to COPD GM-MDM. This study shows that phosphoinositide-3-kinase (PI3K) activity controls *IL-36RN* expression; PI3K is increased in COPD monocytes and macrophages and thus *in vitro IL-36RN* expression is likely further suppressed.

Nasosorption is a non-invasive sampling technique of the nasal epithelial lining fluid, previously used in asthma, allergy and viral illness studies. This is the first study using nasosorption in COPD patients. Eotaxin-1 is raised in COPD, especially those with high blood eosinophil counts: it should be studied further as a potential biomarker for TH2 inflammation in COPD. IL-36α, IL-36β and IL-36γ were all present at relatively high concentrations, but with no difference between COPD and controls. IL-36 agonist levels remained unchanged during exacerbations.

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Abbreviations

AECOPD	Acute exacerbation of COPD
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CAT	COPD Assessment Tool
CCL	Chemokine (C-C) motif ligand
cDNA	Complementary DNA
CFU	Colony forming units
CRP	C-reactive protein
COPD	Chronic obstructive pulmonary disease
CT	Computerised tomography
CXCL	chemokine (C-X-C motif) ligand
DALY	Disability-adjusted life years
DLCOc	Diffusing Capacity of Lung for Carbon Monoxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
D-PBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
eos	Eosinophils
ERK	Extracellular signal-regulated kinases
FBC	Full blood count
FBS	Foetal bovine serum
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage-colony stimulating factor
GOLD	Global Initiative for Obstructive Lung Disease
GPP	Generalised pustular psoriasis
HBSS	Hank's balanced salt solution
HDAC	Histone deacetylase
HRCT	High-resolution computed tomography
ICAM	Intercellular adhesion molecule
ICHT	Imperial college healthcare trust
ICS	Inhaled corticosteroid

iEos	Inflammatory eosinophils
IFN	Interferon
IL	Interleukin
IL-1RaP	IL-1 receptor accessory protein
IL-36R	IL-36 receptor
IL-36RA	IL-36 receptor antagonist
ILC2	Type 2 innate lymphoid cells
IQR	interquartile range
ITU	Intensive therapy unit
JAK	Janus-activated kinase
JNK	Jun N-terminal kinase
LABA	Long acting β2-adrenoceptor agonists
LAMA	Long-acting muscarinic antagonist
LPS	Lipopolysaccharide
LTB4	Leukotriene B_4
MAC-1	Macrophage-antigen 1
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
MDM	Monocyte-derived macrophages
MHRA	Medicines Healthcare products Regulatory Agency
miR	microRNA
MMP	Matrix metalloproteinases
mMRC	Modified Medical Research Council dyspnoea score
MMT	Methylthiazolydiphenyl-tetrazolium bromide
MSD	Mesoscale discovery
mTOR	Mammalian Target of Rapamycin
MTT	methylthiazoyldiphenyltetrazolium bromide
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-B
NHLI	National Heart and Lung Institute
NHS	National Health Service
NICE	National institute of clinical excellence
NK	Natural killer
NSm	Healthy non-smoker

OD	Optical density
PBMC	Peripheral blood monocyte cells
PDE4	Phosphodiesterase-4
PI3K	Phosphatidylinositol 3 kinase
PMN	Polymorphonuclear
qPCR	Quantitative polymerase chain reaction
RCT	Randomised control trial
rEos	Resident eosinophils
RFU	Relative fluorescence units
RNA	Ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640 medium
RV	Residual volume
SAEC	Small airway epithelial cells
SAF	Small airway fibroblasts
SAM	Synthetic absorptive matrix
SASP	Senescent associated secretory phenotype
SEM	standard error of the mean
SGRQ	St George's Respiratory Questionnaire
SIRT	Sirtuin
SLPI	Secretory leukocyte protease inhibitor
TGF-β	Transforming growth factor-β
TH2	T helper type 2
TNF	Tumour necrosis factor
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factors

Prizes

British Thoracic Society Young Investigator of the Year Award (2023)

National Heart and Lung Institute Research Away Day: Best oral presentation award (2021)

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Baker, J. R., P. S. Fenwick, C. K. Koss, **H. B. Owles**, S. L. Elkin, J. Fine, M. Thomas, K. C. El Kasmi, P. J. Barnes, and L. E. Donnelly. 2022. IL-36 receptor agonist and antagonist imbalance drives neutrophilic inflammation in COPD, *JCI Insight*: 15.

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HLB Owles, JR Baker, P Fenwick, PJB Barnes, LE Donnelly. A novel drug target: IL-36 signalling drives inflammation and poor bacterial clearance in COPD. *Annual Winter Meeting of the British Thoracic Society.* Nov 2023.

- ***Awarded British Thoracic Society Young Investigator of the Year Award (2023)***

Owles HLB, Baker JR, Fenwick P, Elkin SL, Kasmi KC, Barnes PJ, Donnelly LE. Non-invasive nasal sampling using nasosorption may identify COPD inflammatory profiles and have a role as a diagnostic tool in exacerbations. *Annual Winter Meeting of the British Thoracic Society*. Nov 2023.

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Chapter 1: Introduction

1.1. Chronic Obstructive Pulmonary Disease

1.1.1 Definition of COPD

Chronic Obstructive Pulmonary Disease (COPD) is a chronic, debilitating lung condition and a major global health burden. It is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as "*a heterogenous lung condition characterized by chronic respiratory symptoms (dyspnoea, cough, sputum production and / or exacerbations) due to abnormalities of the airways (bronchitis / bronchiolitis) and / or alveoli (emphysema) that cause persistent, often progressive, airflow obstruction*" (GOLD 2025). The diagnosis of COPD is confirmed by the presence of poorly-reversible airflow obstruction on spirometry. This is defined by a post-bronchodilator forced expiratory volume in 1 second (FEV_1) / forced vital capacity (FVC) less than 0.7 (FEV_1/FVC <0.7) (GOLD 2025). Previously COPD severity and treatment guidance has been classified by severity of obstruction as corrected for age, ethnicity, gender and height (FEV_1 % predicted), however there is an increasing understanding that spirometric values alone do not correlate with clinical severity or treatment response. This discrepancy is likely due to the heterogeneity within the disease as discussed in Section 1.1.3. As such, the 2024 GOLD guidance stratifies patients according to symptom burden, exacerbation frequency, basic biomarkers and hospital admissions as a step towards personalising management (see Section 1.1.4). Despite this, treatment options remain limited with those available being aimed at symptom control and reduction of exacerbation frequency. In the last year there have been positive Phase 3 randomised control trials (RCT) showing promise for three biologic drugs targeting Type 2 (T2) eosinophilic inflammation, which is found in a minority of patients, but as yet there is no data to confirm long term effects on disease progression or mortality (Bhatt 2024, Ramakrishnan, Russell 2025, Sciurba 2025). Ongoing research is desperately needed in order to further identify novel disease modifying drug targets.

1.1.2 Epidemiology of COPD

COPD affects over 10% of people over the age of 45 years old (Adeloye 2022, GOLD 2025) and is the third leading cause of death worldwide causing over 3 million deaths in 2019 (WHO 2023). Within the European Union, COPD is thought to account for 56% of the healthcare costs of all respiratory disease, totalling 38.6 billion Euros (GOLD 2025). Its incidence is expected to increase over the coming decades due to ongoing exposure to the environmental risk factors and an aging population, with estimations that prevalence of the disease will double between now and 2060 (GOLD 2025). COPD is not just associated with significant healthcare costs but also societal burdens with significant disability-adjusted life years (DALY) and productivity costs due to patients, and often a carer, being unable to continue in the workplace (Asthma+LungUK 2023, GOLD 2025).

The main risk factor for COPD is tobacco smoking but other environmental factors such as biomass fuels (especially in developing countries), ambient ozone and air pollution also have a significant contribution to global rates of the disease (Alter 2020). It is noteworthy that although smoking is a major causative factor, only ~20% of smokers develop COPD, with genetics and epigenetics playing a key part in disease development (Terzikhan 2016): Indeed a smoker with a first degree relative with COPD is three times more likely to develop COPD than a smoker within the general population (Agusti 2017). Alpha-1 anti-trypsin deficiency is a well understood autosomal co-dominant syndrome due to mutations in the *SERPINA1* gene that lead to early onset severe emphysematous COPD. But beyond this, there are also a large number of genetic loci which have been associated with COPD, each contributing a small fraction to the overall risk (Agustí 2019): These include Family with Sequence Similarity 13 Member A (FAM13A), Transforming Growth Factor β2 (TGF-β2), and Hedgehog Interacting Protein (HHIP) (Agustí 2019, Chen 2021). Smoking promotes DNA methylation, and as such epigenetic changes also contribute to an individual's susceptibility to COPD (Agustí 2019). Meanwhile insults in early life such as nutritional status, socio-economic status, respiratory infections and chronic asthma have been shown to contribute heavily to disease development (Barnes 2015).

1.1.3 Pathophysiology of COPD

There are three main pathological processes which contribute toward COPD: small airways disease, chronic bronchitis and emphysema (Barnes 2015). The combination of these pathologies all contributes to the airflow obstruction which defines COPD (Figure 1.1). These pathologies often co-exist but the contribution of each is highly variable between individuals with COPD thus accounting for high degree of heterogeneity within clinical presentation. It is not yet fully understood what determines this differential disease pathology and progression.

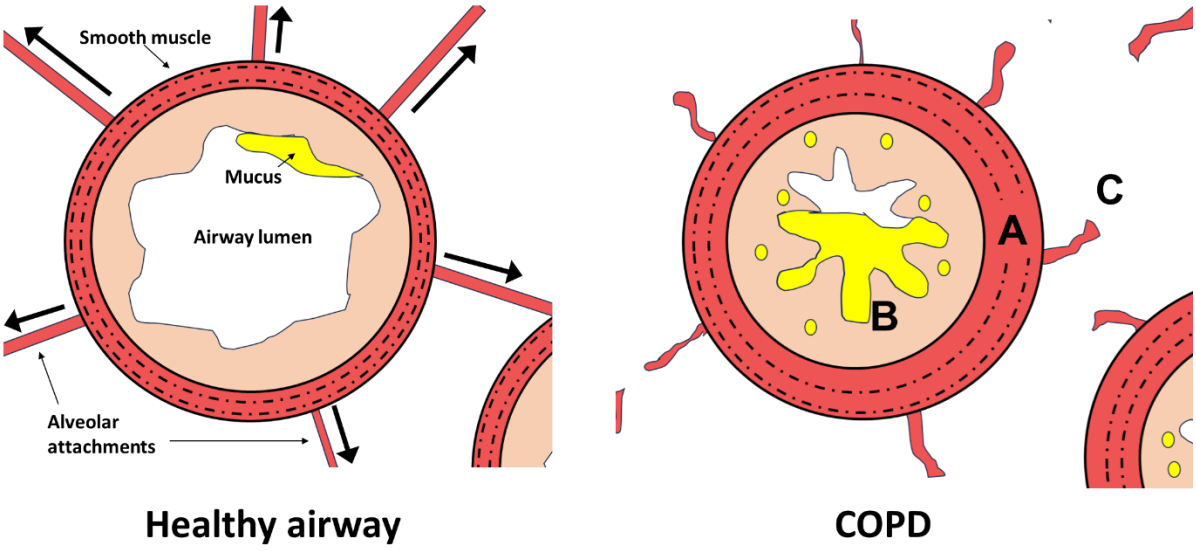

Figure 1.1: Overview of COPD pathophysiology causing small airway obstruction
In health the small airways are held open by elastin alveolar attachments, the lumen is wide and unobstructed by mucus. In COPD small airway obstruction is the results of (A) Small airways disease: inflammation and peribronchiolar fibrosis of smooth muscle causing fixed narrowing (B) Chronic bronchitis: increased inflammation of epithelium and mucus hypersecretion obstructing airway further (C) Emphysema: degradation of alveolar attachments resulting in dynamic collapse of small airways in expiration.

Figure adapted from: (Barnes 2015)

1.1.3.1 Small airways disease

The term “small airways” refers to all the terminal respiratory bronchioles measuring less than 2mm in luminal diameter. In health, these small airways contribute to less than 10% of airways resistance due to their very high number (Barnes 2019). However, in COPD there is widespread narrowing of small airways due to infiltration with inflammatory cytokines and inflammatory cells, as well as peribronchiolar fibrosis. In severe COPD there are up to 80% fewer terminal and transitional bronchioles which further adds to the increased airways resistance (Agustí 2019). It is not known whether smoking causes this reduction in number of airways, or whether the number of airways reflects abnormal lung development and therefore a predisposition to developing COPD later in life (Agustí 2019).

Small airways disease is thought to be an early feature of COPD often preceding any development of emphysema (Galban 2012, Hogg 2017). For both clinical and research purposes, it is difficult to accurately determine the degree of small airways disease as traditional spirometry is a better representation of large and medium sized airways rather than small airways disease itself. Instead, some studies have used forced oscillometry, and inspiratory and expiratory phased high-resolution computed tomography (HRCT) to be more representative of this disease pathology (Galban 2012, Barnes 2019).

1.1.3.2 Chronic bronchitis

Chronic bronchitis is defined clinically as chronic cough and sputum production for more than three months in two consecutive years (GOLD 2025). Pathologically it represents an excessive build-up of mucus as a result of goblet cell hyperplasia and mucus hypersecretion; change in composition of sputum due to increased mucins (MUC5A and MUC5B); and reduced mucociliary clearance due to reduced number, length and co-ordination of cilia on epithelial cells (Ramos 2014, Kesimer 2017). This excessive mucus contributes to small airway obstruction. Clinically chronic bronchitis is associated with increased exacerbation frequency, increased dyspnoea, lower health related quality of life and accelerated decline in lung function (Kim 2016, Maselli 2019). Current smoking status has been identified as a major driver of chronic bronchitis. Kim et al showed that smoking conferred an odds ratio of 3.53 for chronic bronchitis (Kim 2016); This risk improved with smoking cessation, meanwhile chronic bronchitis can worsen or develop if smoking is restarted. As such, smoking cessation should be particularly emphasised in any treatment consultation.

1.1.3.3 Emphysema

Emphysema refers to the destruction of the alveoli. Pathologically, emphysema is the result of increased recruitment of macrophages and neutrophils in the COPD lung, which in turn release

excessive proteases (e.g. matrix metalloproteinases (MMP)8, MMP9, and neutrophil elastase) resulting in degradation of extracellular matrix components such as elastin and collagen (Russell 2002, Shapiro 2003). Cigarette smoke also drives emphysema more directly via the release of oxygen species causing secretion of cytokines and proteases, and inhibition of antiproteases such as α-1-antitrypsin (Barnes 2016). The resultant loss of alveoli causes a decrease in gas exchange. Meanwhile, the loss of attachments that hold the alveoli open results in a loss of elastic recoil and in turn airway closure during expiration, gas trapping and hyperinflation (Barnes 2016). This dynamic hyperinflation is a major contributor to breathlessness in COPD.

1.1.4 Pharmacotherapy of COPD

Whilst GOLD describe COPD as a "preventable and treatable disease" there are as yet no pharmacological treatments which reverse or even stop disease progression (GOLD 2025). Beyond pharmacological therapies, smoking cessation, pulmonary rehabilitation and vaccination are core components of COPD treatment. Indeed, smoking cessation is the only intervention that has been shown to cause a slowing of the rate of decline in lung function over time (Anthonisen 1994).

Initial pharmacological treatment of COPD is based on assessing symptoms using modified Medical Research Council dyspnoea score (mMRC) and the COPD Assessment Tool (CAT score), and exacerbation risk based on past history of exacerbations (Figure 1.2).

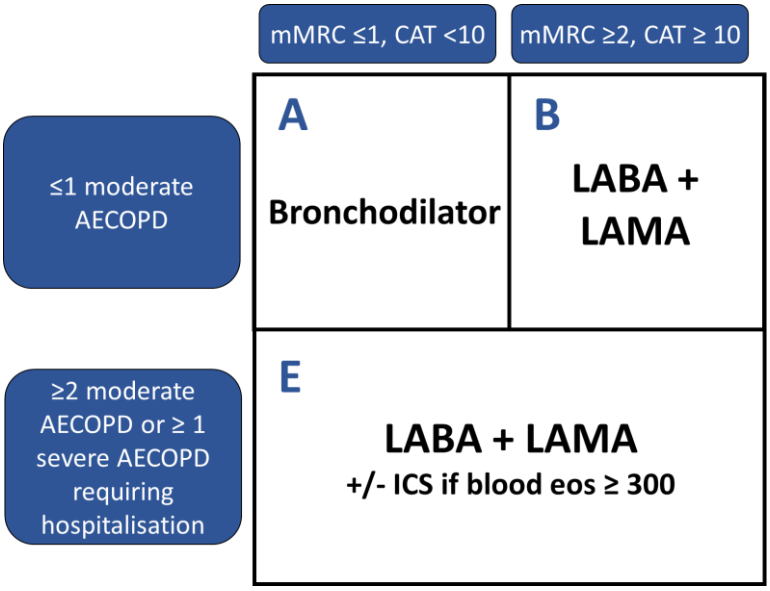

Figure 1.2: Treatment algorithm for COPD maintenance therapy
Patients are stratified in terms of low (Groups A/B) and high (Group E) exacerbation risk. Those of low risk are further classified according to symptom burden as measured by modified medical research council dyspnoea score (mMRC) and COPD assessment test (CAT). Group A should be treated with either a short or long-acting bronchodilator. Dual bronchodilation with long-acting beta agonist (LABA) and long-acting muscarinic antagonist (LAMA) is recommended for Groups B and E. Addition of inhaled corticosteroid (ICS) should be considered in Group E patients with blood eosinophil count (eos) ≥ 300 cell/µl. AECOPD = acute exacerbation of COPD. Moderate AECOPD are defined as those requiring treatment with oral corticosteroids; Severe AECOPD defined as those requiring A&E attendance or hospitalisation

Figure adapted from: (GOLD 2025)

Long-acting bronchodilators (β_2-adrenoceptor agonists and muscarinic receptor antagonists) are a mainstay of COPD pharmacotherapy. By relaxing smooth muscle tone, bronchodilators improve symptoms, lung function and also reduce exacerbation frequency (Tashkin 2008). Dual bronchodilation with long-acting beta agonist (LABA) and long-acting muscarinic (LAMA) combined therapy (LABA-LAMA) is more effective than monotherapy and thus should be used in those either with high symptom burden and/or frequent exacerbations (GOLD 2025).

In the context of frequent exacerbators, anti-inflammatory agents also have a role in pharmacotherapy (Figure 1.3). Inhaled corticosteroids (ICS) reduce exacerbation frequency, improve quality of life and reduce mortality. Post hoc analyses suggest that this effect is greatest in patients with high blood eosinophil counts (Agusti 2018). As such, ICS are recommended alongside LABA-LAMA in frequent exacerbators with a blood eosinophil count ≥ 300 cells/µl, or those with moderate blood eosinophil count (100-300 cells/µl) who continue to exacerbate despite adherence with LABA-LAMA therapy (GOLD 2025). However, ICS use has been associated with increased risk of pneumonia

especially in those with low blood eosinophil counts, and therefore they are not recommended in COPD patients with blood eosinophil counts of <100 cells/µl (Agusti 2018). COPD patients who continue to exacerbate despite optimal inhaled therapy (LABA-LAMA with blood eosinophil count <100 cells/µl; LABA-LAMA-ICS with blood eosinophil count ≥ 100 cells/µl) consideration of either roflumilast, a phosphodiesterase-4 inhibitor, or a macrolide such as azithromycin is recommended (GOLD 2025). Roflumilast has broad anti-inflammatory activity and clinically reduces exacerbation frequency and improves lung function in frequent exacerbators with severe obstruction and chronic bronchitis (Martinez 2016). Macrolides reduce exacerbation frequency and hospitalisations. This therapeutic effect is not just due to their antimicrobial properties but via an immunomodulatory function: Macrolides decrease pro-inflammatory cytokine production, reactive oxygen species (ROS) and improve macrophage phagocytosis (Segal 2018, Baker 2021). Macrolides do not appear to be effective in active smokers (Han 2014) and should therefore be used preferentially in current non-smokers (GOLD 2025).

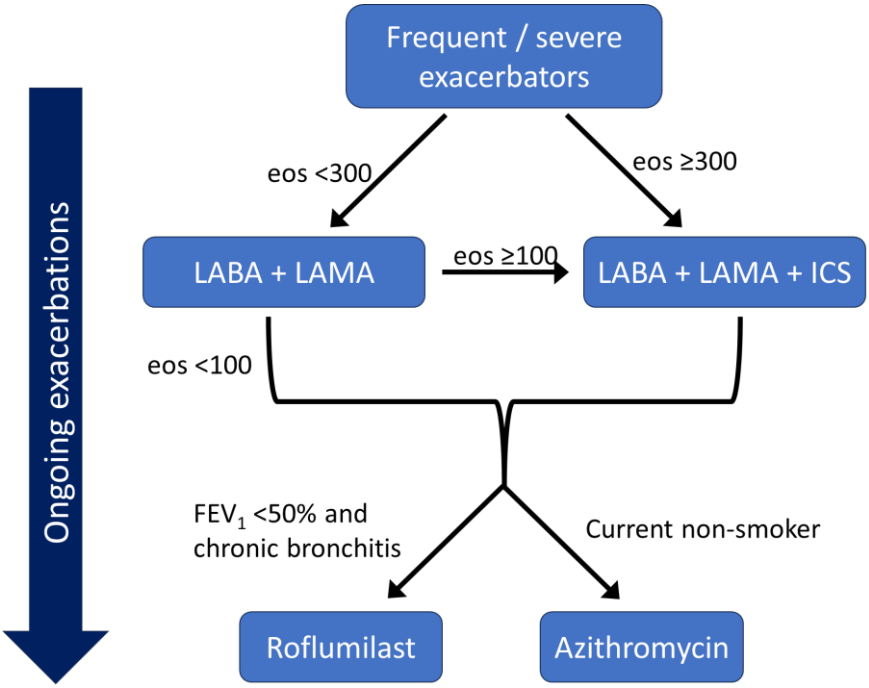

Figure 1.3: Treatment escalation for COPD maintenance therapy in the context of frequent exacerbations

All COPD patients with high exacerbation risk (Group E) should be on dual bronchodilator with combined long-acting beta agonist (LABA) + long-acting muscarinic antagonist (LAMA). If blood eosinophil count is ≥ 300 cell/µl, or ≥ 100 cell/µl with ongoing exacerbations despite LAMA-LABA therapy, inhaled corticosteroids (ICS) should be added to treatment regime. If despite the above treatment exacerbations persist then roflumilast (for patient with sever obstruction and chronic bronchitis) or azithromycin (for former smokers) should be considered.

Figure adapted from: (GOLD 2025)

There have been multiple studies aiming to re-purpose asthma biologics in COPD, given the seeming similarity between COPD and asthma as inflammation driven obstructive airway diseases. Only dupilumab, a monoclonal antibody targeting IL-4/IL-13, and mepolizumab (anti IL-5 mAb) have shown success, with reduced exacerbation rates and an improvement in FEV_1 in the eosinophilic frequent exacerbator phenotype {Bhatt 2024, Bhatt 2023, Beech 2024, Pavord 2017, Sciurba 2025) . Dupilumab has been approved by the UK Medicines healthcare products regulatory agency (MHRA), but at the time of writing is not yet approved by National institute of clinical excellence (NICE) (Lipanovic 2024), meanwhile both dupilumab and mepolizumab have received regulatory approval by the Food and Drug administration (FDA) for use in eosinophilic COPD in the US.

Part of the challenge of developing new drugs in COPD is the heterogeneity of COPD, as well as its late presentation by which time lung damage is often already severe. Furthermore, the slow rate of clinical progression in the COPD and lack of biomarkers to measure disease activity mean that RCTs in COPD are lengthy and expensive (Belz 2024).

1.2 COPD exacerbations

1.2.1 Definition of exacerbations

Acute exacerbations of COPD (AECOPD) are defined as an increase in breathlessness and/or cough and sputum which occur within a 2 week time period (GOLD 2025). They are often associated with other clinical features such as change in colour and viscosity of sputum, fevers, wheeze, tachycardia, tachypnoea and hypoxia (GOLD 2025). AECOPD are an important feature of COPD as they are associated with significant morbidity, increased hospital admissions, accelerated decline in airflow obstruction and increased mortality (Donaldson 2002, Soler-Cataluña 2005, Pavord 2016). Consequently, AECOPD also have large financial costs accounting for £1.6 billion/year and a further £1 billion/year due to adverse events associated with exacerbations such as pneumonia, pulmonary emboli or cardiac events (Asthma+LungUK 2023).

1.2.2 Causes of exacerbations

AECOPD are caused by external environmental stressors such as viral or bacterial infections, pollution or other inhaled irritants, with viruses being the most common of these. 29-64% of AECOPD are associated with viral infections, of which rhinovirus, respiratory syncytial virus (RSV) and influenzae predominate (Seemungal 2001, Bafadhel 2011, Zwaans 2014). Of note during the Covid-19 pandemic, there was a 50% decrease in admissions due to AECOPD at a time when the circulating respiratory viruses in the population decreased due to social distancing and shielding of the vulnerable (Alsallakh 2021). Whilst admissions avoidance (both healthcare initiated and patient-driven healthcare avoidance) as well as other lifestyle changes associated with the pandemic may have impacted these

results to some degree, overall this statistic adds weight to the significant epidemiological importance of viruses as a causative agent for exacerbations.

The high incidence of viral AECOPD is due to both an increased susceptibility of COPD patients to be infected by respiratory viruses and an altered inflammatory response to viruses once infected. Indeed, in chronic bronchitis there is upregulation of intercellular adhesion molecule (ICAM)-1, a transmembrane protein facilitating adherence of respiratory viruses to airway epithelial cells (Di Stefano 1994). Meanwhile, COPD patient lungs also show a deficient interferon response to viruses (Mallia 2011, Singanayagam 2019): Type 1 and type 3 interferons (IFNs) are key proteins in limiting viral replication, protein synthesis and trafficking, and so reduced levels in COPD patients increase respiratory viral loads. Once infected by rhinovirus, COPD patients have a heightened and prolonged pro-inflammatory response with increased secretion of cytokines and chemokines, resulting in increased neutrophil recruitment, mucus hypersecretion and worsened airway obstruction (Mallia 2011).

Bacterial infections are present in up to half of AECOPD (Bafadhel 2011). The most frequent pathogens are *Haemophilus influenzae, Moraxella catarrhalis, Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, and these are associated with increased systemic inflammation (Wedzicha 2013). Impaired bacterial phagocytosis and an exaggerated pro-inflammatory response to bacteria by COPD macrophages contributes to rates of bacterial AECOPD (Patel 2002, Singh 2021). Roughly a quarter of AECOPD have viral and bacterial co-infection (Wilkinson 2017)**.** In COPD, but not healthy subjects, respiratory viruses impair both phagocytosis of bacteria and the release of inflammatory cytokines involved in the innate immune response (Finney 2019). COPD patients with secondary bacterial infections also have a reduction of antimicrobial peptides, secretory leukocyte protease inhibitor (SLPI) and elafin, possibly due to increased degradation by virally-induced neutrophil elastase (Mallia 2012). Together these are likely to contribute to the increased rates of secondary bacterial infections after viral infection seen in COPD. Viral and bacterial co-infection is clinically important as these exacerbations are associated with more severe AECOPD in terms of severity of obstruction, symptom burden, and prolonged clinical recovery (Wilkinson 2006, Wark 2013).

Beyond infection, other environmental triggers also result in AECOPD. Air pollution is increasingly recognised as causing AECOPD, with increased ambient air pollution correlating with increased AECOPD hospitalisation rates (Finney 2024).

1.2.3 Cellular pathophysiology of exacerbations

During exacerbations there is heightened airway inflammation. One of the common ways of clustering exacerbations is by inflammation type: neutrophil predominant; eosinophilic predominant; and

paucicellular. 12-31% of COPD exacerbations have a predominant eosinophilic-inflammatory phenotype (Bafadhel 2011, Gao 2013, Bafadhel 2016). These exacerbations are not associated with bacterial or viral infection (Bafadhel 2011) and respond well to systemic corticosteroids (Bafadhel 2012, Bafadhel 2016).

Approximately 60% of exacerbations are neutrophilic, with neutrophil predominant exacerbations associated with the frequent exacerbator phenotype (Lee 2016, Lu 2021). Viral and bacterial infections are associated with recruitment and activation of neutrophils to the lungs. In turn there is increased secretion of pro-inflammatory cytokines such as interleukin (IL)-6, granulocyte macrophage-colony stimulating factor (GM-CSF), tumour necrosis factor-α (TNF); aberrant neutrophil extracellular trap (NET)osis; degranulation of neutrophils and release of proteases such as MMP9; mucus hypersecretion; and damage to airways (Gao 2013, Finney 2014, Winslow 2021). Neutrophilic AECOPD show prolonged recovery times compared to eosinophilic or paucicellular AECOPD (Gao 2013). Furthermore, a high neutrophil-to-lymphocyte ratio is associated with worse outcomes in severe AECOPD, such as non-invasive ventilation, intensive therapy unit (ITU) admission and death (Lu 2021). Neutrophilic AECOPD are less responsive to corticosteroids than eosinophilic or paucicellular AECOPD (Gao 2013) and this may in part explain the poor outcomes associated with this inflammatory phenotype.

1.2.4 Prognosis and Current treatments of exacerbations

Treatment of AECOPD currently focusses on minimising the severity of the current exacerbation and preventing risk of future exacerbations. The mainstay of treatment involves bronchodilation, corticosteroids and antibiotics with the majority of AECOPD being managed in an outpatient setting.

- **Bronchodilators:** Bronchodilation relieves symptoms of breathlessness and wheeze and improves ability to expectorate sputum. As such, it is recommended that bronchodilators are continued during an AECOPD. Nebulised forms of the drugs are often preferentially prescribed during more severe exacerbations for ease of administration (GOLD 2025).
- **Corticosteroids:** Oral corticosteroids shorten recovery time, improve lung function (FEV_1) and reduce risk of early relapse (Niewoehner 1999). However, systemic corticosteroids have significant side-effects and even acute use has been associated with increased risk of pneumonia, sepsis and death and therefore their use is reserved for moderate to severe exacerbations (Waljee 2017). Corticosteroids are more effective in treating AECOPD with higher blood eosinophil counts (Bafadhel 2012, Bafadhel 2016). This supports the earlier hypothesis that there a variety of different inflammatory processes are at play to account for different exacerbations. However, given that there is currently no alternative effective

treatment option for those with lower levels of blood eosinophils, the decision to treat with steroids is currently based on signs and symptom severity alone rather than on biomarkers.

- **Antibiotics:** Antibiotics reduce short-term mortality and treatment failure in patients hospitalised with AECOPD but not in community exacerbation cohorts (Ram 2006, van Velzen 2017, Vollenweider 2018). Treatment with antibiotics have significant side effect profiles and also overuse results in antimicrobial resistance. As such, GOLD advises that antibiotics are used only in those with presumed bacterial infection or with life threatening AECOPD requiring mechanical ventilation (GOLD 2025).

With the above measures the majority of patients fully recover within 9 days, but some have a more prolonged recovery with just under a quarter of patients continuing to have increased symptoms at 5 weeks and others never returning to their pre-exacerbation health (Perera 2007). 22% patients have a recurrent exacerbation within 50 days (Perera 2007) and this likely represents a frequent exacerbator phenotype as described in Section 1.2.5.

More recently there have been trials in biologic therapy for acute exacerbations. The ABRA study investigated the use of benralizumab, an IL-5 receptor antibody, to treat acute eosinophilic exacerbations of asthma and COPD (Ramakrishnan 2025). In this eosinophilic cohort, benralizumab resulted in a significant reduction of re-exacerbation within 90 days and improvement in patient reported symptoms at 28 days compared with prednisolone treatment. Given the poor outcomes associated with AECOPD and lack of effective therapies especially for non-eosinophilic exacerbations, more research is needed in order to develop more targeted therapies for both the acute exacerbation, and exacerbation prevention in frequent exacerbators.

1.2.5 Frequent exacerbator Phenotype

Approximately 1 in 4 COPD patients have frequent (≥ 2 per year) exacerbations (Finney 2024). The number of exacerbations in previous year is highly predictive of future exacerbations (Hurst 2010): Thus, frequent exacerbations appear to represent a persistent and easily recognisable COPD phenotype. The reason behind an individual's susceptibility to frequent exacerbations is largely unknown and likely to be a complex mix of socio-economic factors, personal health perception and biological predisposition. Low income, cold and damp housing and previous occupational exposure to airborne pollutants have all been shown to be associated with increased frequency of exacerbations (Parris 2022). Perception of breathlessness on CO_2 rebreathing is higher in frequent exacerbators than infrequent exacerbators despite similar minute ventilation, suggesting that in this group some reported exacerbation-like episodes might be down to heightened perception of symptoms rather than physiological cause (Scioscia 2017). From a physiological perspective, frequent exacerbations are

associated with increased severity of obstruction, blood eosinophils, chronic bronchitis, bronchial wall thickening (with or without bronchiectasis), emphysema and bacterial colonisation (Patel 2002, Han 2011, Han 2017, Yun 2018). Extra-pulmonary comorbidities such as cardiovascular disease, diabetes, gastro-oesophageal reflux disease and sarcopenia are also associated with frequent AECOPD (Finney 2024).

Several studies have investigated other biomarkers or new drug targets for frequent exacerbators. Thus far however, treatment options in this frequent exacerbator group are limited to inhaled corticosteroids, Phosphodiesterase-4 (PDE4) inhibitors, long term macrolides and mucolytics as described in Section 1.1.4.

1.3 Sampling in COPD

One of the key challenges which limit large COPD cohort and phenotyping studies is traditional sampling methods. Bronchoalveolar lavage (BAL), the most common technique for sampling the lower respiratory tract, is an invasive test with associated risk especially in older and co-morbid patient population (Babb 2001). The utility of BAL is also limited due to poor tolerability, variable yield (especially in COPD patients due to loss of elastic recoil) and unknown dilution factors, with many inflammatory mediators being below the lower limit of detection of assays (Thwaites. 2018). Sputum is often used as a less invasive test, but the sample contains dead and dying cells suspended in mucus, there is salivary contamination, and mediators often become degraded (bacterial, enzymic and dithiothreitol (Nightingale 1998). Furthermore 30-40% COPD patients do not spontaneously produce sputum on a daily basis and in these patient induced sputum is required (Kessler 2011, Choate 2020). Induced sputum is unpleasant for the patient, carries a risk of bronchoconstriction and causes acute neutrophilic inflammation meaning it cannot be repeated within 48 hours (Nightingale 1998). Many studies rely on peripheral blood as an easily accessible non-invasive and repeatable sampling method. By its very nature however it is a measurement of systemic inflammation rather than specific to lung inflammation and therefore likely to be affected by other diseases non-specific to the lung. Exhaled breath has issues because samples are influenced by saliva and the upper gastrointestinal and respiratory tracts; while condensed water vapour causes some protein biomarkers to become denatured. In light of this, Chapter 5 investigates the utility of a novel non-invasive sampling technique in which a synthetic absorptive matrix (SAM) is used to directly absorption of concentrated fluid from respiratory tract mucosa.

1.4 Cellular and molecular mechanisms of inflammation in COPD

COPD is a disease of chronic inflammation. Both the adaptive (B lymphocytes, T lymphocytes) and innate immune immunity (macrophages, neutrophils, eosinophils, natural killer (NK) cells and

dendritic cells) are involved in this increased inflammation (Barnes 2016). There is also increased activation of the structural cells of the lung including epithelial cells, endothelial cells and fibroblasts which in turn release proinflammatory mediators. The main cell types contributing to COPD pathogenesis are discussed below, with particular emphasis on macrophages as these are the main focus of Chapters 3 and 4.

1.4.1 Neutrophils

Neutrophils are produced in the bone marrow and are the main granulocyte in circulation. They are short lived cells with a half-life of just 19 hours (Baker 2021). Their main role is to rapidly respond to inflammation or infection by migrating into the relevant tissue and phagocytose bacteria (Jasper 2019). They also remove infection via formation of extracellular matrices called neutrophil extracellular traps (NETs). NETs bind to the pathogens and enable the pathogen to be degranulated by proteases whilst minimising damage to host cells (Jasper 2019).

Neutrophils are increased in both sputum and BAL from COPD patients, with increased numbers correlated to disease severity (Keatings 1996). There are a number of factors responsible for the neutrophilia seen in COPD: increased secretion of neutrophil chemoattractants (chemokine (C-X-C motif) ligand (CXCL)1, leukotriene (LT)B_4 and CXCL8) from epithelial cells and macrophages (Keatings 1996, Traves 2002); increased migratory speed towards these chemoattractants (albeit with less accuracy) (Dunne 2019); and an increase in E-selectin and macrophage-antigen (MAC)-1 in the lung resulting facilitating migration into the airways (Di Stefano 1994, Yamagata 2007). Neutrophils secrete serine proteases (neutrophil elastase, cathepsin G and proteinase-3) as well as matrix metalloproteases (MMP), MMP2, MMP8 and MMP9 (Figure1.4). These cause alveolar destruction (emphysema), stimulate mucus production from goblet cells (chronic bronchitis) and increase oxidant stress, thus driving COPD pathogenesis (Barnes 2014). Furthermore in COPD, neutrophil numbers are not only increased but they also have impaired phagocytosis and NET formation (Baker 2021): It is believed that excessive and potentially aberrant NET formation results is increased protease release, NET associated inflammation and reduced pathogen clearance (Grabcanovic-Musija 2015, Jasper 2019).

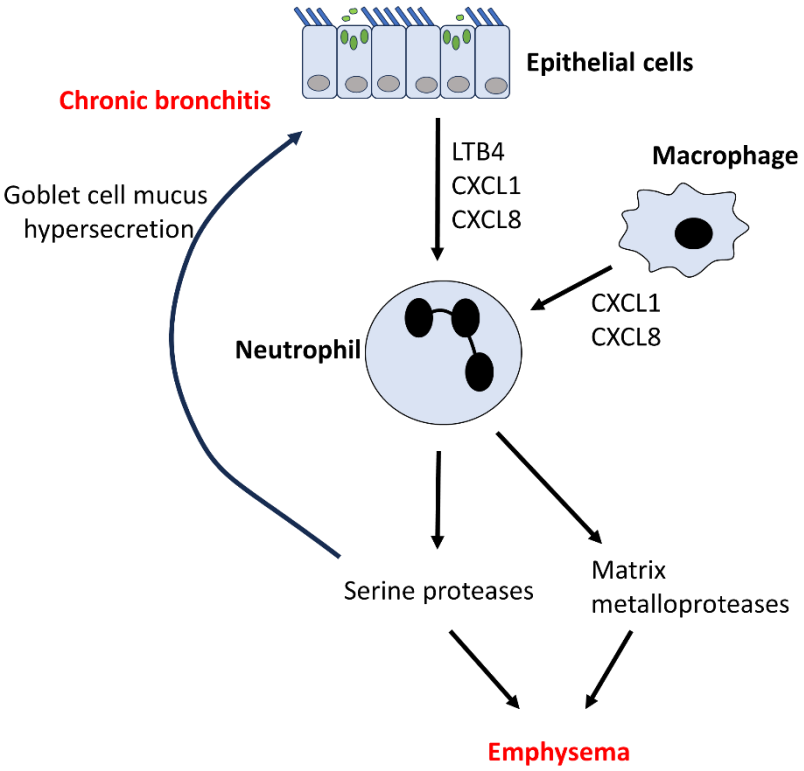

Figure 1.4: Overview of role of neutrophils in COPD pathogenesis
Epithelial cells and macrophages release neutrophil chemotactic factors which promote neutrophil recruitment into the lung. Neutrophils release elastolytic enzymes which drive emphysema and chronic bronchitis.

1.4.2 Eosinophils

Eosinophils are bone marrow derived granulocytes. They contribute to 1–3% of circulating leucocytes, whilst the majority reside in tissues such as the thymus and gastrointestinal tract. Under normal conditions eosinophils are not resident in the lung, and their presence is usually indicative of abnormal inflammation in diseases such as asthma, eosinophilic pneumonias, or eosinophilic granulomatosis (Cabrera López 2023).

In the lung, in response to allergens, pollutants or other stimuli, epithelial cells secrete IL-33 and thymic stromal lymphopoietin (TSLP), these in turn activate T helper type 2 cells (TH2 cells) and type 2 innate lymphoid cells (ILC2) as shown in Figure 1.5 (Barnes 2019). As such IL-4, IL-5 and IL-13 are released which in turn promote growth, maturation and migration of eosinophils. Chemokine ligand (CCL)-5 is released from epithelial cells and directly recruits eosinophils, whilst GM-CSF, which is raised in COPD promotes survival of eosinophils (Barnes 2019). In asthma, the role of eosinophils is relatively well understood and monoclonal antibodies targeting TH2 driven inflammation via targeting IL-5 directly (mepolizumab, reslizumab), IL-5 receptor (benralizumab), IL-4/IL-13 (dupilumab) and TSLP (Tezepelumab) are licenced in the UK (Kavanagh 2003). In COPD however the role of eosinophils in COPD is less well defined.

15-40% COPD patients have high blood eosinophil counts and airway eosinophils (Segal 2018). These patients have higher exacerbation rates and lower levels of emphysema and are more responsive to inhaled corticosteroids (Bafadhel 2018). Despite this apparent evidence that an eosinophilic subtype of COPD exists, various RCTs aimed at re-purposing TH2 directed therapies have had inconclusive results. Mepolizumab (IL-5 mAb) has been shown to reduce AECOPD rate in an eosinophilic COPD population (eos >300cell/µl) in phase 3 trials (Sciurba 2025, Pavord 2017) , however benralizumab (anti-IL-5R mAb) did not reach statistical significant despite similar inclusion criteria and primary endpoints (unpublished, press release of RESOLUTE trial). Astegolimab an anti-ST2R mAB (ie blocking IL-33 signalling) showed promising results in phase IIb trials but failed to meet its primary endpoint in phase III trial (ARNASA) of reduction in AECOPD rate at 52 weeks (unpublished data). Meanwhile there has been success in both phase 2 and phase 3 trials for dupilumab (anti-IL4/IL-13 antibody) decreased exacerbation frequency and improved FEV_1 in a chronic bronchitic COPD cohort with high blood eosinophil counts (Bhatt 2024, Bhatt 2023) .

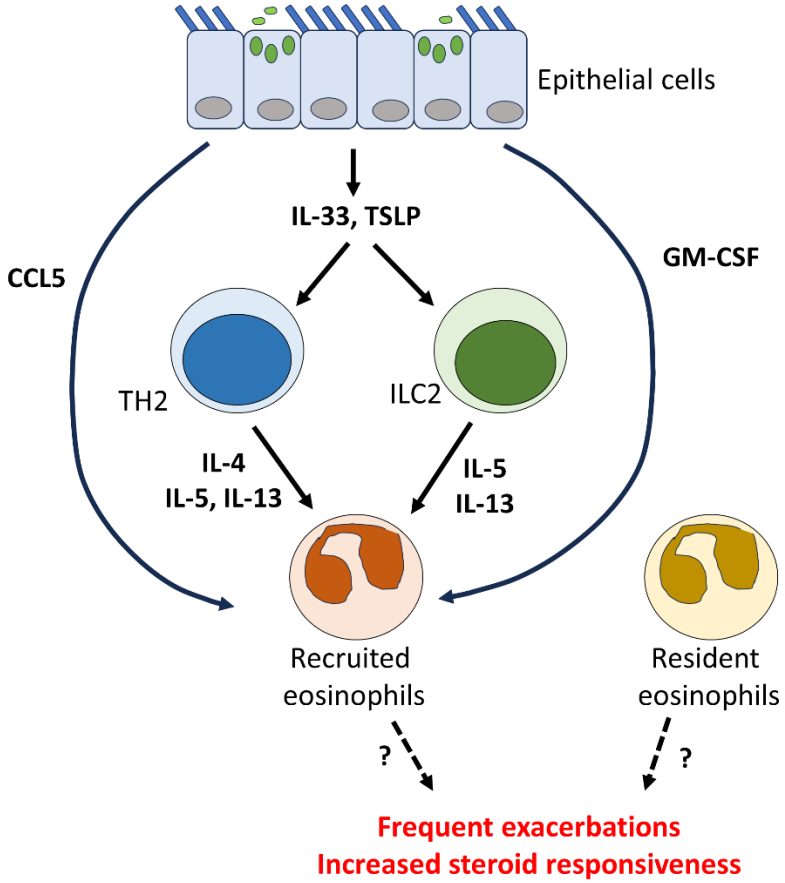

Figure 1.5: Overview of role of eosinophils cells in COPD pathogenesis
In response to insults e.g. smoking, epithelial cells release IL-33, TSLP which activate T helper cells (TH2) and type 2 innate lymphoid cells (ILC2). In turn these cells release IL-4, IL-5 and IL-13 which promote the recruitment, activation and growth of eosinophils. Epithelial cells secrete eosinophil chemoattractant CCL5, and GM-CSF which promotes eosinophil survival. Resident eosinophils are also present in COPD in large numbers, but little is known about these beside being relatively unresponsive to IL-5. Increased eosinophils are associated with frequent exacerbations and steroid responsiveness.

One reason for the lack of significant outcomes of these biologic trials might be difficulty identifying the correct COPD cohort for eosinophilic targeted therapies: Blood eosinophil counts have been predominantly used as a biomarker in RCTs, but there is significant temporal variation of blood eosinophils and whilst there is a group of patients which consistently exhibit low blood eosinophil counts, those with high blood eosinophils move between groups and therefore may be missed with a one off cell count (Baraldi 2024, Beech 2024). Another possible explanation why repurposing of drugs might have had limited success is that eosinophils in COPD are pathogenic by a different mechanism and not mediated by IL-5. Cabrera López et al. suggested that there are two main subtypes of COPD; inflammatory eosinophils (iEos) which are responsive to IL-5 and drive T2 inflammation, and resident

eosinophils (rEos) which have less IL5 receptors and play a more homeostatic role. They showed that iEos are prominent in asthma pathology, but in COPD iEos are very low in number (<5% total eos) and rEos predominate (Cabrera López 2023). Given lack of iEos in COPD, one could postulate that the positive results in the NOTUS trial may be due to other (non-eosinophilic) IL-4/IL-13 actions such as IL-13 mediated goblet cell hyperplasia and mucus secretion (Kavanagh 2001). The anti-IL5R antibody, benralizumab, has shown benefit in eosinophilic AECOPD (Ramakrishnan 2025). More research is needed to examine eosinophil phenotype and their role during exacerbation versus stable state.

1.4.3 Epithelial cells

The airway epithelium acts an important immune barrier within the airways. It includes ciliated epithelial cells, mucus secreting goblet cells and basal cells. Epithelial cells are activated by cigarette smoke, other inhaled irritants and in turn direct an immune response by secreting numerous pro-inflammatory mediators including TNF, IL-1β, IL-6, GM-CSF, and CXCL8 (Barnes 2014). Small airway epithelial cells (SAEC) secrete transforming growth factor-β (TGF-β) which results in peribronchiolar fibrosis and small airways disease (Takizawa 2001). Epithelial cell secretion of vascular endothelial growth factors (VEGF) is reduced in smokers and COPD subjects and this in turn reduces alveolar integrity and drives emphysema (Kanazawa 2014). Chronic airway irritation by cigarette smoke in COPD effects basal cell differentiation, promoting goblet cell hyperplasia and mucus hypersecretion as well as a reduction of ciliated cells and impaired mucociliary clearance (Schamberger 2015). These changes in airway epithelium contribute to the development of chronic bronchitis. The role of epithelial cells in COPD pathogenesis is shown in Figure 1.6.

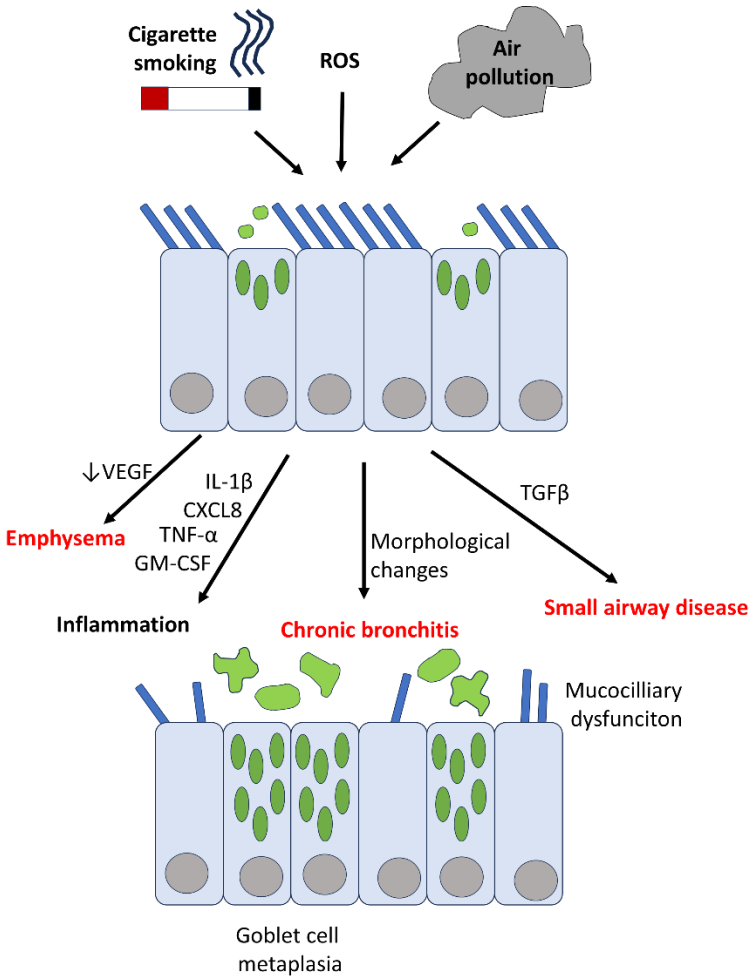

Figure 1.6: Overview of role of epithelial cells in COPD pathogenesis
Cigarette smoke and other reactive oxygen species cause epithelial cells to release more pro-inflammatory cytokines, driving emphysema and small airways disease. Goblet cell metaplasia and mucociliary dysfunction result in chronic bronchitis.

1.4.4 Small airway fibroblasts

Fibroblasts are a major structural cell type which respond to damage by inducing repair and remodelling. Fibroblasts are found in both the parenchymal and airways, and they display different phenotypes in each of these locations: Parenchymal fibroblasts are more myo-fibroblast-like, displaying contractile properties with higher levels of α-smooth muscle actin; by contrast airway fibroblasts are stellate in appearance, synthesising more collagen in response to TGF-β (Kotaru 2006). Whilst parenchymal fibroblasts have been implicated in COPD pathogenesis, it is likely that the small airway fibroblasts (SAF) play a more crucial role in the development of small airways disease which characterises COPD. Cigarette smoke stimulates epithelial cells and macrophages, to secrete TGFβ, in turn this activates SAF (Takizawa 2001). Once activated, COPD SAF show a more pro-inflammatory and senescent associated secretory phenotype (SASP) releasing cytokines and recruiting inflammatory

cells to the airways as discussed in Section 1.4.6 (Wrench 2023). The SAF from COPD patients have also been shown to secrete more collagens including COL1A1, COL3A1 and fibronectin (Barnes 2019, Eapen 2021, Wrench 2023). As such, through increased inflammation, infiltration of leucocytes and peribronchiolar fibrosis, COPD SAF are key to small airway disease progression (Figure 1.7).

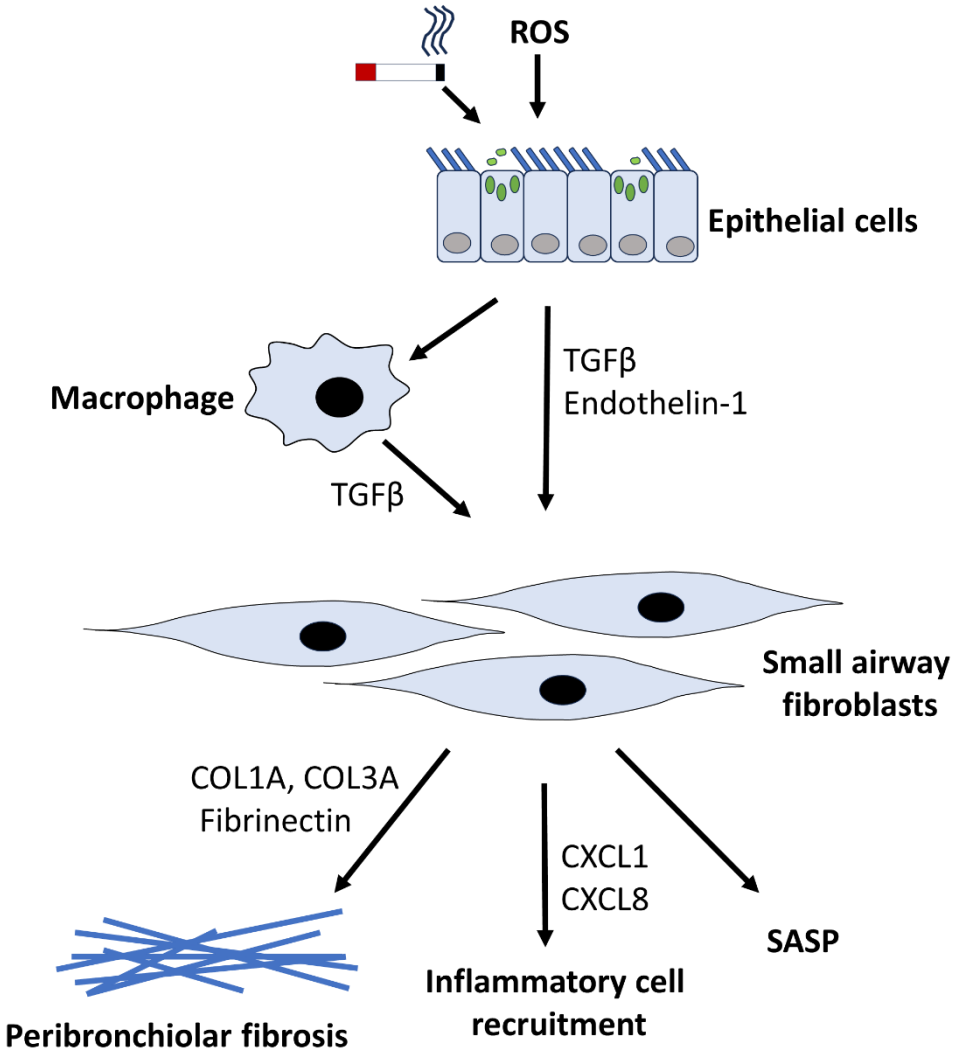

Figure 1.7: Overview of role of small airway fibroblasts cells in COPD pathogenesis
Cigarette smoke and other reactive oxygen species cause epithelial cells to release TGFβ and endothelin-1. Epithelial cells stimulate macrophages to release TGFβ. Consequently, small airway fibroblasts become pro-inflammatory resulting in tissue inflammation and infiltration of leucocytes. Small airway fibroblasts also release collagens and fibronectin which drive peribronchiolar fibrosis.

1.4.5 Macrophages

Macrophages are found throughout the lung in the interstitium and alveolar compartments. They play a key role in regulating lung inflammation and responding to lung insult and infection. There are two major sources of macrophages within the lung: tissue resident and circulating monocyte-derived cells. Tissue resident macrophages originate during embryonic development from the yolk sac and foetal liver and through self-replication persist throughout adulthood (Baker 2021). Meanwhile at the time of injury or insult, circulating blood monocytes are recruited into the lung (by CXCL1, CXCL8 and CCL2) where they subsequently differentiate into mature macrophages (Baker 2021). Studies on lung transplant recipients show that in humans the majority of mature alveolar macrophages are derived from donor circulating blood monocytes (Byrne 2020).

Traditionally macrophages were considered to have two major phenotypes: M1 (classically activated) and M2 (alternatively activated). M1 were thought to be more pro-inflammatory producing a variety of cytokines and chemokines in response to an inflammatory stimulus or pathogen, meanwhile M2 were anti-inflammatory and responsible for phagocytosis of potentially harmful inflammatory material (Murray 2011). More recently it has been acknowledged that macrophage phenotyping is likely to be a more complex spectrum of subtypes, and in COPD a distinct macrophage phenotype predominate, as discussed in Section 1.4.5.1 (Chana 2014). Macrophages also have functional plasticity allowing them to adapt to their environment complicating the picture yet further (Hussell 2014, Kulikauskaite 2020).

1.4.5.1 The role of macrophages in COPD pathogenesis

Macrophage numbers are increased 5 to 10-fold in the tissues, bronchoalveolar fluid and sputum of patients with COPD (Barnes 2016). Macrophage number is correlated to disease severity COPD with increased numbers found in sites of most severe emphysema (Barnes 2016). In COPD there is a particular macrophage phenotype, which have different expression of cell-surface markers compared to healthy smokers and healthy non-smokers as outlined in Figure 1.8 (Chana 2014). Compared to healthy subjects, alveolar macrophages from COPD patients release more pro-inflammatory cytokines (e.g. GM-CSF), chemokines (e.g. CXCL8) and elastolytic enzymes (e.g. MMP9) (Russell 2002, Culpitt, 2003, Chana 2014). Macrophages from COPD patients also show reduced capacity to phagocytose bacteria, apoptotic cells and fungal spores (Hodge 2003, Taylor 2010, Wrench 2018). This defective phagocytosis is associated with more frequent exacerbations (Singh 2021) and may be a factor in determining the chronic bacterial colonisation of lower airways seen in 50% of COPD patients (Patel 2002). It is not yet fully understood the reason for this defect in macrophage phagocytosis. Reduced expression of scavenger receptors reduces ability recognise and engage with prey (Tanno 2020). Meanwhile defective cytoskeleton (Anders 2019) and dysfunctional macrophage mitochondrial

function (Belchamber 2019) are also thought to contribute. Macrophages also release CXCL9, CXCL10 and CXCL11, attracting cytotoxic CD8+ T-cells and CD4+ T-cells. T cells release proteases such as granzyme B and perforin, thus contributing to emphysema (Devulder 2024).

Approximately one third of COPD macrophages show an altered response to glucocorticoids with no suppression of pro-inflammatory cytokines, CXCL8 and TNFα, but ongoing inhibition of anti-inflammatory IL-10 (Chana 2014). As such steroids may in fact accentuate the pro-inflammatory state of COPD macrophages. A likely mechanism behind this steroid insensitivity is oxidant mediated downregulation of histone deacetylase (HDAC) 2 activity in the lung tissue of COPD patients. Reduced HDAC2 is associated with increased cytokine production, reduced steroid sensitivity and increased severity of airway obstruction (Ito 2005). Corticosteroids are currently a mainstay of treatment for both stable and exacerbating COPD patients but given steroid-insensitivity macrophage induced inflammation is likely to be unaltered. Further treatments are necessary to target this particularly prominent source of inflammation in COPD.

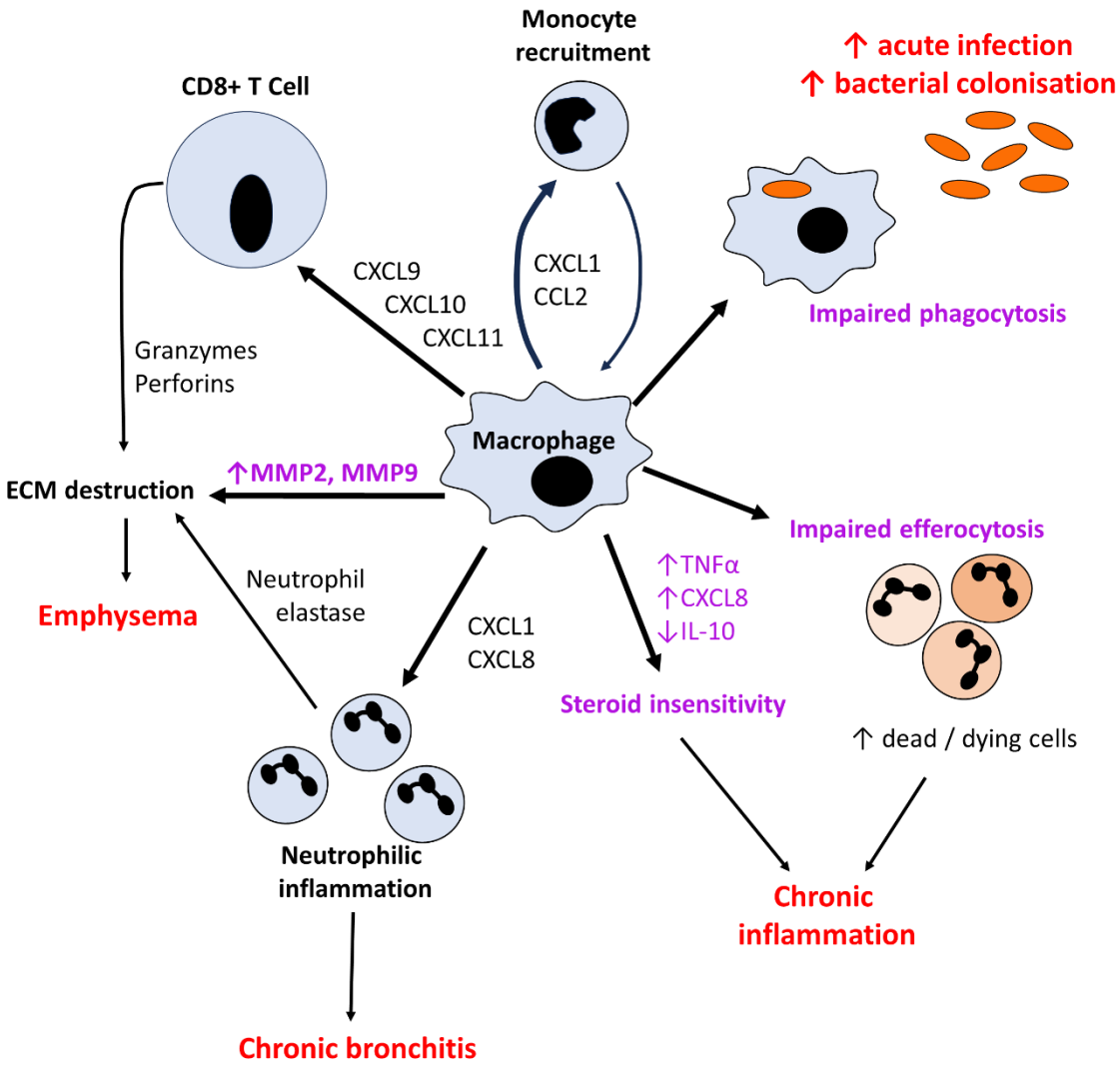

Figure 1.8: Overview of role of macrophages in COPD pathogenesis
Circulating monocytes are recruited to the lung and differentiate into macrophages. Macrophages recruit neutrophils, cytotoxic T cells and monocytes to the lung: These contribute to lung inflammation and, through the release of proteases, cause extracellular matrix degradation leading to emphysema. COPD macrophages have a particular phenotype (**marked in purple**) secreting more pro-inflammatory cytokines and MMPs; reduced phagocytosis and efferocytosis resulting in increased rates of infection and inflammation. Macrophage induced inflammation in COPD is steroid insensitive accentuating this pro-inflammatory state further.

1.4.5.2 Studying lung macrophages in vitro

Lung macrophages can be retrieved at bronchoscopy, surgical lung resection or via sputum. BAL and lung resection are invasive tests, meanwhile sputum often is not produced spontaneously by COPD patients, thus each has its practical limitations. Given that the majority of macrophages within the lung are derived from circulating monocytes (Byrne 2020), a monocyte-derived macrophage (MDM) model has been successfully developed (Winkler 2008, Belchamber 2019). Unlike macrophages retrieved at bronchoscopy, surgical lung resection or via sputum, MDM have the advantage of providing a reliable high yield of macrophages via a minimally invasive manner, allowing for larger scale studies on a wider range of respiratory patients (Finney 2019). In this method, peripheral blood monocytes are differentiated with GM-CSF (resulting in more M1-like polarisation) or Macrophage colony stimulating factor (M-CSF) (M2-like polarisation) for 10-12 days to create monocyte-derived macrophages (MDM) (Belchamber 2019). Using this method, GM-MDM have been shown to be similar in properties to alveolar macrophages in terms of cell-surface molecule expression and functional response to oxidative stress (Winkler 2008). MDM show a similar cytokine response to stimuli and impaired phagocytosis when compared to matched bronchoalveolar fluid derived alveolar macrophages of COPD and non-smoking participants (Finney 2019). The MDM model is therefore useful for investigation of abnormal macrophage behaviour in COPD. As such this study will use MDM as a surrogate for alveolar macrophages in the majority of experiments.

1.4.5.3 Macrophage cell signalling

Macrophages respond dynamically to intrinsic and extrinsic signalling (Kulikauskaite 2020). Therefore, when considering novel drug targets, it is important to understand the major cell signalling pathways that facilitate this. Phosphatidylinositol 3 kinase (PI3K), mitogen activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB) are major parts of macrophage inflammatory signalling cascades, which respond to stimuli and affect cellular survival, function, and cell-to-cell interaction (Wang 2020).

1.4.5.3.1 Phosphatidylinositol 3 kinase (PI3K)

PI3K regulate key cellular functions such as cell growth, proliferation, differentiation, and apoptosis. Environmental stressors such as ROS, *H. influenzae* upregulate PI3K signalling in epithelial cells (Moradi 2021). There are three main classes of PI3K of which class 1 is the most studied and most relevant to the lung. Class 1 PI3K consist of a heterodimer in which there are 4 different catalytic subunit isomers (p110α, p110β, p110γ, p110δ) (Barnes 2016, Wang 2020). The α and β subunits are fairly ubiquitously expressed, meanwhile γ and δ are more specific to leucocytes including macrophages. PI3Kγ activity in macrophages results in immune suppression decreasing inflammation but whilst increasing neoplastic growth (Kaneda 2016). PI3Kδ causes increased oxidative stress in

peripheral blood monocyte cells (PBMC) and alveolar macrophages, as well as steroid resistance via suppression of HDAC2 (Barnes 2016, Moradi 2021). More generally in COPD, increased activation of PI3K contributes to cellular senescence via mTOR and miR-34a, mucin overproduction, impaired response to viral infection and production of inflammation and further ROS (Wang 2020).

1.4.5.3.2 Mitogen-activated protein kinase (MAPK)

There are three major MAPK pathways: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38 MAPK. MAPK are usually inactive in resting cells, but in COPD ERK, JNK and p38 show increased activity (Barnes 2016). ERK is the predominant MAPK in lung macrophages. Increased ERK1/2 activity in COPD leads to increased IL-1 and IL-6 release. Elsewhere in the lung, ERK activity increases mucus secretion via upregulation of MUC5A and EGFR (Barnes 2016). JNK plays a role in allergic inflammation contributing to mucus hypersecretion, smooth muscle proliferation, collagen synthesis and fibrosis and steroid resistance in macrophages (Barnes 2016). p38 is activated in airway epithelial cells and macrophages by lipopolysaccharide (LPS), rhinovirus and cigarette smoke (Ahmadi 2023). Its activity signals increased cytokine release (CXCL8, IL-6, TNFα, GM-CSF), neutrophilic inflammation, release of MMPs and affects phagocytosis (Ahmadi 2023). There are four different p38 MAPK isomer (α, β, γ and δ) of which lung macrophages predominantly express the α and δ isoforms (Smith 2006, Renda 2008). LPS stimulates p38 MAPK phosphorylation to its active form that in turn stimulates release of inflammatory cytokines such as TNF from macrophages (Smith 2006). Phosphorylated p38 levels in alveolar macrophages have been shown to be inversely correlated with FEV_1 and FEV_1/FVC (Renda 2008).

1.4.5.3.3 Nuclear factor kappa-B (NF-κB)

In COPD, NF-κB is predominantly active in airway epithelial cells and macrophages (Barnes 2016).Its activity has been shown to be increased in COPD sputum macrophages during exacerbation, and *in vitro* is activated by viruses, bacterial infection, oxidative stress and pro-inflammatory cytokines (TNFα, IL-1β) (Edwards 2009). NF-κB results in cytokine and chemokines secretion, reduced MUC5A expression (Edwards 2009). In macrophages from healthy subjects, corticosteroids inhibit NF-κB by histone deacetylation via HDAC2, but in COPD macrophages show reduced HDAC activity and as such may contribute to steroid insensitivity in COPD (Cosio 2004, Barnes 2016).

1.4.6 Senescent associated secretory phenotype

In healthy individuals lung function normally peaks at about 25 years of age and then there is a gradual decrease in lung function with narrowing of the airways, decreased lung elasticity and enlargement of alveolar spaces, known collectively as senile emphysema (Barnes 2015). In COPD there is accumulation of senescent cells driving accelerated again, and increasingly senescence is recognised as a key process within the disease pathogenesis (Barnes 2019). Cellular senescence is characterised by irreversible cell cycle arrest and is associated with several characteristic features including telomere shortening, activation of DNA damage response, mitochondrial dysfunction and stem cell exhaustion. All these features are described in lungs of COPD patients (Barnes 2019).

Oxidative stress is increased in COPD and activates the cyclin dependent kinase inhibitor $p21^{CIP1}$ leading to cell cycle arrest. This in turn activates NF-κB, p38MAPK and JAK pathways, resulting in the secretion of a characteristic pattern of pro-inflammatory cytokines, chemokines and MMPs known as the senescent associated secretory phenotype (SASP) as shown in Figure 1.9 (Barnes 2019). The SASP not only results in structural changes of the tissue but also induces further senescence itself. The PI3K - Mammalian Target of Rapamycin (MTOR) pathway regulates cellular senescence directly and also by reducing the anti-ageing molecules sirtuins (SIRT) 1 and SIRT 6. Both SIRT1 and SIRT6 are reduced in COPD lungs thus further amplifying senescence (Barnes 2019).

COPD is commonly associated with other chronic diseases thought to be diseases of accelerated aging. Cluster analyses suggest there may be a COPD phenotype associated with cardiovascular and metabolic disease (Burgel 2010, Vanfleteren 2013). Indeed, transfer of mi-34a between cells via extracellular vesicles, propagates senescence to recipient cells and may be responsible for co-morbidities and multimorbidity in the elderly (Devulder 2025). Targeting cellular senescence has been propose as a potential pharmacological approach to COPD, with the aim of reduce disease progression, mortality and severity of co-morbidities (Barnes 2017).

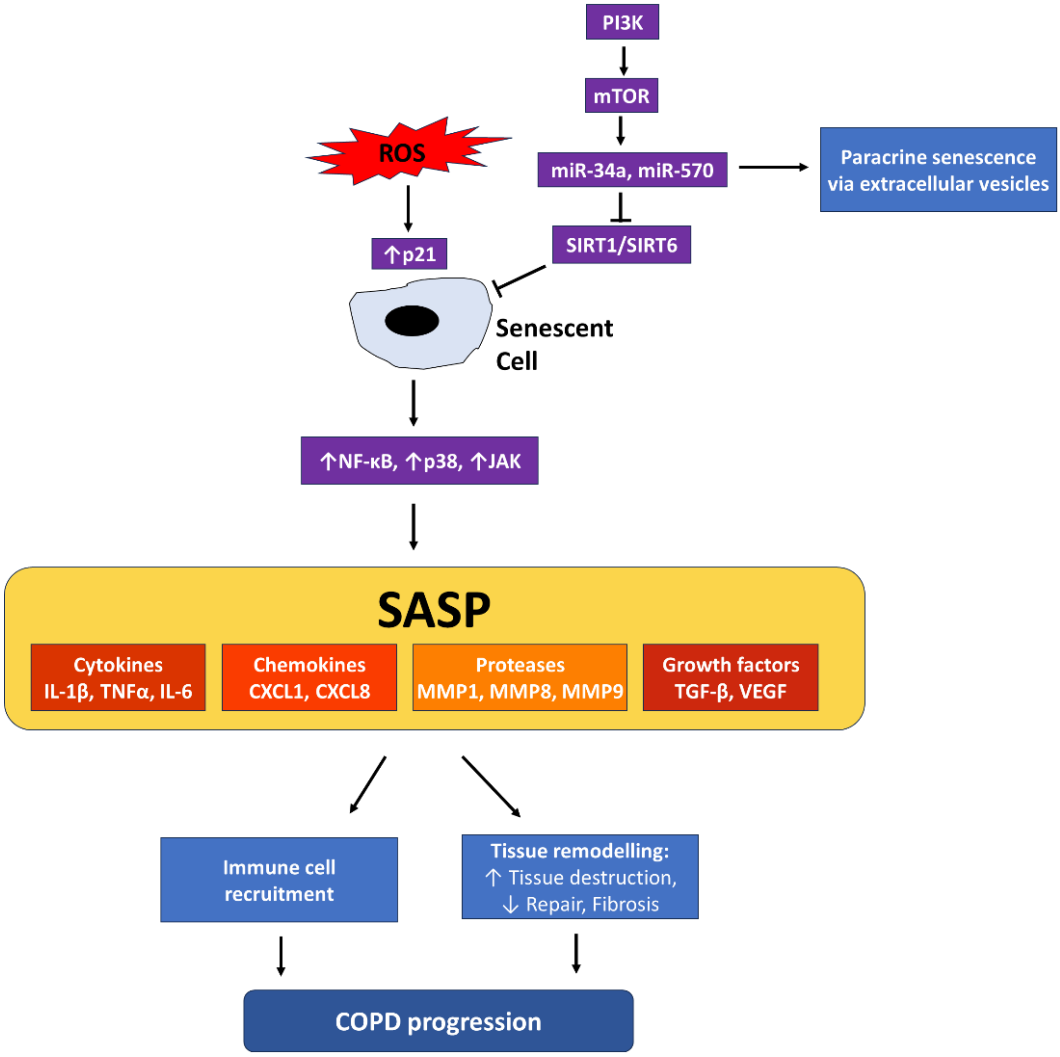

Figure 1.9: Senescence in COPD

Reactive oxygen species (ROS) promote $p21^{CIP1}$ resulting in cell cycle arrest and cell senescence. Senescent cells have increased NF-κB, p38 MAPK, and JAK activity resulting in the secretion of a characteristic group of inflammatory proteins known as the senescence-associated secretory phenotype (SASP). The SASP drives COPD pathogenesis. PI3K activated mTOR which in turn activates miR-34a and mR-570. microRNAs inhibit sirtuin-1 and sirtuin-6 which usually regulate senescence. miRNAs are released from cells in extracellular vesicles and promote senescence in other cell types.

Figure adapted from: (Barnes 2019)

1.4.7 Role of bacteria

In COPD, bacteria play a key role in pathogenesis and disease progression both through lower airway colonisation at stable state and acute bacterial infection (Tiew 2024). In COPD, the lower airway microbiome is different in COPD compared to health, with reduced species diversity and increased presence of pathogenic organisms, most notably *H. influenzae*, *M. catarrhalis*, *S. pneumoniae* (Tiew 2024). The reason for this altered microbiome is unclear, but impaired mucociliary clearance, parenchymal structural damage and defective neutrophil and macrophage phagocytosis likely contribute to the persistence of pathogenic organisms (Finney 2014). Treatment with inhaled corticosteroids, a mainstay of severe COPD treatment, further accentuates the bacterial dysbiosis already present in COPD (Tiew 2024). Bacterial colonisation is clinically important as it is associated with increased symptom burden, more frequent exacerbations and disease progression (Patel 2002, Singh 2015). *H. influenzae* is the most common bacteria to colonise COPD lungs and present in almost half of COPD patients at stable state (Singh 2021). *H. influenzae* promotes neutrophilic inflammation, stimulates NET formation and in turn causes inflammatory cytokine release. As such colonisation by *H. influenzae* promotes COPD disease progression (Marin 2009, Winslow 2021).

As discussed in Section 1.2, approximately half of AECOPD are associated with bacterial infection, of which *H. influenzae* is the most common organism (Wedzicha 2003). In COPD, the ability of macrophages to phagocytose of *H. influenzae* negatively correlates with exacerbation frequency (Singh 2015), perhaps explaining the frequency of bacterial induced exacerbations. Macrophages release increased pro-inflammatory cytokines when phagocytosing *H. influenzae* and *S. pneumoniae* during AECOPD compared to phagocytosis at stable state (Singh 2021). This increased inflammatory response to bacteria during AECOPD may contribute to disease progression associated with acute exacerbations.

Given the above, a potential aim of future COPD treatments would be to modulate the COPD microbiome, reduce bacterial colonisation by pathogenic organism such as *H. influenzae*, and decrease incidence of acute bacterial infection.

1.5 IL-36 as a potential target in COPD

Recently, the IL-36 subgroup of the IL-1 cytokine family has been recognised as potentially important within the pathogenesis of COPD (Kovach 2021, Baker 2022). This thesis explores further the role of IL-36 signalling in COPD and regulation, with the aim of considering whether the IL-36 receptor might be a useful drug target in COPD.

1.5.1 The IL-36 family

The IL-36 family sits within the IL-1 superfamily (Bassoy 2018). There are three isoforms of the IL-36 receptor agonist (IL-36α, IL-36β and IL-36γ) and two antagonists (IL-38 and IL-36 receptor antagonist (IL-36RA)). Each requires N-terminal cleavage from its secreted form in order to reach full potency as an IL-36 receptor agonist (Bassoy 2018). A variety of neutrophil proteases are thought to be responsible for cleavage and activation of the IL-36 cytokines including neutrophil elastase, cathepsin G and proteinase-3 (Clancy 2018).

The IL-36 receptor is a heterodimer receptor comprising of the IL-36 receptor (IL-36R) and the IL-1 receptor accessory protein (IL-1RaP). Activation of this receptor results in a signalling cascade via NF-κB and MAPK signalling pathways, and this in turn results in release of pro-inflammatory cytokines such as IL-6 and CXCL8 (Zhang 2017).

Dysregulation of the IL-36 pathway has been shown to be important in several chronic inflammatory diseases including osteoarthritis, inflammatory bowel disease and systemic lupus erythematosus (Neurath 2020). In generalised pustular psoriasis (GPP), a mutation in *IL-36RN*, the gene encoding IL-36RA, drives this severe form of skin disorder. Phase 1 trials of spesolimab, an IL-36 receptor antibody, showed efficacy and safety as a biologic therapy in GPP (Bachelez 2019).

1.5.2 IL-36 in the lung

IL-36 receptor signalling plays a role in lung inflammation. IL-36γ is released from small airway and bronchial epithelial cells in the early phase response to viruses (Ombredane 2023). Other noxious stimuli have also been variably shown to increase IL-36 agonist secretion including: cigarette smoke (Kovach 2021); bacteria (Ahsan 2018, Aledamee 2020, Kovach 2021); and allergens such as house dust mites (Ramadas 2011). IL-36γ stimulates SAF which in turn release pro-inflammatory cytokines, elastolytic proteins and neutrophil chemoattractants (Baker 2022). In mouse studies, IL-36 receptor signalling appears to have a role in host defence in the lung, promoting both bacterial and viral clearance (Aoyagi 2017, Wein 2018, Nanjo 2019). IL-36 receptor signalling, however, has variably been associated with both decreased (Wein 2018) and increased (Aoyagi 2017) mortality in response to viral infection. This may be in part due to different experimental infection doses, the degree of IL-36-induced inflammation and thus the amount of inflammation induced lung injury.

1.5.3 IL-36 in COPD

There is dysregulated IL-36 receptor signalling in COPD: IL-36γ is increased in BAL from healthy smokers and COPD patients compared to healthy non-smokers (Kovach 2021, Baker 2022) with small airway epithelial cells secreting more IL-36γ in COPD patients compared to those of healthy non-smokers (Baker 2022). The increased neutrophilic inflammation in COPD, enables more efficient cleavage of IL-36γ to its active form by neutrophil proteases (Henry 2016, Baker 2022). There is further dysregulation of the IL-36 signalling in COPD patients, with lower levels of IL-36RA in BAL and lung homogenates from COPD patients than those from healthy smokers and non-smokers (Baker 2022).

IL-36 signalling appears to play a role in many aspects of COPD pathology as shown in Figure 1.10. IL-36γ affects myofibroblast differentiation and pushes these cells towards a more pro-inflammatory and proteolytic phenotype (Baker 2022) which in turn drives small airway remodelling and emphysema. IL-36γ stimulates the release of neutrophil chemo-attractants such as CXCL1 and CXCL8 from SAF (Baker 2022). In turn, the resulting neutrophilic inflammation leads to the release of proteases, mucus hypersecretion and increased oxidative stress (Barnes 2019). Furthermore, IL-36 agonists appear to promote bronchoconstriction, with IL-36RA reducing airway hyperresponsiveness and inflammation in an asthma mouse model (Liu 2020).

As yet there is no data regarding IL-36 in terms of how concentrations in the lung correlate with COPD severity or clinical phenotype. Given that IL-36γ secretion is virally induced, it would be interesting to assess *in vivo* any association between IL-36γ and AECOPD.

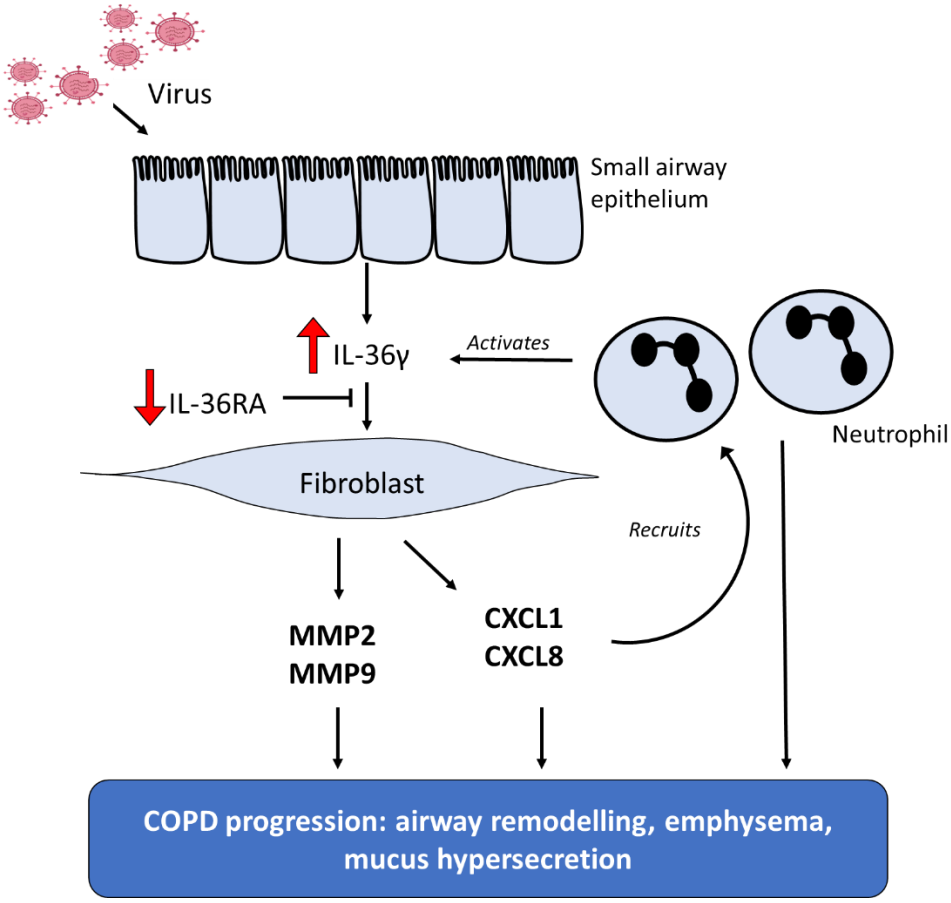

Figure 1.10: Role of IL-36γ in COPD pathogenesis
IL-36γ is increased in COPD whilst IL-36RA is reduced. Viruses further stimulate SAEC to release IL-36γ. IL-36γ acts on SAF leading to the release of elastolytic enzymes (MMP2, MMP9) and pro-inflammatory cytokines which drive emphysema and small airway remodelling. Neutrophils are recruited to the lung in response to CXCL1 and CXCL8, these directly cause COPD progression via mucus hypersecretion, protease production and oxidative stress. Neutrophil derived proteases cleave IL-36 into its more active form, further increasing the above inflammatory cascade.

Figure adapted from: (Baker 2022)

1.6 Hypotheses

IL-36γ accentuates the abnormal COPD macrophage phenotype promoting secretion of pro-inflammatory cytokines and inhibiting bacterial phagocytosis. Abnormal regulation of *IL-36RN* expression by COPD macrophages means that pathogenic effects of IL-36γ continue relatively unregulated. As such, IL-36γ levels are correlated with COPD severity and acute exacerbations.

1.7 Aims

In order to address this hypothesis, the following aims were developed:

1. To investigate the effect of IL-36 receptor signalling on macrophage secretory and phagocytic functions.
2. To examine the expression of *IL-36RN* in monocytes and macrophages to determine any differences between health and COPD.
3. To investigate the mechanism regulating *IL-36RN* expression in healthy and COPD macrophages.
4. To assess the utility of nasosorption for examining inflammation in health and COPD *in vivo*.
5. To measure nasal IL-36γ levels in health and disease, and examine any correlations with disease severity, phenotype or acute exacerbation in COPD.

Chapter 2: Materials and Methods

2.1 Materials

Materials	Supplier
3-(4,5-Dimethylthiazol-2-yl)-tetrazolium bromide (MTT)	Sigma-Aldrich, Dorset, UK
7500 Real Time PCR system	Applied Biosystems, Paisley, UK
Alexa-fluor 488	Fischer Scientific, Loughborough, UK
amphotericin B	Gibco, Invitrogen, Paisley, UK
AS252424	Tocris, Biotechne, US
Bovine serum albumin (BSA)	Sigma-Aldrich, Dorset, UK
Brain Heart Infusion broth	Sigma-Aldrich, Dorset, UK
CAL-101 (idelalisib)	APExBio, Houston, US
Columbia agar plates	Fisher Scientific, Loughborough, UK
Costar 24/96-well tissue culture plates	Fisher Scientific, Loughborough, UK
Costar 96-well black tissue culture plates	Fisher Scientific, Loughborough, UK
Costar Spin-X filter cup	Sigma-Aldrich, Dorset, UK
CXCL1 capture, detection, standard antibodies	R&D Systems, UK
CXCL8 capture, detection, standard antibodies	R&D Systems, UK
Dextran	Sigma-Aldrich, Dorset, UK
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Dorset, UK
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich, Dorset, UK
Dulbecco's phosphate buffered saline (D-PBS)	Sigma-Aldrich, Dorset, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Dorset, UK
Falcon tubes 15/50ml	Sarstedt, Numbrecht, GER
Foetal bovine serum (FBS)	BioSera, Heathfield, UK
Granulocyte macrophage colony-stimulating factor (GM-CSF)	R&D Systems, Abingdon, UK
GraphPad Prism 8.0	GraphPad Software, California, USA
Hank's balanced salt solution (HBSS)	Sigma-Aldrich, Dorset, UK
Hunt Developments UK Ltd	Hunt Developments UK Ltd, Midhurst, UK
IL-36RA capture, detection, standard antibodies	AdipoGen life sciences, Switzerland
IL-36RN primer (Hs00104220)	Applied Biosystems, Paisley, UK

IL-36α capture, detection, standard antibodies	R&D Systems, UK
IL-36β capture, detection, standard antibodies	R&D Systems, UK
IL36γ primer (HS00219742)	Applied Biosystems, Paisley, UK
IL-36γ capture, detection, standard antibodies	AdipoGen life sciences, Switzerland
IL-6 capture, detection, standard antibodies	R&D Systems, UK
L-glutamine	Sigma-Aldrich, Dorset, UK
Macrophage colony stimulating factor-1 (M-CSF)	R&D Systems, UK
Micro Amp Optical 96 Well reaction plate	Applied biosystems, Thermo Fisher, USA
MicroAmp Optical Adhesive Film	Applied biosystems, Thermo Fisher, USA
MSD discovery workbench version 4	Meso Scale Diagnostics, USA
MSD instrument	Meso Scale Diagnostics, USA
Nasopharyngeal PCR Media Uni Swab Sample Packet	Cobas, Roche Molecular Systems, USA
Non-typeable *H. influenzae* (strain 1479)	ATCC, Teddington, UK
NUNC Maxisorp 96-well plates	Sigma-Aldrich, Dorset, UK
Pasteur pipette	Sarstedt, Numbrecht, GE
penicillin/streptomycin	Invitrogen, Paisley, UK
Percoll	GE Healthcare, Buckinghamshire, UK
Qiagen RNeasy Mini Kit	Qiagen, Manchester, UK
RACK1 primer (Hs00272002_m1)	Applied Biosystems, Paisley, UK
Recombinant Human IL-1β	R&D Systems, Biotechne, UK
Roswell Park Memorial Institute 1640 medium	Sigma-Aldrich, Dorset, UK
SDS software	Applied Biosystems, California, USA
Sodium azide	Sigma-Aldrich, Dorset, UK
Sodium chloride (0.9%)	Baxter Healthcare Ltd, Norfolk, UK
Sodium chloride (9%)	Sigma-Aldrich, Dorset, UK
Spirometer	Vitalograph, Buckingham, UK
Streptavidin-horseradish peroxidase	Abcam, Cambridge, UK
Sucrose	Sigma-Aldrich, Dorset, UK
sulphuric acid	Sigma-Aldrich, Dorset, UK
Syringe (various sizes)	Beckton Dickinson, Oxford, UK
T25 tissue culture flask	Fisher Scientific, Loughborough, UK
TaqMan Gene Expression Master Mix (TGE)	Life-Technologies, Paisley, UK
TaqMan normal RNA Reverse Transcription Kit	Life-Technologies, Paisley, UK

TMB reagent A and B	BD, Oxford, UK
Trypan blue	Sigma-Aldrich, Dorset, UK
Tween®-20	Sigma-Aldrich, Dorset, UK
U-plex custom assay 10-spot kit	Meso Scale Diagnostics, USA

2.2 Methods

2.2.1 Participant recruitment

COPD patients were recruited from Imperial College Healthcare Trust (ICHT) and integrated community respiratory clinics from North-West London. Healthy smokers, ex-smoker and never smokers were recruited from pre-existing research databases at the National Heart and Lung Institute and through word of mouth. All participants gave written informed consent as approved by the London-Stanmore Research Ethics Committee (reference 18/LO/0571). NHS permission was granted by ICHT. Clinical assessments and procedures initially took place at the Imperial Clinical Respiratory Research Unit and St Mary's Hospital. Due to COVID-19 pandemic shutting research facilities in 2020, the study's ethics was amended in June 2020 to allow for sampling to also take place in participants' own homes.

2.2.1.1 Non-smokers

Healthy non-smoking volunteers were defined as people aged 40 – 85 years with no smoking history, no history of respiratory disease, no respiratory symptoms, normal respiratory examination, and normal spirometry as predicted for sex, age, height and weight (Table 2.1).

2.2.1.2 Healthy smokers

Healthy smokers were defined as people aged 40 – 85 years with a significant (≥10 pack years) smoking history, no history of respiratory disease, no respiratory symptoms, normal respiratory examination, and normal spirometry as predicted for sex, age, height and weight. This group is subdivided into current and ex-smokers during some analysis in this thesis.

2.2.1.3 COPD patients

COPD patients were defined as per GOLD criteria (GOLD 2025) with an FEV_1/FVC ratio of less than 0.7, with a significant (≥10 pack years) smoking history. All 4 GOLD COPD stages were included in terms of severity of obstruction. Exclusion criteria included patients with concomitant asthma or significant bronchodilator reversibility, defined as greater than 200ml or more than 12% change in FEV_1 after salbutamol inhaler compared to baseline spirometry.

Participant group	Inclusion criteria	Exclusion criteria
Non-smoker	• Age 40-85 years of both sexes • Life-long non-smokers • Normal spirometry	• History of respiratory disease • Respiratory symptoms or abnormal respiratory examination • Upper respiratory tract infection within the last 4 weeks • Significant immunosuppression • Pregnancy or breast feeding • Subjects unable to consent
Healthy smoker	• Age 40-85 years of both sexes • Smokers and ex-smokers (≥10 pack year history) • Normal spirometry	
COPD	• Age 40-85 years of both sexes • Smoking history ≥10 pack years • Confirmed obstruction on spirometry ($FEV_1/FVC < 0.7$)	• No significant bronchodilator reversibility • No history of other respiratory disease • Significant immunosuppression • Pregnancy or breast feeding • Subjects unable to consent • Stable state visit: upper respiratory tract within the last 4 weeks • Exacerbation visit: antibiotics / steroids started > 48h previously.

Table 2.1: Summary of inclusion and exclusion criteria

2.2.2 Study Visits

2.2.2.1 Clinical assessment

At enrolment all participants had an up-to-date clinical assessment of any respiratory or nasal symptoms; exacerbation history (for COPD cohort); past medical history; current medication; smoking history (including inhaled recreational drug use) and occupational history. A clinical examination was performed including pulse oximetry and spirometry (see Section 2.2.2.4). Validated research questionnaires were used to quantify the COPD participants' symptoms including the modified Medical Research Council (mMRC) dyspnoea score and COPD assessment tool (CAT). In addition to the above, most COPD patients had extensive investigation as outpatients prior to enrolment including: full lung function including gas transfer, lung volumes and bronchodilator reversibility if clinical indicated; HRCT; blood tests and microbiology. Notes were made of results in order to allow further clinical phenotyping of the cohort. Table 2.2 summarises assessment and sampling at each visit.

Participants attended for regular blood donation and nasal swabs with a minimum interval between visits of 6 weeks. Participants were required to be at least 4 weeks clear of any type of infection, including mild upper respiratory tract infections, when stable state bloods or nasal samples were taken.

	Recruitment	Stable state visits	Exacerbation visit	Post-exacerbation
Consent	X			
Clinical assessment: symptoms	X		X	
Medical history: co-morbidities, drug history, smoking history, occupational exposures, family history	X			
Update on smoking status and medical history		X	X	X
Assessment of radiology	X			
Note made of other investigations: eosinophil trend, lung function, microbiology	X			
CAT score	X			
mMRC score	X			
Spirometry	X			
Bloods (PBMC)	X	X		X
Bloods (FBC, CRP)	X		X	
Nasosorption	X	X	X	X
Nasopharyngeal swab (viral PCR)			X	
Sputum MCS			X	

Table 2.2: Summary of study visit assessments and samples obtained
CAT = COPD assessment test; mMRC = modified medical research council dyspnoea score; PBMC = Peripheral blood monocyte cells; FBC = Full blood count; CRP = C reactive protein; PCR = polymerase chain reaction; MCS = microbiology culture and sensitivities

2.2.2.2 COPD exacerbations

COPD patients were asked to contact the team in the event of acute exacerbation of COPD (AECOPD) (Figure 2.1, panel A). Exacerbations were defined as an increase in two major symptoms (dyspnoea, sputum purulence or sputum volume) or one major and one minor symptom (cough, wheezing, cold, nasal discharge, sore throat) for at least two days as per the criteria used in the London COPD cohort work (Seemungal 1998). On confirmation of AECOPD a home visit was made within the first 72h of symptom onset and within 48h of any steroid or antibiotics treatment being initiated. Repeat visit was made at least 6 weeks after recovery from AECOPD to collect a baseline sample. In cases when a second exacerbation between initial sampling and follow up visit, recovery sampling was postponed until 6 weeks after second exacerbation (Figure 2.1 Panel B).

2.2.2.3 Spirometry

Forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were measured using a spirometer (Vitalograph, Buckingham, Bucks, UK). The best of three reproducible attempts was noted

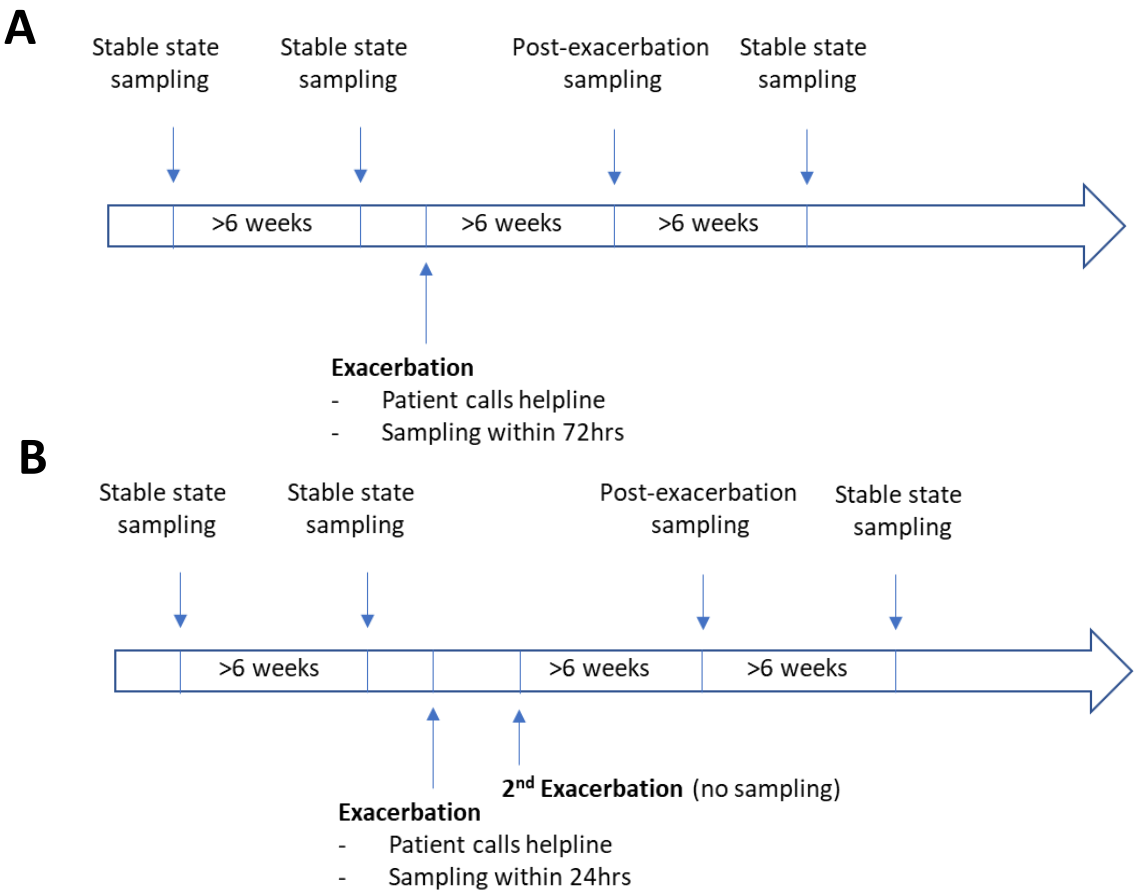

Figure 2.1: Timeline for stable state and exacerbation sampling
(A) Stable state sampling happened with a minimal interval of 6 weeks. When COPD patients had AECOPD they contacted helpline. Exacerbation visit done within 24h of call. Post-exacerbation recovery samples taken 6 weeks later. (B) When a 2nd exacerbation occurs, post-exacerbation sampling delayed until 6 weeks after most recent exacerbation.

both in terms of absolute volume (L) and as percentage predicted based on sex, weight and height as calculated by spirometers software.

2.2.2.4 Sampling methods

The following sampling was performed as outlined in Table 2.2:

- Venepuncture (see Section 2.2.2.5.1)
- Nasosorption (see Section 2.2.2.5.2)
- Exacerbation visits only: Nasopharyngeal viral swab for viral PCR, Spontaneous sputum for microbiology, culture and sensitivity (MCS). These were processed by ICHT microbiology lab.

2.2.2.4.1 Venepuncture

For all stable state visits, whole blood was aspirated using a 21G needle into 3 x 20ml syringes pre-filled with 500µl of 0.5M ethylenediaminetetraacetic acid (EDTA). These were transported to the research laboratory for monocyte isolation and cell culture (see Section 2.2.3). At enrolment and all AECOPD visits, venepuncture with the appropriate vacutainer tube attached was performed to collect samples for a full blood count and C-reactive protein measurement. These were processed by the ICHT laboratory.

2.2.2.4.2 Nasosorption

Nasosorption™ FX·I (Hunt Developments UK Ltd, UK) synthetic absorptive matrices (SAM) were passed into each nostril to oppose the inferior turbinate. After 1min the SAM was removed and transported to the lab on ice. The SAM was placed in a Costar Spin-X filter cup (Sigma-Aldrich, UK), eluted in 300µl buffer (Dulbecco's phosphate buffered saline (D-PBS) with 1% ($^{w}/_{v}$) Bovine serum albumin (BSA), 0.05 % ($^{v}/_{v}$), Tween®-20 and 0.05% ($^{w}/_{v}$) sodium azide) and centrifuged for 20min at 16,000 x *g* at 4°C (Thwaites 2018). Samples from the two nostrils were pooled and stored at -80°C.

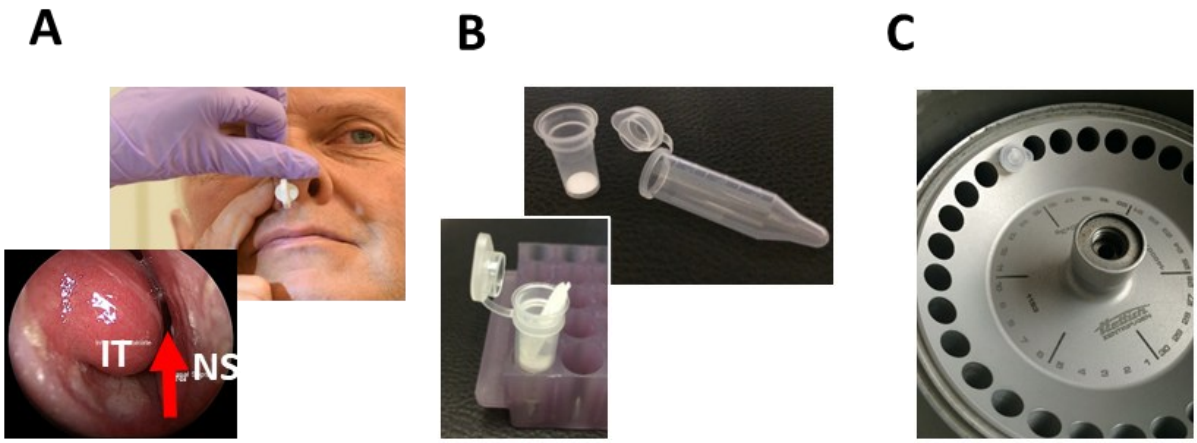

Figure 2.2: Schematic showing nasosorption method and processing
(A) Synthetic absorptive matrix (SAM) is inserted into nasal cavity under direct visualisation to the point where it lies opposing the inferior turbinate (IT) and the nasal septum (NS). (B) SAM is detached and placed in the filter cup of an Eppendorf tube with pore size 0.22µm, where it is immersed in 300µl elution buffer. (C) The samples are then centrifuged at 16,00 x *g* for 20min and elutes stored at -80°C.

2.2.2.4.3 Microbiological samples

At AECOPD visits, nasopharyngeal swabs were taken using a cotton swab which was then placed into appropriate viral transport medium (Cobas PCR media). A respiratory viral PCR panel was performed by the ICHT virology lab. If patients were productive at AECOPD visit, patients were asked to expectorate into sterile specimen pot. This was sent to the ICHT microbiology lab for microbiological culture and sensitivities (MC&S).

2.2.3 Cell isolation and culture

2.2.3.1 Peripheral blood mononuclear cells

PBMCs were isolated from 60ml of whole blood. 20ml aliquots of blood were mixed with 10ml 6% ($^{w}/_{v}$) dextran and 20ml 0.9% ($^{w}/_{v}$) saline in 3 separate Falcon tubes. These were left for 30 min at room temperature to allow red blood cell sedimentation. The top leucocyte rich layer was aspirated off and

transferred to 3 new Falcon tubes. 20ml 0.9% ($^{w}/_{v}$) saline was added and the suspension centrifuged at 500 x *g* for 5min.

Peripheral blood mononuclear cells (PBMC) were separated from the polymorphonuclear (PMN) cell fraction in the resulting cell pellet, using a discontinuous Percoll gradient. 22.5ml Percoll was mixed with 2.5ml 9% ($^{w}/_{v}$) saline to create a 100% Percoll solution. From this 81% ($^{v}/_{v}$), 67% ($^{v}/_{v}$) and 55% ($^{v}/_{v}$) Percoll solutions were prepared using 0.9% ($^{w}/_{v}$) saline. 4ml of 81% ($^{v}/_{v}$) Percoll, followed by 4ml of 67% ($^{v}/_{v}$) Percoll was transferred into a two 15ml Falcon tubes. The cell pellet was resuspended in 8ml 55% Percoll and then 4ml transferred as the top layer of each gradient. The cell fractions were then separated by centrifugation for 30min (750 x *g*, room temperature) as shown in Figure 2.3.

The PBMC fraction lies at the 55% ($^{v}/_{v}$) / 67% ($^{v}/_{v}$) interface. This cell layer was aspirated using a Pasteur pipette into a new 50ml Falcon tube and resuspended using 5ml complete media (Roswell Park Memorial Institute (RPMI) 1640 medium, 10% ($^{v}/_{v}$) foetal bovine serum (FBS), 10mg/ml (1% ($^{v}/_{v}$)) penicillin/streptomycin and 2mM (1% ($^{v}/_{v}$) L-glutamine) and then topped up with 0.9% ($^{w}/_{v}$) saline to make up to total volume of 50ml. Cell count was performed using a Neubauer haemocytometer after staining 1:1 with Kimura stain (0.05% ($^{v}/_{v}$) toludine blue, 0.03% ($^{v}/_{v}$) light green, 10% ($^{v}/_{v}$) saponin, 0.7M phosphate buffer). The PBMCs were centrifuged for 10min (500 x *g*, room temperature) and the cells resuspended in complete media at a concentration of $4x10^{6}$ PBMC/ml (equivalent of ~$1x10^{6}$ monocytes/ml).

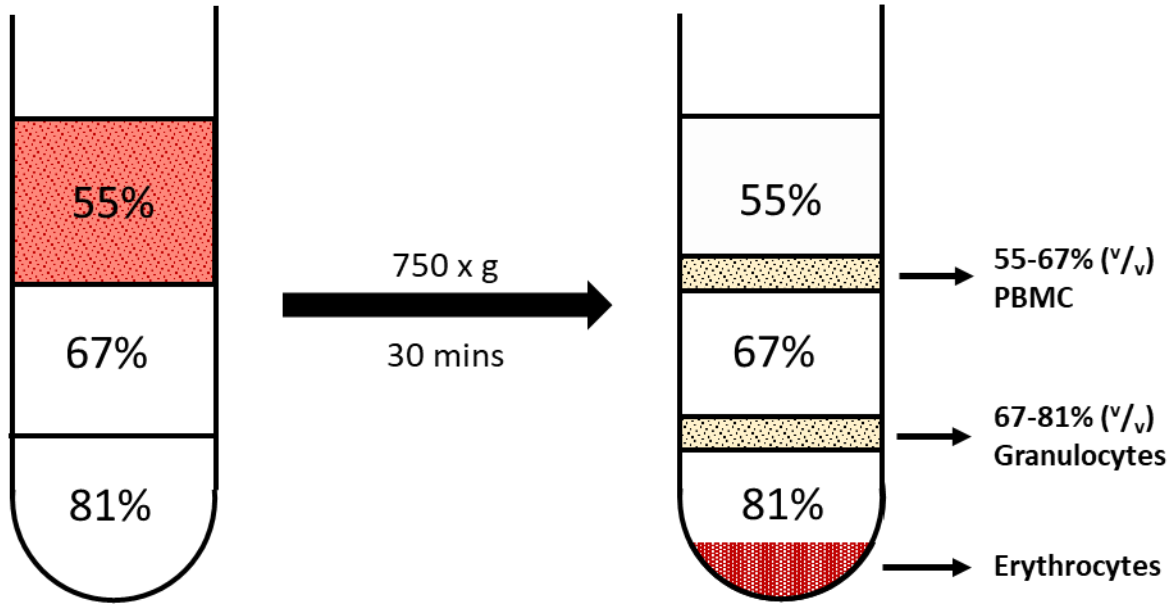

Figure 2.3: Isolation of PBMC using discontinuous Percoll gradient
81%, 67% and 55% Percoll solutions were prepared. The leucocyte rich layer was resuspended in 55% Percoll. The gradient was prepared in a 15ml Falcon tube by layering 81%, 67% and 55% ($^{v}/_{v}$) bottom to top. The column was centrifuged for 30min at 750 x *g*. PBMC layer was then extracted from the 55-67% interface.

2.2.3.2 Separation of monocytes by adherence

The cell suspension was seeded in 24 or 96 well plates at 100µl ($1x10^5$ monocytes / well) or 500µl ($5x10^5$ monocytes / well) respectively and incubated at 37° C, 5% (v/v) CO_2 for 2h to allow adherence of monocytes. Black 96 well plates were used for phagocytosis assays.

2.2.3.3 Monocyte derived macrophages

PBMC were cultured in media supplemented with either 100ng/ml M-CSF or 2ng/ml GM-CSF for 10-14 days at 37°C and 5% (v/v) CO_2 to obtain M-MDM and GM-MDM respectively. Media was replaced twice weekly during this time.

2.2.3.4 Isolation of tissue macrophages

Lung parenchyma tissue from non-smoker, ex-smoker and COPD patients was retrieved from tissue resection for lung cancer or emphysema. Tissue was assessed by the pathologist as being non-cancerous. Tissue was lavaged by injecting 120ml of complete media (Roswell Park Memorial Institute (RPMI) 1640 medium, 10mg/ml (1% (v/v)) penicillin/streptomycin, 2mM (1% (v/v)) L-glutamine, 0.25µg (w/v) amphotericin B, and 50 mmol/l EDTA). The flushed fluid was then centrifuged for 5min (300 x *g*, room temperature) and the pellet resuspended in 2ml 10% Percoll (v/v). A discontinuous Percoll gradient composed of 2ml of the following: 60% (v/v), 50% (v/v), 40% (v/v), 30% (v/v), 20% (v/v) Percoll was prepared in a 15ml falcon tube. The resuspended cell pellet was layered on top, and the column centrifuged for 30min (1000 x *g*, room temperature). The top two layers contain dead macrophages and therefore were removed and discarded. A Pasteur pipette was used to collect the other gradient interface layers (30-40%, 40-50%, 50-60%) and the suspension made up to 50ml with PBS. Macrophages were counted using a Neubauer haemocytometer after staining 1:1 with Kimura stain (0.05% (v/v) toludine blue, 0.03% (v/v) light green, 10% (v/v) saponin, 0.7M phosphate buffer). Cells were resuspended at $1x10^6$ macrophages/ml in complete media. 100µl or 500µl macrophage suspension was seeded into 96 or 24 well plates respectively. Cells were incubated overnight at 37°C and 5% (v/v) CO_2 to allow macrophages to adhere.

2.2.3.5 Small airway fibroblasts

Lung tissue was flushed using Hank's balanced salt solution (HBSS) to remove blood and macrophages as described in Section 2.2.3.4. Visible airway walls were extracted using forceps and scissors. Approximately 3-5 x $2mm^2$ tissue samples were placed in T25 cell culture flask with fibroblast complete medium (Dulbecco's Modified Eagle Medium (DMEM), 10% (v/v) FBS, 1% (v/v) L-glutamine, 1% (v/v) penicillin/streptomycin, 1% (v/v) amphotericin). Flasks were incubated for 2-3 weeks at 37°C, 5% CO_2 (v/v) until cells were confluent. Tissue sections were then removed, and cells subcultured using 1x (v/v)

trypsin-EDTA for experiments or frozen in freezing medium (90% ($^{v}/_{v}$) FBS, 10% ($^{v}/_{v}$) dimethyl sulfoxide (DMSO)) in liquid nitrogen for later use.

2.2.3.6 Preparation of heat killed and fluorescently-labelled Haemophilus influenzae

2.2.3.6.1 Culture of *Haemophilus influenzae*

Non-typeable *H. influenzae* (stain 1479) was isolated from exacerbating COPD patients. The bacteria were streaked onto Columbia agar plates and incubated for 24h at 37°C, 5% CO_2 ($^{v}/_{v}$). 20ml Brain Heart Infusion broth was inoculated with single colonies of bacteria from the plate and then incubated on an orbital shaker incubator at 37°C, 200 rpm. Bacterial growth rate was assessed hourly via optical density (OD) readings at λ600nm on a spectrophotometer and bacteria harvested when OD reached 1.2. Colony forming units (CFU) were calculated:

$$\text{CFU (ml)} = \frac{(\text{colony number} \times \text{dilution factor})}{(\text{amount plated (ml)})}$$

2.2.3.6.2 Heat-killing

The bacteria were heat killed by incubation for 2h at 70°C. The bacteria were then washed twice by centrifugation and the pellet resuspended in fresh PBS. Confirmation of heat killing was show by demonstrating an absence of any colonies after overnight culture of bacteria on agar.

2.2.3.6.3 Fluorescent labelling

Heat killed bacteria were washed in HBSS, sonicated and centrifuged (300 x *g*) for 3min. The pellet was resuspended in 1ml sodium bicarbonate buffer (8.4g $NaHCO_3$ in 100ml distilled water). 10µl Alexa-fluor 488 (1mg lyophilised dye in 1ml dimethyl sulfoxide (DMSO)) (ex λ488nm/em λ520nm) was added and rotated overnight, in the dark, at room temperature. Bacteria were centrifuged for 3min (300 x *g*) and resuspended in 1ml HBSS, three times to remove unbound dye. Finally, bacteria were resuspended in HBSS and stored at -20°C.

2.2.4 Phagocytosis assays

Black 96 well plates were used for phagocytosis assays and all samples performed in triplicate. Fluorescently-labelled heat-killed *Haemophilus influenzae* were thawed at room temperature and sonicated in a water bath for 2min prior to use to disrupt any clumping of bacteria. Bacteria were diluted in RPMI to give a final concentration of $5x10^8$ bacteria/ml which is known to be an optimum

concentration for phagocytosis (Taylor 2010). Bacteria were added to wells containing $1x10^5$ macrophages and incubated at 37°C, 5% ($^v/_v$) CO_2 for 4h. MDMs were washed with D-PBS and then 200µl of Trypan blue 1% ($^v/_v$) was added to each well for 2min to quench florescence of any extracellular particles. The Trypan blue was removed, and the fluorescence of internalised bacteria determined by fluorometry using FLUOstar Optima pate reader (excitation λ480nm, emission λ520nm). Phagocytosis was calculated by subtracting autofluorescence of untreated cells in each condition.

2.2.5 Cell viability assay

Cell viability was assessed by adding 50µl of methylthiazoyldiphenyltetrazolium bromide (MTT) solution (1mg/ml) to each well. Plates were incubated at 37°C for 40min. The MTT was aspirated and 50µl DMSO added to each well to lyse the cells. Absorbance was then measured at λ550nm using Spectramax microplate reader. Data were normalised to the viability of control untreated MDMs (assumed to be 100% viable) and expressed as a percentage of this.

2.2.6 Enzyme-linked immunosorbent assay (ELISA)

IL-6, CXCL8, CXCL1, IL-36α, IL-36β, IL-36γ and IL-36RA concentrations in cell media were measured using commercially available ELISA kits according to the manufacturer's instructions.

2.2.6.1 Measurement of IL-6

IL-6 mouse anti-human capture antibody (R&D Systems, UK) was diluted in PBS at a concentration of 1µg/ml. 100µl was transferred to each well of a 96-well NUNC Maxisorp plate. The plate was left covered overnight at room temperature. The following morning the plate was tapped out and blocked with 120µl/well blocking buffer (1% ($^w/_v$) BSA, 5% ($^w/_v$) sucrose and 0.05% ($^w/_v$) sodium azide in PBS). The plate was left for 2h at room temperature, then tapped out and washed three times using a wash buffer (PBS with 0.05% ($^v/_v$) Tween®-20) ready to be loaded with samples.

An assay buffer of PBS with 0.5% ($^w/_v$) BSA was used for all dilutions below unless otherwise stated. The IL-6 standard (R&D Systems, UK) was diluted to a top concentration of 2000ng/ml. The standard was performed in duplicate down the plate in 2-fold serial dilutions (100µl/well) with the final wells being assay buffer alone. Samples were diluted in assay buffer and loaded in duplicate to individual wells (100µl/well). The dilution factor was specific to experiments and indicated in the appropriate chapter. The plate was then incubated for 1h at 37°C.

The detection antibody (R&D Systems, UK) was diluted to 0.2µg/ml. The plate was washed three times using the wash buffer and detection body added (100µl/well). The plate was incubated for 1h at 37°C, before being tapped out, washed three times and streptavidin-horseradish peroxidase (HRP) (1µg/ml)

added (100µl/well). The plate was incubated for a final 30min at room temperature then washed three times.

100µl of a substrate solution was subsequently added to each well (equal volumes of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine)). The ensuing reaction was then stopped by 1M sulphuric acid (100µl/well).

Absorbance was measured using Spectramax microplate reader at λ450nm and λ590nm wavelengths. A standard curve was drawn (Figure 2.4 Panel A) and concentrations of IL-6 in the samples derived by interpolation. The lower limit of detection was 31.25 pg/ml

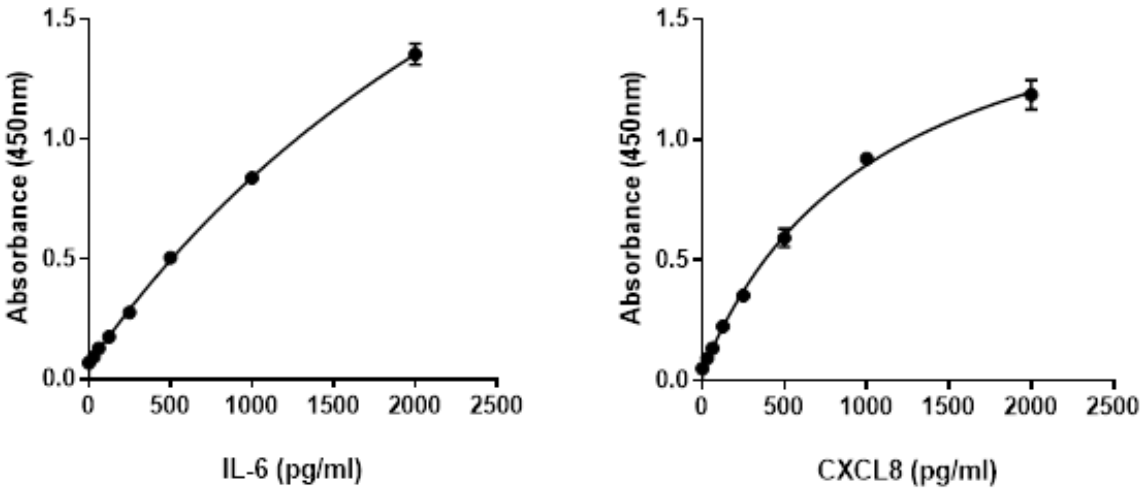

Figure 2.4: IL-6 and CXCL8 standard curves from ELISA
A standard curve of recombinant human (A) IL-6 and (B) CXCL8. Absorbance was read at λ450nm and λ590nm. λ590nm was subtracted from λ450nm to account for imperfections in the plate and background noise.

2.2.6.2 Measurement of CXCL8

Measurement of CXCL8 by ELISA was performed following similar methodology described in section 2.2.6.1. CXCL8 mouse anti-human capture (0.5µg/ml) and detection (0.4µg/ml) antibodies were used (R&D Systems, UK). The CXCL8 standard (R&D Systems) was diluted to a top concentration of 2000 pg/ml. Samples were diluted as per the specific experiment. The lower limit of detection was 31.25 pg/ml.

2.2.6.3 Measurement of CXCL1

Measurement of CXCL1 by ELISA was performed following similar methodology described in section 2.2.6.1. CXCL1 mouse antihuman capture (0.5µg/ml) and detection (0.4µg/ml) antibodies were used (R&D Systems, UK). The CXCL1 standard (R&D Systems) was diluted to a top concentration of 2000 pg/ml. Samples were diluted as indicated in each chapter. The lower limit of detection was 31.25 pg/ml.

2.2.6.4 Measurement of IL-36α

Measurement of IL-36α by ELISA was performed following similar methodology described in section 2.2.6.1. IL-36α mouse antihuman capture (2µg/ml) and detection (125ng/ml) antibodies were used (R&D Systems, UK). The IL-36α standard (R&D Systems) was diluted to a top concentration of 800 pg/ml. Samples were diluted as indicated in each chapter. The lower limit of detection was 12.5 pg/ml.

2.2.6.5 Measurement of IL-36β

Measurement of IL-36β by ELISA was performed following similar methodology described in section 2.2.6.1. IL-36β mouse antihuman capture (400ng/ml) and detection (100ng/ml) antibodies were used (R&D Systems, UK). The IL-36β standard (R&D Systems) was diluted to a top concentration of 800 pg/ml. Samples were diluted as indicated in each chapter. The lower limit of detection was 12.5 pg/ml.

2.2.6.6 Measurement of IL-36γ

Measurement of IL-36γ by ELISA was performed following similar methodology described in section 2.2.6.1. PBS with 0.2% ($^{w}/_{v}$) BSA and 0.05% ($^{v}/_{v}$) Tween®-20 was used as the assay buffer for all stages of ELISA. IL-36γ mouse antihuman capture (2µg/ml) and detection (0.1µg/ml) antibodies were used (AdipoGen life sciences, Switzerland). The IL-36γ standard (AdipoGen life sciences, Switzerland) was diluted to a top concentration of 2000 pg/ml. Samples were diluted as indicated in each chapter. The lower limit of detection was 31.25 pg/ml.

2.2.6.7 Measurement of IL-36RA

Measurement of IL-36RA by ELISA was performed following similar methodology described in section 2.2.6.1. IL-36RA mouse monoclonal capture (1mg/ml) and detection (0.5mg/ml) antibodies were used (AdipoGen life sciences, Switzerland). The IL-36RA standard (AdipoGen life sciences, Switzerland) was diluted to a top concentration of 50 ng/ml. Samples were diluted as indicated in each chapter. The lower limit of detection was 780 pg/ml.

2.2.7 Meso Scale Discovery (MSD)

U-plex custom assay 10-spot, 96-well plates were used. Each plate contains its own linkers, capture antibodies, detection antibodies and calibrators (Meso Scale Diagnostics, USA). MSD was performed according to manufacturer's instructions.

In summary, 200µl biotinylated antibody was added to 300µl of the assigned linker. This was vortexed and incubated for 30min at room temperature. 200µl stop solution (Meso Scale Diagnostics, USA) was added and the solution incubated for a further 30min at room temperature. 600µl of each U-plex Link-coupled antibody solution was combined in a single tube and where necessary the total solution made up to 6ml with MSD stop solution. 50µl of this multiplex coating solution then added to each well. The plates were incubated at room temperature for 1h. A wash buffer of PBS with 0.05% ($^{v}/_{v}$) Tween® -20 was made. After 1h incubation the plates were washed three times with the wash buffer (150µl/well).

The MSD lyophilised calibrators were reconstituted with 250µl Assay Diluent 57 (Meso Scale Diagnostics, USA). The calibrator vials were inverted 3 times and incubated at room temperature for 30min before being vortexed: For some plates more than one calibrator solution was needed. 50µl of each calibrator solution was combined and then the total volume made up to 250µl using Assay Diluent 57. The calibrator solution was then serially diluted 4-fold to create the standard, with the bottom of the standard being Assay Diluent 57 alone.

The plates were loaded with 25µl Assay diluent 57, and 25µl of the specific Calibrator Standard or clinical sample into each well. The plates were incubated for 1h at room temperature. The plate was then washed 3 times with 150µl/well wash buffer.

The detection antibody solution was prepared by mixing 60µl of each unique detection antibody. The solution was then adjusted to 6ml using Diluent 3 (Meso Scale Diagnostics, USA). 50µl detection antibody solution was added to each well, and the plates incubated for a further 1h. The plates were then washed 3 times with 150µl/well wash buffer. Finally, 150µl MSD GOLD Read Buffer B (Meso Scale Diagnostics, USA) was added to each well. Electrochemiluminescence was quantified using an MSD instrument and cytokine concentrations were then interpolated from standard curves using MSD discovery workbench analysis software version 4 (Figure 2.5). For each analyte a different upper and lower limit of detection was specified.

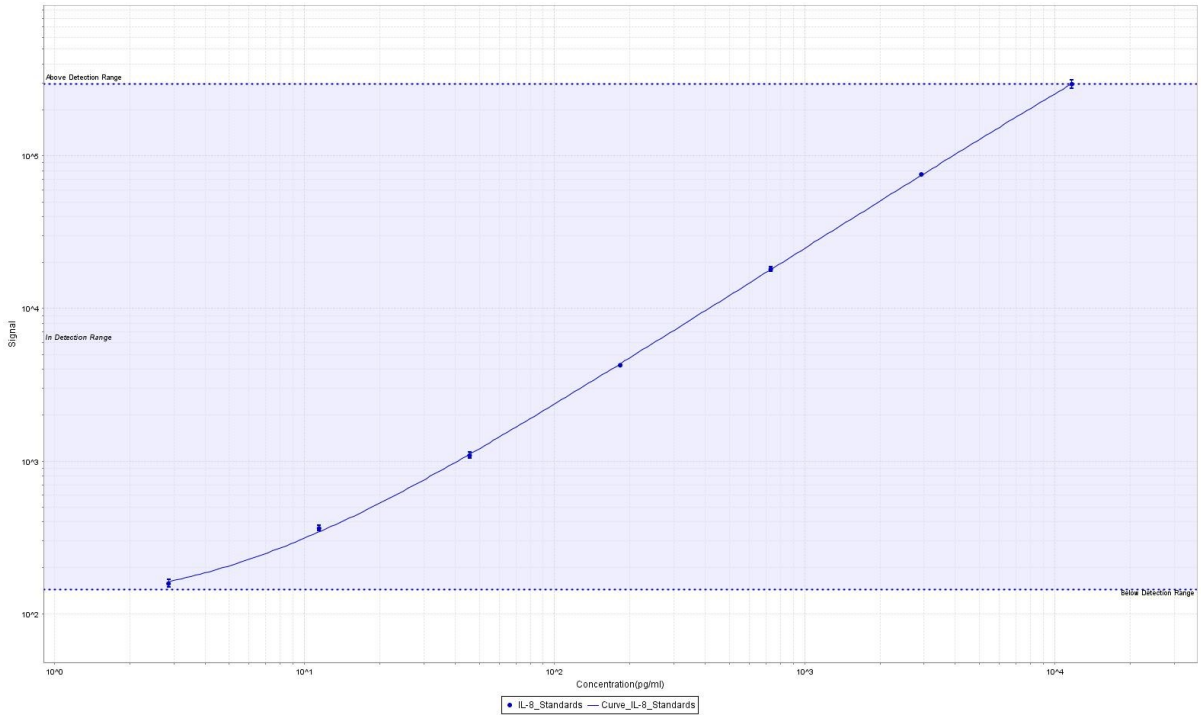

Figure 2.5: Standard curve used to derive unknown concentrations of CXCL8 via MSD
MSD performed as described in 2.2.7. Lyophilised calibrators reconstituted with Assay diluent 57 and then serially diluted 4-fold. Standards were performed in duplicate. Electroluminescence quantified on MSD instrument using MSD GOLD Read buffer B. Upper and lower limits of detection shown on standard curve.

2.2.8 Real time quantitative polymerase chain reaction (rt-PCR)

2.2.8.1 RNA extraction

Total RNA was isolated using Qiagen RNeasy Mini Kit. Briefly, cells were lysed with 350µl Buffer RLT. 350µl 70% ethanol was then added to the lysate and mixed well by pipetting. The sample was transferred to a RNeasy Mini spin column, placed in 2ml collection tube, centrifuged for 15s (≥8000 x *g*) and the effluent discarded. 700µl Buffer RW1 was added to the spin column, it was centrifuged for 15s (≥8000 x *g*), and the effluent discarded. 500µl Buffer RPE then added to the spin column, centrifuged for 15s (≥8000 x *g*) and the effluent discarded. A further 500µl Buffer RPE was added to the spin column, this time centrifuged for 2min (≥8000 x *g*). The spin column was transferred to a new 1.5ml collection tube and 40µl RNase-free water added to the spin column. The column was centrifuged for 1min (≥8000 x *g*) to elute the RNA. The RNA was then stored at -80°C until used for further experiments.

2.2.8.2 *Reverse transcription*

RNA was reverse transcribed using the TaqMan normal RNA Reverse Transcription Kit. Briefly for each RNA sample a master mix was prepared containing: random primers (2µl), dNTPs (0.8µl), multiscribe (1µl), 10x RT buffer (2µl) and nuclease free water (4.2µl). 10µl of master mix solution and 10µl RNA were mixed in PCR tubes. These were then loaded into a thermal cycler and heated for 10min at 25°C followed by 120min at 37°C then 5min at 85°C. The cDNA was then stored at -80°C until used for further experiments.

2.2.8.3 *Real time quantitative PCR*

Gene expression was quantified by TaqMan real time PCR with all samples run in duplicate. Briefly, 20µl cDNA was diluted with 80µl DNase free water. A primer master mix was created with TaqMan PCR master mix (4.5µl) and gene of interest (0.5µl). The following primers were used: IL-36RN (HS00104220), IL36γ (HS00219742), RACK1 (HS00272002). 5µl of cDNA and 5µl primer master mix were loaded into a MicroAmp optical 96 well reaction plate. Plates were covered with MicroAmp optical adhesive film and briefly centrifuged (700 x *g* for 20s). The covered plate was then loaded into 7500 Real Time PCR system (Applied Biosystems Life Technologies, Paisley, UK) for analysis. SDS software (Applied Biosystems, California, USA) provided graphs and data analysis for each well of the plate. From this the relative expression of the gene of interest against the housekeeping gene RACK1 (HS00272002) was calculated using the $2^{-\Delta\Delta CT}$ method.

2.2.9 Statistical analysis

Data were analysed using GraphPad Prism 10.4 (GraphPad Software, California, USA). All data were expressed as means ± standard error of the mean (SEM) or mean ± interquartile range (IQR) unless otherwise stated. Mann-Whitney U test (unpaired data) or Wilcoxon signed rank test (paired data) were used to determine any statistical differences between two data sets where appropriate. Comparisons between three of more subject groups or treatment conditions used Friedman's test with Dunn's post hoc analysis (matched data) or Kruskal-Wallace with Dunn's post-hoc test (independent data). Differences were considered significant if $p < 0.05$ where *=$p<0.05$, ** = $p<0.01$ and *** = $p< 0.001$. Correlations were performed using Spearman's rank correlation coefficient and presented with associated p values.

Chapter 3: Effect of IL-36γ on macrophages

3.1 Introduction

IL-36 receptor (IL-36R) signalling is increased in COPD compared to healthy controls with increased levels of the receptor agonists and reduced IL-36 receptor antagonist (IL-36RA) within the lung (Baker 2022). IL-36α, IL-36β, IL-36γ have all been reported to be elevated at mRNA level in COPD, but only IL-36γ is consistently measurable as a protein in lung tissue, BAL and sputum suggesting that it the most clinically important of the IL-36 receptor agonists in lung disease (Kovach 2021, Baker 2022). With this in mind, IL-36γ has been used as the stimuli for this study.

A major source of IL-36γ in the lung is from small airway epithelial cells (SAEC) (Chustz 2011, Baker 2022). Within the human lung IL-36R expression is present on small airway fibroblasts (SAF), airway epithelial cells as well as myeloid cell types such as dendritic cells, eosinophils and macrophages (Zhang 2017, Qin 2019). Of these, SAF are thought to be the main effector cells causing a release of pro-inflammatory cytokines (IL-6, GM-CSF), neutrophil chemoattractants (CXCL1, CXCL8) and proteases (MMP2, MMP9) in response to IL-36γ stimulation, with cytokine release 25-fold higher than that from SAEC (Chustz 2011, Baker 2022).

Macrophages are a key cell type driving COPD pathology. COPD macrophages are more pro-inflammatory and have reduced phagocytic function than those in healthy non-smokers (Taylor 2010, Chana 2014). The role of macrophages in the dysregulated IL-36R signalling pathway seen in COPD remains relatively undefined. Macrophages express IL-36γ mRNA, but they do not appear to secrete IL-36γ extracellularly (Baker 2022). Instead macrophages are thought to be the major contributor of IL-36RA levels in the lung (Baker 2022) and this will be explored further in Chapter 4. Human lung macrophages and MDM express IL-36R (Dietrich 2016, Zhang 2017) and as such are likely to respond to heightened IL-36γ levels seen in COPD. Knockout mouse studies suggest that in response to viral triggers, IL-36 signalling may drive polarisation of lung macrophages away from an M2-like phenotype and protect against macrophage apoptosis (Wein 2018). Phagocytosis function may also be affected, with IL-36$^{-/-}$and IL-36R$^{-/-}$ mice showing worse bacterial clearance, increased lung damage and mortality after infection with *Streptococcus pneumoniae, Klebsiella pneumoniae and Legionella pneumonia* (Kovach 2017, Nanjo 2019) but conversely an improved survival with *Pseudomonas aeruginosa* infection than wildtype mice (Aoyagi 2017). In the context of viral infection, administration of wildtype alveolar macrophages to IL-36γ$^{-/-}$ mice results in better survival after influenza infection (Wein 2018).

This chapter investigates how IL-36γ affects human alveolar macrophages in health and COPD. GM-CSF differentiated MDM from healthy non-smokers, healthy smokers and COPD donors are predominantly used in this study as MDM have been shown to reliably represent lung macrophages and are more easily sourced (Section 1.2.1.4) (Taylor 2010, Finney 2019). Two major macrophage cell

functions will be studied: phagocytic ability and secretion of inflammatory cytokines. *Haemophilus influenzae* was used to assess phagocytosis as it is the most common cause of both acute bacterial infection and chronic colonisation in COPD (Patel 2002, Wark 2013). 100ng/ml IL-36γ was used for all treatments, as this concentration had previously been shown to stimulate SAF cytokine release (Baker 2022). IL-6, CXCL1 and CXLC8 were selected to represent secretory function of macrophages as these cytokines are secreted in measurable quantities by alveolar macrophages and MDM in stable state, with levels responsive to change of environment. Furthermore, these cytokines are also known to be released from other lung cell types in response to IL-36γ (Baker 2022).

3.2 Hypothesis

IL36γ results in impaired phagocytosis of *H. influenzae* and increased secretion of pro- inflammatory cytokines by macrophages.

3.3 Aims

Using an MDM model I aim to:

- Investigate the effect of IL-36γ stimulation of macrophages on phagocytosis of *H. influenzae,*
- Investigate the effect of IL-36γ stimulation on cytokine secretion by macrophages,
- Explore how IL-36γ stimulated SAF affect macrophage phagocytic and secretory function.

3.4 Methods

3.4.1 Subject selection

Healthy never smokers, Smokers and ex-smokers without lung disease and COPD patients were recruited as described in Section 2.2.1.

3.4.2 Isolation and culture of MDM

60ml of blood was taken from participants as described in section 2.2.2.4.1. PBMCs were isolated, plated in black 96 well plates $1x10^5$ monocytes / well and cultured in the presence of GM-CSF (2ng/ml) for 10-14 days to generate MDM as described in section 2.2.3.

3.4.3 Isolation of tissue macrophages

Tissue macrophages were isolated from surgical lung tissue from COPD patients as described in section 2.2.3.4. Macrophages were plated in black 96 well plates $1x10^5$ monocytes / well and incubated overnight at 37°C and 5% ($^v/_v$) CO_2 to allow macrophages to adhere.

3.4.4 Identifying the effect of IL-36γ on macrophage function

Macrophages (MDM/tissue macrophages) were cultured with 100ng/ml IL-36γ at 37°C, 5% ($^v/_v$) CO_2 for 24h. Media from macrophages were aspirated and stored at -80°C for later analysis by ELISA (See section 3.4.6). A 4h phagocytosis assay with *H. influenzae* was performed as described in section 2.2.5. Cell viability was confirmed with an MTT assay as described in section 2.2.6.

To confirm if changes seen were direct effect of IL-36γ MDMs were pre-treated with 25µl of 0.5µg/ml IL-36RA for 1h prior to IL-36γ stimulation (Figure 3.1).

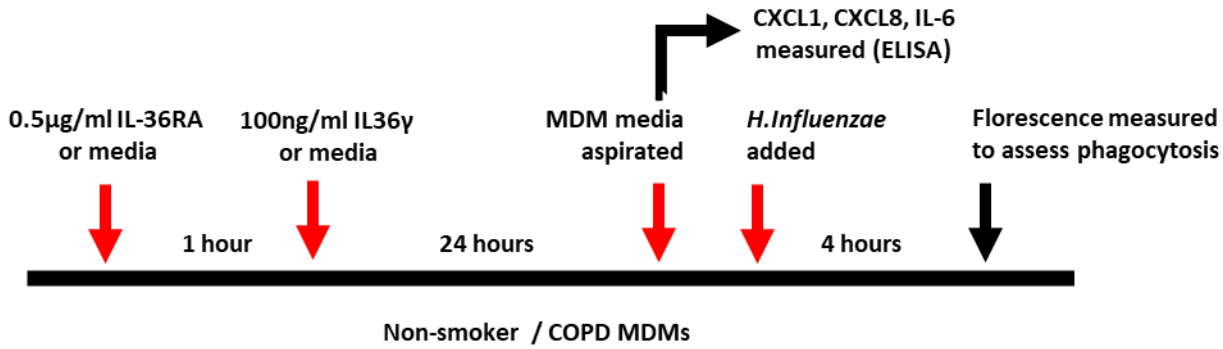

Figure 3.1: Experimental procedure assessing how IL-36γ effects MDM function
To investigate the effects of IL-36γ on MDM function, MDM were pre-treated for 1h with 0.5µg/ml IL-36RA or media alone, then treated for 24h with 200ng/ml IL-36γ or media. Media from treated MDM were aspirated and analysed for cytokine concentrations by ELISA. MDM were then treated with *H. influenzae* for 4h and internalised bacteria determined by fluorometry using FluoSTAR Optima plate reader.

3.4.5 Fibroblast culture and treatment with IL-36γ

SAF were isolated from lung parenchyma obtained following surgery as described in Section 2.2.3.5. SAF from non-smokers (n=4) and COPD patients (n=4) were cultured in media (DMEM, 10% ($^{v}/_{v}$) foetal bovine serum, 10mg/ml (1% ($^{v}/_{v}$)) penicillin/streptomycin and 2mM (1% ($^{v}/_{v}$)) L-glutamine). Once confluent SAF were cultured with 100ng/ml IL-36γ or media alone for 24h. Cell media from non-smoker and COPD SAF under each condition were pooled and stored at -80°C.

3.4.6 Assessing cross talk between IL-36γ stimulated SAF affect MDM function

100µl media from untreated and IL-36γ stimulated SAF (as described in Section 3.4.4) were incubated with MDMs at 37°C, 5% ($^{v}/_{v}$) CO_2 for 24h. Fibroblast media was aspirated after 24h and stored at -80°C for later analysis by ELISA (See section 3.4.7). A 4h phagocytosis assay with *H. influenzae* was performed as described in section 2.2.5. Cell viability was confirmed with an MTT assay as described in section 2.2.6.

To confirm if changes seen were direct effect of IL-36γ or indirect via IL-36γ's effect on SAF, MDMs were pre-treated with 25µl of 0.5µg/ml IL-36RA for 1h prior to the SAF media being transferred (Figure 3.2).

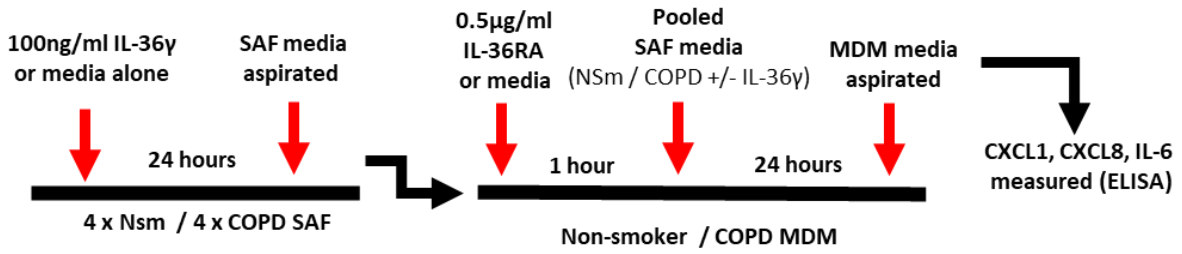

Figure 3.2: Experimental procedure assessing crosstalk between IL-36γ stimulated small airway fibroblasts (SAF) and monocyte derived macrophages (MDM)
Non-smoker and COPD SAF were treated for 24h with media (NT) or 200ng/ml IL-36γ. Media from 4 non-smoker and 4 COPD SAF were pooled. MDM were pre-treated with fresh media or 0.5µg/ml IL-36RA for 1h, this was aspirated and 100µl of the pooled SAF media (non-smoker NT, non-smoker IL-36γ, COPD NT and COPD IL-36γ) were added to MDMs for 24h. Media from treated MDM were aspirated and cytokines measured by ELISA.

Media from pooled non-smoker and COPD SAF (NT and IL-36γ stimulated) were also analysed by MSD as described in section 2.2.9 using V-plex pre-coated 10 spot assay plates (Meso Scale Diagnostics, Maryland US). Each plate contains its own calibrators. Samples were run in duplicate at a 1 in 2 dilution with recommended diluent. Electrochemiluminescence was quantified and cytokine concentrations were then interpolated from standard curves using MSD discovery workbench analysis software.

3.4.7 Measurement of inflammatory mediators

CXCL1, CXCL8 and IL-6 concentrations in the media from SAF and MDM were measured by ELISA using commercially available ELISA kits (R&D Systems, UK) as described in section 2.2.8. All samples were run in duplicate. Media from MDM were diluted 1 in 30, 1 in 300, 1 in 30 with 0.5% ($^{w}/_{v}$) BSA in D-PBS for measurement of CXCL1, CXCL8 and IL-6 respectively. Each ELISA plate contains its own set of standards and concentrations of samples were interpolated from this standard curve. The lower limit of detection was 31.25ng/ml. Absorbance was measured using Spectramax microplate reader at 450nm and 590nm wavelengths.

3.4.8 Statistics

All data were expressed as mean ± SEM unless otherwise stated and analyses performed using GraphPad Prism software (version 9.4.1). Statistical analyses are detailed in figure legends.

3.5 Results

3.5.1 Subject demographics

The characteristics of healthy and COPD blood donors used in this study are shown in Table 3.1. Participants were age-matched across all groups. There were significant differences in lung function and smoking pack years between COPD and healthy groups. Of note, healthy smokers had fewer pack years than COPD participants, but this was not statistically significant.

	Non-smoker (n=12)	**Smokers** (n=7)	**COPD** (n=17)
Age	61.9 ± 2.3	62 ± 4.9	66.7 ± 2.6
Gender (male:female)	9:6	2:5	10:7
FEV1 (L)	3.17 ± 0.28	2.83 ± 0.27	1.44 ± 0.16 *** ##
FEV1 (%)	107.0 ± 6.7	101.6 ± 6.1	54.3 ± 4.7 *** ##
FVC (L)	4.23 ± 0.38	3.70 ± 0.31	3.11 ± 0.20 *
FVC (%)	120.3 ± 7.0	103 ± 5.1	94.0 ± 4.6 **
FEV1/FVC	0.75 ± 0.16	0.76 ± 0.02	0.46 ± 0.03 **
Smoking history (pack years)	0.0 ± 0.0	23.1 ± 5.7 *	48.6 ± 7.8 ***

Table 3.1: Subject demographics for Non-smoker and COPD MDM
FEV1: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for one year. Data are presented as mean ± SEM. Kruskall-Wallis with Dunn's post-test was performed to test for significant differences between subject groups. * $p<0.05$, ** $p< 0.01$, *** $p< 0.001$ compared to healthy non-smoking controls. # $p<0.05$, ## $p< 0.01$, ### $p< 0.001$ compared to smokers without lung disease.

3.5.2 Effect of IL-36γ on MDM phagocytosis

To assess whether IL-36γ effects macrophage phagocytic function, a 4h phagocytosis assay with heat killed *H. influenzae* was performed on non-smoker and COPD MDM. There was no effect of IL-36γ on phagocytosis of *H. influenzae* in MDM from healthy non-smokers or healthy smokers (Figure 3.3, panels A and B). However, IL-36γ reduced phagocytosis in COPD MDM by ~22 % p<0.01 (Figure 3.3, panel C).

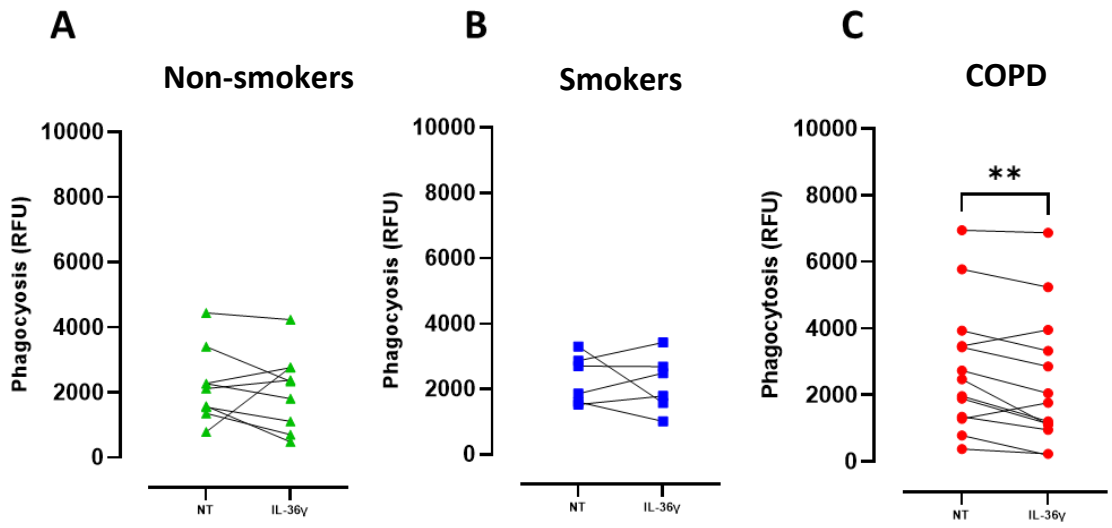

Figure 3.3: Effect of IL-36γ stimulation on MDM phagocytosis
(A) MDM from non-smoker (▲) and (B) Smokers without lung disease (■) and (C) COPD (●) donors were stimulated for 24h with media (NT) or 200ng IL-36γ. MDM were then exposed to fluorescently-labelled heat-killed *H. influenzae* for 4h and phagocytosis measure by fluorometry. Data analysed using Mann-Whitney U to compare phagocytosis by treated and untreated MDM. **: p< 0.01, n=9 non-smokers, n=6 smokers and n=13 COPD

To confirm that the reduction in phagocytosis seen in COPD MDM was a direct effect of IL-36R signalling, MDM were pre-treated with 25µl of 0.5µg/ml IL-36RA for 1h prior to incubation with IL-36γ. Pre-treatment of COPD MDM with IL-36RA prevented any IL-36γ mediated reduction in phagocytosis of *H. influenzae* confirming a direct effect of IL-36γ on MDM phagocytosis (Figure 3.4). There was no effect on *H. influenzae* phagocytosis with when MDM were treated with IL-36RA alone in the absence of IL-36γ (NT: 3449 ± 834.6 RFU vs. IL-36RA: 2939 ± 920.4 RFU, p = 0.312, n=6).

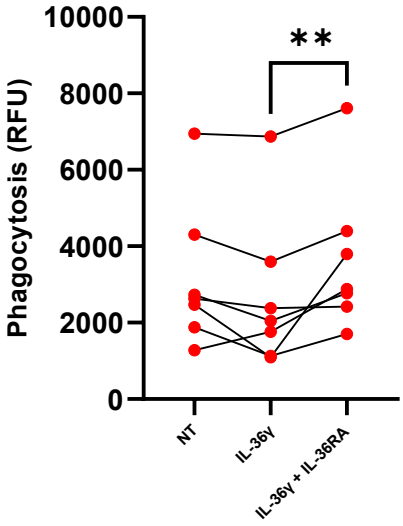

Figure 3.4: Blocking effect of IL-36γ on MDM phagocytosis with IL-36RA pre-treatment
COPD GM-MDM (•) were pre-treated with IL-36RA or media alone for 1h prior to 24h stimulation with media (NT) or 200ng IL-36γ. A 4h phagocytosis assay with *H. influenzae* was then performed. Data analysed using Friedman test with Dunn's post hoc test to compare treatment groups. *p<0.05, ** p< 0.01, n=7.

3.5.3 Effect of IL-36γ on MDM viability

It was important to determine whether the effect of IL-36γ on COPD phagocytosis was due to alterations in cell viability. Figure 3.5 shows that treatment with neither IL-36γ nor IL-36RA affected MDM viability.

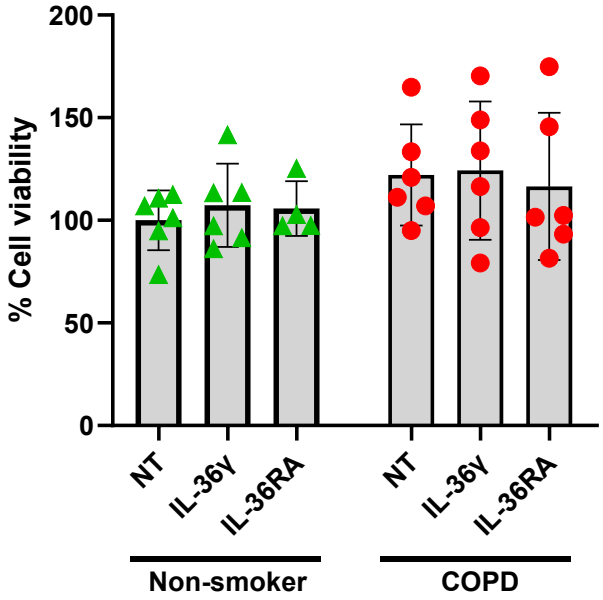

Figure 3.5: Effect IL-36γ inhibition of MDM viability
MDM from non-smoker (•) and COPD (•) donors were stimulated for 24h with media (NT) or 100ng IL-36γ. MDM were then exposed to fluorescently-labelled heat-killed *H. influenzae* for 4h. An MTT assay was performed, and absorbance measured. Data presented as % cell viability (as measured by absorbance) compared to untreated non-smoker cells. n=6

3.5.4 Effect of demographic variables on IL-36γ inhibition of MDM phagocytosis

Given that IL-36γ appeared to negatively affect phagocytosis in COPD patients, it was of interest to determine whether this was associated with subset of COPD patients. Phagocytosis of fluorescent *H. influenzae* after IL-36γ stimulation was calculated as a percentage change compared to baseline phagocytosis in the same patient. The percentage change in phagocytosis was then compared to clinical demographic parameters. Across all cohorts there was no effect of age or sex on MDM phagocytosis post IL-36γ stimulation (Figures 3.6 and 3.7).

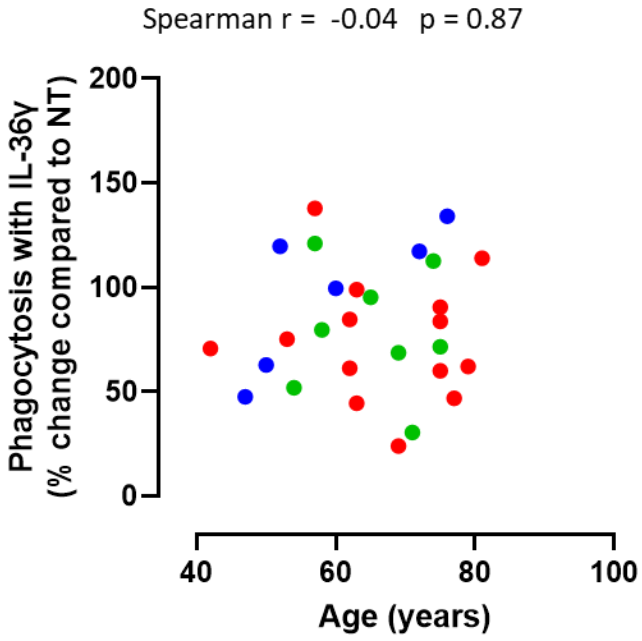

Figure 3.6: Effect of age on IL-36γ inhibition of MDM phagocytosis
MDM from non-smoker (•), Smokers without lung disease (•) and COPD (•) donors were treated for 24h with media (NT) or 100ng IL-36γ. MDM were then exposed to fluorescently-labelled heat-killed *H. influenzae* for 4h and phagocytosis measure by fluorometry. Data shown as percentage phagocytosis with IL-36γ treatment normalised to NT MDM from the same donors. Data presented as Spearman rank correlation coefficients and associated p values (subject groups analysed together). n=9 non-smokers, n=6 smokers and n=13 COPD.

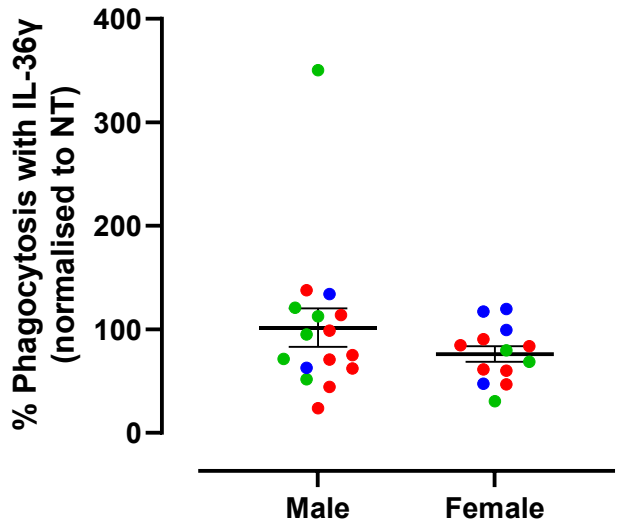

Figure 3.7: Effect of gender on IL-36γ inhibition of MDM phagocytosis
MDM non-smoker (•), Smokers without lung disease (•) and COPD (•) were treated for 24h with media (NT) or 100ng IL-36γ. MDM were exposed to fluorescently-labelled heat-killed *H. influenzae* for 4h and phagocytosis measure by fluorometry. Data presented as percentage phagocytosis with IL-36γ treatment normalised to NT MDM from the same donors. Data analysed using Mann-Whitney U to compare effect of treatment in male and female donors. n=16 males, n=13 females

In those donors with a smoking history, neither smoking status (current vs ex-smoker, 71.3 ± 12.9% vs 88.0 ± 9.0%, p=0.28) nor number of smoking pack years had any correlation with the effect of IL-36γ on MDM phagocytosis (Figure 3.8).

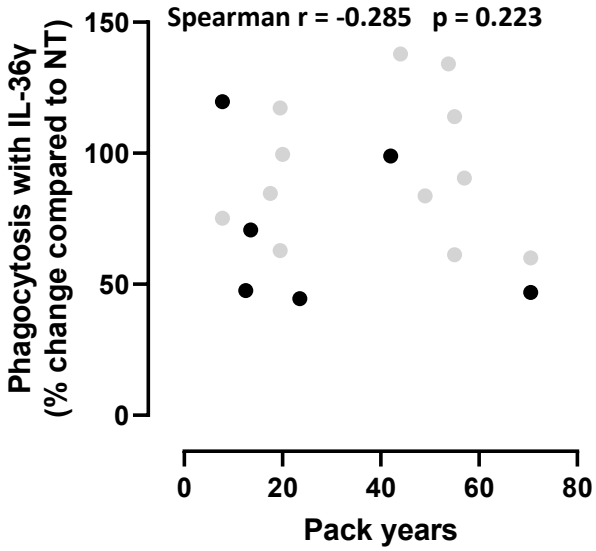

Figure 3.8: Effect of smoking history on IL-36γ inhibition of MDM phagocytosis
MDM from current (•) and ex-smokers (•) with or without COPD were stimulated for 24h with media (NT) or 200ng IL-36γ. MDM were then exposed to fluorescently-labelled heat-killed *H. influenzae* for 4h and phagocytosis measure by fluorometry. Data shown as percentage phagocytosis with IL-36γ treatment normalised to non-treated MDMs from the same donors. Data presented as Spearman rank correlation coefficients and associated p values (subject groups analysed together). n=6 current smokers, n=14 ex-smokers

IL-36γ appears to have a greater inhibition of MDM phagocytosis in those with less severe COPD in terms of obstruction as shown by significant correlation in FEV_1% and trend in FEV_1/FVC (Figure 3.9 panel A and B). Positive correlations in residual volume and a strong negative trend in $TLCO_c$ suggest that those with less severe emphysema also show greater inhibition of phagocytosis in the presence of IL-36γ (Figure 3.9 panel B and C).

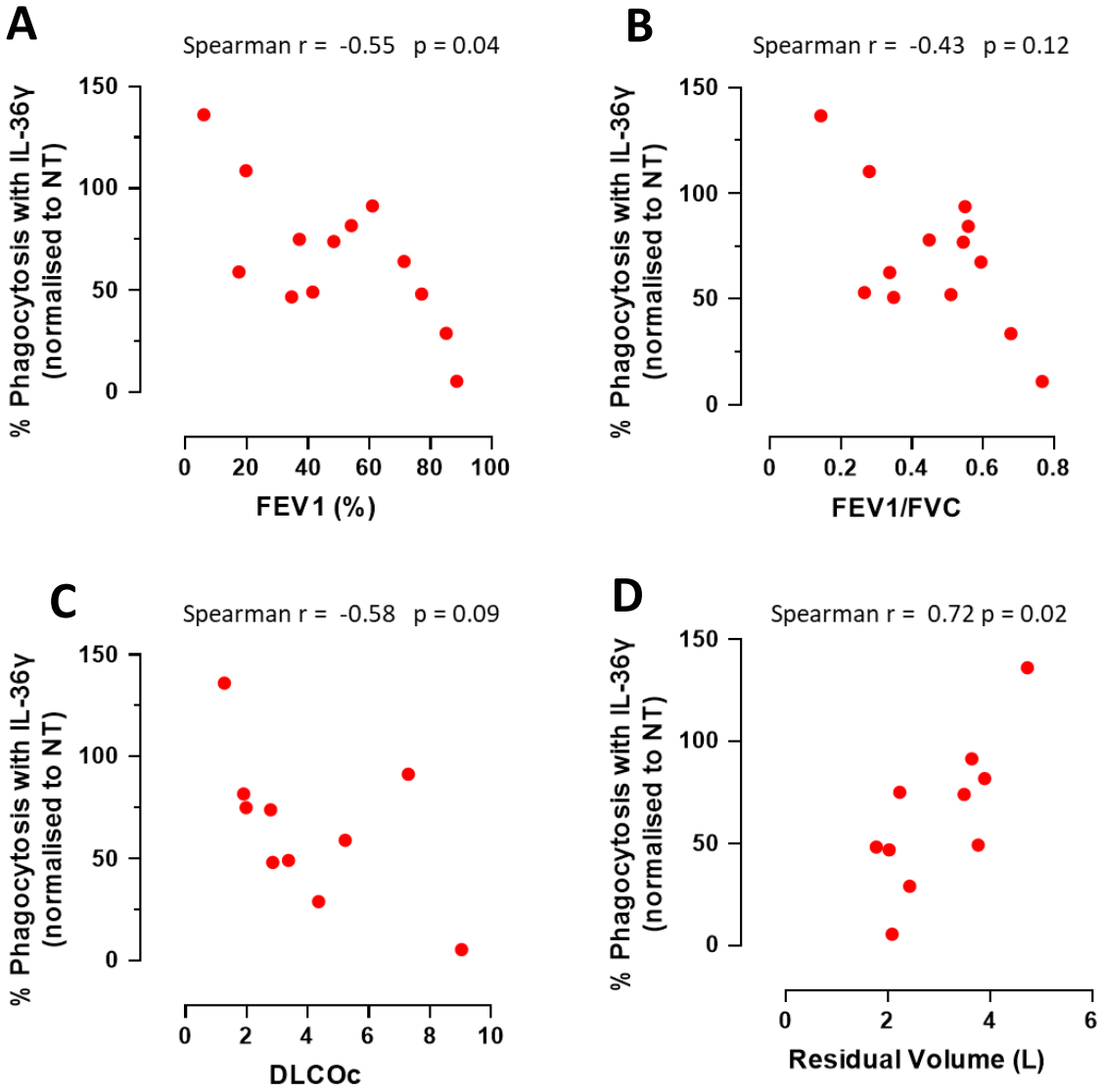

Figure 3.9: Correlation between lung function and effect of IL-36γ inhibition of COPD MDM phagocytosis

MDM from COPD patients were stimulated for 24h with media (NT) or 100ng IL-36γ. MDM were then exposed to fluorescently-labelled heat-killed *H. influenzae* for 4h and phagocytosis measure by fluorometry. Data shown as percentage phagocytosis with IL-36γ treatment normalised to non-treated MDMs from the same donors. Phagocytosis correlated with patient lung function (A) FEV_1% (B) FEV_1/FVC (C) Diffusing Capacity of Lung for Carbon Monoxide (DLCOc%) (D) Residual Volume (RV). Data presented as Spearman rank correlation coefficients and associated p values. n=10-13. Of note only 10/13 patients had data available for DLCO and RV.

There was no significant correlation between number of COPD exacerbations in preceding year and effect of IL-36γ on MDM phagocytosis (Spearman r = 0.38, p = 0.21). However, when COPD patients were grouped into infrequent (<2 exacerbations / year) and frequent exacerbators (≥ exacerbations / year), IL-36γ causes a significant decrease in phagocytic ability of MDMs in the infrequent exacerbator group (p<0.05) but not in the frequent exacerbator group (p=0.31) (Figure 3.10).

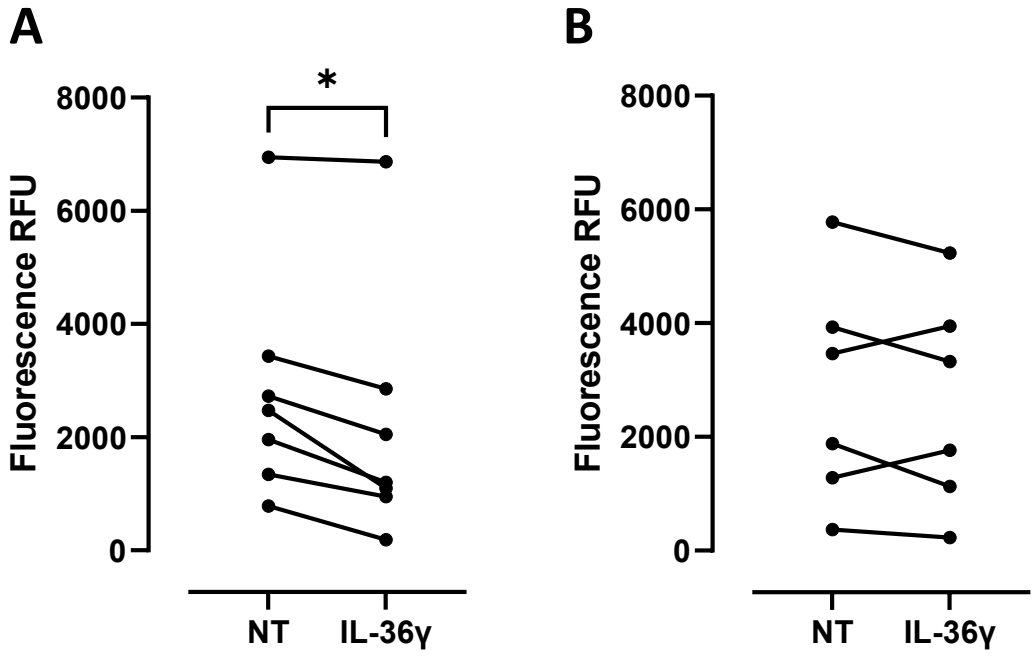

Figure 3.10: Effect of IL-36γ on MDM phagocytosis of infrequent and frequent exacerbators
MDM from (A) infrequent (<2/year) and (B) frequent exacerbators (≥2/year) COPD patients were treated for 24h with media (NT) or 100ng IL-36γ. MDM were then exposed to fluorescently-labelled heat-killed *H. influenzae* for 4h and phagocytosis measure by fluorometry. Data analysed using Mann-Whitney U to compare phagocytosis by treated and untreated MDM. *: p< 0.05, n=7 infrequent exacerbators, n=6 frequent exacerbators.

3.5.5 Effect of IL-36γ on tissue macrophage phagocytosis

To confirm the above findings in MDM occur at an organ level, tissue macrophages from COPD patients were isolated. The demographics of these patients can be seen in Table 3.2. As in COPD MDM, lung tissue macrophages isolated from the lung tissue of COPD patients showed a significant decrease in phagocytosis following culture with IL-36γ n compared to untreated cells ($p<0.05$) (Figure 3.11 Panel A). However, the magnitude of this effect is smaller than that seen in COPD MDM (6.9±3.8% vs 22.6 ±0.8%). IL-36γ did not affect tissue macrophage cell viability (Figure 3.11 Panel B).

	COPD (n=7)
Age (years)	71 ± 2.62
Gender (male:female)	4:3
FEV1 (L)	2.02 ± 0.61
FEV1 (%)	82.7 ± 6.7
FVC (L)	3.43 ± 0.34
FVC (%)	110.2 ± 4.2
FEV1/FVC	0.59 ± 0.04
Smoking history (pack years)	34.1 ± 12.0

Table 3.2: Subject demographics for tissue macrophages
FEV_1: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for one year. Data are presented as mean ± SEM.

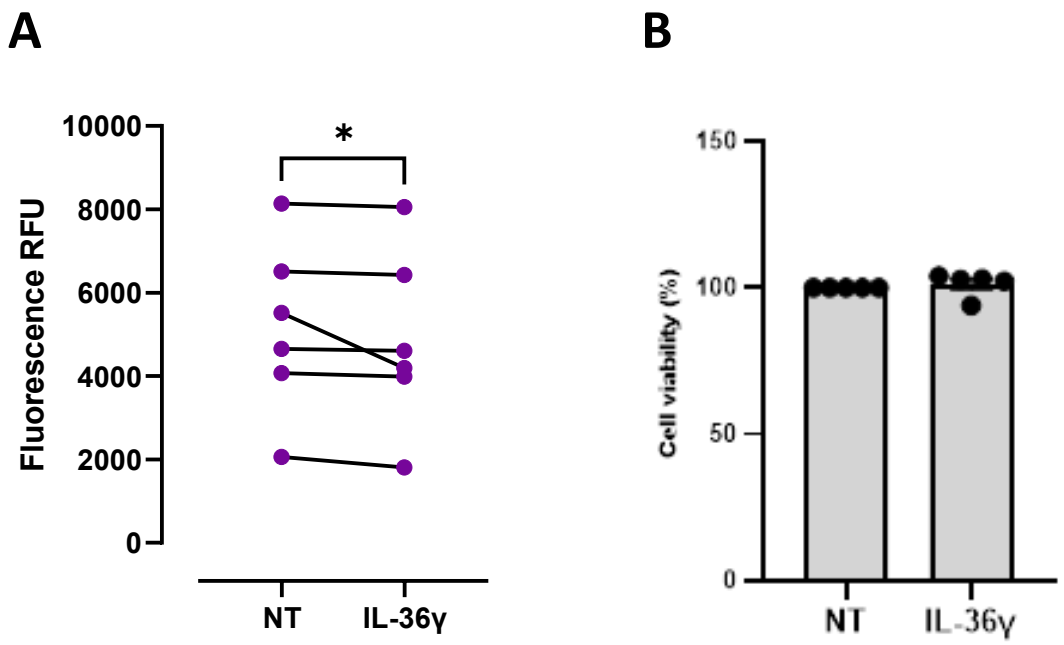

Figure 3.11: Effect of IL-36γ on COPD tissue macrophage phagocytosis
Macrophages were isolated from COPD lung tissue and treated for 24h with media (NT) or 100ng IL-36γ. (A) MDM were then exposed to fluorescently-labelled heat-killed *H. influenzae* for 4h and phagocytosis measure by fluorometry *: $p < 0.05$, n=6 (B) Cell viability was measured using a MTT assay. n=5

3.5.6 Effect of IL-36γ on MDM secretory function

To assess any effect of IL-36γ on secretory function of macrophages, CXCL1, CXCL8 and IL-6 were measured in the media of MDM following culture for 24h in the presence of IL-36γ. IL-36γ had no effect on secretion of these cytokines in either non-smoker or COPD MDM (Figure 3.12).

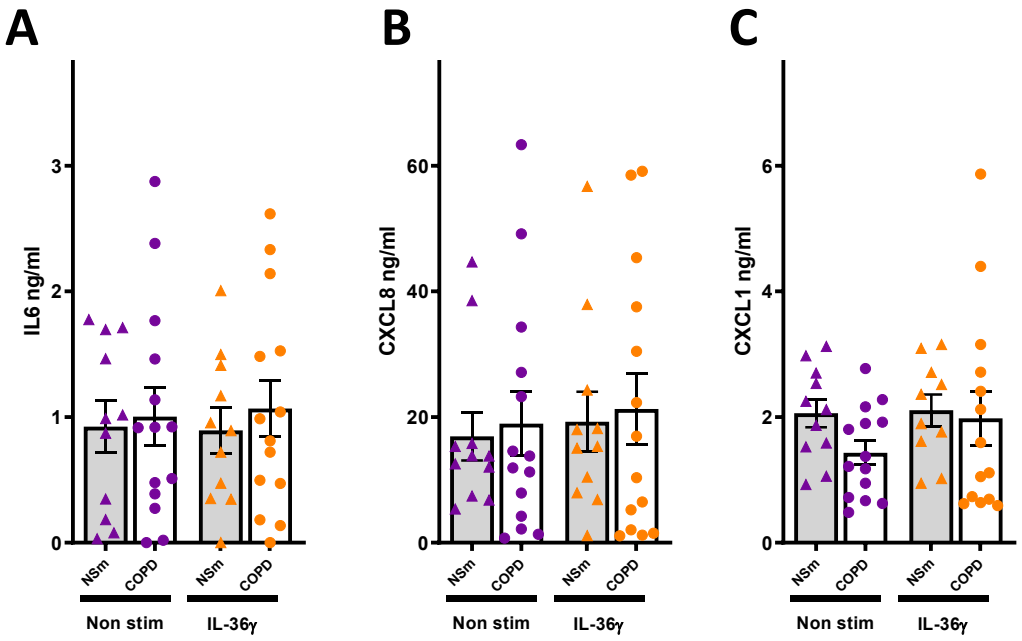

Figure 3.12: Effect of IL-36γ stimulation on MDM secretory function
Non-smoker (Δ grey columns) and COPD (O white columns) MDM were stimulated for 24h with media (▲/●) or IL-36γ (▲/●). Media from MDMs was aspirated and (A) IL-6 (B) CXCL8 (C) CXCL1 levels measured by ELISA. Data presented as mean ± SEM, n=11 non-smokers and n=14 COPD.

3.5.7 Crosstalk between IL-36γ stimulated SAF and macrophages

SAF are considered the main effector cell of IL-36γ (Chustz 2011, Baker 2022). Therefore, it was important to determine whether the IL-36γ effect on SAF was in turn affecting macrophage secretory function. As such, media from untreated SAF and SAF cultured with IL-36γ were transferred onto MDM as described in Section 3.4.6. The demographics of the SAF donors are listed in Table 3.3: These confirm that the patients were age matched, with spirometric values appropriate to their respective cohorts.

	Non-smoker (n=4)	**COPD** (n=4)
Age	66.8 ± 3.7	66.8 ± 3.8
Gender (male:female)	1:3	2:2
FEV1 (L)	2.24 #	1.43 ± 0.63
FEV1 (%)	93.2 #	63.4 ± 23.2
FVC (L)	3.00 #	2.76 ± 0.80
FVC (%)	101.0 #	94.3 ± 9.4
FEV1/FVC	0.75 #	0.50 ± 0.15
Smoking history (pack years)	0 ± 0	34.5 ± 8.5

Table 3.3: Subject demographics for small airway fibroblast (SAF) obtained from non-smoker and COPD lung tissue
FEV1: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for one year. Data are presented as mean ± SEM. #: Demographic data was not available for all subjects.

3.5.7.1 *Effect of IL-36γ on SAF*

Measurements of cytokine concentration in SAF media by ELISA and MSD confirmed that culture with IL-36γ for 24h induced cytokine release compared to basal conditions (Table 3.4).

A

	Non-smoker SAF NT	Non-smoker SAF IL-36γ	COPD SAF NT	COPD SAF IL-36γ
CXCL1 (ng/ml)	3.23	18.7	1.99	15.9
CXCL8 (ng/ml)	1.63	41.9	0.63	31.7
IL-6 (ng/ml)	0.53	2.01	0.54	0.59
GM-CSF (pg/ml)	18.50	183.83	11.16	124.41

B

	NSm fibro	NSm fibro + IL36 stim
Eotaxin 3	0.00	1.33
TARC	0.00	1.54
IP10	0.55	5.60
MIP1a	0.00	4.57
IL8	51.51	2579.25
MCP1	336.94	7757.81
MDC	46.31	60.62
MCP4	0.00	3.53
IFNg	0.78	5.72
IL1b	0.34	4.68
IL2	0.00	2.82
IL4	0.06	0.56
IL6	23.71	782.66
IL8	192.14	2338.39
IL10	0.20	1.55
IL12p70	0.00	1.79
IL13	13.88	56.25
TNFa	0.13	2.76
GM CSF	1.67	6.84
VEGF	30.17	51.65

Table 3.4: Effect of IL-36γ on SAF cytokine secretion.
(A) Non-smoker (n=4) and COPD (n=4) SAF were left in media (NT) or with 200ng IL-36γ for 24h. Media from non-smokers SAF and those from COPD SAF were pooled and cytokine levels measured by ELISA. (B) 20 analytes were also measured by MSD in pooled media from non-treated SAF, and NSm SAF treated with 200ng IL-36γ for 24h . All data is shown as ng/ml. Statistics were not performed as data is a single measurement on pooled samples.

3.5.7.2 Effect of SAF on MDM function

Conditioned media from SAF from untreated non-smoker or COPD SAF was transferred to wells containing MDM and cells were cultured for a further 24h. Transfer of media from unstimulated SAF had no effect on MDM IL-6 secretion (Figure 4.13 Panel A). However, when the same experiment was performed with media from SAF that were cultured for 24h with IL-36γ, there was an increase in IL-6 release from MDMs (40-fold with Non-smoker SAF, 20-fold with COPD SAF). The levels of IL-6 were above that measured in the SAF media used as treatments (shown by red dotted lines in Figure 3.13 panel A). A similar but less pronounced pattern was seen when CXCL8 and CXCL1 were measured (Figure 3.13 Panel B and C respectively), with media from IL-36γ stimulated SAF causing an increased in cytokine release from MDM. There was no difference in the responsiveness of MDM from controls or COPD subjects and none of the treatments altered MDM viability (Figure 3.13D).

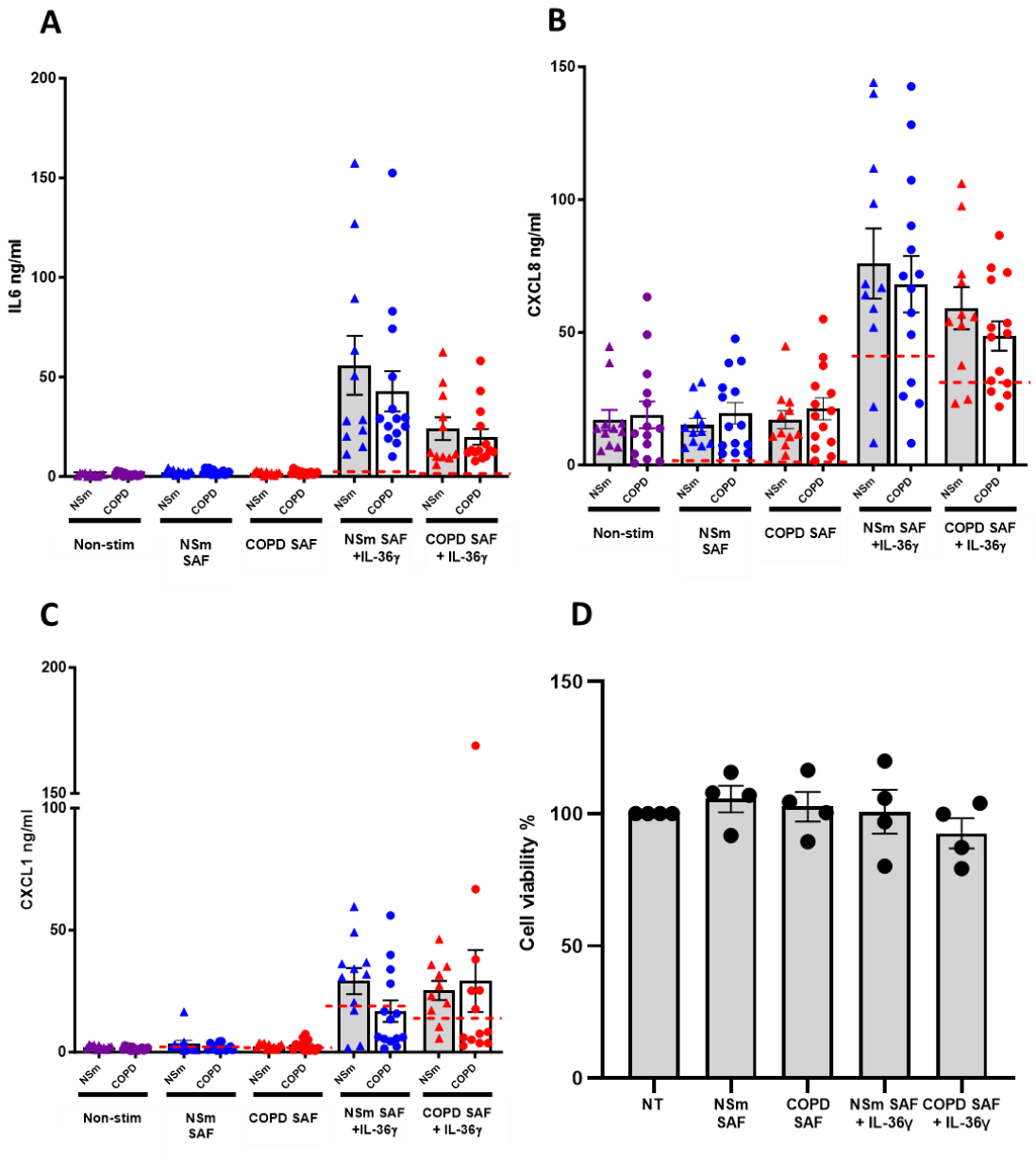

Figure 3.13: Effect of IL-36γ on MDM secretory function via crosstalk with small airway fibroblasts (SAF)

Non-smoker MDM (Δgrey columns) and COPD MDM (O white columns) were stimulated for 24h with media from untreated NSm (▲/●) and COPD (▲/●) SAF; and with media from SAF which had been treated with 100ng/ml IL-36γ. MDM in media alone (▲/●) were used as a control for the experiment. Media from MDM were aspirated and (A) IL-6, (B) CXCL8 and (C) CXCL1 levels measured by ELISA. Red dotted line shows levels of respective cytokines in baseline SAF media that was used as treatments (ng/ml). (D) Cell viability of MDM under each of the above conditions was measured using a MTT assay and data presented as percentage normalised to non-stim (NT). Data are expressed as mean ± SEM. n=11 non-smoker, n=14 COPD MDM.

3.5.7.3 Determining whether crosstalk between SAF and MDM is IL-36R dependent

In order to determine whether the crosstalk between SAF and MDM was dependent on MDM IL-36R signalling, MDM were pre-treated for 1h with IL-36RA before media from IL-36γ stimulated SAF were transferred as described in Section 3.4.6. Pre-treatment with IL-36RA did not affect MDM secretory function (Figure 3.14). This shows that the inflammatory cross talk between SAF and macrophages was not driven by IL-36R activation on MDM, but rather due to an independent mediator released by SAF in response to IL-36γ.

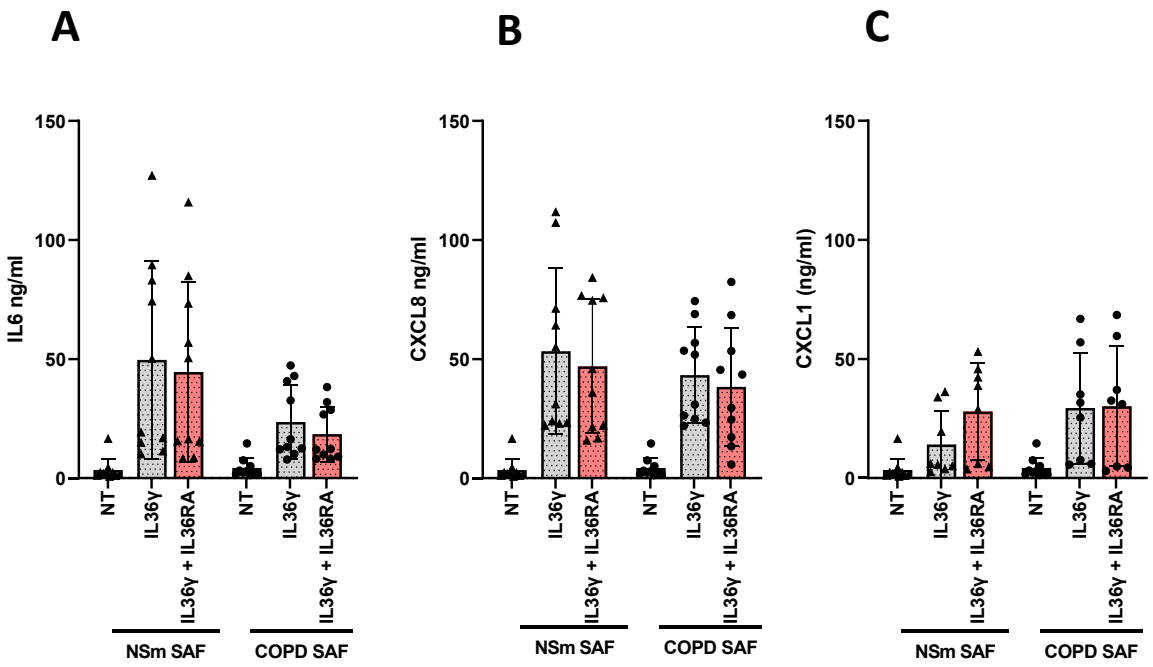

Figure 3.14: Cytokine release from MDM after 24h treatment with IL-36γ stimulated small airway fibroblasts in the presence or absence of IL-36RA

MDM were pre-treated with IL-36RA (pink column) or media alone (grey columns) for 1h. Media from IL-36γ stimulated non-smoker (▲) and COPD (●)SAF were then added for 24h. MDM media was aspirated and ELISAs performed to measure (A) IL-6, (B) CXCL8 and (C) CXCL1. Data are expressed as mean ± SEM, n=10.

3.6 Discussion

Macrophage function is known to be affected by microenvironment and external stimuli. This is the first study looking at how IL-36R signalling effects macrophage function in humans and more specifically in COPD. COPD macrophages are known to have defective phagocytosis of bacteria, fungi and apoptotic cells (Hodge 2003, Taylor 2010, Wrench 2018). This macrophage phenotype contributes to increased bacterial colonisation, acute infection/exacerbation, and heightened inflammation due to impaired clearance of pathogens and necrotic cells (Singh 2021). In knockout mouse studies, IL-36R signalling appears to effect pulmonary macrophage phagocytosis variably depending on prey. IL-36R signalling is associated with improved phagocytosis of *S. pneumonia* and *K. pneumoniae* (Kovach 2017) but has no effect on phagocytosis of *M. tuberculosis* (Ahsan 2018) or *P. aeruginosa* (Aoyagi 2017), with IL-36γ instead impairing bactericidal activity against *P. aeruginosa* (Aoyagi 2017). In contrast to these mouse studies, the present study shows that IL-36γ significantly impairs phagocytosis of *H. influenzae* by macrophages from COPD patients but has no effect on macrophage phagocytic function from healthy non-smokers. The phagocytosis data for healthy smokers is not powered to test for significance. Phagocytosis of *H.* influenzae in COPD MDM pre-treated with IL-36RA prior to IL-36γ slightly higher (non-significantly) than phagocytosis of the same untreated MDM. Further studies further investigated whether treatment with IL-36RA effects non-smoker, healthy smoker and COPD macrophage phagocytosis independent of IL-36γ. Taylor et al. showed that in COPD patients the defect in phagocytosis is a recognition of prey as COPD macrophages show normal phagocytosis of manufactured beads (Hodge 2003, Taylor 2010). This is partly thought to be the result of a change in surface receptor molecules on COPD macrophages (Belchamber 2017)**.** It would be interesting to confirm whether IL-36γ affects phagocytosis of latex beads and if there are any changes in cell surface receptors on COPD macrophages induced by IL-36γ. In this study, only phagocytosis of *H. influenzae* has been studied: Given that mouse studies suggest that the effect of IL-36R signalling on phagocytosis might vary depending on bacterial species, further work is needed to look at how IL-36γ effects human pulmonary macrophages across a range of common respiratory pathogens (bacteria and fungi) as well as efferocytosis.

The negative effect of IL-36γ on phagocytosis of *H. influenzae* is seen in both MDM and tissue macrophages from COPD patients. This supports the use of MDM as a suitable surrogate for COPD tissue macrophages. Indeed, the results in MDM suggest that pulmonary macrophages are systemically primed in their abnormal response to IL-36γ before reaching the lung. There is a smaller overall percentage reduction in phagocytosis induced by IL-36γ in tissue macrophages than MDM (6.9±3.8% vs 22.6 ±0.8%), this may be due to desensitisation of tissue macrophages to IL-36γ having been resident for some time in the lung and thus exposed to high levels of IL-36γ. Furthermore,

different populations of macrophages have been shown to express varying levels of IL-36R (Queen 2019). There is also likely heterogeneity within individuals (Chana 2014, Kulikauskaite 2020) and it may be that some clusters of macrophages respond differently to IL-36γ, thus making the overall effect of IL-36γ less than in a more homogenous GM-CSF differentiated MDM population.

Given that bacterial colonisation and acute exacerbations are associated with more severe COPD, rapid lung function decline and a worse prognosis (Barnes 2021), one might expect degree IL-36γ induced inhibition of phagocytosis to be positively correlated with severity of disease. Instead, the data presented here suggest the opposite, whereby the effect of IL-36γ to reduce phagocytosis is more marked in the milder group of patients. More data is needed to confirm this association but it maybe that in the more severe patients, the phagocytic response cannot be suppressed further. Indeed, greater impairment of phagocytosis was not associated with the frequent exacerbator phenotype: However, numbers in each of the frequent and infrequent exacerbator groups are low. In a future study it would be good to look at any association with rates of bacterial AECOPD (rather than overall AECOPD incidence) given only half of AECOPD are associated with bacterial infection (Belchamber 2017) or at any link to bacterial colonisation.

Aside from the effect on macrophage phagocytosis, this study does not show any direct effect of IL-36γ on macrophage secretory function. Instead, there appears to be an indirect effect via crosstalk with SAF. Indeed, IL-36γ causes SAF to be more pro-inflammatory (Baker 2022). This study shows that a substance released from SAF which have been cultured in IL-36γ causes both non-smoker and COPD macrophages to be more pro-inflammatory. Pre-treatment of macrophages with IL-36RA does not affect cytokines secretion thus showing that the cellular crosstalk does not prime MDM to be more responsive to IL-36γ but rather stimulate them via an independent, SAF-derived mediator. The pro-inflammatory effect of IL-36γ on pulmonary macrophages is supported by mouse studies which suggesting that IL-36R signalling within the lung causes a differentiation of alveolar macrophages away from an M2-like phenotype (Wein 2018).

In contrast to results from macrophage phagocytosis, the proinflammatory effect on MDM of increased IL-36γ in the lung is not disease specific. A healthy smoking cohort was not completed for these experiments as there was no difference between healthy non-smokers and COPD cohorts in order to need to differentiate whether it was smoking related or disease specific. There is also no significant correlation between any demographics (sex, age, smoking history) and MDM cytokine secretion. The concentrations of cytokines secreted have high intersubject variability. This heterogeneity in the data may reflect heterogeneity of COPD population seen in clinical practice. There is however no correlation between cytokine secretion and any COPD clinical parameters such

as lung function, exacerbation frequency or eosinophil count. More numbers are needed to look for look any potential phenotype effect.

Three cytokines (IL-6, CXCL1, CXCL8) were chosen for this study. These are all associated with neutrophilic inflammation in COPD. For a future study it would be interesting to expand this to determine if other mediators relevant to COPD pathogenesis are effected by IL-36γ crosstalk between SAF and macrophages e.g. GM-CSF and CCL2 (neutrophil and monocyte chemoattractants), MMP2 and MMP9 (proteases key in driving emphysema) (Finlay 1997), IL-10 and TGFβ (anti-inflammatory cytokines downregulated in COPD); or CXCL9, CXCL10 and CXCL11 (inflammatory cytokines increased in COPD) (Finlay 1997, Belchamber 2017). This study has not investigated what the intermediate mediator(s) from IL-36γ stimulated SAF is, that drives the pro-inflammatory effect on macrophages. The data from ELISA and MSD of IL-36γ stimulated SAF media suggests that there is a large inflammatory effect on SAF with multiple analytes raised. However, this data is on pooled SAF media and therefore cannot be used to look for any significance in results. Trying to differentiate what causes the crosstalk between SAF and macrophages would be a prolonged process of pre-treating macrophages with various receptor blockers before adding the SAF media. This would be complicated yet further by the fact that there may be more than one mediator at play.

3.7 Conclusion

In summary, IL-36γ promotes two of the key features of the COPD macrophage phenotype: IL-36γ, via its action on the IL-36R on macrophages, directly impairs bacterial phagocytosis, meanwhile it indirectly increased secretion of pro-inflammatory mediators of neutrophilic inflammation (IL-6, CXCL1 and CXCL8) via crosstalk between SAF and macrophages (Figure 3.15). As such the hypothesis "IL36γ results in impaired phagocytosis of *H. influenzae* and increased secretion of pro- inflammatory cytokines by macrophages" can be accepted.

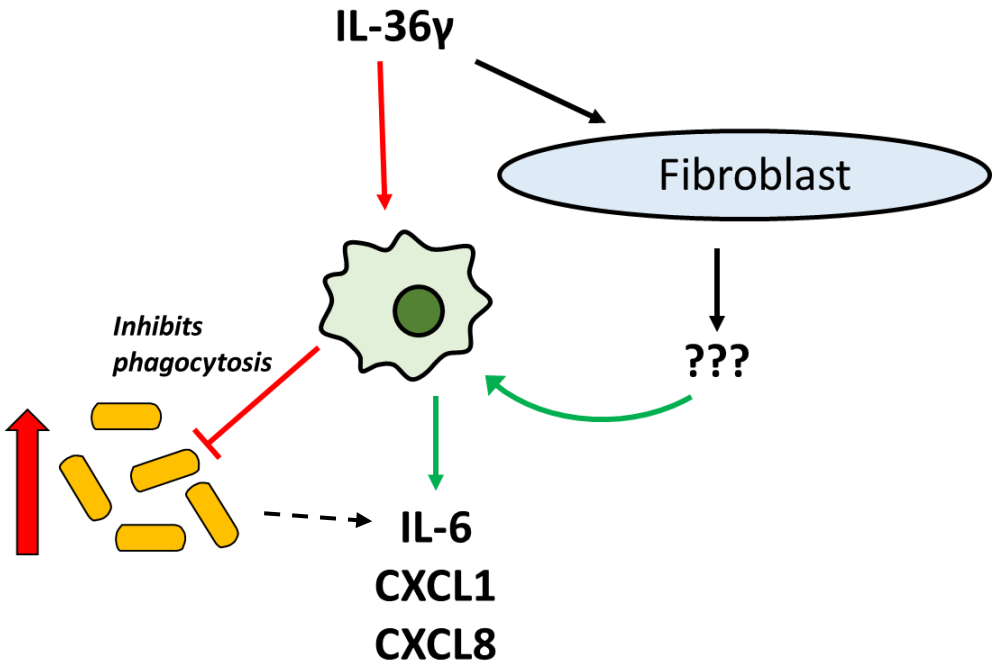

Figure 3.15: Schematic summarising IL-36γ effect on macrophages
IL-36γ via direct stimulation of IL-36R on macrophages results in impaired phagocytosis of *H. influenzae* in COPD (red arrows). IL-36γ also stimulates small airway fibroblasts. Unknown mediator(s) in the resultant SAF media in turn stimulates macrophages to release IL-6, CXCL1 and CXCL8 (green arrows). Bacteria in turn induce IL-6 expression.

The findings presented above are likely to be clinically relevant as pulmonary IL-36γ levels are raised in COPD compared to health subjects (Kovach 2021, Baker 2022). In turn increased IL-36R signalling results in increased neutrophilic inflammation and may contribute to bacterial colonisation through reduced bacterial phagocytosis: Both of which are known to contribute to COPD severity (Belchamber 2017, Pavord 2018). Furthermore, viral infection is known to stimulate SAEC to secrete increased IL-36γ (Baker 2022). Over 60% of AECOPD are driven by neutrophilic inflammation (Finney 2024) and therefore IL-36γ driven neutrophilic inflammation within the lung may be a key causative cytokine for virally induced exacerbations. Meanwhile inhibition of phagocytosis as a direct effect of IL-36γ may contribute to secondary bacterial infections. Given the above potential implications on both stable state and acute exacerbations of COPD, IL-36R signalling appears to be a promising target for novel COPD therapies.

Chapter 4: Regulation of IL-36RA in macrophages

4.1 Introduction

Chapter 3 demonstrated that increased IL-36γ levels in COPD cause macrophages to be less efficient at clearing bacteria and indirectly via SAF, drive inflammatory mediator release. These data provide additional mechanisms by which IL-36R signalling drives COPD pathogenesis. There are two IL-36 receptor antagonist molecules: IL-38 and IL-36RA. Of these IL-36RA is the endogenous inhibitor of IL-36R signalling in the lung. Indeed IL-38 is not detectable at a mRNA level in BAL, tissue homogenate or individual lung cell types (Baker 2022). It is important to understand more about regulation and expression IL-36RA when considering IL-36 as a potential new therapeutic target.

The IL-36 receptor antagonist, IL-36RA, is encoded by the *IL-36RN* gene. Once cleaved into its active form, IL-36RA competes with IL-36α, IL-36β and IL-36γ for IL-36R. IL-36 signalling is dependent on a heterodimer receptor comprising of IL-36R and IL-1RAcP. But IL-36RA, on binding to the IL-36R, does not induce the recruitment of the accessory protein. In the absence of the IL-36R/IL1RAcP complex, there is no downstream NF-κB signalling (Zhou 2018). IL-36RA binds with a higher affinity to IL-36R than IL-36α or IL-36γ and has a slower release rate and as such is effective at inhibiting IL-36 dependent inflammation in a concentration dependent fashion (Vigne 2011, Zhou 2018).

The concentration of IL-36RA is reduced in BAL, sputum and lung homogenates from COPD patients compared to healthy smoking and non-smoking controls (Baker 2022). This deficiency in IL-36RA exaggerates the imbalance in IL-36 receptor signalling already set by increased levels of IL-36α and IL-36γ in the lung (Kovach 2021, Baker 2022). Within the lung *IL-36RN* is expressed by tissue macrophages, small airway epithelial cells and small airway fibroblasts. Of these cell types, only macrophages mirror what is seen on an organ basis with *IL-36RN* expression lower in COPD compared to non-smokers and healthy smokers (Baker 2022). This suggests that macrophages are the principal source of *IL-36RN* in the lung. The ontology of alveolar macrophages is complex, but a proportion of mature alveolar macrophages are derived from circulating blood monocytes, which then differentiate to macrophages on reaching the lungs (Byrne 2020). Outside the lung, *IL-36RN* is expressed in monocytes as well as both M1-like and M2-like macrophages but the relative expression of *IL-36RN* in each of these cell types in health compared with COPD is not known (Boutet 2016).

In health, macrophages respond to intrinsic and extrinsic stimuli via various cell signalling pathways (as outlined in Section 1.4.5.3) which allows adaptation to their environment. In COPD, increased activation of kinase signalling pathways such as PI3K and MAP pathways alter macrophage phenotype and contribute to COPD pathogenesis (Barnes 2016). Class 1 PI3K consist of a heterodimer in which there are 4 different catalytic subunit isomers (p110α, p110β, p110γ, p110δ) (Barnes 2016, Wang. 2020). The α and β subunits are ubiquitously expressed, whilst γ and δ are more specific to leukocytes

including macrophages (Barnes 2016). Increased activity of these signalling pathways in COPD macrophages promotes increased release of pro-inflammatory cytokines, chemokines and proteases as well as contributing to the steroid resistance typically seen in COPD (Barnes 2016, Wang 2020, Moradi 2021). The data presented in this chapter examines how the activity of these cell signalling pathways regulate *IL-36RN* expression in healthy and COPD lung macrophages.

4.2 Hypothesis

IL-36RN expression is reduced in COPD MDM due to dysregulated response to increased IL-36 agonists.

4.3 Aims

Using an MDM model, I aim to:

- Investigate the differential expression of *IL-36RN* in monocytes, M-MDM and GM-MDM, comparing healthy participants to COPD patients.
- Investigate whether members of the IL-1 family regulate *IL-36RN* expression in macrophages via negative/positive feedback loops
- Investigate transcriptional regulation of *IL-36RN* in macrophages with the aim to restore *IL-36RN* expression to “healthy” levels in COPD.

4.4 Methods

4.4.1 Subject selection

Healthy non-smokers and COPD patients were recruited as described in Section 2.2.1.

4.4.2 Isolation and culture of MDM

60ml of blood was taken from participants as described in section 2.2.2.5.1. PBMC were isolated, plated in 24 well plates $5x10^5$ monocytes / well. Once adhered, monocytes were either directly lysed for RNA or cultured in the presence of M-CSF (100ng/ml) or GM-CSF (2ng/ml) for 10-14 days to generate M-MDM and GM-MDM as described in section 2.2.3.

4.4.3 Isolation of tissue macrophages

Tissue macrophages were isolated from surgical lung tissue from COPD patients as described in section 2.2.3.4. Macrophages were plated in 24 well plates $5x10^5$ monocytes / well and incubated overnight at 37°C and 5% ($^v/_v$) CO_2 to allow macrophages to adhere.

4.4.4 Investigating the effect of IL-1 cytokines on *IL-36RN* expression

Macrophages (GM-MDM) were incubated with either media (NT), or 1ng/ml IL-1β, 100ng/ml IL-33, 100ng/ml IL-36α, 100ng/ml IL-36β and 100ng/ml IL-36γ at 37°C, 5% (v/v) CO_2 for 24h. Cells were lysed with 175µl RLT buffer per well and then stored at -20°C for later RNA extraction (see section 4.4.6).

4.4.5 Investigating the effect of pathway inhibitors on *IL-36RN* expression

Macrophages (GM-MDM/tissue macrophages) were incubated with media (NT), or DMSO (control), 1µM PIK75 (PI3K inhibitor), 10µM UO0126 (ERK inhibitor), 100µM VX745 (p38 inhibitor), 10µM SP600125 (JNK inhibitor) and 1µM TPCA1 (NF-κB inhibitor) at 37°C, 5% ($^v/_v$) CO_2 for 24h. Cells were lysed with 175µl RLT buffer per well and then stored at -20°C for later RNA extraction (see section 4.4.6). MTT assays were performed to assess cell viability as described in section 2.2.5.

4.4.6 RNA extraction, reverse transcription and real time quantitative PCR

RNA was extracted from cells as described in section 2.2.8.1. RNA was reverse transcribed (see section 2.2.8.2) and then real-time quantitative PCR performed on cDNA (see section 2.2.8.3). The relative expression of IL-36RN against the housekeeping gene RACK1 was calculated using the $2^{-\Delta\Delta CT}$ method.

4.4.7 Statistics

All data were expressed as mean ± SEM unless otherwise stated and analysis performed using GraphPad Prism software (version 9.4.1). Statistical analyses are detailed in figure legends.

4.5 Results

4.5.1 Subject demographics

The characteristics of non-smoking and COPD blood donors used in this study are shown in Table 4.1. Participants were age matched across groups. There were significant differences in obstructed lung function (FEV_1, FEV_1 %, FEV_1/FVC) and smoking history between COPD and non-smoking participants.

	Non-smoker (n=11)	**COPD** (n=17)
Age (years)	62.7 ± 2.0	65.9 ± 2.4
Gender (m:f)	7:4	10:7
FEV1 (L)	2.81 ± 0.29	1.56 ± 0.17 **
FEV1 (%)	103.2 ± 7.1	58.0 ± 5.04 ***
FVC (L)	3.69 ± 1.29	3.01 ± 0.79
FVC (%)	107.6 ± 6.9	91.2 ± 5.0
FEV1/FVC	0.77 ± 0.02	0.50 ± 0.03 ***
Smoking (pack years)	0.0 ± 0.0	53.1 ± 9.2 ***

Table 4.1: MDM demographics table
FEV_1: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year = 20 cigarettes a day for one year. Data are presented as mean ± SEM. Mann-Whitney was performed to test for significant differences between subject groups. * $p<0.05$, ** $p< 0.01$, *** $p< 0.001$ compared to healthy non-smoking controls.

4.5.2 Baseline *IL-36RN* expression in non-smoker and COPD monocytes

IL-36RN expression was measured by RT-qPCR in healthy non-smoker and COPD monocytes. *IL-36RN* significantly lower in monocytes from COPD patients compared to those from healthy non-smokers (0.32±0.33 vs 1.52±1.19), see figure 4.1.

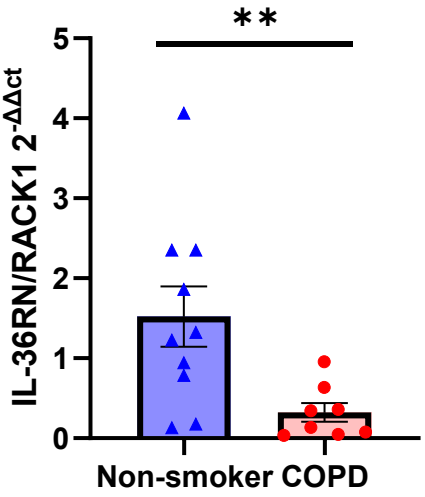

Figure 4.1: *IL-36RN* expression in non-smoker and COPD monocytes
Monocytes isolated from whole blood from non-smoker (▲) and COPD (●) subjects were lysed, RNA extracted and *IL-36RN* expression measured by RT-qPCR. Data was normalised to *IL-36RN* expression in non-smoker monocytes. Data expressed as mean ± SEM. Data analysed using Mann-Whitney U to compare *IL-36RN*. **: p< 0.01, n=10 non-smokers, n=8 COPD.

4.5.3 Effect of demographic variables on *IL-36RN* expression in monocytes

It was important to understand if any demographic variables other than COPD affected *IL-36RN* expression in monocytes. In this experiment, there was no effect of gender (Figure 4.2A). Of note, healthy non-smokers were younger than COPD patients (62.9±1.65 vs 70.3±1.66, p<0.01) and there was a negative correlation between age and *IL-36RN* expression when groups were analysed together (Figure 4.2 Panel B). *IL-36RN* expression was not correlated with age however when groups were analysed separately (non-smokers: r = -0.14, p = 0.70; COPD: r = -0.25, p = 0.54). There was no correlation between *IL-36RN* expression and smoking history (pack years) in COPD patients (Figure 4.3).

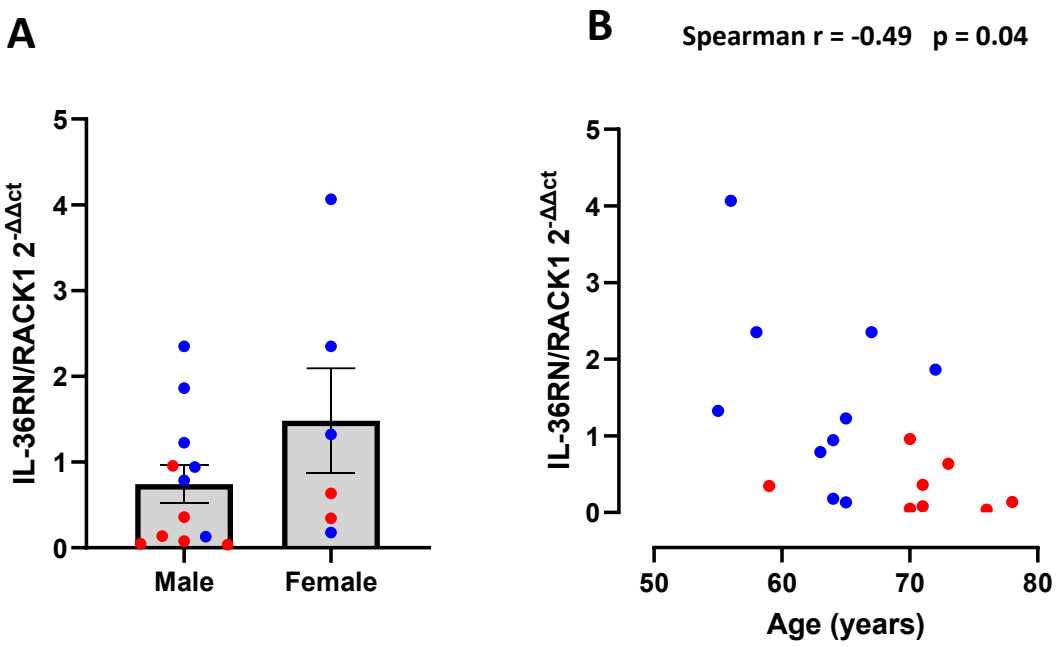

Figure 4.2: Effect of gender and age on *IL-36RN* expression in monocytes
Monocytes from non-smoker (•) and COPD (•) donors were lysed, RNA extracted and *IL-36RN* expression measured by RT-qPCR. Effect of (A) gender (B) age on *IL-36RN* depicted. In (B) data presented as Spearman rank correlation coefficients and associated p values (subject groups analysed together). n=10 non-smokers, n=8 COPD.

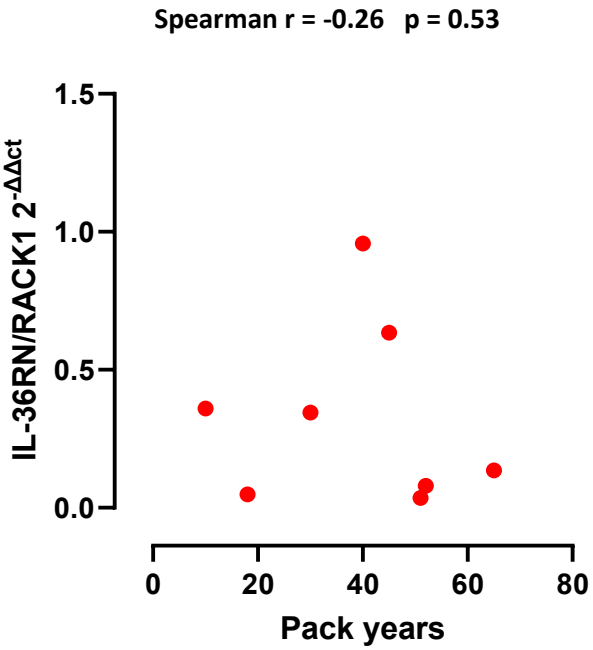

Figure 4.3: Effect of smoking history on *IL-36RN* expression in COPD monocytes
Monocytes from COPD patients were lysed, RNA extracted and *IL-36RN* expression measured by RT-qPCR. Data presented as Spearman rank correlation coefficient and associated p value. n=8

Despite IL-36 signalling causing increased inflammation in COPD, circulating monocyte expression of *IL-36RN* was not associated with severity of obstruction in COPD patients (Figure 4.4).

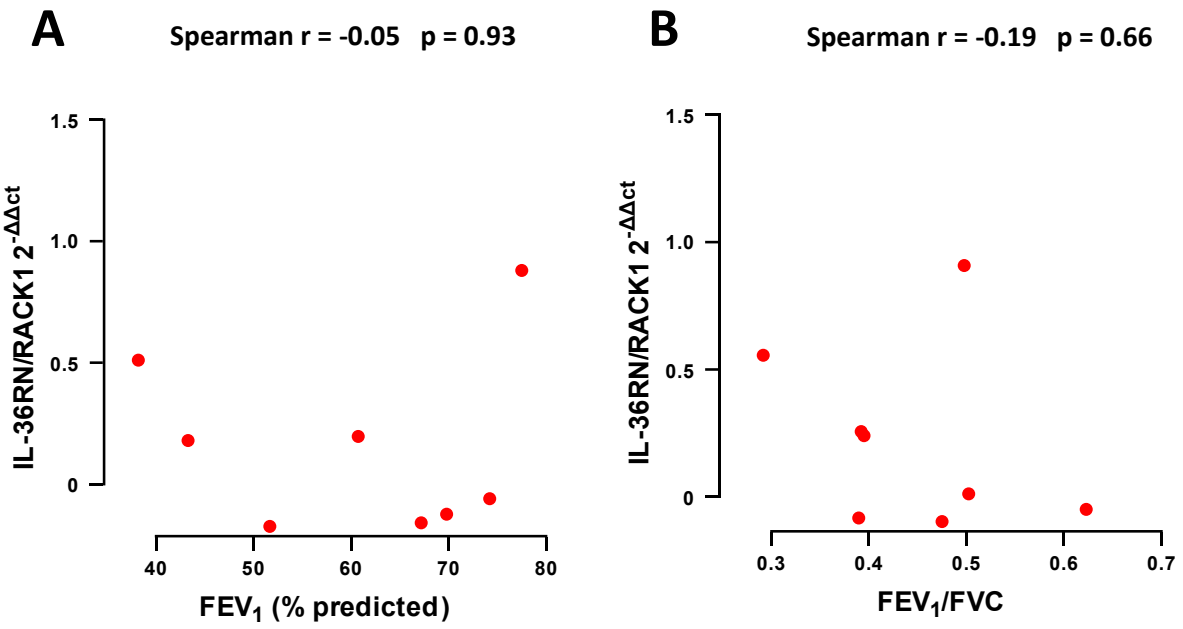

Figure 4.4: Correlation between *IL-36RN* expression in COPD monocytes and severity of obstruction
Monocytes from COPD patients were lysed, RNA extracted and *IL-36RN* expression measured by RT-qPCR. (A) FEV_1 % predicted and (B) FEV_1/FVC were plotted against *IL36RN* expression. Data presented as Spearman rank correlation coefficient and associated p value. n=8

4.5.4 *IL-36RN* expression on differentiation to monocyte derived macrophages in healthy and COPD subjects

Monocytes differentiate into macrophages when they reach the lungs, thus *IL-36RN* expression was measured in M1-like, GM-CSF differentiated MDM (GM-MDM), and M2-like, M-CSF differentiated MDM (M-MDM). Differentiation of monocytes to M-MDM did not cause any change in *IL-36RN* expression either in non-smoker or COPD donors (Figure 4.5 panel A). Meanwhile there was a significant increase in *IL-36RN* expression when non-smoker monocytes were differentiated into GM-MDM (13.6-fold, $p<0.01$). This increase in *IL-36RN* expression was not seen in COPD GM-MDM (Figure 4.5 panel B). As a result, *IL-36RN* expression is significantly higher in GM-MDM than M-MDM in healthy subjects (20.76 ± 7.24 vs 6.50 ± 2.72, $p<0.01$). Meanwhile COPD GM- MDM have significant lower *IL-36RN* expression than GM-MDM from healthy non-smoking donors (3.72 ± 1.20 vs 20.76 ± 7.24, $p<0.01$).

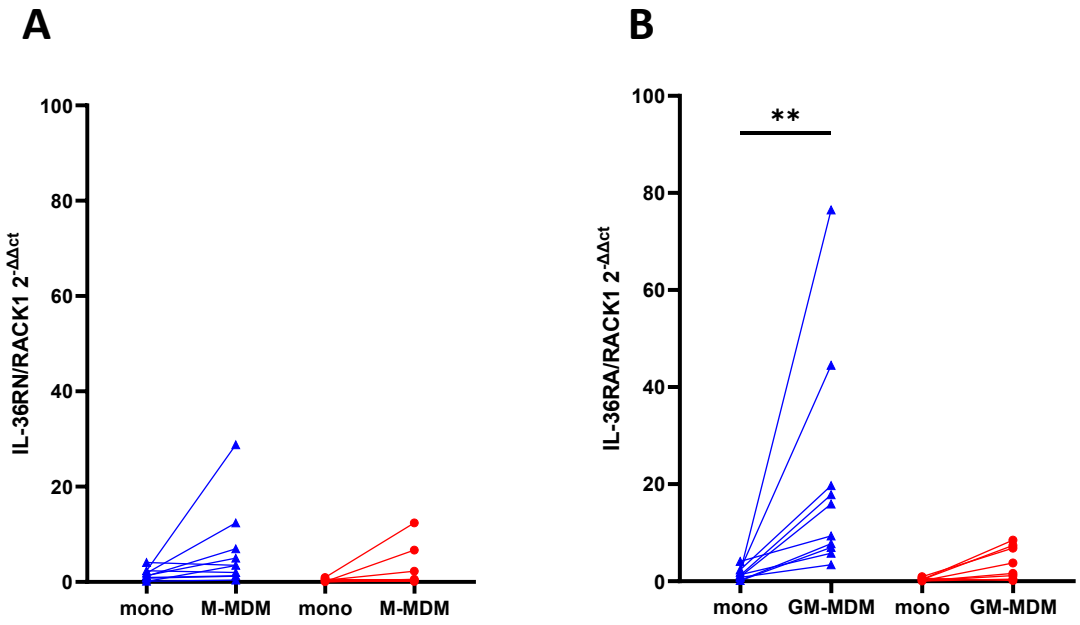

Figure 4.5: *IL-36RN* expression in non-smoker and COPD Monocytes, M-CSF and GM-CSF differentiated MDM

PBMCs from non-smoker (▲) and COPD (●) patients were separated from whole blood. PBMC were differentiated for 10-14 days in (A) M-CSF or (B) GM-CSF. Monocytes, M-MDM and GM-MDM were lysed, RNA extracted, reversed transcribed and *IL-36RN* expression measure by RT-qPCR. Data was normalised to IL-36RN expression in non-smoker monocytes. Data analysed using Kruskal-Wallis with Dunn's post hoc test to compare *IL-36RN* expression. **: p< 0.01 non-smokers GM-MDM vs COPD GM-MDM. n=10 non-smokers, n=8 COPD.

4.5.5 Effect of demographic variables on *IL-36RN* expression in MDM

Having shown that GM-MDM are the major MDM phenotype expressing *IL-36RN* it was important to determine whether any demographic variables effected this expression. In healthy GM-MDM there was a trend towards a positive correlation between age and expression of *IL-36RN* whereas in the COPD derived cells, expression of *IL-36RN* trended to negatively correlate with age (Figure 4.6).

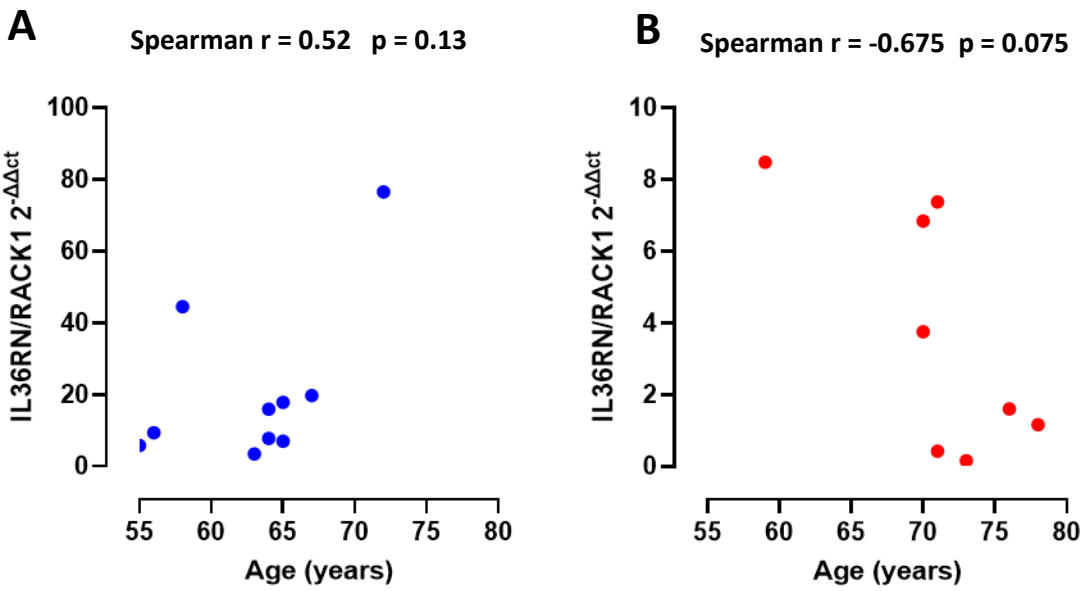

Figure 4.6: Effect of age on *IL-36RN* expression in GM-MDM
IL-36RN expression was measured in GM-MDM from (A) Healthy non-smokers (•) and (B) COPD patients (•). Data was normalised to *IL-36RN* expression in non-smoker monocytes. Data presented as Spearman rank correlation coefficients and associated p values. n=10 non-smokers and n=8 COPD

In contrast to monocytes, smoking history was negatively associated with expression of *IL-36RN* in GM-MDM, albeit not significantly (p<0.06) (Figure 4.7)

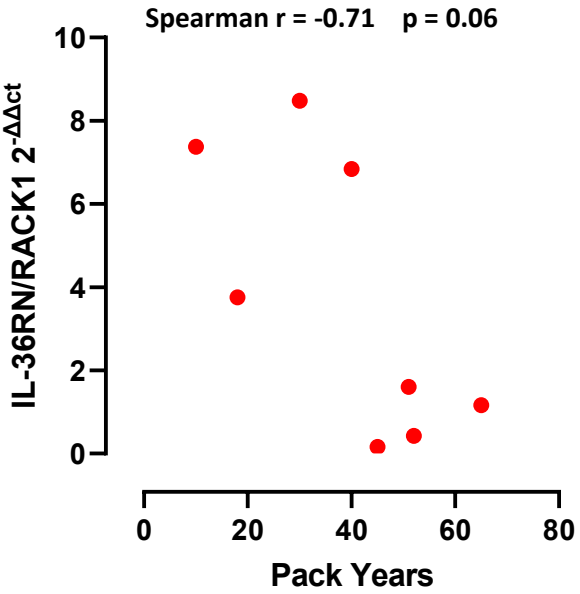

Figure 4.7: Effect of smoking history on *IL-36RN* expression in GM-MDM
IL-36RN expression was measured in GM-MDM from COPD patients. Data presented as Spearman rank correlation coefficients and associated p values n=8.

There was no correlation between *IL-36RN* expression in GM-MDM and severity of obstruction in COPD patients (Table 4.2). In the cohort of COPD patients used for this part of the study, there was limited data with regards to exacerbation frequency, symptom burden or radiological findings and as such, further analysis was not possible.

	Spearman r	**p value**
FEV1 (L)	0.048	0.935
FEV1 (%)	0.024	0.977
FVC (L)	0.071	0.882
FVC (%)	0.262	0.536
FEV1/FVC	-0.119	0.793

Table 4.2: Correlation between *IL-36RN* expression in COPD GM-MDM and spirometry
IL-36RN expression was measured in GM-MDM from COPD patients. Correlation matrix performed between*IL-36RN* and spirometry values. Data presented as Spearman rank correlation coefficients and associated p values, n=8.

4.5.6 Investigating the pathway regulating *IL-36RN* expression in macrophages

4.5.6.1 Effect of IL-1 family cytokines on IL-36RN expression

Having shown that GM-MDM appear to be the main subtype of MDM expressing *IL-36RN*, it was then examined whether other members of the IL-1 family of cytokines affected its expression. GM-MDM were stimulated for 24h with IL-1 family members and *IL-36RN* expression measured by RT-qPCR. Treatment with IL-1 family cytokines (IL-1β, IL-33, IL-36α, IL-36β and IL-36γ) did not significantly affect *IL-36RN* expression in GM-MDM derived from either non-smoker or COPD subjects (Figure 4.8).

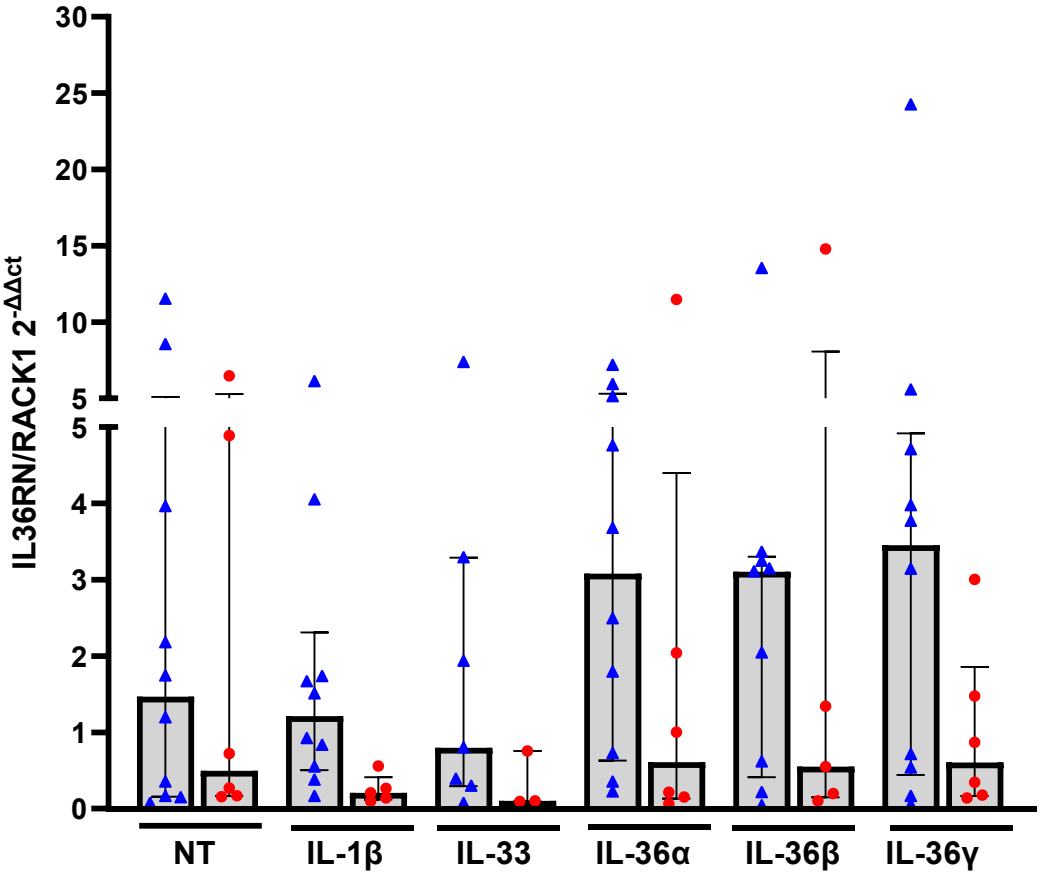

Figure 4.8: Effect of IL-1 family cytokines on MDM effects *IL-36RN* expression
Non-smoker (▲) and COPD (●) GM-MDM were treated for 24h with media (NT), 1ng/ml IL-1β, 100ng/ml IL-33, 100ng/ml IL-36α, 100ng/ml IL-36β and 100ng/ml IL-36γ. Cells were then lysed, RNA extracted, and RT-qPCR performed. Data presented normalised to non-smoker untreated MDM IL-36RN expression. Data expressed as median ± IQR. n=10 non-smoker, n=6 COPD.

4.5.6.2 *Effect of pathway inhibitors on IL-36RN expression*

To investigate how *IL-36RN* expression is controlled in macrophages, GM-MDM were treated with drugs inhibiting the common upstream cell signalling pathways present in macrophages. Treatment with PIK75, a PI3K inhibitor, significantly increased *IL-36RN* expression in both non-smoker and COPD MDM (Figure 4.9). The effect of PI3K inhibition was greater in non-smoker cells compared to COPD cells (approximately 6-fold vs 2-fold increase). However, ERK, p38 or JNK MAP kinase pathway inhibitors had no effect. There was similarly no effect of an IKKα inhibitor (Figure 4.9) suggesting that these pathways were not regulating expression of *IL-36RN*. MTT assay confirmed that none of these treatments affected cell viability (Figure 4.10).

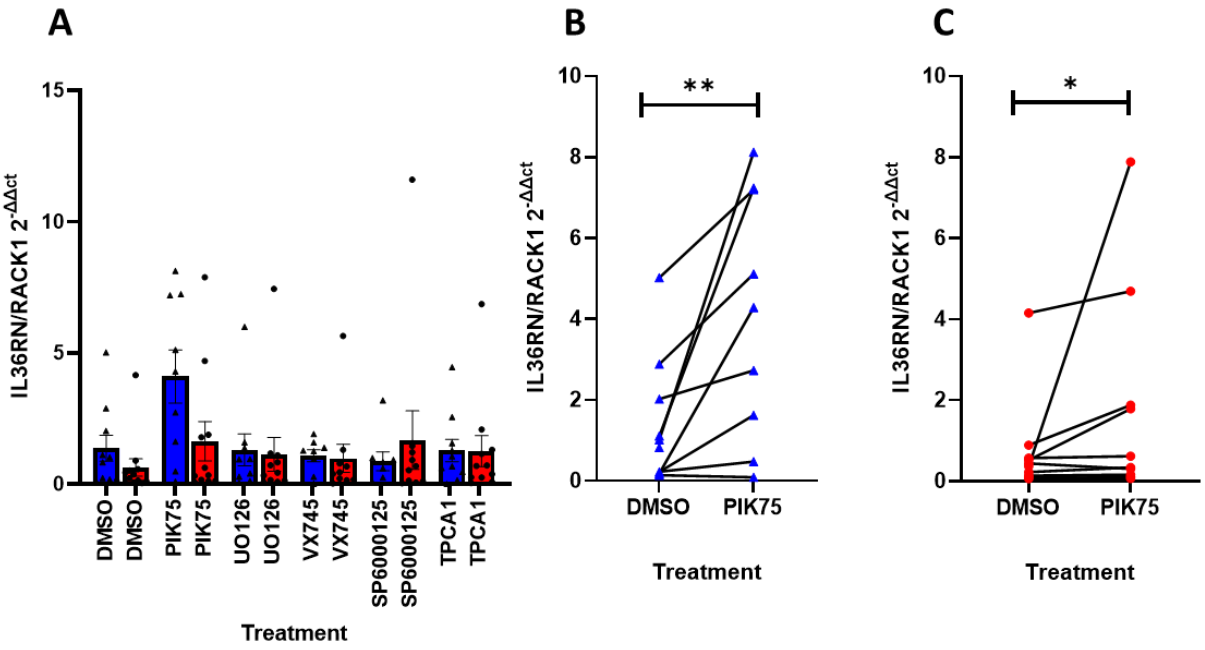

Figure 4.9: Using pathway inhibitors to understand what controls *IL-36RN* expression in MDM
(A) Non-smoker (▲) and COPD (●) GM-MDM were treated for 24h with DMSO (control), 1μM PIK75 (PI3K inhibitor), 10μM UO0126 (ERK inhibitor), 100μM VX745 (p38 inhibitor), 10μM SP600125 (JNK inhibitor), 1μM TPCA1 (NF-κB inhibitor). Cells were then lysed, RNA extracted, and RT-qPCR performed. Data presented normalised to non-smoker GM-MDM with DMSO, data expressed as mean ± SEM. Paired comparison of IL-36RN expression between DMSO and PIK75 in (B) Non-smoker (▲) and (C) COPD (●) GM-MDM. Data analysed using the Wilcoxon Sign test, *: p< 0.05, **: p<0.01 DMSO vs PIK75, n=10 for all groups.

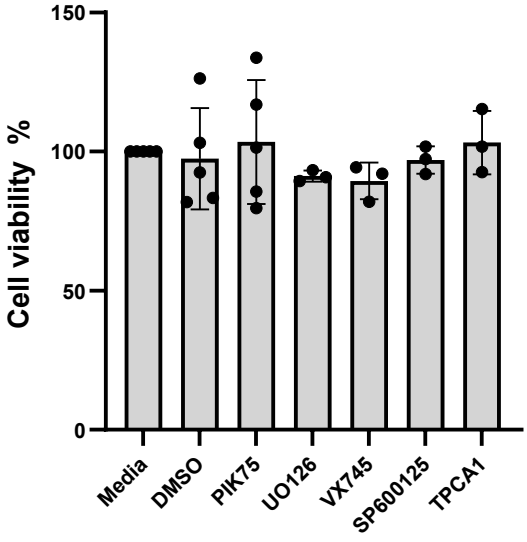

Figure 4.10: Cell viability of MDM after treatment with pathway inhibitors
GM-MDM were treated for 24h with media, DMSO (control), 1µM PIK75 (PI3K inhibitor), 10µM UO0126 (ERK inhibitor), 100µM VX745 (p38 inhibitor), 10µM SP600125 (JNK inhibitor), 1µM TPCA1 (NF-κB inhibitor). MTT assay performed to assess cell viability. Data presented normalised to untreated cells. n=3-5

MDM are a model *in vitro* system that enables investigation of putative pathways regulating macrophage dysfunction in COPD. However, it is important to understand if similar pathways are also altered in macrophages resident in the lung. Therefore, experiments were performed using macrophages isolated from human lung tissue. Table 4.3 shows the demographics for tissue macrophages used. Macrophages were incubated with either PIK75, UO0126, VX745, SP600125 or TPCA1 and expression of *IL-36RN* measured. PIK75 increased *IL-36RN* expression in tissue macrophages suggesting that the observation in GM-MDM was also reflected in lung tissue macrophages (Figure 4.11). There was no effect of the on *IL-36RN* expression with any of the other pathway inhibitors examined (Figure 4.11).

	n=5
DOB/ age	63.2 ±5.0
Sex M:F	3:2
FEV_1 (L)	2.07 ± 0.38
FEV_1 (%)	79.52 ± 6.13
FVC (L)	3.46 ± 0.68
FVC (%)	101.9 ± 6.51
FEV_1/ FVC	0.62 ± 0.05
Smoking (pack years)	35.4 ± 10.11

Table 4.3: Demographics table of tissue macrophages
FEV_1: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year = 20 cigarettes a day for one year. Data are presented as mean ± SEM.

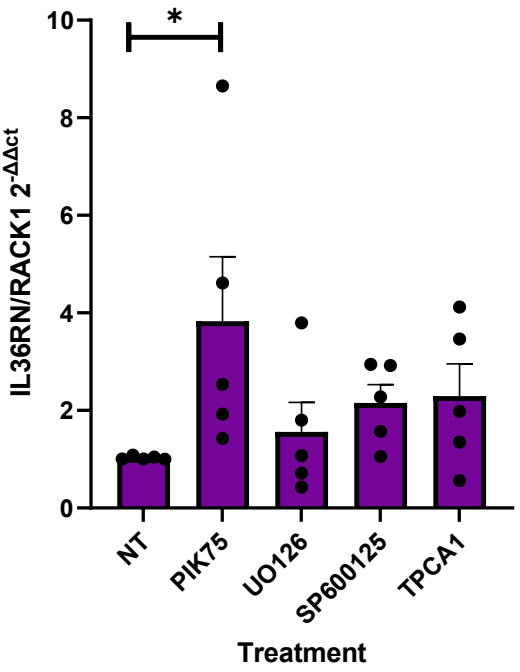

Figure 4.11: Using pathway inhibitors to understand what controls *IL-36RN* expression in tissue macrophages
Tissue macrophages were treated for 24h with DMSO (control), 1µM PIK75 (PI3K inhibitor), 10µM UO0126 (ERK inhibitor), 100µM VX745 (p38 inhibitor), 10µM SP600125 (JNK inhibitor), 1µM TPCA1 (NF-κB inhibitor). Cells were then lysed, RNA extracted, and RT-qPCR performed. Data expressed as mean ± sem. Analysed by Friedman test with Dunn's post hoc test *: $p<0.05$ vs NT, n=5

There are three main classes of PI3K of which class 1 (p110α, p110β, p110γ, p110δ) is the most studied and most relevant to the lung (Barnes 2016, Wang 2020). Of these subtypes, the p110γ and p110δ subunits are the main PI3K expressed by macrophages and other leucocytes, whilst p100α and p110β are more ubiquitous (Moradi 2021). PIK-75 although marketed as a p110α inhibitor but also inhibits p110γ and p110δ (IC_{50} 5.8nM, 76nM, 510nM respectively) (Chaussade2007). To ascertain which of the more leucocyte specific PI3K isoforms regulates *IL-36RN* expression, GM-MDM were incubated with either AS-252424, a selective PI3K p110γ inhibitor (IC_{50} 30nM, 30-700 fold selectivity over other PI3K classes), or CAL-101 a selective PI3K p110δ inhibitor (IC_{50} 2.5nM, 40-300 fold selectivity over other PI3K classes). 1nM CAL-101 and 1µM AS252424 concentration were selected for experiments as these were the highest concentrations which did not affect MDM cell viability (Figure 4.12). The selective p110γ inhibitor, AS-252424, caused an increase in *IL-36RN* expression. This was not significant when analysed separately in non-smokers (p=0.09, n=6) and COPD patients (p=0.29, n=7) but showed significance when combining data irrespective of disease cohort (p<0.05, n=13). The p110δ inhibitor, CAL-101, had no effect on IL-36RN expression when analysed in any of healthy non-smoking cohort, COPD cohort or overall combined data (Figure 4.13).

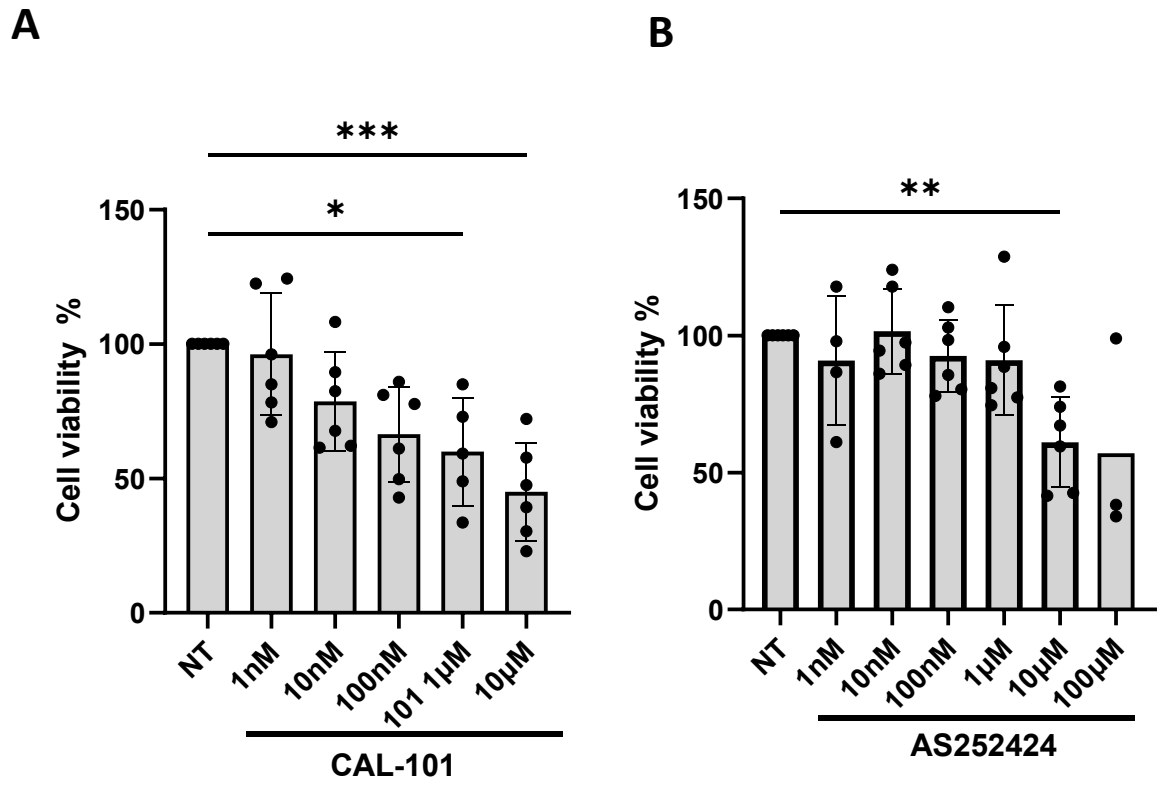

Figure 4.12: Cell viability after treating MDM with selective PI3Ki, CAL-101 and AS252424
GM-MDM from non-smoker and COPD donors were treated for 24h with DMSO (NT), [A] 1µM AS-252424 (p110γ inhibitor) and [B] 1nM CAL-101 (p110δ inhibitor) at serial concentrations from 1nM to 100µM. MTT assay performed to assess cell viability. Data presented as % compared to NT for each individual subject and expressed as mean ± sem. Analysed by Kruskal-Wallis test. *: P<0.05, **: p<0.01, ***:p<0.001 vs NT, n=3-6

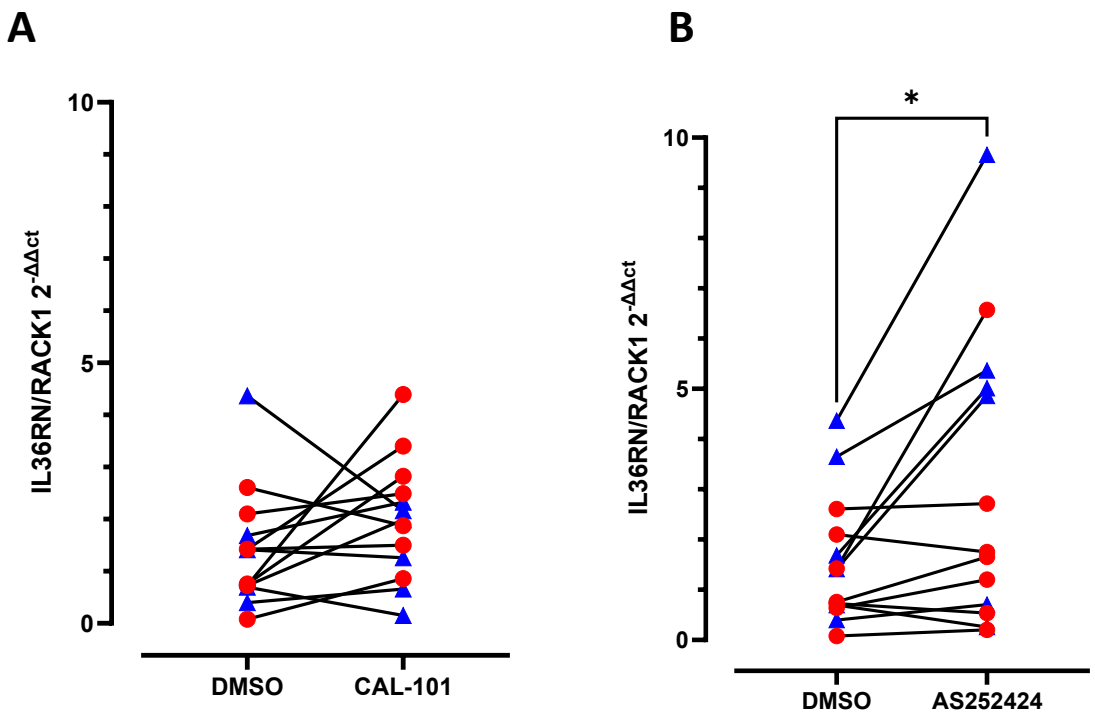

Figure 4.13: Effect of selective PI3K inhibitors on *IL-36RN* expression in MDM
GM-MDM from non-smoker and COPD donors were treated for 24h with DMSO (NT), [A] 1µM AS-252424 (p110γ inhibitor) and [B] 1nM CAL-101 (p110δ inhibitor). Cells were then lysed, RNA extracted, and RT-qPCR performed. (▲) NSm GM-MDM and (•) COPD GM-MDM. Data expressed as mean ± sem. Analysed by Wilcoxon test. *: P<0.05 vs NT, n=13.

4.6 Discussion

The main cause of COPD is undoubtedly inhalation of toxic substances such as tobacco smoke, biomass fuels and pollutants, but there is also known to be an epigenetic effect with only 50% of heavy smokers developing COPD (Rennard 2006). Immune cells in the lung are crucial to COPD pathophysiology with environmental stimuli affecting their function (Barnes 2016), but there are also changes in circulating cells that may be associated with disease (Kulikauskaite 2020). In this study, there were differences in *IL-36RN* expression between healthy and COPD circulating monocyte cells. Considering that IL-36R signalling has an important role in COPD pathogenesis, the defective *IL-36RN* expression in these bone marrow derived cells, may represent an innate pre-disposition in certain individuals to develop COPD. A previous study showed no difference between IL-36RA levels in healthy smokers and non-smokers lung samples (Baker 2022): In this study, contemporaneous recruitment of healthy smokers was challenging due to the timing of the covid pandemic. Figure 4.7 shows a negative correlation between smoking pack years and *IL-36RN* expression raising the possibility of a smoking-related effect. A future study should measure *IL-36RN* expression in monocytes from age-matched smokers without lung disease would help to confirm that the reduced *IL-36RN* expression is disease specific rather than due to smoking history. It is important to note that the non-smoker blood donors used for this part of the study were significantly younger than COPD donors, although the mean was ~60 years of age and as such were not considered 'young'. There was, however, no correlation between age and *IL-36RN* expression when non-smoker and COPD groups were analysed separately. This implies that the difference between COPD and healthy *IL-36RN* expression in monocytes is unlikely to be due to lack of age-matching between groups.

In healthy donor cells, there was significant upregulation of *IL-36RN* upon differentiation of monocytes with GM-CSF but not M-CSF. The higher *IL-36RN* expression in GM-MDM rather than M-MDM is consistent with previous findings in which M-1 like, not M2-like, macrophages are a major source of *IL-36RN* (Boutet 2016). These findings are particularly relevant to the lung as GM-MDM, rather than M-MDM, are more representative of alveolar macrophages in terms of cell surface receptors, resistance to oxidative stress, phagocytic capacity and susceptibility to pathogens (Winkler 2008). In COPD, monocytes do not show any significant upregulation of *IL-36RN* upon differentiation to GM-MDM further exaggerating the deficiency in *IL-36RN* expression seen at a monocyte level. As such *IL-36RN* expression is almost 2-fold higher in healthy GM-MDM compared to those from COPD patients. There is a trend towards negative correlation between *IL-36RN* expression and smoking history, measured by pack years. This suggests that oxidative stress may suppress *IL-36RN* expression in fully differentiated macrophages.

Given that IL-36 signalling contributes to neutrophilic inflammation in COPD (Koss 2021, Baker 2022), it would be reasonable to hypothesise that lower *IL-36RN* in monocytes or GM-MDM are correlated with severity of disease. However, there was no correlation between *IL-36RN* expression and lung function. Of note, spirometry is only one basic measure of COPD severity in terms of small airway disease. Unfortunately, other clinical parameters such as exacerbation frequency, symptom burden or radiographic findings which may be more representative of chronic bronchitis and emphysematous phenotypes respectively were not known for the patients used for this part of the study. For a future study the ratio of IL-36γ:IL-36RA on an organ level (e.g. via BAL or tissue homogenate) is likely to be more representative of IL-36R signalling than *IL-36RN* expression alone and thus it would be interested to see if this correlated with COPD severity.

This is the first study which shows that PI3K activity controls *IL-36RN* expression. PI3K activity is increased in the lungs and PBMC of COPD patients and is activated by reactive oxygen species (ROS) (Barnes 2019). As such *in vivo IL-36RN* expression is likely suppressed yet further beyond that which already exists as an innate defect in unstimulated cells *in vitro*. More specifically that of the two PI3K isomers (p110δ and p110γ) which predominate in macrophages, the p110γ catalytic subunit inhibits *IL-36RN* expression. The PI3Kγ inhibitor, AS252424, caused smaller increase in *IL-36RN* expression (197%) than the non-specific PI3K inhibitor, PIK75 (272%). This may be due to non-equivalent dosing: The concentration of AS-252424 was limited to 1μM as higher concentrations affected cell viability. An alternative explanation is that p110α and p110β isomers may also affect *IL-36RN* expression.

PI3K activity controls many essential cellular processes such as cell differentiation, growth, proliferation, DNA repair and apoptosis (Moradi 2021). In COPD, a key PI3K dependent mechanism contributing to disease pathogenesis is cellular senescence (Barnes 2019). ROS cause activation of the PI3K-mammalian target of rapamycin (mTOR) signalling pathway which in turn inhibits expression of SIRT1 and SIRT6. Reduced SIRT1 and SIRT6 levels cause increased NF-κB signalling and promote the release of senescence associated secretory proteins (SASP). The SASP includes cytokines (IL-1β, TNFα, IL-6), chemokines (CXCL1, CXCL8, CCL2), proteases (MMP2, MMP9) and growth factors (TGFβ, VEGF), all of which are elevated in COPD (Barnes 2019, Baker 2020). Outside the lung, increased IL-36R signalling (either due to increased IL-36 agonists or reduced *IL-36RN*) has been noted in many different diseases including osteoarthritis, coronary artery disease, metabolic dysfunction, inflammatory bowel disease and renal fibrosis (Neurath 2020, El-Awaisi 2022). Many of these diseases are associated with accelerated aging and are known co-morbidities of COPD (Barnes 2019). Meanwhile in lung cancers, where cellular senescence is largely protective, reduced IL-36γ and upregulation of IL-36RA and IL-38 are associated with higher grade tumours and increased mortality (Liang 2015, Lv 2016, Takada 2017, Qu 2020). In rat cardiomyocytes, IL-36R knockout activates SIRT1 and in turn results in with decreased

secretion of inflammatory cytokines, reduced myocardial injury, and improved cardiac function (Luo 2020). This supports the idea that upregulated IL-36 signalling might contribute to cellular senescence and drive senescent related diseases. Further work should investigate which cell signalling pathways, miRNA or other molecules downstream of PI3K affect *IL-36RN* expression in MDM and tissue macrophages, or whether by upregulating *IL-36RN* there is any change in markers of senescence in these cells.

It is not known what happens to IL-36R signalling with age in the lung. In mouse heart, IL-36α, IL-36β and IL-36R expression increase with age and this is associated with myocardial ischaemia (El-Awaisi 2022). In the present study there is a trend toward *IL-36RN* expression increasing with age in healthy subjects, but not in COPD. This suggests that in health, lung macrophages have a protective feedback loop or signalling pathway (additional to the PI3K inhibition demonstrated above) that upregulates *IL-36RN* in age. In COPD any protective pathway associated with aging appears to be lost, with *IL-36RN* expression decreasing with age.

A major limitation to the results in this study is that IL-36RA was only measured by *IL-36RN* gene expression and not at protein level due to the poor selectivity and availability of antibodies. At present there is not a sensitive IL-36RA antibody to measure IL-36RA by ELISA. Once a suitable IL-36RA antibody has been developed it would be important to confirm patterns of secretion of IL-36RA in healthy and COPD monocytes, GM-MDM and tissue macrophages as well as the effect of PIK inhibitors on its secretion.

4.7 Conclusion

This chapter demonstrates that *IL-36RN* expression is reduced in COPD monocytes and macrophages compared to health. In healthy subjects there is upregulation of *IL-36RN* expression on differentiation from monocytes into GM-differentiated macrophages, but this does not happen in cells from COPD patients. As such the hypothesis that *IL-36RN* expression is reduced in COPD MDM can be accepted. *IL-36RN* expression by macrophages is controlled by PI3K activity. The p110γ isoform of PI3K downregulates *IL-36RN* expression (Figure 4.14). In COPD, cigarette smoking upregulates PI3K activity and therefore is likely to contribute further to reduced *IL-36RN* expression. Increasing age and pack year history are associated with lower levels of *IL-36RN* expression in COPD.

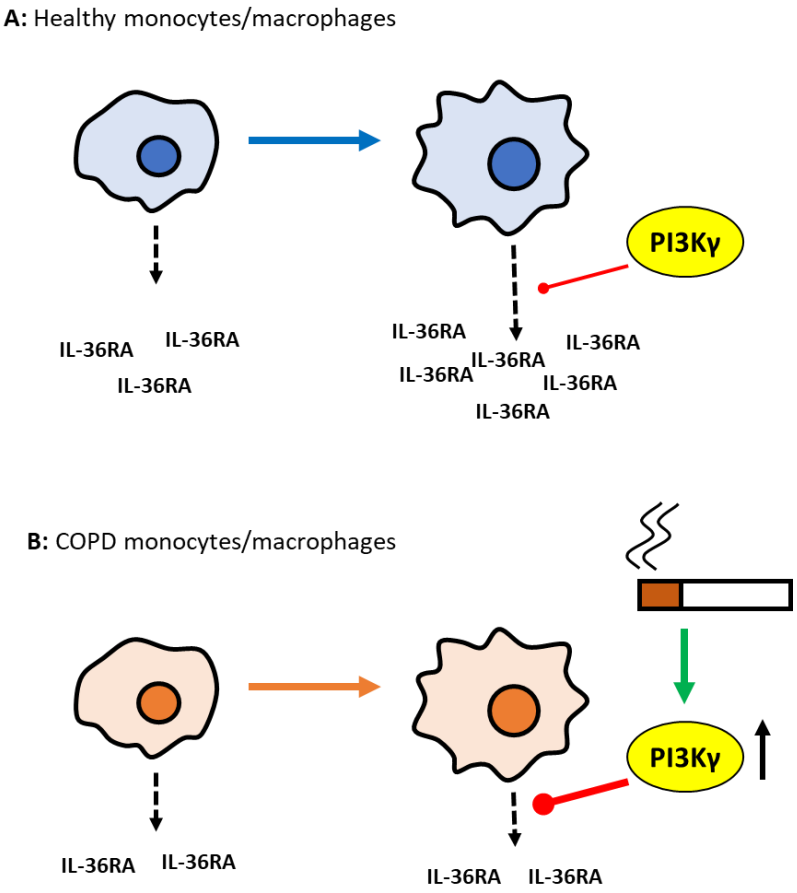

Figure 4.14: Schematic showing regulation of *IL-36RN* expression in non-smoker and COPD macrophages

(A) Non-smoker monocytes upregulate expression of *IL-36RN* on differentiation to macrophages. PI3Kγ activity inhibits *IL-36RN* expression. (B) At baseline COPD monocytes show less *IL-36RN* expression compared to non-smoker monocytes. There is no upregulation of *IL-36RN* on differentiation to macrophages. Cigarette smoking causes ROS which activates PI3K. Upregulated PI3Kγ activity inhibits *IL-36RN* expression.

Chapter 5: Using non-invasive nasal sampling to investigate inflammation in COPD at stable state and during exacerbation

5.1 Introduction

COPD is defined and diagnosed on the basis of an obstructive FEV1/FVC ratio (<0.7) on spirometry. Despite this, it well recognised that severity of obstruction has very little correlation with symptom burden, prognosis or response to treatment. This is partly because of the large heterogeneity which is encompassed by the term COPD. There have been multiple large multicentre international longitudinal studies including ECLIPSE, COPDgene, SPIROMICS which all aimed to phenotype COPD (Rennard 2015, Keene 2017, Vanfleteren 2023). Some common themes are seen between these cohorts in terms of clinical clusters including: (a) patients with mild symptoms and mild impairment of FEV_1 and relatively good prognosis; (b) patients with high cardiac comorbidity and high mortality; (c) frequent exacerbators with high symptom burden, severe FEV_1 impairment, emphysema and high mortality (Garcia-Aymerich 2011, Rennard 2015, Burgel 2017, Vanfleteren 2023). Despite these common themes, there remains some variation in clinical characteristics of clusters and there is a significant failure to show reproducibility in biomarkers between comparative groups (Keene 2017). As such the medical and scientific world is in no doubt of the heterogeneity within COPD, but the disease continues to fall behind other respiratory diseases such as asthma in terms of clear treatable phenotypes. Indeed, besides the clearly defined α1-AT deficiency, there are at present there are only two identifiable phenotypes which are commonly accepted and affect pharmacological management of COPD: eosinophilic COPD and frequent exacerbator phenotypes.

15-40% of COPD patients show the presence of eosinophilic airway inflammation (Segal 2018). These patients are more corticosteroid responsive in terms of health status, lower FEV_1 and exacerbation frequency than neutrophilic counterparts (Agusti 2018, Bafadhel 2018). This T2 driven inflammatory phenotype has been given extra credence recently as dupilumab, a monoclonal antibody targeting IL-4 and IL-13 receptor (IL-4Rα), and mepolizumab (anti IL-5 mAb) have shown efficacy in COPD patients with high blood eosinophils and high exacerbation rates (Bhatt 2024, Bhatt 2023, Sciurba 2025). Meanwhile benralizumab, an anti-IL-5 antibody, has shown promise in improving outcomes from exacerbations in asthma and COPD patients with high exacerbation blood eosinophil counts (Ramakrishnan 2025). Blood eosinophil counts are often used as a substitute biomarker for eosinophilic airway inflammation in COPD in research studies and RCTs despite mixed reports as to whether airway and blood eosinophil counts correlate (Negewo 2016, Papaioannou 2017, Turato 2017) and an acknowledgement that blood eosinophil counts are very variable over time especially amongst patients with high blood eosinophils >0.3 $x10^9$cells/litre (Brightling 2016, Turato 2017, Beech 2024). A more representative biomarker for TH2 driven eosinophilic airway inflammation may help identify cohorts who would benefit from treatment most.

The frequent exacerbator phenotype is another very reproducible and commonly accepted phenotype affecting approximately 1 in 4 COPD patients (Finney 2024). The ECLIPSE cohort showed that the strongest predictor of future AECOPD was the number of previous exacerbations (Vestbo 2014). In various cohort studies frequent exacerbators (≥2/year) have been shown to have a higher inflammatory status even in a relatively stable state; however, no one biomarker appears to be a reliable indicator of exacerbation risk (Perera 2007, Vestbo 2014, Rennard 2015). Other clinical predictors of exacerbation risk besides previous history of exacerbation include chronic bronchitis, bacterial colonisation, bronchiectasis, emphysema, gastro-oesophageal reflux disease, diabetes and cardiovascular disease (Finney 2024). AECOPD are very clinically important: They are associated worse quality of life, accelerated disease progression, increased hospitalisation and increased mortality and as such carry a large economic burden (Seemungal 1998, Donaldson 2002, Soler-Cataluna 2005, Maselli 2019). AECOPD may be caused by respiratory infections (viral, bacterial or co-infection), environmental pollutants or an unknown factor (Vogelmeier 2017). As with the disease overall, there appears to be heterogeneity in inflammatory profiles associated with exacerbations. Bafadhel identified 4 main exacerbation clusters: bacterial, viral, eosinophilic and pauci-inflammatory (Bafadhel 2011). Of the cytokines measured in this study sputum IL-1β, serum CXCL10, and blood eosinophil count were the best predictors of bacterial, viral and eosinophilic exacerbations respectively (Bafadhel 2011). Other studies have differentiated exacerbations by cell type: neutrophilic, eosinophilic, mixed granulocytic or pauci-granulocytic (Gao 2013). Currently the mainstay of treatment of exacerbation is: (a) prevention via vaccination, inhaled corticosteroids and bronchodilators (b) treatment of AECOPD with oral prednisolone with or without antibiotics. Better understanding of COPD susceptibility to exacerbations and pathogenesis of the exacerbation itself is needed in order to have more targeted therapies.

One of the limiting factors of large COPD cohort studies to phenotype and find clinically useful biomarkers is finding a suitable sampling method. Directly sampling the respiratory tract via bronchoscopy or surgical resection is invasive with risks to patient. It is also often unfeasible in large cohort studies due to requirement of highly skilled operators, time and cost restraints. Blood, sputum and breath samples have all been extensively used to measure biomarkers of inflammation in patients with respiratory disease: but each of these sampling methods has a different set of inherent problems. Blood is too far from the site of airways disease, thus mediators become dilute in the circulatory volume, in an environment that is maintained by homeostasis via metabolism in the plasma and liver and excretion into the urine. Sputum has issues of oral contamination, and mediator degradation as well as inherent challenges to obtain samples as it not spontaneously produced by many COPD patients and healthy volunteers. Exhaled breath has issues because samples are influenced by saliva

and the upper gastrointestinal and respiratory tracts; while condensed water vapour causes some protein biomarkers to become denatured.

These issues with blood, sputum and breath samples have provided the impetus to study biomarkers in mucosal lining fluid (MLF) absorbed from the surface of the airway mucosa (Thwaites 2018). The Hansel group has developed precision techniques to absorb concentrated airway mucosal lining fluid [MLF], using synthetic absorptive matrix (SAM) strips. The SAM used consist of medical grade material, with defined absorption characteristics and low protein binding: giving high and consistent levels of eluted biomarkers (Thwaites 2018). SAMs can be used to sample the endobronchial MLF via a bronchoscopy (bronchosorption), the nasal MLF by inserting the SAM under direct visualisation up the nostril (nasosorption), or to abso sputum (sputosorption) (Thwaites 2018). The absorbent materials are soft and comfortable for patients and can obtain MLF even from inflamed noses at frequent intervals over extended periods of time. Levels of cytokines and chemokines, prostanoids, leukocyte granule proteins, complement, antibodies and viruses have all been measured from nasosorption and bronchosorption samples without the issues of unknown dilution factors present in nasal or bronchial lavage.

Nasosorption has been used to successfully study airway responses in asthma, allergy and respiratory infection:

- **Asthma:** The first description of nasosorption with SAM showed differences in type 2 cytokines (IL-5 and IL-13) in allergic and non-allergic children (Chawes 2010). In adults with asthma, it has been shown that nasosorption IL-5 is correlated with markers of lower respiratory tract TH2 inflammation including sputum eosinophils and sputosorption IL-5 levels (Melo 2019).
- **Allergy:** Nasal allergen challenges have demonstrated that nasosorption can be used to study phased response including the molecular basis of early mast cell activation and complement activation (up to 1 hour) and late type 2 (eosinophilic) inflammation (from 2 to 12 hours) in response to timothy grass pollen in atopic individuals (Thwaites 2018), as well as the effect of drug treatment (anti-IL-13 mAb and steroids) on suppressing inflammatory response to birch pollen (Nicholson 2011).
- **Respiratory viruses:** Nasosorption has been used to study immune response to Human rhinovirus (HRV), respiratory syncitial virus (RSV), influenza and COVID-19 in both real world situations, human challenge models (live attenuated influenza virus) and vaccine responses (covid-19 vaccine): measuring study cytokine response, nasal IgA and IgG antibodies and viral load (Mallia 2011, Hansel 2017, Thwaites 2017, Jha 2019, Baker 2022, Liew 2023).

A number of studies have shown that nasal inflammation is increased in COPD patient at stable state (Vachier 2004, Hurst 2010) and that levels of upper and lower airway inflammation within an individual also correlate (Hurst 2005). Batista showed in a small study (n=12) a good correlation between endobronchial and nasal CXCL8 concentrations taken using a SAM via bronchosorption and nasosorption (Batista 2016). As such, nasosorption is particularly attractive in the context of investigating elderly and co-morbid COPD patients in which it is difficult to justify the risks of invasive bronchoscopies. As a quick and safe sampling method, nasosorption allows for large numbers of patients to be sampled without limitations of severity of disease, age or clinical co-morbidity and also allows sampling during exacerbation when other sampling techniques such as BAL are relatively contra-indicated. As described above, nasosorption has shown promise in asthma, atopy, viral exacerbation and smoking studies (Ross 2015, Hansel 2017, Leaker 2017, Jha 2018) but this is the first study looking at the utility of nasosorption in investigating COPD inflammation.

Figure 5.1 outlines some of the major inflammatory pathways involved in COPD pathogenesis. In order to investigate inflammatory profiles, cytokines were measured in nasosorption samples by MSD and ELISA. 22 cytokines were chosen on the MSD panel to represent:

- Neutrophilic inflammation: CXCL1, CXCL8, GM-CSF, IL-1β, IL-6, IL-17A, IL-36
- Eosinophilic inflammation: IL-4, IL-5, IL-13, IL-33, Eotaxin-1, TSLP, (CCL17 (TARC)
- Virally induced inflammation: CXCL10 (IP-10), CXCL11 (I-TAC), IFNα-2a, IFNβ, IFNγ, MCP-1, MIP-1β, IL-10
- Bacterially induced inflammation: CXCL8, GM-CSF, IL-1β, IL-17A, MCP-1

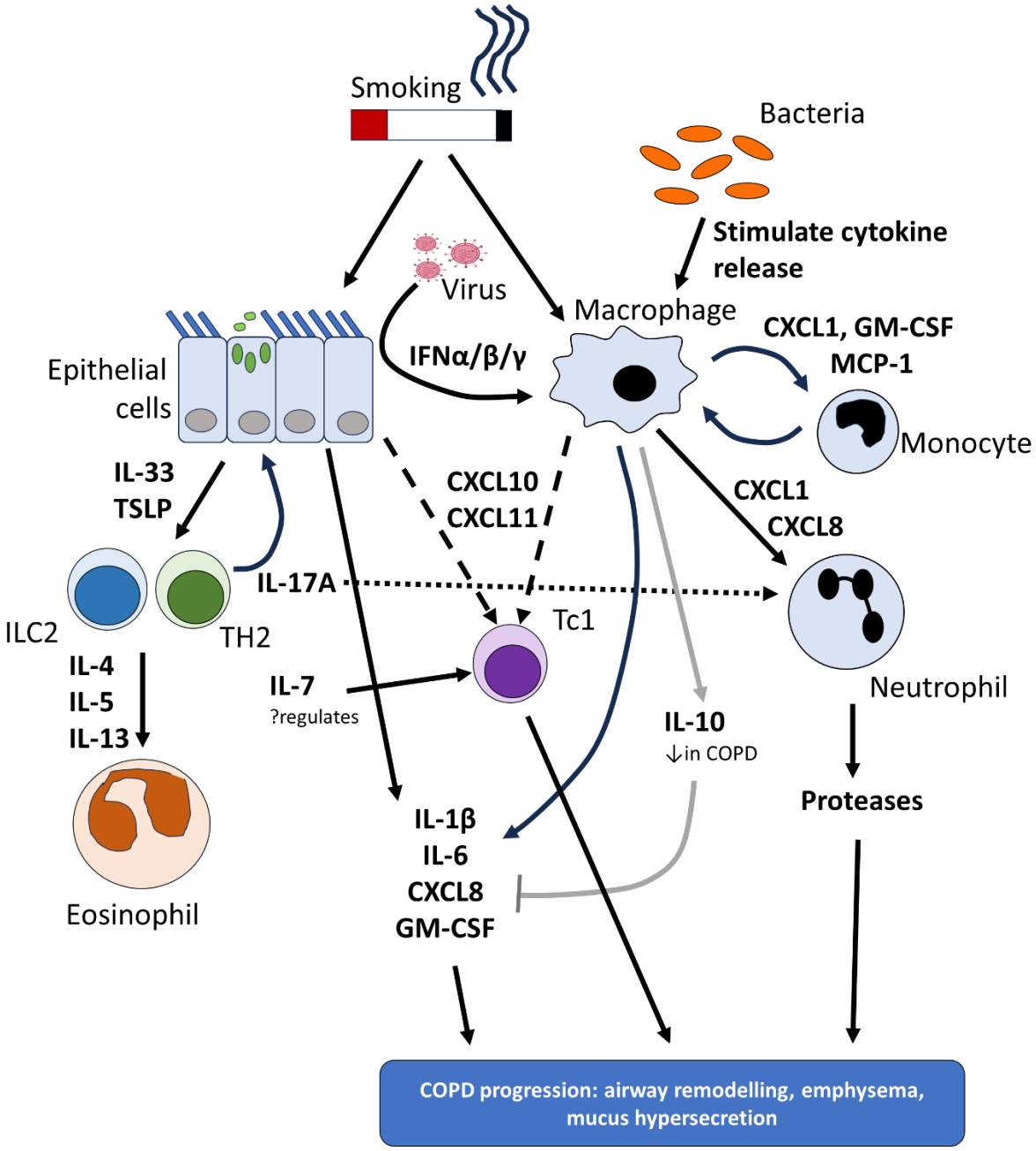

Figure 5.1: Inflammatory pathways in COPD
Smoking activates small airway epithelial cells to secrete IL-33 and TSLP which in turn activates innate lymphoid cells (ILC) 2 and T helper (TH2) cells to recruit eosinophils to the lung. Stimulation of SAEC by viruses causes an interferon (IFN) response and this activates macrophages to be more pro-inflammatory. COPD macrophages are more pro-inflammatory than healthy macrophages releasing pro-inflammatory cytokines, and neutrophil chemoattractants, as well as chemotactic factors (CXCL10 and CXCL11) which recruit $CD8^+$ T cells (Tc1). Bacterial infection promotes the release of pro-inflammatory cytokines by macrophages.

As investigated in Chapters 3 and 4, IL-36 family have been shown *in vitro* to be important within COPD pathogenesis. IL-36γ is released by small epithelial cells and its levels significantly elevated in COPD compared to healthy non-smokers (Baker 2022) . To understand whether nasosorption might be a useful sampling method for investigating IL-36 *in vivo,* IL-36α, IL-36β, IL-36γ and IL-36RA were measured in nasosorption samples by ELISA. This chapter compares nasal IL-36 cytokines in healthy controls and COPD patients at baseline as well as COPD in stable state and exacerbation.

5.2 Hypothesis

Nasosorption can detect differences in COPD compared to health.

5.3 Aims

- Identify whether nasosorption can detect differences in common inflammatory cytokines known to be elevated in COPD compared to health.
- Investigate whether nasosorption can be used to identify different inflammatory signal in common COPD clinical phenotypes
- Investigate changes in nasal inflammation during acute exacerbation of COPD
- Investigate whether nasosorption can be used to study IL-36 family cytokines *in vivo* in health and COPD (stable state and exacerbation)

5.4 Methods

5.4.1 Subject recruitment

Healthy non-smokers, smokers and ex-smokers without lung disease and COPD patients were recruited as described in Section 2.2.1. All patients were clinically phenotyped in terms of symptom burden (CAT score, mMRC), exacerbation frequency (number in last 12 months), degree of airway obstruction (FEV1, FEV1/FVC). Drug history was noted. Where available full lung function, cross sectional imaging, eosinophil trend was noted and analysed. See Section 2.2.2 for full details of clinical assessment. All patients had nasosorption performed at stable state (at least 4 weeks clear of respiratory tract infections or exacerbations of COPD) as described in Section 2.2.2.1.

5.4.2 Exacerbation study recruitment

COPD patients were asked to telephone an acute exacerbation helpline in the context of worsening of their symptoms. Where acute exacerbation was suspected a home visit was performed within 24h of the call, patient was assessed and if AECOPD was confirmed nasosorption, blood test (FBC, CRP) and nasopharyngeal swab for respiratory viral PCR was performed as described in Section 2.2.2.2. Sputum was collected if COPD patients were productive at exacerbation visit.

A recovery visit was made at least 6 weeks after initial exacerbation visit during which nasosorption was repeated. In cases where a second exacerbation had occurred during this time, the recovery visit was postponed until 6 weeks clear of most recent exacerbation. Of note, not all subjects in the exacerbation study had baseline nasosorption done prior to exacerbation visit.

5.4.3 Sample processing

Nasosorption samples were transferred to the lab on ice, diluted in 300µl nasosorption buffer, centrifuged for 20min (16,000 x *g* at 4°C) and then stored for later use at -80°C as described in Section 2.2.2.5.2. Full blood count, C reactive protein, respiratory viral PCR panel and sputum microscopy, culture and sensitivities were performed by the Imperial College Healthcare NHS Trust laboratory.

5.4.4 Mesoscale discovery

Nasosorption samples were analysed by MSD as described in Section 2.2.7 using a customised U-plex 10-spot assay, 96-well plates (Meso Scale Diagnostics, Maryland US). Each plate contains its own calibrators. Electrochemiluminescence was quantified and cytokine concentrations were then interpolated from standard curves using MSD discovery workbench version 4 analysis software. Table 5.1 outlines the 22 analytes measured, with the upper and lower limits of detection for each. When sample concentrations were below or above the lower limit of

detection, the value of lower or upper limit of detection was used in place of an accurate number.

Analyte	Lower limit of detection (pg/ml)	Upper limit of detection (pg/ml)	Analyte	Lower limit of detection (pg/ml)	Upper limit of detection (pg/ml)
CCL17 (TARC)	22.37	15400.00	**IL-5**	14.69	21550.00
CXCL10 (IP-10)	13.61	55500.00	**IL-6**	8.56	9200.00
CXCL11 (I-TAC)	0.16	7500.00	**IL-7**	22.71	34300.00
CXCL8 (IL-8)	1.94	11700.00	**IL-10**	0.0012	47650.00
Eotaxin-1	4.51	33250.00	**IL-13**	0.75	47650.00
GM-CSF	0.00027	47650.00	**IL-17A**	23.14	153500.00
IFNα-2a	13.06	189000.00	**IL-33**	22.54	48950.00
IFNβ	129.39	530000.00	**MCP-1**	7.53	30500.00
IFNγ	0.01	47650.00	**MIP1b**	56.56	12050.00
IL-1β	2.71	22800.00	**TSLP**	10.40	36350.00
IL-4	0.82	9950.00			

Table 5.1: MSD analytes with respective upper and lower limits of detection
22 analytes were measure on MSD. Each of these had its own upper and lower limits of detection.

5.4.5 Measurement of inflammatory mediators via ELISA

IL-36α, IL-36β, IL-36γ and IL-36RA concentrations in the SAF and MDM supernatants were measured by ELISA using commercially available ELISA kits as described in Section 2.2.6. All samples were run in duplicate. Nasosorption samples were diluted 1 in 15, 1 in 15, 1 in 20 and 1 in 2 with 0.5% ($^{w}/_{v}$) BSA in D-PBS for measurement of IL-36α, IL-36β, IL-36γ and IL-36RA respectively. Each ELISA plate contains its own set of standards and concentrations of samples were interpolated from this standard curve. Optical density was measured using Spectramax microplate reader at λ450nm and λ590nm.

The CXCL1 standard did not read correctly on the MSD, therefore CXCL1 was measured by ELISA. Samples were run at a 1 in 10 dilution with 0.5% ($^{w}/_{v}$) BSA in D-PBS as described in Section 2.2.6.3.

5.4.6 Statistics

Generally, categorical variables are presented as numbers (percentages), parametric continuous variables are presented as the mean ± standard deviation (SD), and nonparametric continuous variables are presented as median and interquartile ranges (25th and 75th percentiles). Analysis performed using GraphPad Prism software (version 9.5.1). Statistical analyses are detailed in figure legends, these included corrections for multiple pairwise comparisons where relevant.

5.5 Results

5.5.1 Subject demographics

The characteristics of healthy and COPD subjects used in this study are shown in Table 5.2. COPD participants were slightly older than those in non-smoker and healthy smoker groups. There were significant differences in obstructive lung function parameters (FEV_1(L), FEV_1 % and FEV_1/FVC) and smoking pack years between COPD and healthy groups. COPD patients also had significantly lower FVC compared to non-smokers, but not healthy smokers this may be due to lower numbers in the healthy smoking group. Healthy smokers had fewer pack years than COPD participants, but this was not statistically significant.

	Non-smokers	Healthy Smokers	COPD
Number	31	7	45
Age (years)	61.1 ± 1.6	62.0 ± 4.9	68.1 ± 1.4**
Sex (male:female)	16:15	01:06	22:23
FEV_1 (L)	2.79 ± 0.15	2.83 ± 0.27	1.37 ± 0.09***$^{###}$
FEV_1 (%)	103.5 ± 3.4	101.6 ± 6.12	55.6 ± 3.0***$^{###}$
FVC (L)	3.77 ± 0.22	3.70 ± 0.31	2.85 ± 0.12**
FVC (%)	112.4 ± 3.6	103.8 ± 5.05	93.4 ± 3.3**
FEV_1/FVC	0.75 ± 0.01	0.76 ± 0.02	0.47 ± 0.02***$^{###}$
Current smokers	0 (0%)	2 (29%)	11 (24%)
Smoking history (pack years)	0.0 ± 0.0	23.14 ± 5.69*	53.92 ± 4.66***

Table 5.2: Subject demographics for non-smoker, healthy smoker COPD study participants
FEV1: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for one year. Data are presented as mean ± SEM. Kruskall-Wallis with Dunn's post-test was performed to test for significant differences between subject groups. * $p<0.05$, ** $p< 0.01$, *** $p< 0.001$ compared to healthy non-smoking controls. $^{\#}$ $p<0.05$, $^{\#\#}$ $p< 0.01$, $^{\#\#\#}$ $p< 0.001$ compared to smokers without lung disease.

5.5.2 Using nasosorption to differentiate health and COPD

Nasosorption was performed on 23 Non-smokers, 7 healthy smokers and 32 COPD patients at stable state. 22 cytokines were measured on each nasosorption sample by MSD using 3 separate multiplex assay plates and the appropriate calibrators. Cytokine concentrations were detected in most samples: However, 3/22 analytes (IFNγ, IL-4, IL-5) were below the lower limit of detection in more than 50% of the samples.

CXCL8, IL-4, IL-5, GM-CSF and IFN-γ were significantly lower in COPD than non-smokers (Figure 5.2). Meanwhile IL-7 and IFN-α2a were higher in healthy smokers compared to COPD. There were no cytokines which were significantly increased in COPD compared to healthy subjects. Direct comparison between COPD and non-smoker groups (excluding healthy smoker cohort given small sample numbers) and analysis using Mann-Whitney test highlighted significantly lower IL-6 and significantly higher eotaxin-1 in COPD samples compared to healthy non-smokers (Figure 5.3).

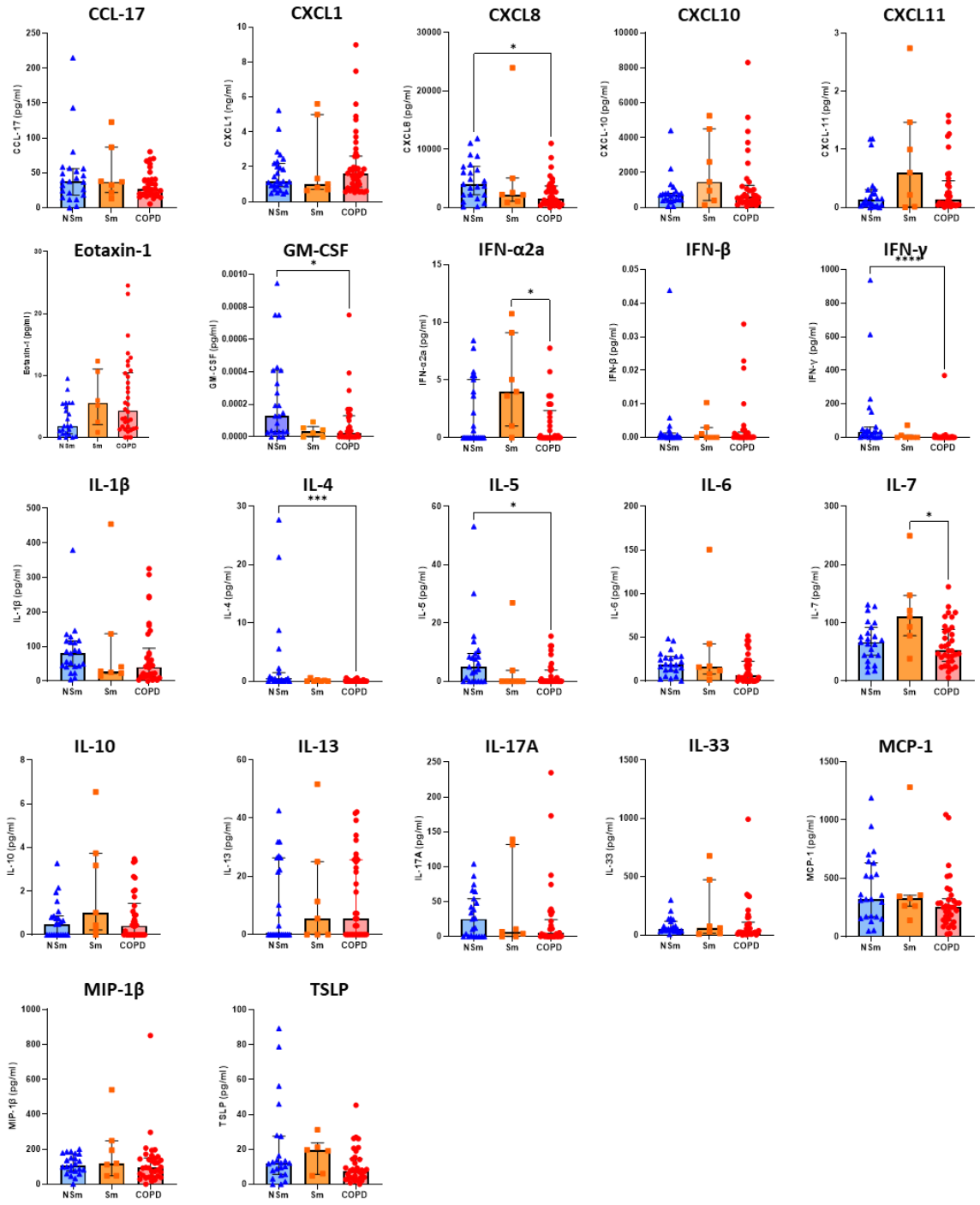

Figure 5.2: Nasal epithelial lining fluid cytokine concentrations in non-smoker, healthy smokers and COPD subjects

Nasosorption was performed in non-smokers, healthy smokers and COPD patients at stable state. Data are presented as mean ± SEM. Kruskall-Wallis with Dunn's post-test was performed to test for significant differences between subject groups. * p<0.05, ** p< 0.01, *** p< 0.001. n=23 non-smokers, n=7 healthy smokers, n=34 COPD.

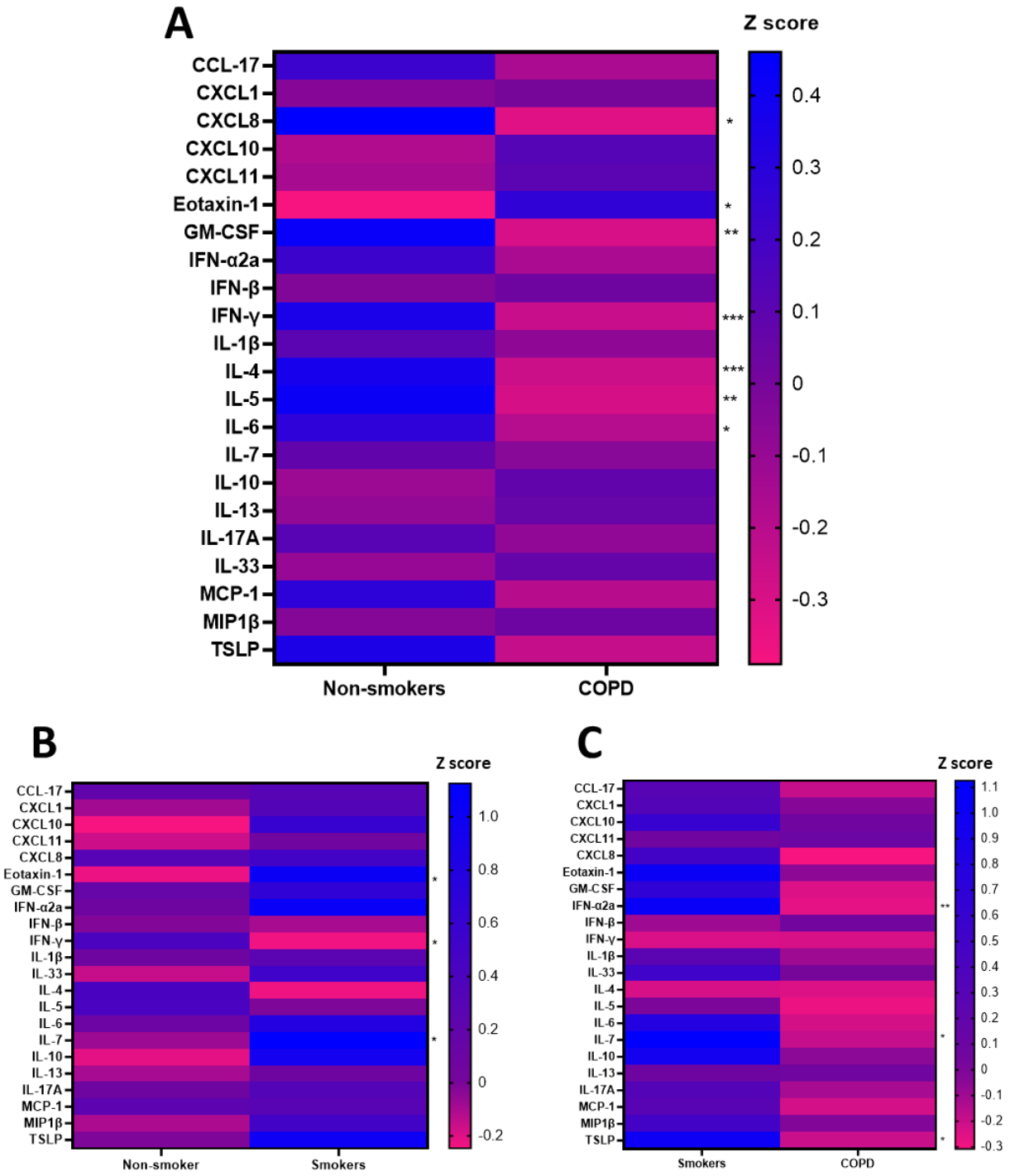

Figure 5.3: Heatmaps of nasal epithelial lining fluid cytokine concentrations

Nasosorption was performed in non-smokers (n=23), healthy smokers (n=7) and COPD (n=34) participants, and 22 analytes measured by MSD. Data are presented as heatmap of (A) Non-smokers vs COPD (B) Non-smokers vs healthy smokers (C) Healthy Smokers vs COPD and analysed using Mann Whitney test and then applying benjamini, krieger and yekutieli to control the false discovery rate. * $p<0.05$, ** $p< 0.01$, *** $p< 0.001$

5.5.2.1 *Effect of nasal corticosteroids on nasal cytokine concentrations*

5/34 (14.7%) COPD were on regular nasal corticosteroids at the time of sampling. Given that corticosteroids are known to affect release of inflammatory mediators, the data were analysed to determine the effect of nasal corticosteroids on cytokine measurements. There was no significant difference between COPD patients on nasal corticosteroids (n=5) or not on nasal corticosteroids (n=29) in any of the cytokine levels measured by MSD (Table 5.3). When COPD patients who were on nasal corticosteroids were excluded, there was no difference in the trends of cytokine concentration between non-smoker, smoker and COPD subjects compared to that displayed in Figures 5.2 and 5.3.

	No nasal steroid	Nasal steroid	p value
n	29	5	
CCL-17	34.42 ± 3.22	28.53 ± 13.18	0.191489
CXCL1	0.84 ± 0.24	2.30 ± 1.65	0.664706
CXCL8	2788.31 ± 487.91	1241.41 ± 581.07	0.148748
CXCL10	1382.33 ± 346.99	787.07 ± 446.87	0.341613
CXCL11	1.144 ± 0.76	0.14 ± 0.09	0.267412
Eotaxin-1	6.44 ± 1.06	6.83 ± 4.53	0.563715
GM-CSF	0.00 ± 0.00	0.00 ± 0.00	0.709796
IFN-α2a	1.37 ± 0.37	0.16 ± 0.12	0.390277
IFN-β	0.00 ± 0.00	0.01 ± 0.01	0.199511
IFN-γ	14.34 ± 12.72	2.16 ± 2.16	>0.999999
IL-1β	71.38 ± 15.33	85.46 ± 61.57	0.663817
IL-4	0.08 ± 0.03	0.05 ± 0.05	0.78509
IL-5	2.48 ± 0.81	2.14 ± 2.14	0.530954
IL-6	13.64 ± 2.77	12.14 ± 10.04	0.332381
IL-7	63.94 ± 6.10	56.18 ± 27.39	0.320622
IL-10	0.77 ± 0.19	1.32 ± 0.70	0.367514
IL-13	12.75 ± 2.73	11.73 ± 6.88	0.895359
IL-17A	23.81 ± 9.99	22.79 ± 15.03	0.802243
IL-33	114.73 ± 38.86	26.94 ± 16.05	0.163403
MCP-1	288.91 ± 36.47	305.58 ± 189.30	0.294592
MIP1β	131.72 ± 28.56	86.70 ± 26.44	0.477474
TSLP	10.25 ± 1.91	12.09 ± 3.19	0.336654

Table 5.3: Nasal cytokine concentrations of COPD patients on nasal steroid and not on nasal steroids spray
Nasosorption performed in COPD patients and cytokine concentrations measure by MSD and ELISA (pg/ml). COPD patients were subcategorised into those not using nasal steroids (n=29) and those using nasal steroids (n=5). Data presented as mean ± sem. Data analysed by Mann-Whitney tests to test for significant differences between subject groups. There were no significant differences found.

5.5.2.2 *Effect of current smoking status on nasal cytokine concentrations*

In order to understand whether current smoking status affected nasal cytokine concentrations, participants were grouped into lifelong non-smokers, ex-smokers (healthy or COPD) and current smokers (healthy or COPD). IL-6 was higher in ex-smokers than current smokers. Other than IL-6, there was no effect of being a current smoker on any other cytokine as measured by nasosorption (Table 5.4). The differences between ex-smokers or current smokers and non-smokers represent a similar pattern seen to differences between health and COPD in light of the small healthy ex-smoker numbers.

Cytokine (pg/ml)	Never smoker	Ex-smoker	Current smoker
CCL-17	46.71 ± 10.36	38.46 ± 4.86	31.19 ± 5.59
CXCL1	1.61 ± 0.22	2.24 ± 0.32	1.82 ± 0.49
CXCL8	**4,440.00 ± 671.50**	3,227.00 ± 813.40	**2,098.00 ± 913.10 ***
CXCL10	858.40 ± 195.60	1,568.00 ± 347.90	1,206.00 ± 541.70
CXCL11	0.27 ± 0.08	1.26 ± 0.76	0.31 ± 0.14
Eotaxin-1	**2.94 ± 0.59**	**12.70 ± 5.76 ***	5.00 ± 1.31
GM-CSF	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
IFN-α2a	2.23 ± 0.61	1.78 ± 0.42	2.06 ± 1.12
IFN-β	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
IFN-γ	**111.60 ± 48.87**	**16.80 ± 12.86 *** **	**2.40 ± 1.44 ****
IL-1β	**83.96 ± 16.48**	84.89 ± 19.97	**53.80 ± 28.16 ***
IL-4	**3.20 ± 1.55**	**0.09 ± 0.03 ****	**0.06 ± 0.05 ****
IL-5	**7.83 ± 2.61**	2.95 ± 1.07	**1.11 ± 1.11 ****
IL-6	**19.49 ± 2.81**	**20.71 ± 5.46**	**6.62 ± 3.71 ** ~**
IL-7	67.96 ± 7.43	75.98 ± 9.87	64.28 ± 9.90
IL-10	0.60 ± 0.17	1.06 ± 0.30	1.22 ± 0.37
IL-13	9.93 ± 2.91	11.26 ± 2.85	17.75 ± 4.39
IL-17A	27.60 ± 6.51	26.86 ± 10.02	20.94 ± 15.55
IL-33	81.19 ± 14.52	103.00 ± 28.07	160.60 ± 93.17
MCP-1	398.40 ± 62.80	346.90 ± 55.73	209.10 ± 25.41
MIP1β	111.10 ± 11.77	130.50 ± 19.56	144.30 ± 71.96
TSLP	20.63 ± 5.31	25.68 ± 14.90	11.78 ± 4.22

Table 5.4: Nasal cytokine concentrations grouped by smoking status
Cytokines measured in nasosorption samples by MSD. Never smokers, ex-smokers (healthy or with COPD) and current smokers (healthy or with COPD) were compared. Data are presented as mean ± SEM. Kruskall-Wallis performed to test for significant differences between subject groups. * p<0.05, ** p< 0.01, *** p< 0.001 compared to never smokers. ~ p<0.05 compared to ex-smokers.

5.5.3 COPD clinical characteristics

Having investigated whether nasosorption could differentiate between health and disease, the COPD cohort was then sub-analysed in order to try and identify phenotypes. All COPD patients had spirometry, clinical assessment of symptoms, exacerbation frequency, blood eosinophil counts and medication history to characterise their disease. 32/45 (71%) had more detailed lung function including transfer factor and / or lung volumes. 38/45 (84%) had cross sectional imaging of the chest.

The COPD cohort in this study represents a range of severities and phenotypes. GOLD stages 1 to 4 are all represented with the majority of patients having moderate (GOLD 2) or severe (GOLD 3) obstruction (Figure 5.4).

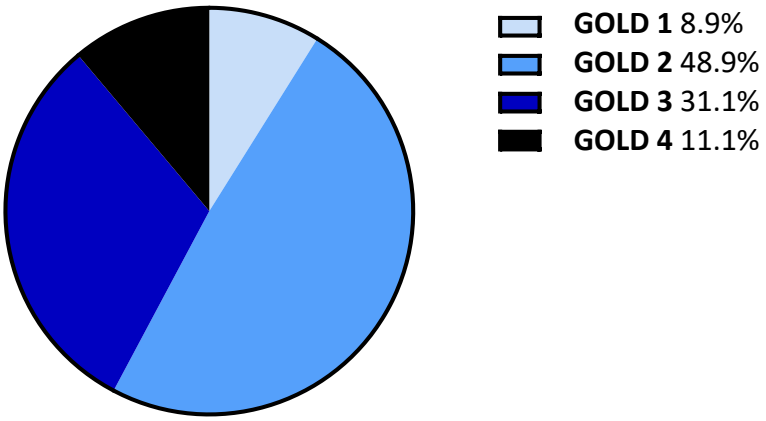

Figure 5.4: Severity of obstruction in COPD study participants as per GOLD criteria
FEV_1 measured in all COPD patients. Participants characterised as per GOLD criteria. GOLD 1: FEV_1 <30%, GOLD 2: FEV_1 = 30 - 50%, GOLD 3: FEV_1 = 50 – 80%, GOLD 4 ≥80%.

In addition to the range in severity of obstruction, the COPD cohort also represented a wide range of symptom severity. The COPD Assessment Test (CAT), in which 8 different symptoms are scored 0-5 (5 representing worse symptoms) showed a range of scores from 4/40 to 38/40 with a mean of 17.2 ± 1.6 (Figure 5.5 Panel A). Looking at subdivisions of CAT score categories patients scored breathlessness significantly higher than all other categories (Figure 5.5 Panel C). The mMRC was also used for patients to grade their breathlessness, with patients representing all 4 scores (Figure 5.5 Panel B).

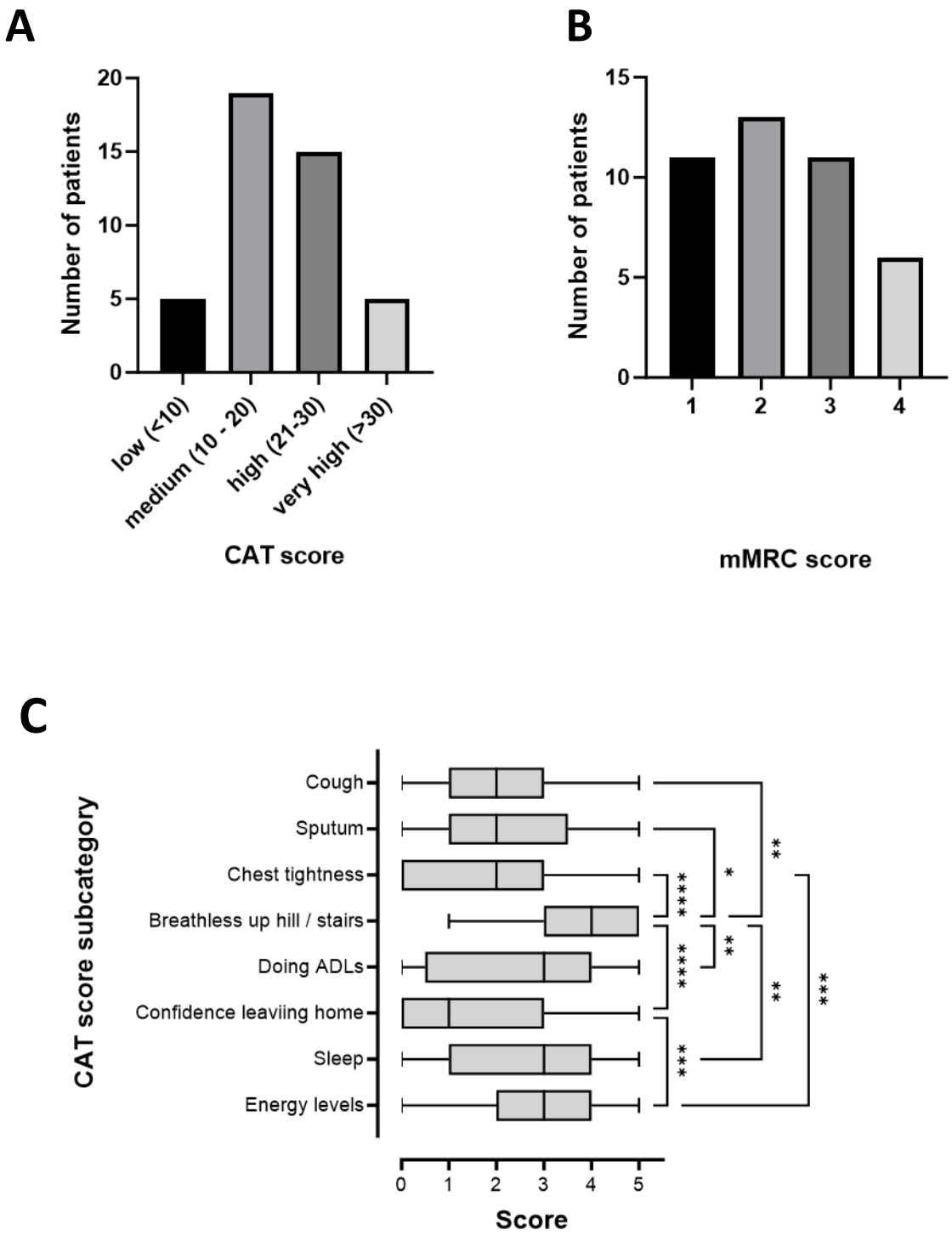

Figure 5.5: Symptom burden in COPD study participants as CAT score and mMRC score
All COPD patients were asked to score their symptoms using CAT and mMRC scores. (A) The distribution of total CAT scores and (B) mMRC scores is shown. CAT scores are compiled of individual score from 8 different categories. (C) The distribution of patients' scores for each of the 8 CAT score subcategories is graphed. n=42

Table 5.5 summarises main clinical characteristics of COPD patients according to GOLD stages 1-4. There was no significant difference between GOLD stages in terms of demographics or smoking history. There was also no significant difference in symptom burden in terms of mMRC, CAT score or exacerbation frequency between GOLD stages 1-4. Indeed, as shown in Table 5.6, overall symptom burden, as measured by CAT score did not correlate with severity of obstruction, or amount of emphysema on CT or as defined by transfer factor. Instead, breathlessness scores (0-5 score on CAT) and mMRC correlated with both severity of obstruction and degree of hyperinflation as measured by Residual Volume divided by the Total Lung Capacity (RV/TLC). Neither measure of breathlessness showed correlation with extent of emphysema on CT or using transfer factor as a surrogate for emphysema.

	GOLD 1	**GOLD 2**	**GOLD 3**	**GOLD 4**
Number (% total)	4 (9%)	22 (49%)	14 (31%)	5 (11%)
Age (y)	68.2 ± 1.9	66.8 ± 2.1	70.3 ± 2.9	67.6 ± 4.2
Male (%)	2 (50%)	7 (32%)	9 (64%)	4 (80%)
BMI (kg/m^2)	24.3 ± 1.8	27.1 ± 1.1	22.6 ± 1.1	25.4 ± 2.9
Current smokers (%)	2 (50%)	7 (32%)	3 (21%)	0 (0%)
Smoking history (pack years)	75.3 ± 26.0	51.2 ± 5.4	53.9 ± 8.6	57.7 ± 7.2
Exacerbations in last 12m (IQR)	0 (0-2)	1 (0-7)	0.5 (0-11)	2 (0-5)
Symptom burden				
mMRC (IQR)	2.75 (1-3)	2 (1-4)	2.5 (1-4)	3 (2-4)
CAT score	14 ± 3.5	19.8 ± 1.8	21.8 ± 2.3	20.25 ± 1.3

Table 5.5: Clinical characteristics of COPD patients according to GOLD stage
Demographics and clinical characteristics of COPD patients when characterised by GOLD stage. Unless otherwise specified continuous variables expressed as mean ± sem and discrete variables as median (interquartile range). * $p<0.05$, ** $p< 0.01$ *** $p<0.001$.

	Total CAT score	Breathlessness CAT subcategory	mMRC
FEV_1 (L)	-0.172	**-0.387 ***	**- 0.400 ****
FEV_1 (%)	-0.224	**-0.372***	**- 0.337 ***
FEV_1/FVC	-0.096	-0.246	**- 0.326 ***
RV/TLC	0.241	**0.401 ***	**0.525 ****
CT emphysema	-0.083	0.030	0.143
$TLCO_c$	-0.087	0.007	-0.164
$TLCO_c$ (%)	-0.031	0.087	-0.252

Table 5.6: Correlation between symptom scores and objective markers of COPD severity
COPD patients self-assessed their symptoms with CAT and mMRC scores. Symptoms in terms of overall CAT, breathless subcategory of CAT score and mMRC were correlated with objective markers of severity in terms of lung function and CT scans. Data presented as Spearman rank correlation coefficients. Statistically significant values shown in bold * $p<0.05$, ** $p< 0.01$ *** $p<0.001$.

Of note on MSD, none of the analytes measured had a significant correlation with severity of symptoms (CAT score or mMRC) or severity of obstruction on spirometry (FEV_1 (l), FEV_1 (%), FEV_1/FVC or GOLD stage 1-4).

5.5.4 Identifying COPD cohorts

As seen the COPD patients represented a wide range of severity and with a wide range of severity of symptoms reflecting the heterogeneity in COPD. This is likely to represent a number of different phenotypes each with different underlying inflammatory processes. As such, COPD sub-cohorts were stratified to see if nasosorption could be useful in differentiating inflammation between different clinical phenotypes.

5.5.4.1 Emphysema

Emphysema is a major disease process within COPD, causing destruction of lung parenchyma, loss of elastic recoil and closure of the small airways due to loss of alveolar attachments. 38/45 COPD patients had cross sectional imaging allowing for the identification of macroscopic emphysema. Of these: 4 (10.5%) had no evidence of emphysema; 3 (7.9%) mild, 12 (31.6%) moderate and 19 (50%) severe emphysema. The degree of emphysema correlated negatively with the transfer factor ($DLCO_c$% r = -0.567 p = <0.001) and severity of obstruction (FEV_1% r = -0.409 p = 0.011). There was no significant correlation between severity of emphysema and symptoms (CAT score r = -0.084 p = 0.623, mMRC r = 0.143 p =0.396) or exacerbation frequency (r = 0.177 p = 0.287).

Comparing COPD patients with no or mild emphysema (n=7) versus those with severe emphysema (n=11) there was non-significant trends towards lower IL-33 and IFN-α2a in severe emphysema (Figure 5.6). None of the other cytokines measured were correlated with degree of emphysema seen on CT or the lung function surrogate of $DLCO_c$.

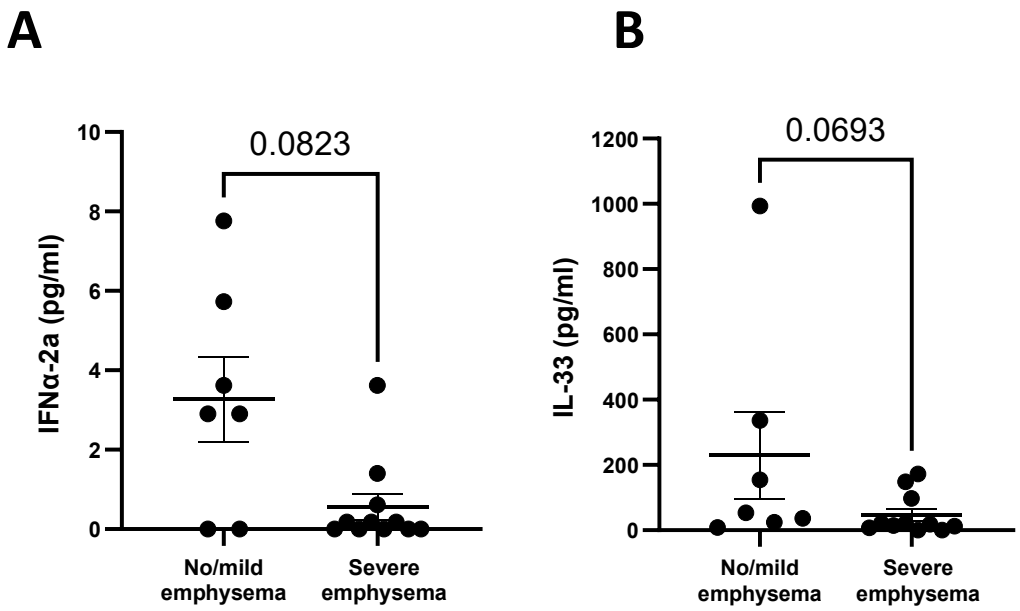

Figure 5.6: Nasal IFNα-2a and IL-33 and CT evidence of emphysema
(A) IFNα-2a and (B) IL-33 were measured in nasosorption samples by MSD. CTs were classified as no, mild, moderate and severe emphysema. Nasal cytokines in no/mild emphysema compared to severe emphysema. Data analysed using Mann-Whitney test. No/mild emphysema = 7. Severe emphysema = 11

Chronic bronchitis

Chronic bronchitis is defined as a productive cough of >3 months duration for more than two successive years. In this study COPD patients scores for cough and sputum subcategories of the CAT score were strongly correlated (r = 0.782, p<0.001). Both cough and sputum correlated with exacerbation frequency (Figure 5.7 panels A and B). Indeed, selecting those with a high cough/sputum burden (total score for the two categories ≥6, n=18) and low cough/sputum (CAT score for cough + sputum <4, n=15) there was a significant increase in exacerbation frequency in those with high cough and sputum burden (Figure 5.7 panel C).

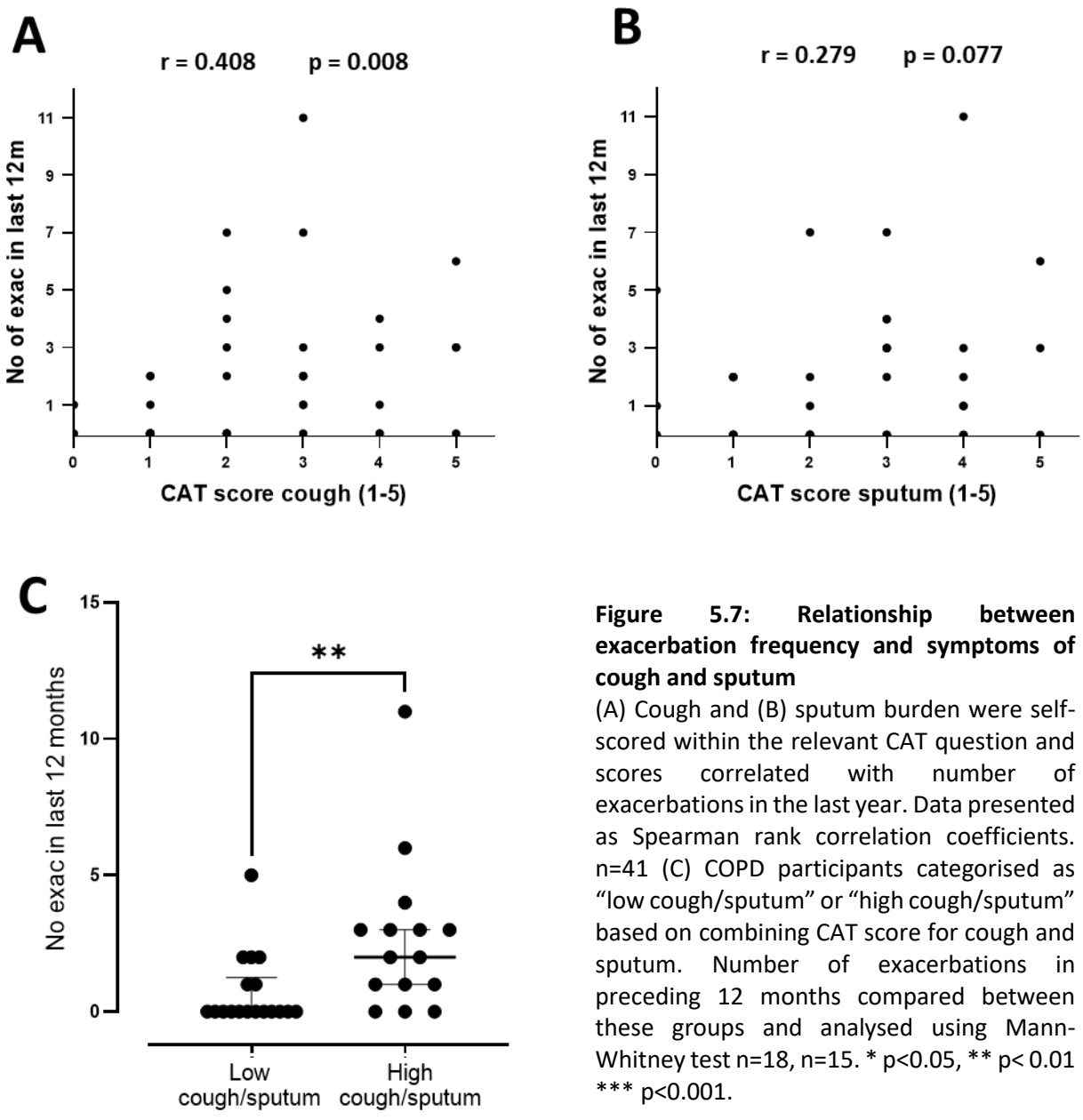

Figure 5.7: Relationship between exacerbation frequency and symptoms of cough and sputum

(A) Cough and (B) sputum burden were self-scored within the relevant CAT question and scores correlated with number of exacerbations in the last year. Data presented as Spearman rank correlation coefficients. n=41 (C) COPD participants categorised as "low cough/sputum" or "high cough/sputum" based on combining CAT score for cough and sputum. Number of exacerbations in preceding 12 months compared between these groups and analysed using Mann-Whitney test n=18, n=15. * p<0.05, ** p< 0.01 *** p<0.001.

Although this study does not specify the duration of cough and sputum, it is a fair assumption that those patients with a “high cough/sputum” score represent a chronic bronchitis cohort. Comparing COPD patients with high cough and sputum symptoms (≥6) and low symptoms (<4), there was no difference in any of the nasal cytokines measured.

5.5.4.2 COPD/bronchiectasis overlap

6/38 (16%) COPD patients showed evidence of bronchiectasis on CT scans. Symptoms of cough and sputum were not associated with presence of bronchiectasis (p=0.88). There was also no difference in number of exacerbations per year in those with bronchiectasis and those without (p=0.67).

Of the MSD nasosorption samples analysed only 4/27 COPD patients with cross sectional imaging had bronchiectasis present. Patients with COPD and co-existent bronchiectasis had higher levels of IL-7, CXCL10, CXCL11 and CCL17 compared to those without bronchiectasis (Figure 5.8).

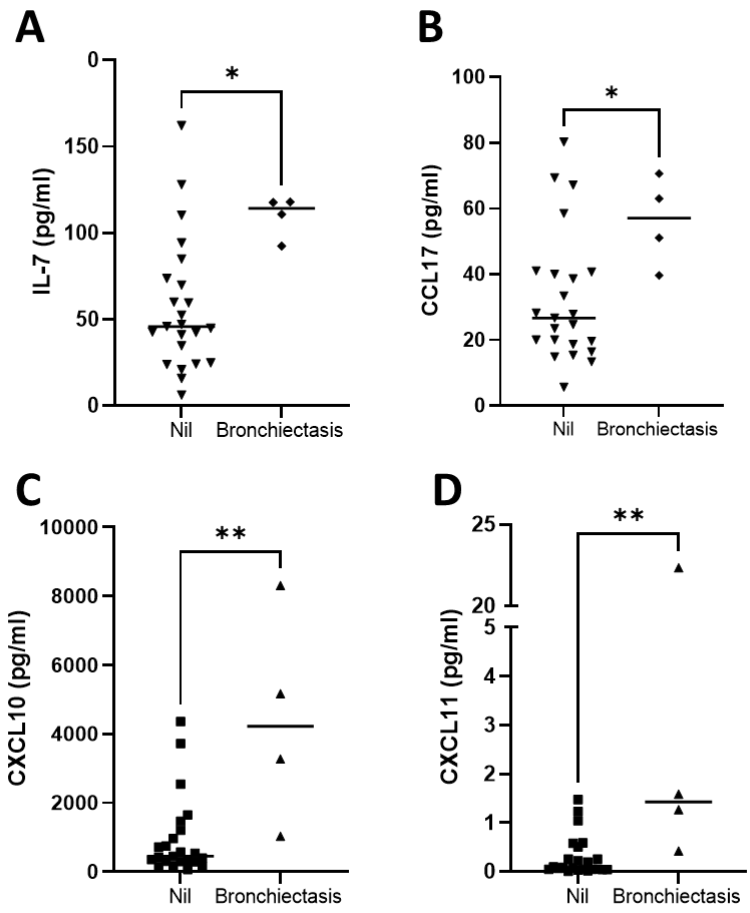

Figure 5.8: Nasal cytokine profile in COPD bronchiectasis overlap
CTs analysed for the presence of bronchiectasis. Nasosorption performed and levels of (A) IL-7 (B) CCL17 (C) CXCL10 (D) CXCL11 compared between COPD patients without bronchiectasis "Nil" and those with bronchiectasis. Median marked as line. Data analysed using Mann-Whitney test. No bronchiectasis n=23, bronchiectasis n=4. * $p<0.05$, ** $p< 0.01$ *** $p<0.001$.

5.5.4.3 Eosinophilic COPD

Eosinophilia is currently viewed as a key phenotype to identify specific patient cohorts as it predicts a more steroid responsive form of COPD, with blood eosinophils ≥ 300 cell/µl (0.3 x10^9cell/litre) used as a cut off in GOLD guidelines. All COPD patients had at least one blood eosinophil count, with all but 5 having >3 separate readings. Looking at blood eosinophils over time, few patients were persistently eosinophil low (<0.3 x10^9cell/litre, n=14) or eosinophil high (≥0.3 x10^9cell/litre, n=3) as shown in Figure 5.9. Indeed, the mean difference between minimum and maximum eosinophils for patients was 0.32 ± 0.004 x10^9cell/litre.

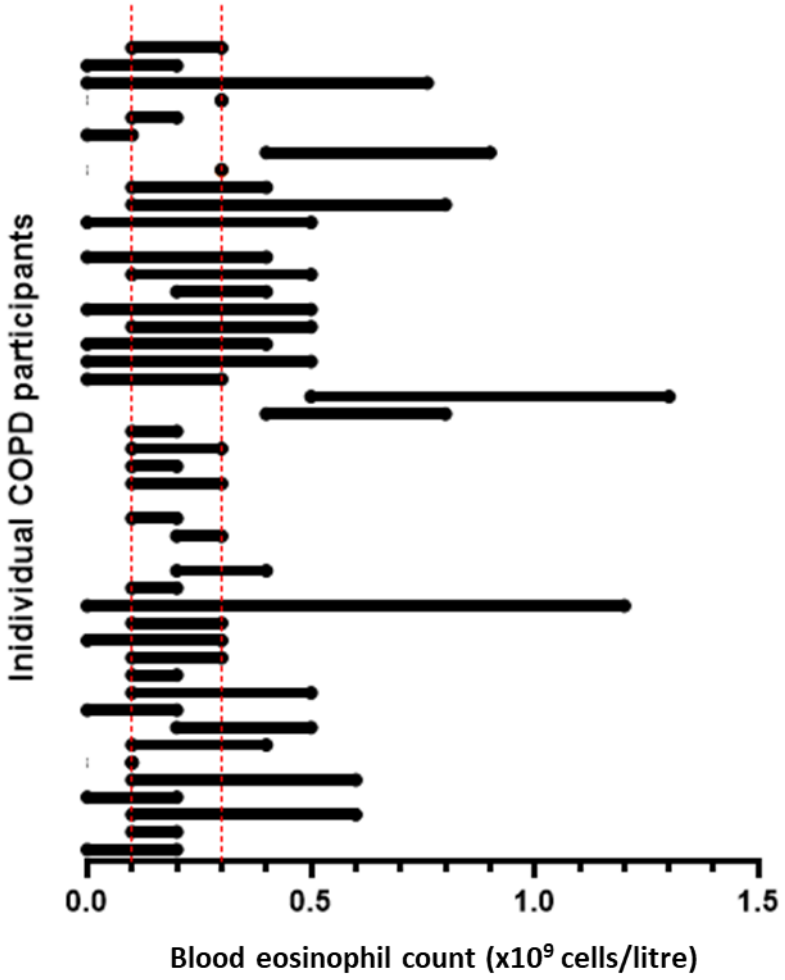

Figure 5.9: Variability of blood eosinophil counts for individual patients
Graph showing maximum and minimum blood eosinophil counts in hospital and GP records over time. Red dashed lines represent GOLD guidelines levels of 0.1 x 10^9 cell/litre and 0.3 x 10^9 cell/litre.

In order to determine whether nasosorption was able to differentiate eosinophilic inflammation in COPD, a "eosinophil high" group was defined as patients with median, mean and mode blood eosinophils ≥0.3 x10^9cell/litre (n=7) and "eosinophil low" group as those patients <0.3 x10^9cell/litre (n=9). Eotaxin-1 was significantly elevated in the "Eosinophil high" group compared to the "eosinophil low" (Figure 5.10). However, other markers of TH2 inflammation were not significantly different between groups. IFNα-2a was also increased in the "eosinophil high" group (1.60pg/ml vs 0.00pg/ml, p = 0.021). There was no significant difference between eosinophil high and eosinophil low groups in cytokine levels associated with neutrophilic inflammation such as GM-CSF, CXCL8, IL-1β and CXCL1.

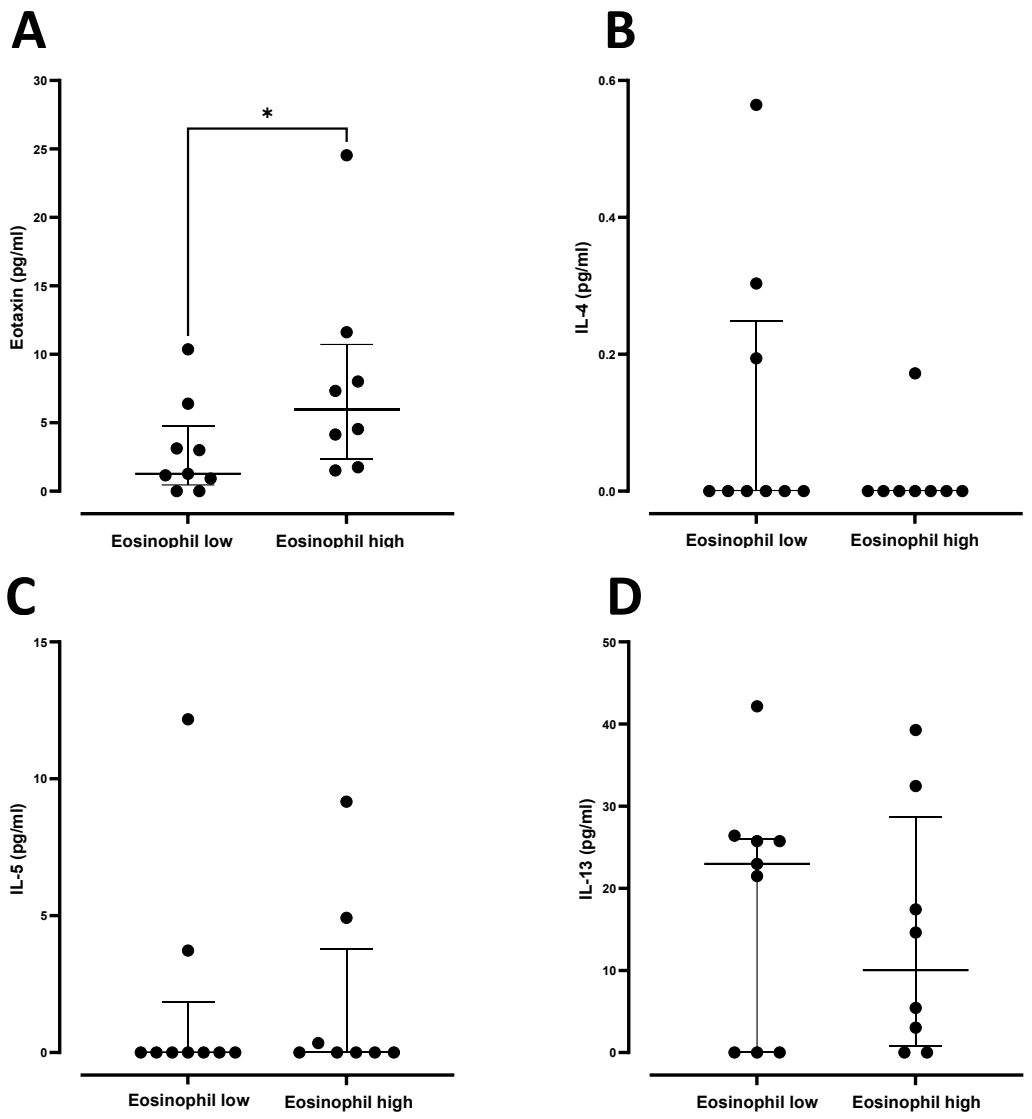

Figure 5.10: TH2 nasal cytokine profile in eosinophilic and non-eosinophilic COPD
COPD patients classified as "Eosinophilic low" and "Eosinophilic high" based on BEC and (A) Eotaxin-1 (B) IL-4 (C) IL-5 (D) IL-13 measure in nasal lining fluid by MSD. Data shown as median ± IQR and analysed using Mann-Whitney test. Eosinophil low n=9, eosinophil high n=8 * $p<0.05$, ** $p< 0.01$ *** $p<0.001$.

In order to assess whether elevated eotaxin-1 might be occurring in COPD without other traditional TH2 cytokines being elevated, heat maps of these cytokine were drawn to look whether there was a relative isolated excess of eotaxin-1 which could potentially be directly be targeted by a monoclonal antibody in eosinophilic COPD. In Figure 5.11 panel A, COPD patients were subgrouped into those with known atopy (based on history only, no routine skin prick or IgE testing performed) and a heatmap between TH2 cytokines created. Of note there was no significant difference between any of the TH2 cytokines in the nose in atopic (n=12) and non-atopic (n=22_) individuals (although sampling did not take place when patients were symptomatic from atopy) when analysed by Mann Whitney test. Eotaxin-1 had a weak correlation with IL-5 but not with IL-4, IL-13 or TSLP in COPD nasal lining fluid as seen in Figure 5.11 panel B.

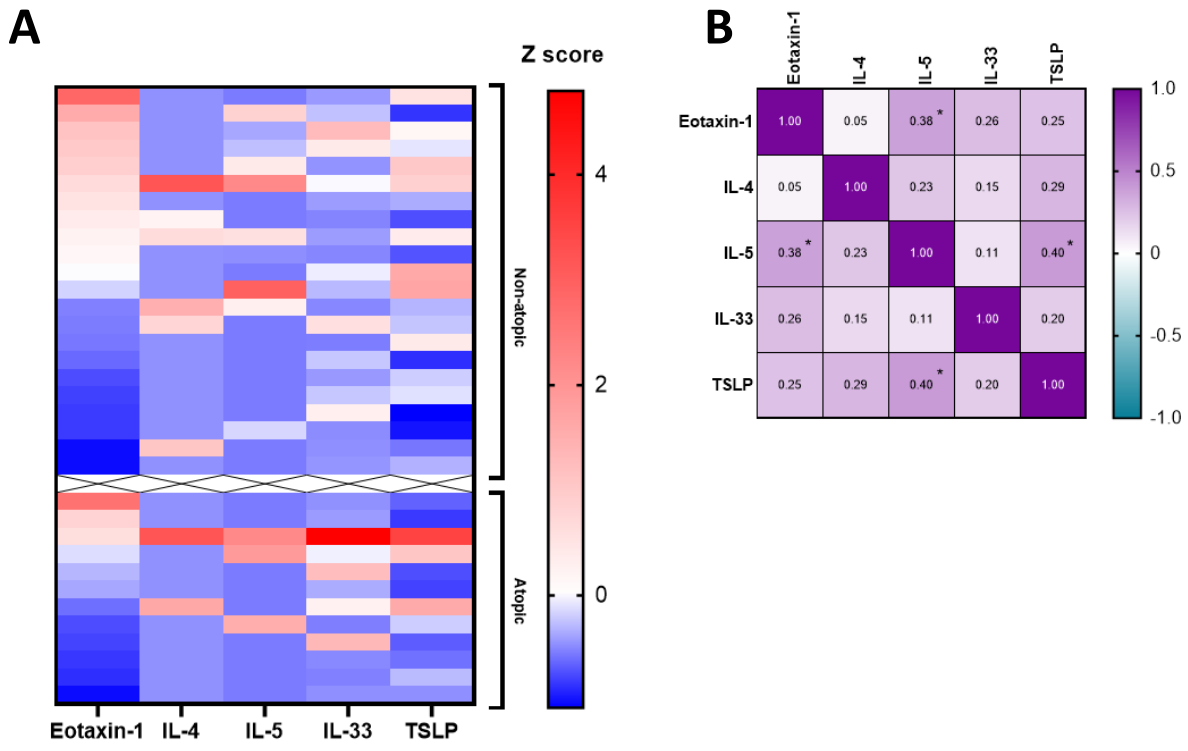

Figure 5.11: Heatmap and correlation of TH2 nasal cytokine profile in COPD
(A) Heatmap showing nasal eotaxin-1, IL-4, IL-5, TSLP concentrations in COPD patients. Data presented as Z scores for each COPD participant. Individuals ordered in high to low concentration of eotaxin-1 (separated into atopic and non-atopic). (B) Correlation matrix of TH2 cytokines in COPD patients. Figures indicate spearman rank co-efficient. n = 34 *: p< 0.05

5.5.4.4 *Frequent exacerbators*

Number of exacerbations in the previous year is the strongest predictor of future exacerbations, and thus it is accepted that a frequent exacerbator phenotype exists within COPD (Hurst 2010). 19/45 (42%) patients had greater or equal to 2 exacerbations in the previous year (i.e. classed as frequent exacerbators. There was no significant difference in demographics between the infrequent (<2/year) and frequent exacerbator (≥2/year) groups in terms of age, spirometry and pack years (Table 5.7).

On MSD of nasosorption samples from COPD patients at stable state, there were no significant differences in nasal cytokine concentrations between frequent (n=17) and infrequent (n=15) exacerbators. Frequent exacerbators had a trend towards increased IL-6 (16.48 vs 2.31 p=0.081) and reduced IL-13 (0.00 vs 17.43, p=0.093) compared to infrequent exacerbators.

	Infrequent exacerbators (<2/year)	**Frequent exacebators (≥2/year)**
Number	25	19
Age (years)	65.3 ± 0.1	71.9 ± 2.2
Sex (male:female)	16:6	6:13
FEV_1 (L)	1.55 ± 0.12	1.19 ± 0.14
FEV_1 (%)	58.9 ± 3.8	50.9 ± 4.7
FVC (L)	3.03 ± 0.16	2.52 ± 0.19
FVC (%)	92.8 ± 4.2	94.3 ± 5.6
FEV_1/FVC	0.50 ± 0.02	0.43 ± 0.03
Current smokers	8 (32%)	4 (21%)
Smoking history (pack years)	50.0 ± 5.8	58.7 ± 6.9

Table 5.7: Demographics and lung function of frequent and infrequent exacerbators
COPD patients classified as "Infrequent exacerbators" (<2 exacerbations in previous 12 months) and "frequent exacerbators" (≥2 exacerbations in previous 12 months) and demographics and spirometry compared. Data analysed using Mann-Whitney test. * p<0.05, ** p< 0.01 *** p<0.001.

5.5.5 COPD exacerbation study

16 COPD patients were sampled during exacerbation and > 4 weeks after recovery from exacerbation. Table 5.8 outlines the patient demographics for this study. The patients represented GOLD stages 2-4. with the majority (14/16) of the being frequent exacerbators with a mean number of exacerbations in the preceding 12 months of 3.7 ±0.72.

	Exacerbation patients
Number	16
Age (years)	70.3 ± 2.52
FEV_1 (L)	1.14 ± 0.13
FEV_1 (%)	48.13 ± 4.71
FVC (L)	2.49 ± 0.22
FVC (%)	86.51 ± 6.73
FEV_1/FVC	0.47 ± 0.04
Current smokers	4 (25%)
AECOPD in last 12 months	3.7 ±0.72

Table 5.8: Demographics and lung function of COPD patient included in the exacerbation study
AECOPD = acute exacerbation of COPD

Patients were sampled within 24 hours of calling the exacerbation hotline with the mean number of days since symptoms starting to the day of being sampled being 4.94 ± 0.91. Exacerbations were assessed clinically through history and examination and also using objective testing via nasopharyngeal swabs for viral PCR, sputum for microscopy and culture, and bloods for systemic markers of inflammation. All patients described a change in sputum and increased breathlessness. 15/16 (93.8%) also complained of increased cough, 14/16 (87.5%) increased chest tightness or wheeze, 10/16 (62.5%) rhinorrhoea, 7/16 (43.8%) fevers and 7/16 (43.8%) increased fatigue.

Table 5.9 summarises the clinical results and overall clinical impression of the 16 individual AECOPD. On clinical assessment 8/16 (50%) exacerbations were virally induced of which 4/8 were confirmed by PCR (2x RSV, 1x rhinovirus, 1x parainfluenza). 6/16 (37.5%) exacerbations had features consistent of

bacterial infection of which 3 had positive sputum cultures (1x *Haemophilus influenzae,* 1x *Haemophilus influenzae* and *Escherichia coli* co-infection, 1x *Serratia marascens*). One patient had positive sputum culture for *Staphylococcus aureus* however clinically this was felt unlikely to be pathogenic. Two patients did not have a blood sample collected at time of exacerbation (1x unable to obtain blood sample; 1x needle phobia). Of the 14/16 patient where blood was collected during AECOPD, the mean blood eosinophil count at exacerbation was 0.22±0.04 $x10^9$ cells/litre. 4/14 (28.6%) had blood eosinophils ≥ 0.3 $x10^9$ cells/litre. COVID-19 was ruled out in all patients by lateral flow test.

At time of sampling 4/16 (25%) patients had commenced 30mg prednisolone (2-48 hours from onset); 5/16 (31.3%) had started antibiotics (2-48 hours from onset) and 1 patient was on maintenance 5mg prednisolone.

Patients were followed up during exacerbation to ensure recovery from the acute illness. 2/16 (12.5%) improved by increasing their short active beta agonist and chest clearance, without the need for additional oral medication. 9/16 (56.3%) improved with steroids +/- antibiotics. 5/16 (31.3%) re-exacerbated within 4 weeks requiring further treatment and in two cases hospitalisation.

Patient	WCC	Neutrophils	Lymphocytes	Eosinophils	Viral Swab	Sputum MCS	Clinical impression
1	7.9	4.8	1.5	**0.7**	negative	***H. influenzae***	Bacterial
2	7.6	5.3	1.3	0.2	negative	X	Viral
3	11.4	8.1	1.6	0.2	negative	X	Bacterial
4	8.8	5.5	2.1	0.2	negative	X	Bacterial
5	8.4	4.5	3.1	0.2	negative	X	Non-infective
6	7.5	6.8	0.5	0	negative	***H. influenzae*** ***E. coli***	Bacterial
7	X	X	X	X	**Rhinovirus**	X	Viral
8	9.1	7.9	0.6	0	**RSV B**	X	Viral
9	5.8	3.5	1.3	**0.4**	negative	X	Viral
10	8.4	5.9	1.3	**0.4**	negative	nil	Viral
11	6.5	4.6	1.6	0.1	negative	***S. marascens***	Bacterial
12	7.3	5.1	0.8	**0.4**	**RSV**	***S. aureus***	Viral
13	10.2	7.8	0.9	0.1	negative	nil	Viral/bacterial coinfection
14	7	5.8	0.8	0.1	**Parainfluenza**	nil	Viral
15	X	X	X	X	negative	X	Non-infective
16	16.4	12.4	3	0.2	negative	X	Non-infective

Table 5.9: Blood, viral PCR and sputum results of individual patients in exacerbation study
COPD patients had full blood count, nasopharyngeal viral PCR and sputum culture (if spontaneously expectorating sputum) during AECOPD. All cell counts expressed as $x10^9$ cells/litre. Overall clinical impression of infective state (non-infective; viral; bacterial; or viral/bacterial coinfection) was made based on clinical assessment and investigation results. Red text indicates positive infection results or high eosinophil counts. WCC = white cell count. Greyed out boxes indicate that investigation was not performed.

5.5.5.1 Use of nasosorption in assessing acute exacerbations of COPD

Of the 22 analytes measured by MSD only 2 showed any significant difference during exacerbation compared to recovery: IFN-γ was higher during exacerbations (729.8±591.2 vs 2.2±1.3pg/ml, p = 0.027) meanwhile TSLP was lower during exacerbation and then increased on recovery (4.81±1.41 vs 12.40±2.79pg/ml, p=0.002).

To determine whether nasosorption might be useful in defining the cause of exacerbations viral, bacterial and eosinophil driven exacerbations were explored. IFNα-2a was significantly higher in viral exacerbations than non-virally induced exacerbations (n=8, p<0.05), with a strong trend also seen in IFNγ (p=0.054) but no difference in IFNβ. CXCL10 was also non-significantly raised in viral exacerbations (p = 0.160) (Figure 5.11).

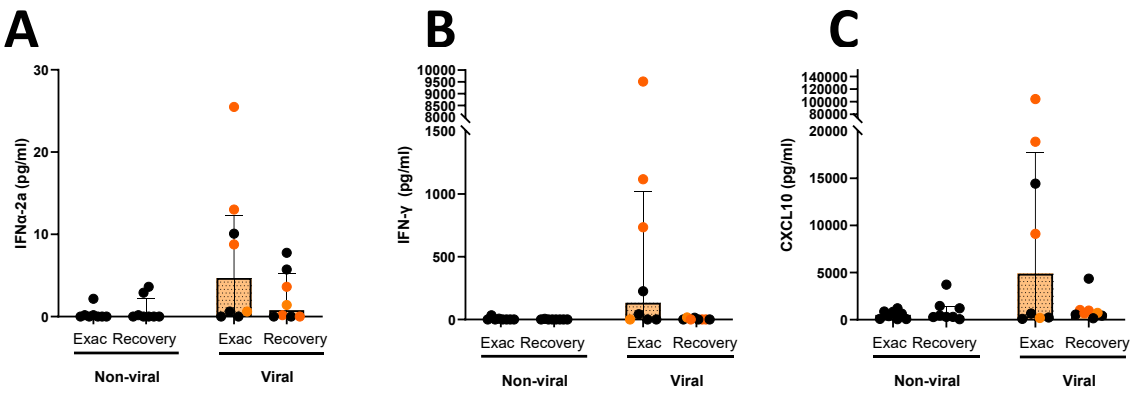

Figure 5.11: Nasal inflammation in viral and non-virally induced AECOPD
COPD patients assessed as non-viral or virally induced through clinical assessment and nasopharyngeal PCR. Nasosorption performed and analytes measured by MSD (A) IFNα-2a (B) IFNγ and (C) CXCL10. • represents those patients confirmed to be virally induced via positive PCR. Data presented as median ± IQR. n=8

AECOPD were clinically determined to be bacterial (n=6) or non-bacterial (n=10). During exacerbation bacterial exacerbations were associated with a lower level of nasal eotaxin-1 than non-bacterial exacerbations (p<0.05) as shown in Figure 5.12. There were no other significant differences between bacterial and non-bacterial groups in the analytes that were measured.

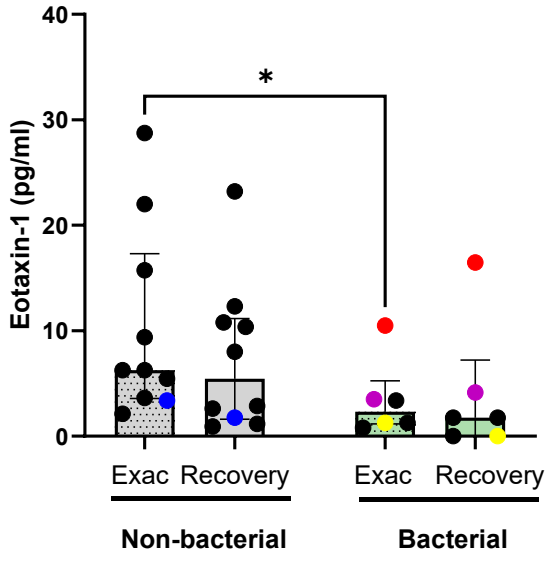

Figure 5.12: Nasal eotaxin-1 concentrations during AECOPD and at recovery in bacterial and non-bacterially induced exacerbations
Nasosorption was performed in AECOPD and > 4 weeks after AECOPD. Eotaxin-1 was measure by MSD. AECOPD classified clinically at time of reviewing and sampling patient as likely non-bacterial and likely bacterially induced exacerbations (Coloured points indicated positive sputum culture • = *S.aureus*, • = *S.marascens*, • = *H.influenzae* + *E.coli*, • = *H.influenzae*). Data presented as median ± IQR and analysed using Mann-Whitney test between AECOPD samples. n=10 non-bacterial, n=6 bacterial. * = p<0.05 ** = p< 0.01 *** = p<0.001

14/16 AECOPD had bloods taken at exacerbation visit. 4/14 had a high blood eosinophil count (BEC) of ≥0.3 x10^9 cells/litre. There was no difference between cytokines at exacerbation between patients with low BEC (≤0.1 x10^9 cells/litre) and high BEC (≥0.3 x10^9 cells/litre) as shown in Figure 5.13.

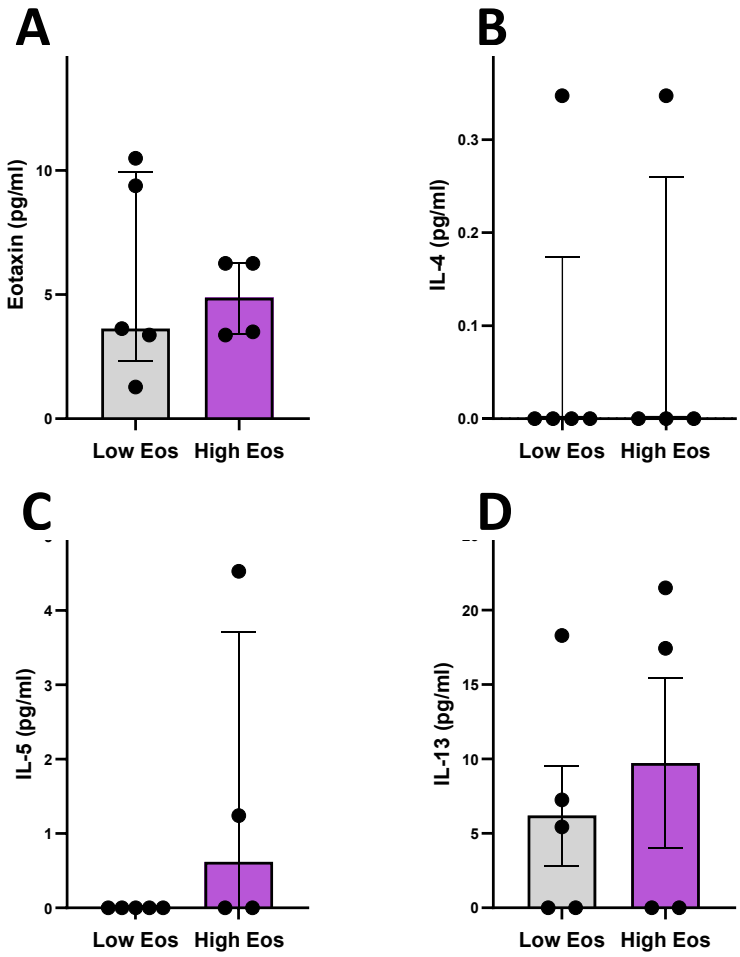

Figure 5.13: Nasal TH2 cytokines during AECOPD comparing those with high and low blood eosinophil count

Nasosorption was performed during AECOPD. (A) Eotaxin-1 (B) IL-4 (C) IL-5 (D) IL-13 were measured by MSD and concentrations compared between "low Eos" groups with BEC ≤0.1x109 cells/litre and "high Eos" with BEC ≥0.3x109 cells/litre. Data presented as median ± IQR and analysed using Mann-Whitney test between AECOPD samples. n=4

Of the 16 patients who took part in the exacerbation study, 5/16 (31%) had repeat exacerbations within the first 4 weeks and recovery sampling was delayed until they were at least 4 weeks clear of exacerbation. Comparing recovery samples, there was significantly higher levels of eotaxin-1 in those that that re-exacerbated compared to those that made a good recovery (Figure 5.14).

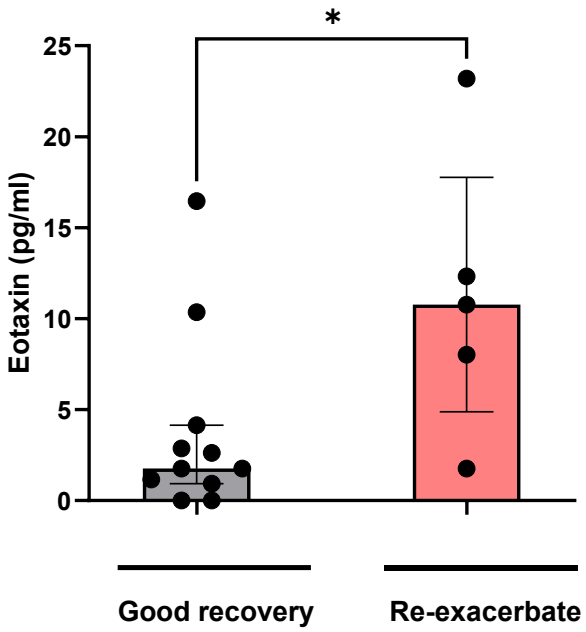

Figure 5.14: Nasal eotaxin-1 concentrations on recovery from AECOPD comparing those with an uncomplicated recovery and those that re-exacerbated
Nasosorption was performed > 4 weeks after AECOPD once symptoms had returned to baseline, in event of re-exacerbation sampling was delayed until >4 weeks free of any exacerbation. Eotaxin-1 was measure by MSD and samples from those with an uneventful "good recovery" and those that "re-exacerbated" within 4 weeks compared. Data presented as median ± IQR and analysed using Mann-Whitney test between AECOPD samples. n=11 good recovery, n=5 re-exacerbate. * = $p<0.05$ ** = $p< 0.01$ *** = $p<0.001$

5.5.6 Using Nasosorption to investigate IL-36 *in vivo*

IL-36α and IL-36γ cytokines have been shown to be increased in COPD compared to healthy non-smokers with a reduction of IL-36RA (Kovach 2021, Baker 2022). Whether nasosorption could be used as a non-invasive and repeatable method to investigate the role of IL-36 cytokines *in vivo* was examined next. 25 non-smokers, 7 healthy smokers and 42 COPD patients at stable state and 16 AECOPD were sampled by nasosorption and IL-36α, IL-36β, IL-36γ and IL-36RA measured by ELISA.

5.5.6.1 *Nasal IL-36 in health and COPD at stable state*

36α, IL-36β and IL-36γ were all detectable in nasal epithelial lining fluid. There was however no significant difference between healthy non-smokers, healthy smokers and COPD patients in nasal IL-36α, IL-36β or IL-36γ concentrations (Figure 5.15). IL-36RA was undetectable in all samples. Nasal IL-36α, IL-36β or IL-36γ levels were not associated with any demographic variables such as age, gender, smoking status or pack years.

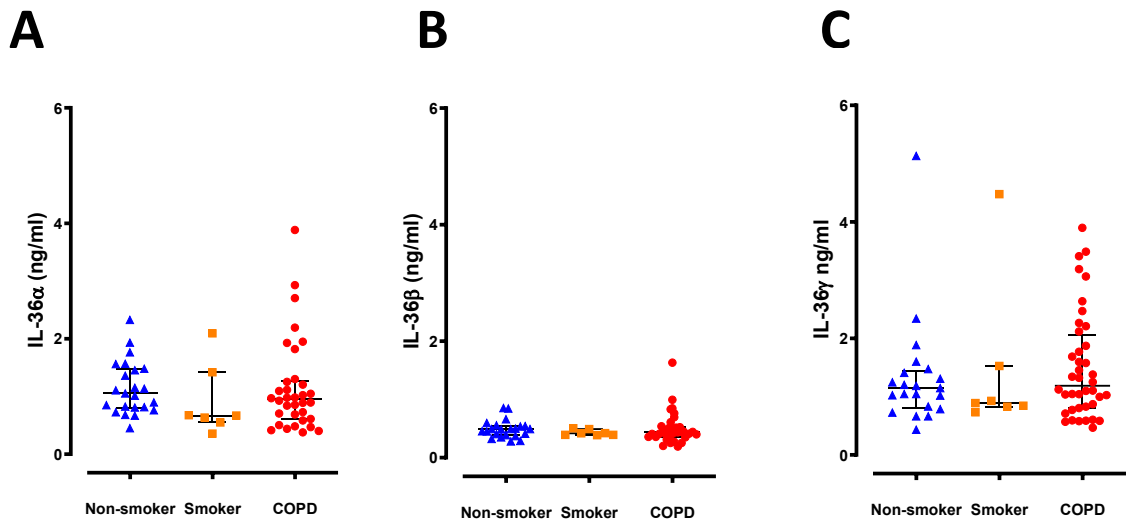

Figure 5.15: IL-36 cytokine concentrations in non-smoker, healthy smokers and COPD subjects nasal epithelial lining fluid
Nasosorption was performed in non-smoker, healthy smoker and COPD patients and (A) IL-36α (B) IL-36β (C) IL-36γ measured by ELISA. Data are presented as median and interquartile range. Kruskall-Wallis with Dunn's post-test was performed to test for significant differences between subject groups. n=25 non-smokers, n=7 healthy smokers, n=42 COPD.

5.5.6.2 Nasal IL-36 in COPD

Although there is no significant difference in IL-36 levels in health and COPD, as shown in Figure 5.15 in COPD patients there is a strong positive skew (Fisher-Pearson coefficient of Skewness IL-36α= +1.82, IL-36β = +3.37, IL-36γ= +1.05). In order to ascertain whether this skew represents a signal coming from a certain phenotype of COPD patients, IL-36γ results were analysed in more detail. There was no correlation between IL-36γ concentrations and markers of symptom burden such as CAT, mMRC and exercise tolerance. There was also no correlation between IL-36 levels and objective markers of COPD severity in terms of lung function parameters or emphysema on CT (Table 5.10).

	Spearman r	p value
CAT score	0.381	0.015
mMRC	-0.078	0.627
Exercise tol (m)	0.290	0.066
FEV_1 (L)	-0.79	0.619
FEV_1 (%)	-0.196	0.213
FVC (L)	0.138	0.382
FVC (%)	-0.191	0.226
FEV_1/FVC	-0.122	0.44
TLC	-0.015	0.942
TV	-0.097	0.639
DLCOc	0.25	0.182
DLCOc %	0.161	0.385
CT emphysema	0.05	0.773

Table 5.10: Correlation nasal IL-36γ concentrations and COPD severity
Patients self-assessed symptoms using CAT score, mMRC and estimated exercise tolerance. Lung function was performed and severity of emphysema on CT was assessed as absent (1) to severe (4). Each variable correlated with nasal IL-36γ concentrations. Data presented as Spearman rank correlation coefficients. n=42

IL-36γ is known to be virally induced (Baker 2022), therefore analysis was performed determine whether nasal concentrations of IL-36γ in COPD patients were associated with exacerbation frequency. IL-36γ at stable state is not associated with previous exacerbation frequency: There was no correlation between number of exacerbations in previous year and IL-36γ at stable state, nor any difference seen when patients were grouped into frequent (≥2 exacerbations/year) or infrequent exacerbators (<2 exacerbations / year) (see figure 5.16).

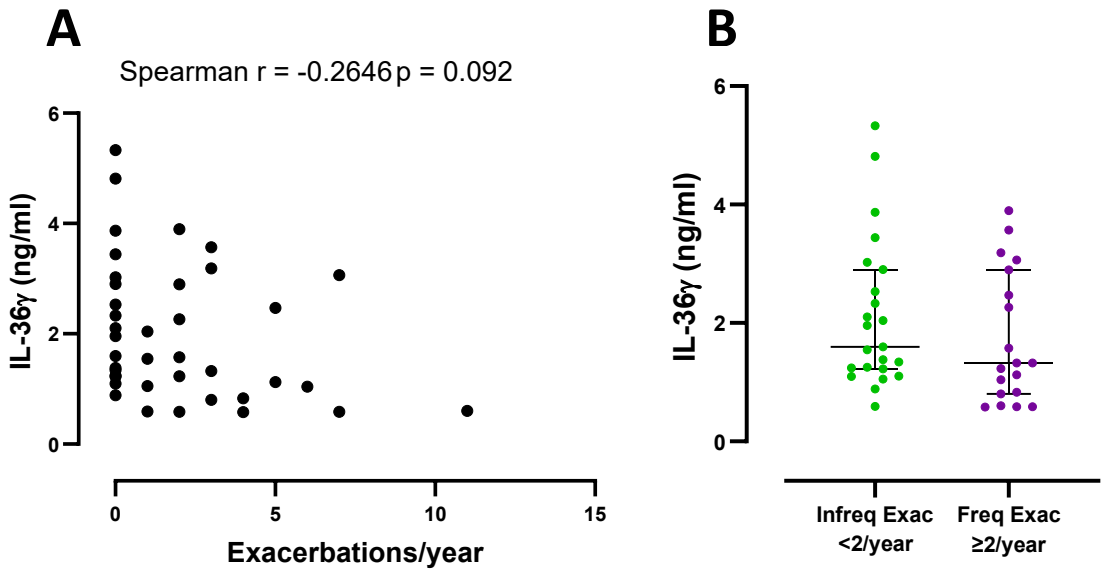

Figure 5.16: Relationship between nasal IL-36γ concentration and exacerbation frequency in COPD patient

Nasosorption was performed in COPD patients and IL-36γ measured by ELISA. Number of acute exacerbations of COPD in the previous 12 months was documented for each patient. (A) Correlation between number of exacerbations/year and IL-36γ. Data expressed as spearman rank co-efficient. n=42 (B) IL-36γ levels in nasosorption samples from infrequent (<2 exacerbations/year) and frequent (≥2 exacerbations/year) exacerbator patients were compared. Mann-Whitney U test used to test for significant differences between subject groups. n=19 infrequent exacerbators, n= 23 frequent exacerbators.

IL-36γ is an inflammatory cytokine thought to be mostly associated with neutrophilic inflammation (Koss 2021, Baker 2022). As such, data were analysed for any correlations with other proteins associated with neutrophilic and eosinophilic inflammation. IL36γ was strongly positively correlated with IL-1β (r = 0.738, p = <0.001), CXCL8 (r = 0.629, p = <0.001), with moderate positive correlations also seen with GM-CSF (r = 0.479, p = 0.004), CXCL1 (r = 0.488, p = 0.009), IL-5 (r = 0.468, p = 0.005), IL17A (r = 0.405, p 0.007), CCL17 (r = 0.408, p 0.016), and TSLP (r = 0.416, p = 0.014).

Given the large range of IL-36γ concentrations, temporal stability of the IL-36γ signal was also assessed. Serial nasosorption samples were taken from 7 COPD patients, at 3-4 different time points, with a minimum of 6 weeks between each sample. Patients were confirmed not to be exacerbating at any of these time points. As shown in Figure 5.17, the IL-36γ concentrations were not stable across time points despite relative stability in symptoms (i.e. no samples were taken during exacerbations). Those with lower IL-36γ levels (participants 2,3,4,6) have less relative variability (coefficient of variation 4.52-36.11%) meanwhile those with higher IL-36γ concentrations (participants 1,5,7) had greater relative variability (30.37-68.10%).

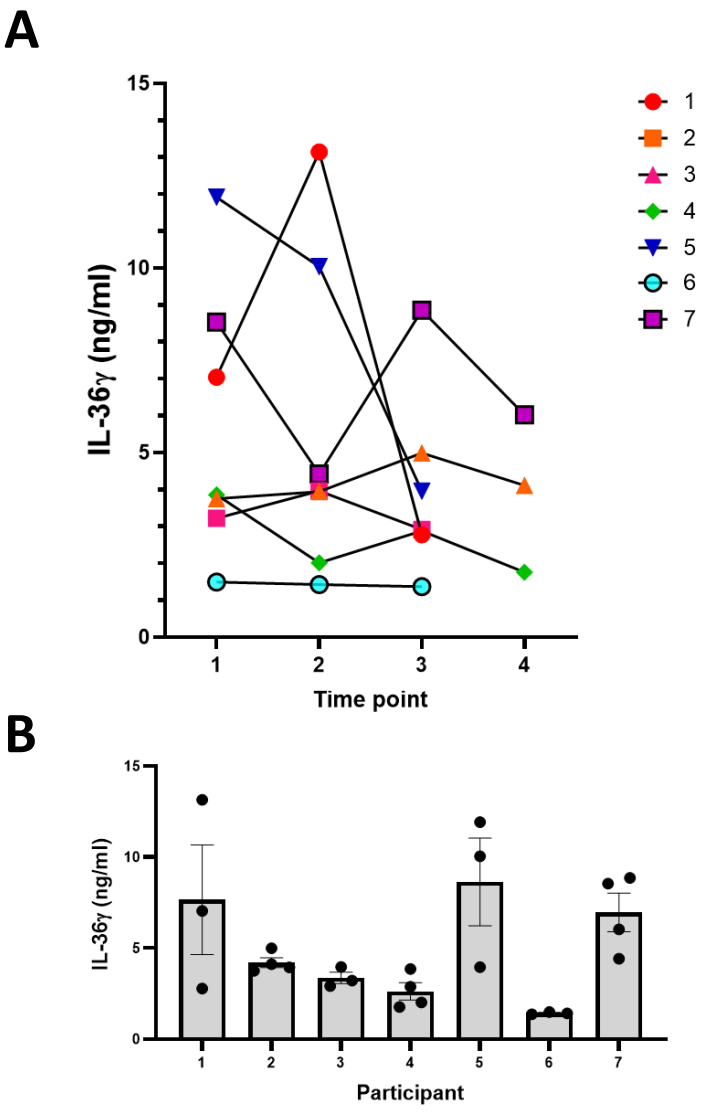

Figure 5.17: Variability in nasal IL-36γ concentration over time
Nasosorption was performed in stable COPD patients at 3-4 different time points and IL-36γ measured by ELISA. (A) Serial measurements of IL-36γ over time for individual COPD patients. (B) Bar chart showing IL-36γ concentrations for individual patients. Data expressed as mean ± sem n=7 COPD patients

5.5.6.3 IL-36 in acute exacerbations of COPD

16 COPD patients were sampled using nasosorption during AECOPD and ≥4 weeks post recovery from exacerbation. There was no difference in levels of IL-36α, IL-36β or IL-36γ between exacerbation samples and those taken on recovery when the patient had returned to baseline (see figure 5.18).

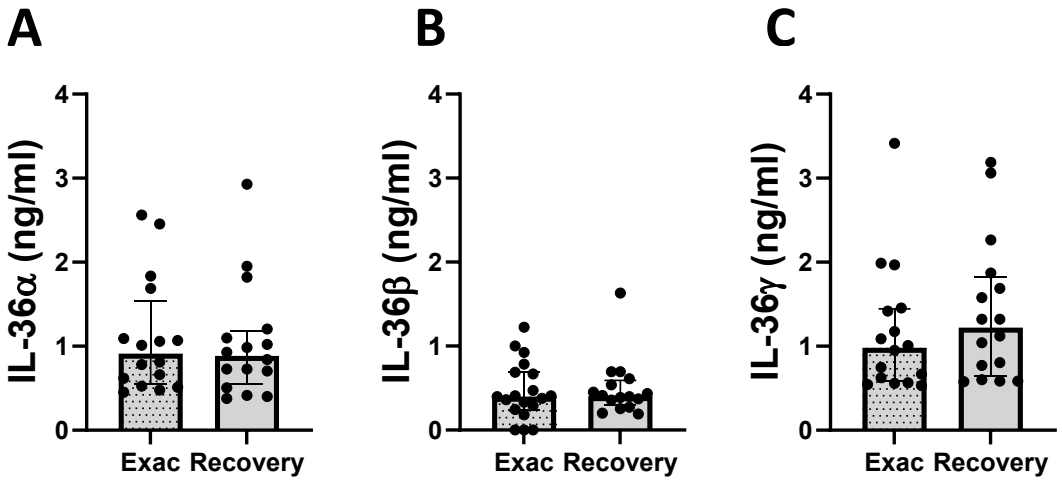

Figure 5.18: Nasal IL-36 concentrations during AECOPD and at recovery
Nasosorption was performed in AECOPD and at least 4 weeks after AECOPD once symptoms returned to baseline. (A) IL-36α (B) IL-36β (C) IL-36γ were measured by ELISA and data compared between exacerbation and recovery using Wilcoxon signed rank test. n=16

AECOPD were sub analysed by causation of AECOPD. There was no difference between nasal levels of IL-36γ during exacerbation in virally induced exacerbations versus non-viral induced exacerbations (Figure 5.19 Panel A). There was also no significant difference in IL-36γ exacerbation levels between likely bacterial and non-bacterial exacerbations (Figure 5.19 Panel B). Separating AECOPD into viral vs non-viral or bacterial vs non-bacterial did not highlight any difference between exacerbation and stable state levels of IL-36γ.

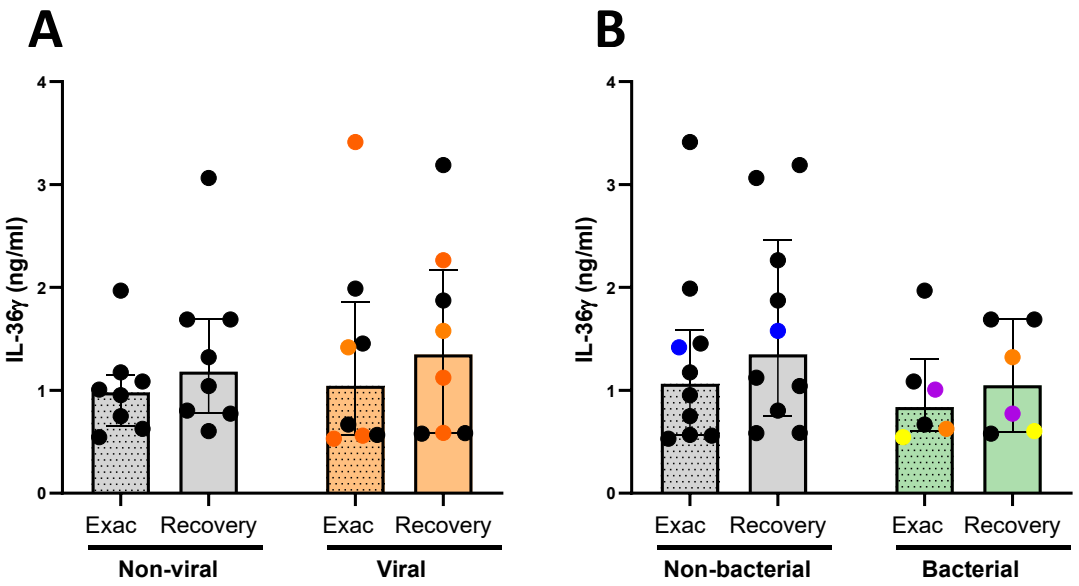

Figure 5.19: Nasal IL-36 concentrations during AECOPD and at recovery in viral and bacterially induced exacerbations

Nasosorption was performed in AECOPD and at least 4 weeks after AECOPD once symptoms returned to baseline. IL-36γ were measured by ELISA. AECOPD classified as (A) likely non-viral and virally induced exacerbations (• = confirmed viral exacerbation on PCR) and (B) likely non-bacterial and bacterial (Coloured points indicated positive bacterial sputum culture • = *S.aureus*, • = *S.marascens*, • = *H.influenzae* + *E.coli*, • = *H.influenzae*) and IL-36γ levels compared. n=16

There was a positive correlation between nasal IL-36γ levels and blood eosinophil count during acute exacerbation (Figure 5.20 Panel A). Participants were grouped into low exacerbation blood eosinophils (≤0.1 x10^9cells / litre) or high exacerbation blood eosinophils (>0.3 x10^9cells / litre) as per GOLD treatment guidelines classifications and levels of IL-36γ compared at exacerbation and on recovery. Numbers were only n=5 and n=4 respectively with a possible trend towards higher IL-36γ at exacerbation in the high eosinophil group p=0.064 (Figure 5.20 Panel B).

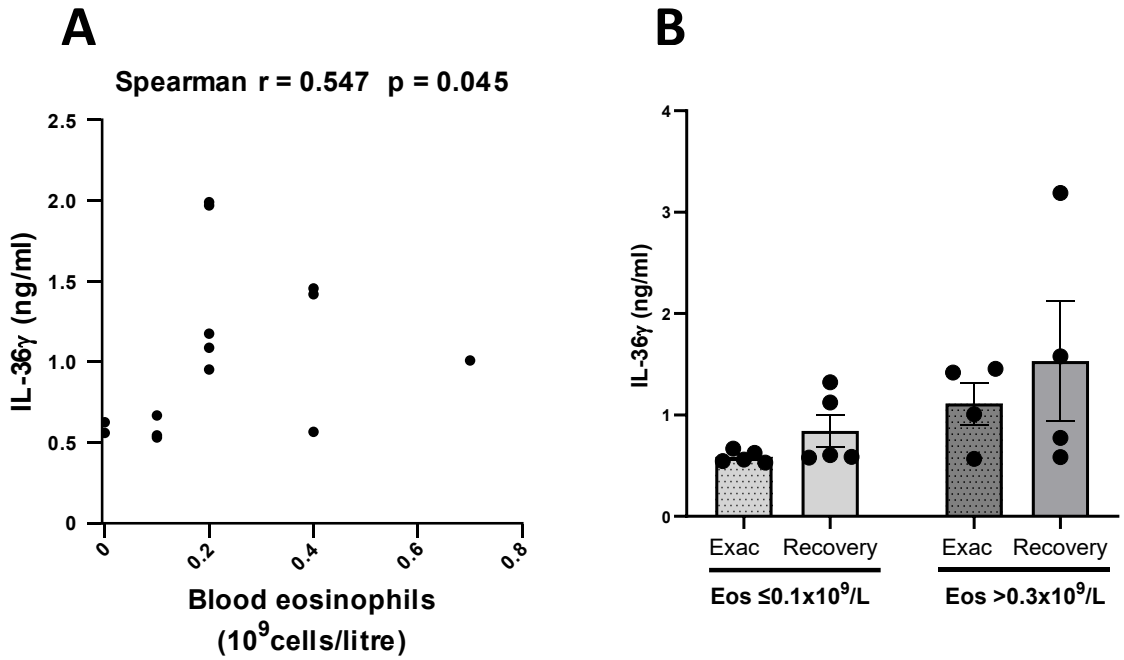

Figure 5.20: Blood eosinophil count and nasal IL-36γ concentrations during AECOPD
(A) Blood eosinophil count was correlated with nasal IL-36γ concentration during acute exacerbation of COPD. Data analysed using spearman rank correlation co-efficient. n=14 (B) COPD participants grouped as low exacerbation eosinophil count (≤0.1 109/l cells) or high (>0.3 109/l cells) and IL-36γ levels at exacerbation and recovery compared in the two groups. Data shown as mean ± sem. n=5 high eosinophils n=4 low eosinophils.

5/16 (31%) had repeat exacerbations within the first 4 weeks after initial exacerbation. There was no difference in levels of IL-36γ at exacerbation between those that made and those that re-exacerbated quickly (1.30±0.87ng/ml vs 0.88±0.38ng/ml, p=0.583). In those that re-exacerbated, there was a non-significant increase in IL-36γ at exacerbation through to recovery samples (0.88±0.38ng/ml vs 1.93±0.52ng/ml, p=0.0625). There was also a trend in higher levels on recovery in these re-exacerbating patients compared to recovery levels of IL-36γ in those who made a good recovery (1.93±0.52ng/ml vs 1.19±0.18ng/ml, p=0.278) see Figure 5.21. More subjects are needed to confirm if there is any significance to these trends.

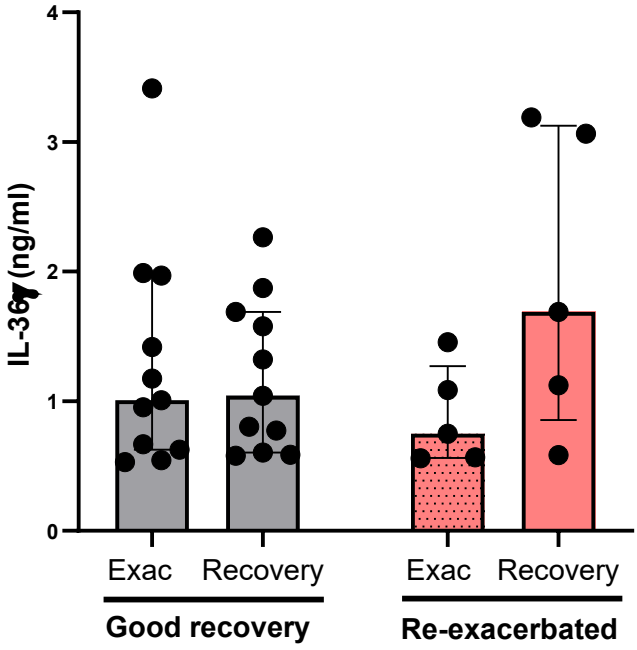

Figure 5.21: Nasal IL-36γ concentrations during AECOPD and on recovery in those that recovered uneventfully and those that had repeat exacerbations

Nasosorption was performed during AECOPD and > 4 weeks after AECOPD once symptoms had returned to baseline, in event of re-exacerbation sampling was delayed until >4 weeks free of any exacerbation. IL-36γ was measured by ELISA and concentrations from those with an uneventful "good recovery" and those that "re-exacerbated" within 4 weeks compared. Data presented as median ± IQR. n=11 good recovery, n=5 re-exacerbated.

5.6 Discussion

This is the first study to date that investigates the use of nasosorption as a method of sampling to measure cytokines and chemokines in patients with COPD. In order to answer whether the above data support the hypothesis "Nasosorption can detect differences in inflammation in COPD compared to health" it is important to consider the integrity of the cohort, before proceeding to discuss the results in terms of the ability of nasosorption to differentiate between (a) COPD vs health, (b) COPD phenotypes and (c) to identify acute exacerbations of COPD.

5.6.1 The study cohort

For this study, healthy non-smoking controls were well matched with COPD patients. However, fewer healthy smokers were recruited than other cohorts. Of the 13 subjects initially screened as healthy smokers for the study, 6 were excluded at recruitment due to either presence of respiratory symptoms or newly found obstruction on spirometry suggestive of undiagnosed COPD; these excluded patients tended to be older with a larger pack year history. Meanwhile, those "healthy smokers" that were included in the study were younger with fewer pack years than those subjects in the COPD cohort although this was not significant. There is much ongoing work into early COPD cohort of 30-45 year old smokers without obstructive lung disease on spirometry but who may have abnormal oscillometry, early CT changes, mild respiratory symptoms and even viral induced exacerbations associated with a decline in FEV_1 (Ritchie 2019, Ritchie 2022, Ritchie 2023). In our study, the high exclusion rate and skewed demographics of the healthy smoker group raises the question as to whether there really is a "healthy smoker" population: Indeed, one cannot be certain "healthy smokers" are truly disease free based on symptoms and spirometry alone. With the above in mind, it is important to interpret any "healthy smoker" results with caution.

The recruitment of patients was deliberately performed using a community setting rather than secondary care. As such, the COPD cohort recruited and sampled appears to be a relatively good representation of the disease in the general population in terms of age (68.1±1.7years) and distribution of airflow obstruction between GOLD stages 1-4 with most being GOLD Stage 2 or 3 (Haughney 2013). Furthermore, in this cohort 22% of patients had a consistently high blood eosinophil count which is aligned with previously quoted figures of 15-40% (Bafadhel 2009, Segal 2018). However, the approximation of chronic bronchitis using the CAT score subcategories may overestimate identifying 40% as "high cough and sputum" burden compared to the 27-35% with chronic bronchitis quoted in literature (GOLD 2025): Any future studies require a more detailed questioning of duration of symptoms rather reliance on a questionnaire at a single time point. Meanwhile the incidence of COPD patients with bronchiectasis is lower than expected in this study

(16% vs 31% in literature (Maselli 2019)). This is possible because participants were selected to have COPD rather than bronchiectasis as the main pathology. Indeed, patients with bronchiectasis and COPD who had a very high exacerbation frequency or excessive sputum burden were excluded prior to enrolment, as such the COPD/bronchiectasis patients in this cohort are likely to be a milder phenotype. By contrast, this study has a slightly higher proportion of frequent exacerbators (42%) due to enrolment both from general COPD clinics but also via the acute COPD exacerbation service (Hurst 2010). These slight differences in phenotypes may skew data when comparing overall COPD to the healthy controls especially in a study such as this with relatively small numbers.

When looking at the clinical characterisation of COPD patients two clear messages are re-emphasised. Firstly, it highlights the important of symptoms as well as objective markers of severity such as lung function, since with the exception of breathlessness these are not correlated. Secondly despite blood eosinophil counts (BEC) being at the heart of categorising patients for standard maintenance therapy or indeed in drug trials for monoclonal antibodies, a one-off blood eosinophil count should be used with caution as there is high temporal variability as demonstrated in Figure 5.9.

A key limitation of the patient selection criteria was a lack of objective evidence of presence of absence of atopy. All subjects (healthy volunteers and COPD patients) were asked regarding atopy, but no allergy testing was performed such as skin prick testing or specific IgE testing on blood were performed. This is particularly relevant as a limitation when reviewing the data of markers of TH2 inflammation discussed later. Indeed, the concentrations of healthy non-smoker IL-5 and IL-33 in this study are much higher than those seen in baseline control group samples from previous papers (Campion 2023); this raises a serious question of whether unknown atopy may be affecting results. It is also notable that the levels of IFNγ are higher than expected in healthy non-smokers based on previous studies with levls <10pg/ml consistently (Hansel 2017, Jha 2021, Jha 2019). All participants were asked whether they had had a viral infection in the last 4 weeks and were asked to delay sampling accordingly. It may however be that healthy controls, under-report mild viral infections. Moreover, previous studies have shown that IFNγ is still significantly raised at 3 weeks after covid-19 infection (Baker 2022). In future, more rigorous checks of stable health should be enforced on healthy controls between study visits and sampling should possibly be delayed until 6-8 weeks post viral infection.

5.6.2 Utility of nasosorption in differentiating and characterising COPD

This study demonstrates that nasosorption can be used to measure an inflammatory profile in the nose and this appears to be independent of current smoking status, use of nasal steroids or major demographics such as age or gender. However, unlike in asthma or atopy (Chawes 2010, Hansel 2017), this study does not support a role of nasosorption in detecting common inflammatory profiles seen in

COPD. Of particular note CXCL8 and GM-CSF two cytokines known to be increased in COPD (Barnes 2009, Bradford 2017) are lower in the nasal MLF of COPD patients compared to healthy non-smokers. Meanwhile IL-6 which has previously been shown to be raised in current smoker (Carpagnano 2003, Di Stefano 2018) is lower in nasal MLF from current smokers compared to both ex-smokers and never smokers. Given that the lower respiratory treat mucociliary escalator brings molecules up from the peripheral small airways to the bronchi, trachea and larynx, meanwhile the mucociliary escalator of the upper respiratory tract moves in the opposite direction from the nose towards the larynx, it is perhaps not surprising that nasal mucosal inflammatory profile does not mirror the inflammatory process associated with the small airway fibrosis and parenchymal emphysema characteristic of COPD.

When considering the utility of nasosorption to phenotype COPD, the results are limited due to small sample size. The most interesting data presented above is in figure 5.10, which suggests that nasosorption may be able to identify those with high TH2 inflammation, with eotaxin-1 levels significantly higher in the nasal samples of those COPD patients with a high blood eosinophil count. This will be discussed in more detail below. There are some suggestions of potentially interesting trends in bronchiectasis/COPD overlap and frequently exacerbator, but these are unable to be validated given the very small sample size.

5.6.2.1 Nasosorption in identifying acute exacerbations

The exacerbation study approximates well to the literature with 50% associated with a viral cause, 37.5% bacterial and 28.6% eosinophilic (Wedzicha 2003, Bafadhel2011, Finney 2014). Nasosorption does not seem to be of use in differentiating between COPD stable state and acute exacerbating state. This study, however, shows that when AECOPD are categorised into cause, nasosorption is likely to be able to detect inflammation associated with virally triggered exacerbations with IFNα-2a being significantly increased and strong trends in CXCL10 and IFNγ (Figure 5.11). This is consistent with previous findings in which Bhafadel et al identified CXCL10 as a biomarker in viral AECOPD (Bafadhel 2011). Meanwhile Type 1 interferons (IFNα and IFNβ) as well as type 2 interferons (IFNγ) are known to be released by the respiratory epithelium in response to viral stimuli (Finney 2024). In COPD there is an impaired interferon release with reduced levels of IFNβ and IFNλ seen in frequent exacerbators both at baseline and during acute exacerbation (Mallia 2011, Singanayagam 2019). If indeed nasosorption is able to identify viral induced inflammation these positive results could be of great importance as the current gold standard, nasopharyngeal PCR, is not 100% sensitive (Wiseman 2024); but perhaps more significantly measuring IFN levels may yield extra information regarding the hosts response to virus, which in turn may have clinical implications. A larger study would be helpful to confirm whether the nasal inflammation seen represents just the presence of a viral upper respiratory tract infection (URTI), or whether levels of inflammation can predict which patients with viral URTI are

likely to go on to have an exacerbation of their airways disease (Future exacerbation study is discussed in more detail in Chapter 6). This would be an important differentiation in determining whether nasosorption could be used to guide physicians about early treatment or "at risk" cases.

With the exception of a relative absence of eotaxin-1 in bacterial AECOPDs, there was no signal seen between bacterial exacerbations versus non-bacterial exacerbations (Figure 5.12). The absence of a signal may be partly reflective of bacterial infections predominately affecting the lower respiratory tract, unlike viral infections which predominate in the upper respiratory tract. Moreover, viral AECOPD which usually have an easily identifiable prodrome, it is more clinically challenging to clinically differentiate bacterial from non-bacterial AECOPD (Miravitlles 2012, Spies 2023) and therefore results may have been misallocated. A larger exacerbation study would be useful in order to be able to look at microbiologically confirmed bacterial exacerbations to see if there are any trends in inflammatory profile in the nose.

5.6.2.2 Nasosorption to assess TH2 inflammation

As mentioned, this study highlights nasal eotaxin-1 as potential novel biomarker for TH2 inflammation in COPD. If one excludes the healthy smokers given concerns regarding true "healthy" status and low numbers or participants and therefore analyse just healthy non-smokers and COPD using Mann Whitney test, eotaxin-1 is significantly increased in COPD compared to healthy non-smokers. This is supported by other studies in which eotaxin-1 has been shown to be elevated in COPD (Miller 2007, Bradford 2017). In this study eotaxin-1 is increased in COPD patients with high blood eosinophil counts unlike other TH2 markers such as IL-4, IL-5 and IL-13 (Figure 5.10). This suggests that nasal eotaxin-1 might be a useful biomarker of eosinophilic inflammation in COPD. This supports Miller et al who also found that eotaxin-1 was the main TH2 cytokine raised in COPD (Miller 2007). Given the instability of blood eosinophil counts which vary with exercise, diet and circadian rhythms an alternative and more stable biomarker of TH2 inflammation is urgently needed to phenotype COPD patients, especially in the current climate of novel biologics in COPD. Unfortunately in this study contemporaneous blood eosinophil levels and nasal eotaxin-1 levels were not always available, and matched nasal and blood samples should be collected in any future studies. It is important to note that in this study patients with high eosinophil level during AECOPD were not associated with raised eotaxin-1. These results are likely distorted by the fact that was a *real world* study and thus 25% of patients were already on prednisolone at time of sampling: As such it is likely that a proportion of eosinophilic exacerbations have been masked and misallocated cohorts.

Nasal eotaxin-1 may also be relevant to recovery from AECOPD. Initial results on just a small sample size comparing recovery levels of eotaxin-1, suggest that it may be relevant to prolonged instability of

patients and early re-exacerbations. Brightling et al showed that an increase in recovery blood eosinophil count from exacerbation was predictive of future exacerbation risk and shorter time to re-exacerbation (Brightling 2016). Given exacerbation rate is a poor prognostic marker for COPD progression, these findings may explain why high eotaxin-1 levels are correlated with worse lung function and emphysematous change on CT (Miller 2007, Bradford 2017). More numbers are needed to look into eotaxin-1 in these recurrent early exacerbators: If a larger study confirmed eotaxin-1 being associated with risk of recurrent exacerbations, it would have clinical relevance when deciding about length of treatment, potential slow weaning of steroids, early interventions such as pulmonary rehabilitation, or in future perhaps a cohort of patients who might be more in need of targeted biologic therapies.

Nasosorption eotaxin-1 levels and TH2 cytokines (IL-4, IL-5 and IL-13) in COPD require further validation. Ideally nasosorption samples should be taken with and compared to contemporaneous blood eosinophil counts, spontaneous sputum eosinophil percentages, and endobronchial epithelial lining fluid samples via bronchosorption. Measurement of leukocyte granule proteins such as eosinophil-derived neurotoxin (EDN) and neutrophil gelatinase-associated lipocalin (NGAL) could be measured in nasosorption samples as a potential further means of differentiation between eosinophil and neutrophil predominant COPD phenotypes. A further study might also look at the comparative temporal stability of eotaxin-1 or other TH2 cytokines, blood eosinophils, and sputum eosinophils in COPD measuring each at regular intervals in stable and exacerbating states, or even in relation to exercise. Nasosorption as a tool for investigating IL-36 *in vivo.*

5.6.2.3 Nasosorption as method of measure IL-36 in vivo

This study assessed whether nasosorption could be a useful sampling method for studying IL-36 *in vivo*. IL-36 agonist cytokines (IL-36βα, IL-36β and IL-36γ) are detectable in nasal epithelial lining fluid when sampled using nasosorption. Meanwhile IL-36RA was not detectable in any samples: This is to be expected as unlike IL-36 agonists, the main source of IL-36RA is lung macrophages and not epithelial cells (Baker 2022). IL-36γ has been consistently shown to be increased in lower respiratory samples (sputum, BAL and lung homogenate) (Kovach 2017, Li 2021, Baker 2022), but using nasosorption as a sampling method of the upper respiratory tract, there was no significant difference between health and COPD in any of the IL-36 agonists. There was however a trend towards increased IL-36γ. Data from repeated samples from the same individuals suggest that nasal IL-36γ levels fluctuate over time particularly in those with higher levels. This variability may disguise any difference between health and COPD in this relatively small sample population taken at a single time point. Further work is needed to increase sample size of non-smokers, healthy smokers and COPD participants at stable state as well as to perform more repeated sampling to assess variability in health and COPD. A future study

should also consider using rhinoprobe nasal curettage to measure IL-36 cytokine mRNA (Dhariwal 2015). In this study nasal IL-36γ correlates with other markers of neutrophilic inflammation (CXCL1, CXCL8, GM-CSF, IL-1β, and IL-17A) in COPD patients which supports other work showing *in vitro* and mouse studies that IL-36γ drives neutrophilic inflammation in COPD (Koss 2021, Baker 2022) .

Earlier it was discussed that nasosorption may identify markers of virally-induced inflammation. IL-36γ secretion is virally induced *in vitro*, despite this in this study there was no difference between nasal IL-36γ exacerbation and recovery levels even when viral exacerbations were sub-analysed. Baker et al showed that IL-36γ was an early responder to RSV, HRV16 and influenza A, with levels from human bronchial epithelial cells peaking at between 48-72 hours post viral inoculation (Ombredane 2023). The incubation period for most viral respiratory infection ranges from 1-7 days (Lessler 2009), and samples in this exacerbation study were taken on average a further 5 days after symptoms started. As such it is almost certain that the AECOPD samples were taken too late to detect any possible virally induced spike in IL-36γ levels. In order to further investigate if IL-36γ signal is present in the nose it would be useful to perform repeated nasosorption in a controlled environment such as an observed viral or topical nasal viral mimetic (e.g. TLR 7 agonist, reisquimod) challenge to healthy and COPD subjects, whilst monitoring for symptom progression. The final results of this study in which recovery levels of IL-36γ are higher in those that re-exacerbate within 4 weeks than those that make a good recovery, suggest that perhaps after initial exacerbation, there may be ongoing inflammation and IL-36γ secretion via a positive feedback loop in a subset of patients who have very frequent exacerbations. Conflicting this argument however is that baseline IL-36γ levels were not elevated in frequent exacerbators overall (Figure 5.16).

5.7 Conclusion

In summary, the hypothesis that nasosorption can detect differences in inflammation in COPD compared to health cannot be accepted. There may however be a role in looking at inflammatory signals (particularly TH2 signal via eotaxin-1) to help differentiate COPD phenotype, as well as inflammatory response in acute exacerbation of COPD and predicting those with good recovery. Further work is needed in a larger cohort at stable state as well as more controlled environment for exacerbation studies.

Chapter 6: Discussion

This thesis has built on existing knowledge about the release of IL-36γ from SAECs and its effect on SAF in COPD and demonstrated that IL-36γ also has both general and disease specific effects on macrophage function (Figure 6.1). Prior to this study being undertaken, it was known that IL-36γ is increased and IL-36RA is reduced in COPD compared to healthy subjects (Baker 2022). IL-36γ causes SAF to be more pro-inflammatory releasing IL-6, MMP2, MMP9 which drive small airway remodelling, mucus hypersecretion, emphysema and neutrophil recruitment (Baker 2022). Chapter 3 demonstrates that macrophages cultured with media from IL-36γ-stimulated SAF are more pro-inflammatory, secreting increased IL-6, CXCL1 and CXCL8: This effect appears to be via an independent mediator(s) released by SAF in response to IL-36γ. Thus, high IL-36γ concentrations in COPD result in increased macrophage driven inflammation, contributing to disease pathogenesis directly and promoting neutrophil recruitment. IL-36γ also directly inhibits macrophage ability to phagocytose *H. influenzae* in COPD patients but not in healthy donor macrophages: This may have a role in the bacterial burden seen in COPD, both in terms of colonisation and acute infection.

IL-36RN is primary expressed by macrophages in the lung with COPD macrophages expressing lower levels of *IL36RN* compared to healthy macrophages (Baker 2022). As such, the pathogenic effects of increased IL-36γ described above remain relatively unregulated. The deficiency of *IL-36RN* expression appears to be an innate defect in COPD subjects. Indeed, monocytes and MDM from COPD subjects, which have never encountered the COPD lung environment, show decreased *IL-36RN* expression compared to healthy subjects' cells. The GM-CSF rich lung, differentiates monocytes to a M1-like alveolar macrophage phenotype (Winkler 2008) and in health this differentiation is associated with an significant increase *IL-36RN* expression as demonstrated in Chapter 4. However, in COPD, the increase in *IL-36RN* expression from monocyte to GM-CSF differentiated macrophage does not occur. As such, healthy GM- MDM show ~5.5-fold higher *IL-36RN* expression than COPD GM-MDM. *IL-36RN* expression is regulated via PI3K activity. In COPD, PI3K activity is increased in the lungs (Moradi 2021), and consequently high PI3K activity will suppress *in vivo IL-36RN* expression beyond that which is seen in GM-MDM experiments *in vitro*. Interestingly GM-CSF also appears to stimulate IL-36γ secretion in the lung (Koss 2021). Thus, high levels of GM-CSF associated with COPD not only induce inflammation directly (Barnes 2017), but may also drive more IL-36γ-induced inflammation, without any protective increase in *IL-36RN* expression by macrophages.

The following discussion will focus on IL-36 receptor signalling and its possible role in the senescence associated secretory phenotype, pathogenesis of acute exacerbations, and chronic bacterial colonisation. Finally, the *in vivo* data will be discussed and the future of IL-36 signalling as a novel COPD drug target.

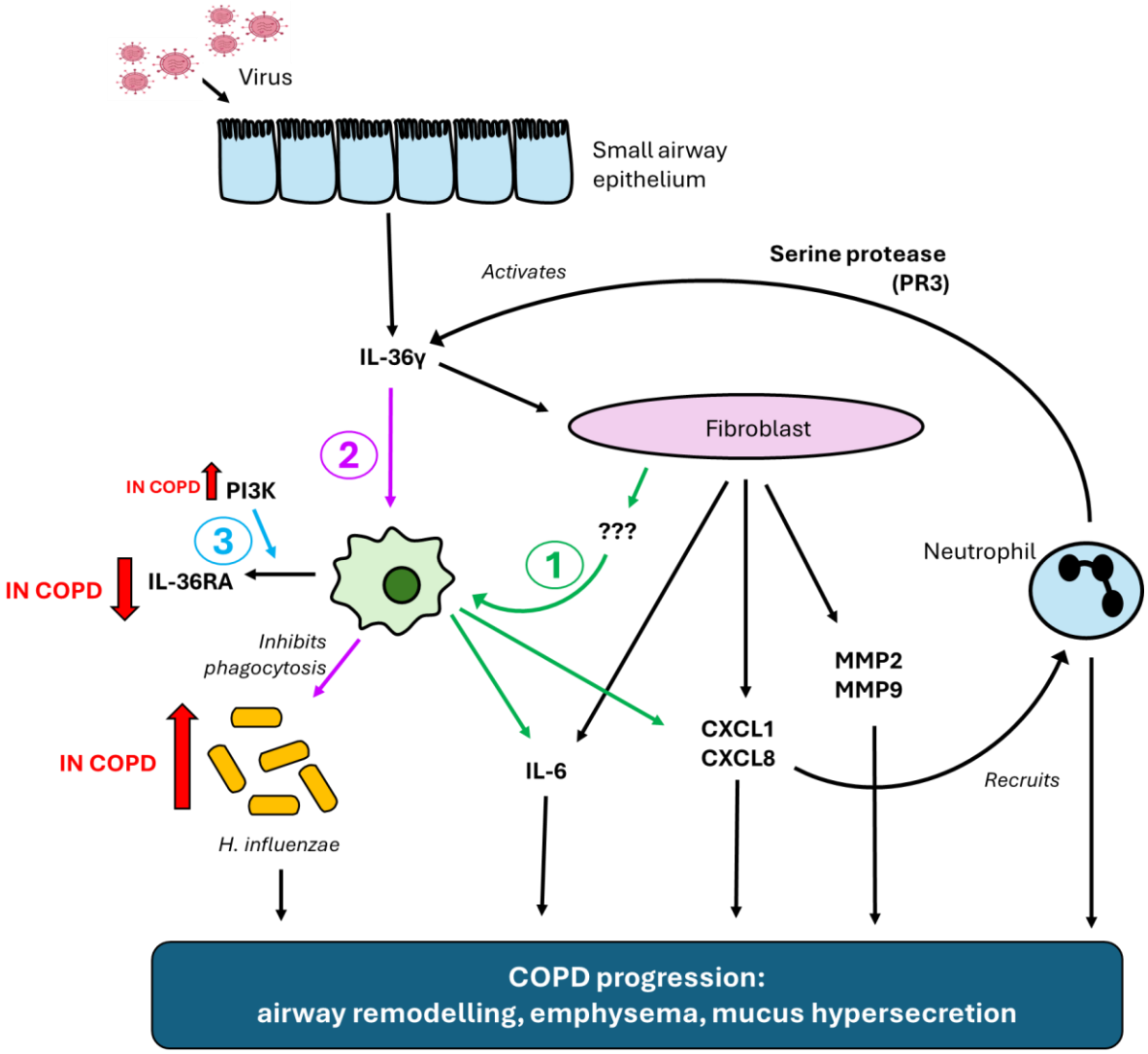

Figure 6.1: Schematic outlining the role of IL-36 signalling in COPD pathophysiology
Viral infection causes IL-36γ to be secreted from SAEC. Neutrophil elastase cleaves and activates IL-36γ which then promotes SAF to release cytokines, chemokines and proteases. These drive COPD progression and recruit neutrophils to the lung. This thesis expands on the above to show: ① IL-36γ stimulated SAF secrete mediator(s) which cause macrophages to be more pro-inflammatory secreting IL-6, CXCL1 and CXCL8. ② IL-36γ directly inhibits COPD macrophage ability to phagocytose *H. influenzae*. ③ *IL-RN* expression is reduced in COPD macrophages compared to healthy macrophages. Increased PI3K activity, raised in COPD, inhibits *IL-36RN* expression.

This thesis builds on data showing that IL-36γ induces the secretion of a range of inflammatory mediators, including IL-1β, IL-6, CXCL1, CXCL8, GM-CSF, TNFα, MMP2 and MMP9 (Kovach 2017, Baker 2022): These molecules are all associated with the SASP (Barnes 2019). There is growing evidence that COPD is due to the accelerated aging of lung with accumulation of senescent cells (Barnes 2019). SIRT-1 and SIRT-6 are two molecules known to inhibit senescence and they are decreased in COPD (Baker 2016). In rats, IL-36R knockout or treatment with IL-38, another IL-36 antagonist, has been shown to increase SIRT-1 expression in cardiomyocytes and synoviocytes respectively (Luo 2020, Pei 2020) This suggests that IL-36 signalling might drive senescence, and consequently IL-36 receptor antagonists could potentially have a role as senolytic agents. Indeed increased IL-36 receptor signalling has been shown to be involved in other diseases associated with increased senescence such as ischemic heart disease, type 2 diabetes mellitus, metabolic bone disease and Alzheimer's disease (Barnes 2017, Masoumi 2020, Neurath 2020, Li 2021, El-Awaisi 2022): Many of these are recognised co-morbidities of COPD (Burgel 2010, Vanfleteren 2013). In this study, *IL-36RN* expression increases (albeit non-significantly) with age in healthy macrophages but is negatively correlated with age and smoking in COPD. Therefore, in COPD there is absence and even reversal of a potentially protective mechanisms for aging. Given the seeming importance of IL-36 signalling in COPD pathogenesis, the innate difference in *IL-36RN* expression in monocytes and MDM suggests certain individuals are pre-disposed to a higher risk of developing COPD. As such it would be interesting to investigate *IL-36RN* expression in a young "pre-COPD" cohort and determine if there was any correlation between *IL-36RN* expression and prospective rate of lung function decline.

Dysregulated IL-36 receptor signalling may be increased during AECOPD. IL-36 agonists are released within the lung as an early warning signal, or alarmin, in response to potentially harmful injury (Macleod 2020, Ombredane 2023). Indeed IL-36γ is secreted by small airway epithelial cells within the first 24-72h after viral challenge (Ombredane 2023). Cigarette smoke and bacterial infection may also increase IL-36 secretion in the lung (Kovach 2016, Kovach 2021). As such, IL-36γ-induced inflammation in response to external stressors, is likely to be contribute to the neutrophilic inflammation present in ~60% of exacerbations (Finney 2024). Furthermore, the imbalance between IL-36γ and IL-36RA may be accentuated during AECOPD compared to stable state. Chapter 4 demonstrates that *IL-36RN* expression is suppressed by PI3K activity. Meanwhile, PI3K is activated by environmental stressors such as cigarette smoke, bacterial and viruses (Moradi 2021). Whilst the present study does not examine the expression of *IL-36RN* by macrophages during AECOPD, Chen et al. demonstrated that IL-36RA is reduced in plasma during AECOPD compared to stable state COPD (Chen 2012). As such there is potentially an accentuation of the already dysregulated IL-36 receptor signalling during AECOPD, with increased IL-36γ and reduced IL-36RA compared to stable state.

After viral infection there is a rise in bacterial load in the COPD lung, especially *H. influenzae*, which is not seen in healthy non-smokers or healthy smokers (Mallia 2012, Molyneaux 2013). *In vitro*, human rhinovirus directly impairs bacterial phagocytosis in COPD macrophages but not healthy macrophages (Finney 2019). The present study shows that another mechanism is also likely to contribute to these secondary bacterial infections. Viruses stimulate IL-36γ secretion from SAEC, which in turn inhibits COPD macrophage ability to phagocytose *H. influenzae*. This negative effect of IL-36γ on macrophage phagocytosis is disease specific and not seen in cells from healthy donors. Acute bacterial infections are present in approximately half of AECOPD. Viral-bacterial co-infection is associated with increased severity of exacerbation, prolonged recovery and increased hospital readmission rates (Wilkinson 2006, Bafadhel 2011, Wark 2013).

Lower airway bacterial colonisation is clinically important in COPD. There appears to be a vicious cycle between AECOPD and colonisation within COPD pathophysiology: During AECOPD, especially neutrophilic or viral induced exacerbations, the bacterial microbiome is altered (Tiew 2024). Meanwhile bacterial colonisation results in an increased sensitivity to viruses and increased rates and severity of AECOPD (Patel 2002, Singh 2015). The relationship between IL-36 concentrations (either in stable state or at AECOPD) and the microbiome have not been studied, but it is plausible that by inhibiting bacterial phagocytosis in COPD macrophages, IL-36γ promotes lower airway bacterial colonisation. IL-36R knockout seems to have variable effects on murine survival of infection with different pathogens (Aoyagi. 2017, Kovach 2017, Ahsan 2018, Nanjo 2019). As such, it is important to examine whether the negative effect of IL-36γ on COPD macrophage phagocytosis of *H. influenzae* is replicated with other common pathogenic bacteria e.g. *S. pneumoniae* and *P. aeruginosa* and fungi e.g. *A. fumigatus*.

6.1 Limitations to study

A major limitation of this study is the absence of positive data regarding IL-36 *in vivo*. Nasosorption has shown to be a useful sampling method in asthma, viral infection and allergy (Hansel 2017, Leaker 2017, Jha 2018). However, the nasosorption data presented in Chapter 5 suggest that many of the common inflammatory signals associated with COPD (e.g. raised CXCL8, GM-CSF, reduced IL-10) are not reflected by this sampling method, and thus its use in investigating inflammation in COPD may be limited. The COVID-19 pandemic closed research bronchoscopy facilities for ~1 year during this study and consequently matched upper and lower airway samples were not able to be taken to determine if nasosorption cytokine concentrations correlate with lung inflammation in COPD. There is a trend towards IL-36γ being raised in nasal epithelial lining fluid in COPD compared to health, but this needs further evaluation, including a better understanding of how IL-36γ concentrations fluctuate within individuals over time. Nasosorption did not detect any change in IL-36γ during AECOPD, perhaps

reflecting the timing of IL-36γ as a very early responder to injury (Ombredane 2023). Future *in vivo* studies should consider using other sampling methods to measure IL-36 cytokine. Although there are challenges in retrieving sputum samples in COPD patients (it is not always spontaneously produced, and induced sputum is unpleasant for patient and carries risk of bronchospasm) sputum may be better suited to examining IL-36 signalling in COPD. IL-36γ is measurable in sputum, and sputum IL-36γ has been shown to be positively associated with a neutrophilic inflammatory phenotype in stable state COPD compared to those with eosinophilic inflammation (Li 2021, Moermans 2021). Only IL-36α has been measured in sputum during AECOPD and was found to be reduced along with IL-36RA (Chen. 2012).

A further limitation is the inability to measure IL-36RA protein due to the poor selectivity and availability of antibodies. Data presented in Chapter 4 on *IL-36RN* expression in monocyte and macrophage populations is robust and presents a mechanism which aligns with existing knowledge of COPD pathogenesis i.e. *IL-36RN* expression is reduced in COPD and its reduced expression is affected by a major signalling pathway (PI3K) increased in COPD. The inability to measure IL-36RA protein, means that there is an absence of direct knowledge about IL-36RA in COPD. For example, it is still not known whether defective *IL-36RN* expression by COPD macrophages correlates with IL-36RA levels, or whether there are other confounding factors such as translation of *IL-36RN*, or packaging and secretion of IL-36RA from macrophages. Inability to measure IL-36RA on nasosorption samples in Chapter 5, is also a major limitation. Indeed, when examining *in vivo* the clinical consequences of dysregulated IL-36 receptor signalling, the ratio between IL-36γ and IL-36RA is likely to be more important than IL-36γ concentration taken alone.

6.2 IL-36 receptor as a future drug target in COPD

Currently COPD is at a pivotal moment in drug development. After many failed attempts to re-purpose biologic therapies, in the last year three biologic treatments have finally shown positive results in COPD. These drugs all target COPD patients with high blood eosinophil counts: Dupilumab (anti-IL4/IL-13) and mepozumab (anti-IL5) are proposed as a maintenance therapy; and benralizumab (anti-IL-5R) as a rescue therapy in moderate/severe eosinophilic AECOPD (Bhatt 2024, Ramakrishnan 2025, Pavord 2017, Sciurba 2025) . But these treatments are not suitable for the majority of COPD patient who demonstrate neutrophilic inflammation both at stable state and during AECOPD. The need for new targeted therapy for neutrophilic inflammation in COPD is not only particularly relevant due its dominance as an inflammatory phenotype, but also because neutrophilic inflammation (unlike eosinophil-associated inflammation) is relatively insensitive to corticosteroids, a mainstay of current COPD treatment (Bafadhel 2012, Gao 2013, Bafadhel 2018). Of note, IL-36γ release from SAEC is also steroid insensitive (Baker 2022).

Given the various mechanisms by which IL-36γ-induced inflammation is thought to drive COPD pathogenesis (Figure 6.1), targeted inhibition of the IL-36 receptor (IL-36R) is an attractive therapeutic proposal. IL-36RA binds to the IL-36R with higher affinity and with a much slower off rate than the IL-36 agonists (Zhou 2018), therefore increasing *IL-36RN* expression is a possible therapeutic strategy. However, PI3K inhibitors are associated with significant side effects including mucositis, pneumonitis, colitis (Fruman 2017). An alternative approach is to use a monoclonal antibody (mAb) that binds specifically to the IL-36R and antagonises IL-36 signalling. Spesolimab is the first IL-36R mAb to be approved by NICE for treatment of GPP (Morita 2023). It has shown significant clinical benefit and a good safety profile in this severe neutrophilic skin disorder which is often associated with *IL-36RN* mutations. Given that IL-36γ drives neutrophilic inflammation, likely promotes bacterial colonisation and contributes to both viral and bacterial AECOPD, there is a good rationale for proposing spesolimab, or an alternative IL-36R mAb, as a maintenance therapy in COPD. If a clinical trial were to go ahead, the following clinical criteria for enrolment should be considered (1) neutrophil predominant inflammation (i.e. persistently low blood eosinophil count <100 cells/µl), (2) with frequent moderate to severe exacerbations (≥2 per year) as these are most likely to derive clinical benefit. Chapter 3 demonstrated that IL-36γ had a smaller effect on phagocytosis in patients with severe airflow obstruction, as such patients with severe airflow obstruction (FEV_1 <50% predicted) should be excluded. A suggested primary endpoint is frequency of AECOPD with secondary endpoints to include pre-bronchodilator FEV_1 (change from randomisation to during treatment), symptom burden (CAT score) and health status (SRGQ). Unfortunately, Chapter 5 data does not support the use of nasosorption as a robust tool for measuring airway inflammation *in vivo* in COPD. As such any proof of mechanism of action of an IL-36R mAb might measure markers of neutrophilic inflammation (e.g. CXCL1 and CXCL8) in sputum samples and also include sputum neutrophil count. More work is needed to show correlation between these and IL-36γ activity *in vivo*.

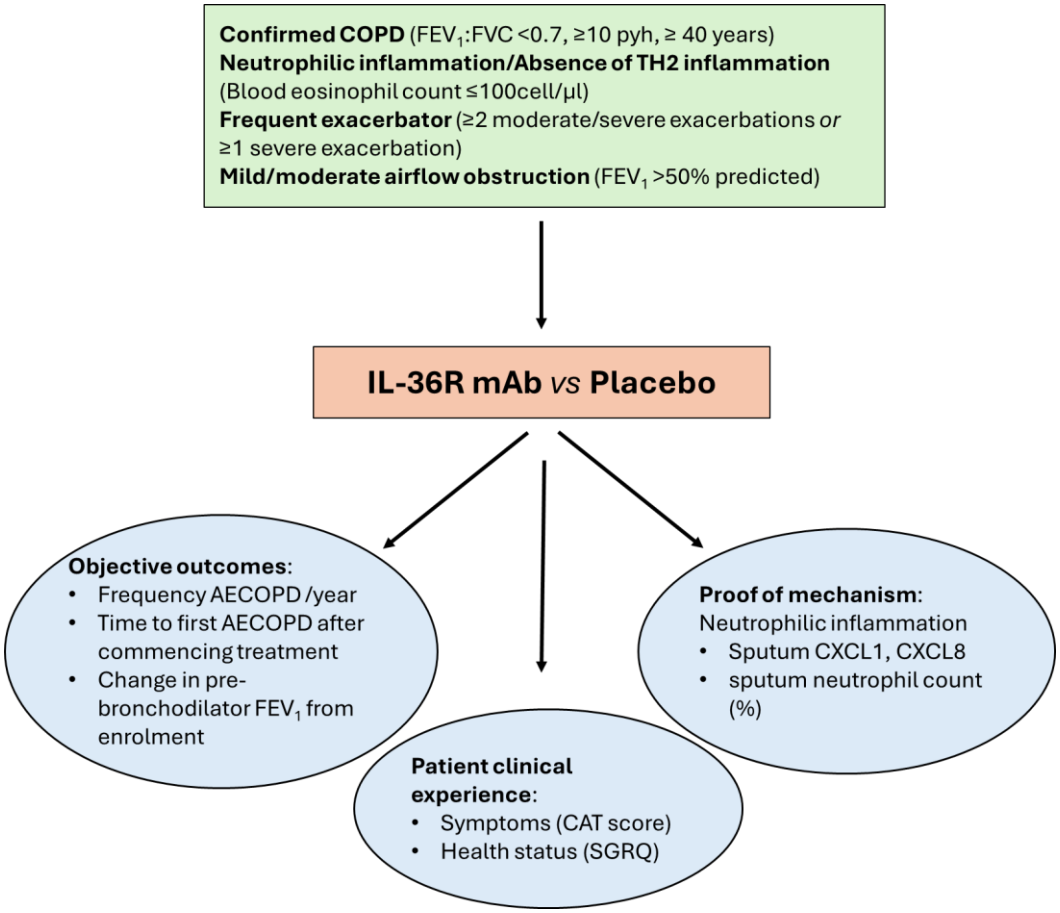

Figure 6.2: Schematic outlining how a clinical trial of IL-36R mAb might be conducted
Randomized, double-blind, placebo-controlled study of IL-36R monoclonal antibody (mAb). Enrolment criteria would include confirmed COPD with absence of TH2 driven inflammation, history of frequent exacerbations (moderate exacerbation = requiring treatment with systemic corticosteroid, severe = requiring hospitalisation) and mild/moderate impairment in FEV_1. Primary end point would be frequency of acute exacerbations COPD (AECOPD) with other objective and patient experience secondary endpoints as outlined. Proof of mechanism of action might include neutrophil chemoattractants (CXCL1 and CXCL8) concentrations and neutrophil count in sputum.

The use of IL-36R mAb as a rescue therapy for AECOPD is a less appealing approach of treatment. IL-36 agonists are early alarmins with peak release 24h post H1N1 influenza, 48h post human rhinovirus, 72h post RSV infection followed by a rapid decline (Ombredane 2023): These timepoints are earlier than the symptom onset for each of these viruses respectively (Lessler 2009). As such by the time a COPD patient presents to healthcare services for viral associated AECOPD, IL-36 receptor signalling is already likely be returning to baseline. Nasosorption data in Chapter 5 suggest that perhaps there is a cohort of AECOPD which have persistently high IL-36γ after recovery from initial exacerbation, and that these patients are more likely to re-exacerbate early. This might reflect that in some AECOPD, there are ongoing feedback loops or prolonged stimulation of IL-36γ secretion beyond the initial epithelial insult causing the acute exacerbation. Indeed, in mice IL-36 receptor signalling appears to positively feedback to cause more IL-36γ release (Koss 2021) and therefore ongoing inflammation. More work is needed to identify which AECOPD subjects are at high risk of early relapse or severe prolonged exacerbation, with the aim of perhaps targeting these patients with acute IL-36R directed mAb in future trials.

6.3 Conclusions

The data presented in this thesis extends understanding of the role of IL-36 receptor signalling in driving the neutrophilic inflammation that characterises the majority of COPD patients. The hypotheses for this thesis were:

1. *IL-36γ accentuates the abnormal COPD macrophage phenotype promoting secretion of pro-inflammatory cytokines and inhibiting bacterial phagocytosis.*
2. *Abnormal regulation of IL-36RN expression by COPD macrophages means that pathogenic effects of IL-36γ continue relatively unregulated.*
3. *As such, IL-36γ levels are correlated with COPD severity and acute exacerbations.*

The first and second of these hypotheses can be accepted. It has been demonstrated that increased IL-36γ in the COPD lung, results in COPD macrophages having impaired ability to phagocytoses *H. influenzae* and (via IL-36γ mediated inflammation from SAF) macrophages display a more pro-inflammatory phenotype. An innate defect in *IL-36RN* expression in both COPD monocytes and macrophages means that IL-36γ induced pathology is relatively unopposed.

The final hypothesis cannot be accepted. Chapter 5 data was unable to confirm that nasosorption reflects the airway inflammation in COPD, and further work is needed to examine *in vivo* how IL-36γ and IL-36RA balance correlates with COPD phenotype and exacerbation pathogenesis.

6.4 Future work

Besides the long-term aim of performing a clinical trial of a monoclonal antibody targeting IL-36R, there are many smaller studies which might be performed in the first instance to continue to understand more about the role of IL-36 receptor signalling *in* vivo and *in vitro* in COPD.

6.4.1 IL-36 induced inflammation

Chapter 3 demonstrates that macrophages secrete more IL-6, CXCL1 and CXCL8 when cultures with media from IL-36γ-stimulated SAF. Further work could examine:

- **What mediator(s) released by IL-36γ stimulated SAF induce the pro-inflammatory state in macrophages?** This is likely to be a lengthy process of pre-treating macrophages with various receptor antagonists to see if any inhibit the pro-inflammatory response to SAF media.
- **What other macrophage secreted mediators are affected by culture with media from IL-36γ stimulated SAF?** e.g. pro-inflammatory cytokine (GM-CSF, TNF), proteases (MMP2, MMP9), anti-inflammatory cytokines (IL-10).
- **Is IL-36γ induced inflammation steroid responsive.** It is known that IL-36γ release from SAEC is not responsive to steroids (Baker 2022) and in general COPD macrophages are also less responsive to steroids than healthy macrophages (Chana 2014). It would be interesting to repeat the experiment described in Section 3.3.6, but with the aim of examining whether pre-treatment of SAF with corticosteroid affected (a) IL-36γ induced secretion of IL-6, CXCL1, CXCL8 from SAF, or (b) macrophage secretion of IL-6, CXCL1, CXCL8 after culture with media from IL-36γ stimulated SAF +/- pretreatment with corticosteroids (Figure 6.3).

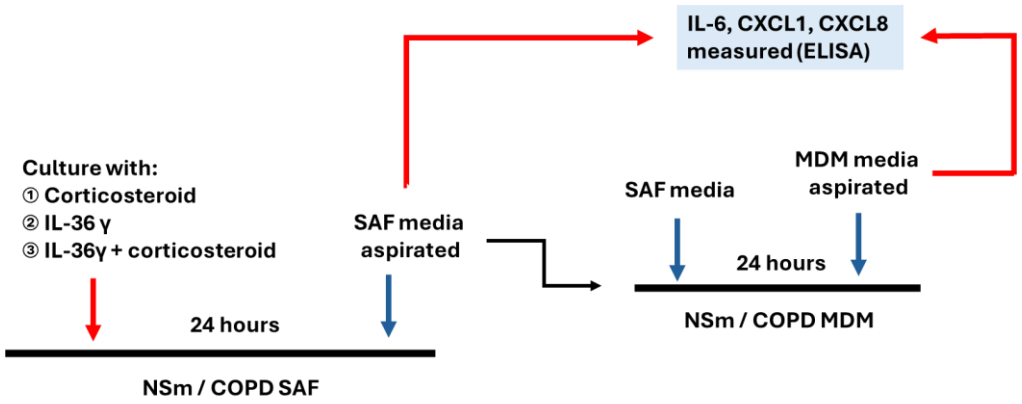

Figure 6.3: Schematic outlining future investigation steroid sensitivity of IL-36γ induced inflammation
Non-smoker (NSm) and COPD SAF are treated with (1) corticosteroid e.g. budesonide (2) 200ng/ml IL-36γ or (3) corticosteroid and 200ng/ml IL-36γ for 24h and media aspirated. IL-6, CXCL1, CXCL8 are measured by ELISA in each of the 3 conditions to assess if IL-36γ-induced inflammation from SAF is steroid responsive. NSm and COPD MDM are then cultured for 24h with the media from SAF aspirated under each of the three conditions and IL-6, CXCL1 and CXCL8 again measured by ELISA.

Another study might look at the role of IL-36 signalling in promotion of senescence. In the first instance, one could examine whether culture of SAF or macrophages with IL-36γ or IL-36RA, effects the expression of key senescence markers e.g. $p21^{CIP1}$, or anti-aging molecules e.g. SIRT-1 and SIRT-6.

6.4.2 IL-36γ effect on macrophage phagocytosis

In COPD macrophage phagocytosis is impaired across various bacterial species, fungi as well as clearance of dead and apoptotic cells (efferocytosis). Data from mouse studies suggests variable effects of IL-36 receptor signalling on clearance of different pathogens (Aoyagi 2017, Kovach 2017, Ahsan 2018). Therefore, it is important to clarify in a human MDM model how culture with IL-36γ affects macrophage phagocytosis of common pathogenic species in COPD (e.g. *S. pneumonia, M. catarrhalis, P. Aeruginosa, A. fumigatus*) and efferocytosis of apoptotic cells. Increase in numbers of COPD subjects would allow one to examine further whether there is any correlation with IL-36γ impairment of phagocytosis and COPD phenotype e.g. frequent exacerbator, chronic bacterial colonisation etc.

6.4.3 *IL-36RN* expression by macrophages

Increased numbers of subjects are needed to confirm correlations with *IL-36RN* expression and age, smoking history. There was no correlation between *IL-36RN* and spirometry, but another study might look at *IL-36RN* expression in macrophages and look for association with other COPD phenotypes such as exacerbation frequency, bacterial colonisation, emphysema or chronic bronchitis.

Further work to understand the pathway downstream of PI3K which controls *IL-36RN* expression could also be undertaken. A starting point might be to investigate involvement of microRNA-34a which is also implicated in COPD in the expression of the anti-aging molecules, SIRT1 and SIRT6 (Barnes 2019).

6.4.4 Examining IL-36 *in vivo*

The work from Chapter 5 could be further expanded to explore what sampling method would be best suited to measure IL-36 associated inflammation in COPD. An increase in size of cohort at stable state of healthy non-smokers, smokers and COPD would allow better ability to see significant trends and phenotype COPD where appropriate. In the first instance matched serum, sputum (including sputosoprtion), nasosorption samples would be taken. Ideally endobronchial sampling either via bronchial wash or bronchosorption would be useful to confirm whether any of the above represent lower respiratory tract IL-36γ concentrations. Microbiological data (either sputum or BAL microscopy and culture) would help understand whether there is an associated between degree of IL-36γ inhibition of macrophage phagocytosis *in vitro* and bacterial colonisation *in vivo.*

As discussed above, a real-life community based AECOPD study has major limitation in capturing any IL-36γ signal, due to IL-36γ being a very early phase alarmin. Human rhinovirus challenges under controlled conditions have been shown to be a safe way to understand more fully inflammatory and physiological responses to viruses in COPD and healthy subjects (Mallia 2011). Indeed, an observed human rhinovirus challenge would allow for regular monitoring from inoculation with virus through to symptoms and recovery and as such, any early IL-36 expression would be captured. If undertaken, this study would also monitor: IL-36 induced inflammation e.g. IL-6, CXCL1, CXCL8, GM-CSF, MMP2, MMP9; viral clearance by PCR of nasopharyngeal swabs and sputum samples; MDM response in terms of phagocytic ability and IL-36RN expression; and clinical complications of viral infection such as symptoms of AECOPD, change in FEV_1, or evidence of secondary bacterial infection. As such, a viral challenge would aim to develop understanding of (a) how COPD vs healthy controls respond to viral infection in terms of IL-36 induced inflammation (b) any variability of IL-36-induced inflammation within COPD patients and (c) whether degree of inflammation predicts clinical outcomes e.g. AECOPD or secondary bacterial infection. This viral challenge study would be invaluable in considering any clinical trial parameters such as patient selection, primary and secondary end points.

6.4.5 Validating nasosorption in COPD

This should concentrate on 2 main avenues:

a) **Type 2 inflammation:** Patients should be well classified in terms of spirometry and bronchodilator reversibility, FeNO, HRCT, and allergy testing (skin prick or specific IgE). Repeated sampling of patients during day to account for changes with circadian rhythm, exercise etc which are known to affect blood eosinophils. Sampling should include: blood eosinophil counts; nasosorption for cytokines (IL-4, IL-5, IL-13, IL-33, TLSP, Eotaxin-1), leucocyte granule proteins (eosinophil derived neurotoxin vs neutrophil lipocalin-2); spontaneous sputosorption for total eosinophil percentage, cytokines and chemokines as above for nasosorption. A one off bronchoscopy for bronchosorption at end of the day of sampling would be done to be able to correlate upper and lower airway inflammation.

b) **Acute Exacerbation**: As per section 6.4.4 a controlled exacerbation challenge would be useful to capture more whether nasal inflammation in response to potential exacerbation trigger is different between healthy and COPD participants. Within COPD participants there would be investigation as to whether levels of inflammation correlate with clinical presentation such as development and severity of exacerbation (measured by symptoms, clinical examination, peak flow). For this study the challenge test might include: inhaled irritant (viral challenge e.g human rhinovirus, viral mimetic e.g. resiquimod, bacterial mimetic e.g. lipopoylsaccharide, or indeed smoke inhalation. Particular areas of interest in terms of nasal serial measurements

would include: viral titres and specific IgG and IgA levels (if relevant); IFN response, TH2 response.

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