NOVEL INSIGHTS INTO THE CLINICAL AND IMMUNOLOGICAL ASPECTS OF EARLY CHRONIC OBSTRUCTIVE PULMONARY DISEASE

by

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Abstract

Chronic obstructive pulmonary disease (COPD) is a leading cause of global morbidity and mortality. Despite this, there has been little progress in disease-modifying therapies, partly due to a poor understanding of the definition and mechanisms surrounding the early COPD disease process before it becomes established. COPD develops only in a proportion of smokers, and underlying pathology is likely to progress before the disease can be detected by spirometry. There is also evidence for the role of neutrophils in early disease, which may form a vital biomarker to investigate the pathophysiological processes of early COPD.

This thesis aimed to investigate changes in a group of smokers who may be at risk of COPD by assessing symptom burden, lung physiology, and evidence of emphysema on chest computed tomography (CT) scanning, with a focus on whether the presence of chronic bronchitis (CB) may influence these changes. Changes in peripheral neutrophil function and phenotype among these smokers were also assessed, using peripheral neutrophils from healthy non-smokers as controls. Smokers with CB had a higher physical and psychological symptom burden than asymptomatic smokers, but no differences were seen between the two groups' lung physiology and emphysema. However, many smokers had evidence of small airway dysfunction and/or emphysema which may be features of early COPD even when there is no evidence of airflow obstruction on spirometry.

In addition, peripheral neutrophils from CB smokers were observed to have impaired migratory function similar to neutrophils from COPD patients, which may result in increased collateral tissue damage in susceptible smokers. No difference in neutrophil degranulation or cell surface marker expression was seen among CB smokers, asymptomatic smokers, and

healthy non-smokers. However, comprehensive neutrophil phenotyping using dimension-reduction algorithms suggested subtle phenotype differences among the three groups. In conclusion, many smokers have evidence of clinical, physiological, and radiological features that could indicate early COPD disease process, which may progress to established disease. Altered neutrophil migration found among CB smokers provides further understanding of the role of neutrophils in early disease and merits further investigation.

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COVID-19 Declaration

My study was significantly impacted by the COVID-19 pandemic, which struck halfway through my second year of PhD. All studies within the hospital were paused as research staff were asked to support clinical work in March 2020. I was also called to support the clinical workforce in the Respiratory Department in Queen Elizabeth Hospital Birmingham between March and July 2020 and again between January and March 2021 as I entered a significant data collection phase. Furthermore, the laboratory where I conducted the translational experiments was closed from March to June 2020.

From March 2020 onwards until the time of writing, the impact of the COVID-19 pandemic meant support within the hospital for physiological testing, and radiological scanning of participants was impossible. Social distancing and hygiene measures meant less testing or scanning could be done daily. Available slots were prioritised for clinical needs and to reduce the backlog which resulted from the departments being closed during the height of the pandemic wave. This resulted in the small number of scans available for analysis, and longitudinal follow-up of physiology data was impossible.

When the laboratory reopened, significant restrictions hampered the use of blood and blood products in the laboratory. Lack of guidance from the HSE caused substantial delays in risk assessment approval. As SARS-CoV-2 is a hazard group 3 agent, work with fresh blood samples was impossible under the law until these assessments were in place. These assessments were approved in August 2020, but a bubble-based system was adopted in the workplace, limiting us to 50% time in the lab and 50% work from home, which continued until June 2021. As a significant part of the study depended on the isolation of neutrophils from peripheral blood,

data collection was impossible, and non-cellular assays using stored samples were used instead. This resulted in some of the results presented in the thesis having low numbers of biological replicates. The impact of the COVID-19 pandemic on data collection is covered in each relevant chapter.

Despite these caveats, I believe the work presented here represents a significant contribution to the field. Data presented here would advance our knowledge of the early COPD disease process and help guide future research direction. When data collection was impossible, I focused on publishing a literature review and contributed to other studies covered in the appendix.

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List of Abbreviations

7AAD	7-aminoactinomycin D
AAT	α1-antitrypsin
AATD	α1-antitrypsin deficiency
ACO	Asthma-COPD overlap
AF700	Alexafluor700
ANOVA	Analysis of variance
APC	Allophycocyanin
ARTP	Association for Respiratory Technology and Physiology
AS	Asymptomatic smokers
AUC	Area under curve
Ax	Reactance area
BAL	Bronchoalveolar lavage
BD	Bronchodilator
BLF	British Lung Foundation
BMI	Body mass index
BOLD	Burden of Obstructive Lung Disease
BSA	Bovine serum albumin
BV	Brilliant Violet
cAMP	Cyclic adenosine monophosphate
CAT	COPD Assessment Test
CB	Chronic bronchitis
CC16	Club cell protein 16
CD	Cluster of differentiation
CEACAM8	CEA Cell Adhesion Molecule 8
CI	Confidence interval
COCOMO	Copenhagen Comorbidity in HIV Infection
COPD	Chronic obstructive pulmonary disease

COPDGene	Genetic Epidemiology of COPD
COVID-19	Coronavirus disease 2019
CRP	C-reactive protein
CRQ	Chronic Respiratory Questionnaire
CT	Computed tomography
CV	Coefficient of variance
CXCL	C-X-C containing ligand
CXCR	C-X-C containing receptor
CXR	Chest X-ray
DELFIA	Dissociation-enhanced lanthanide fluorescence immunoassay
DICOM	Digital Imaging and Communications in Medicine
DNA	Deoxyribonucleic acid
EB-OCT	Endobronchial optical coherence tomography
ECLIPSE	Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints
ECM	Extracellular matrix
ECRHS	European Community Respiratory Health Survey
ELISA	Enzyme-linked immunosorbent assay
ESL-1	E-selectin ligand-1
FCS	Flow Cytometry Standard
FcγRIII	Fragment of crystallisation (Fc)-gamma receptor III
FEF	Forced expiratory flow
FEV1	Forced expiratory volume in the first second
FITC	Fluorescein isothiocyanate
fMLP	N-Formylmethionine-leucyl-phenylalanine
FOT	Forced oscillometry technique
FPR	Formyl peptide receptor
FVC	Forced vital capacity
GAD-7	Generalised Anxiety Disorder Scale
G-CSF	Granulocyte-colony stimulating factor

GesEPOC	Spanish Guidelines for Management of COPD
GHQ-28	General Health Questionnaire
GLI	Global Lung Initiative
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GORD	Gastroesophageal reflux disease
HADS	Hospital Anxiety and Depression Scale
HDAC2	Histone deacetylase 2
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen – DR isotype
HNS	Healthy non-smokers
HS	Healthy smokers
HU	Hounsfield unit
ICAM	Intracellular adhesion molecules
ICC	Intra-class correlation coefficient
ICS	Inhaled corticosteroids
IL	Interleukin
IMD	Index of multiple deprivation
IQR	Interquartile range
IRR	Incidence rate ratio
ISCO	International Standard Classification of Occupations
ISOLDE	Inhaled Steroids in Obstructive Lung Disease in Europe
KCO	Carbon monoxide transfer coefficient
KOLD	Korean Obstructive Lung Disease
LAA-950HU%	Low attenuation areas less than a threshold of -950HU
LABA	Long-acting beta2-agonists
LAMA	Long-acting antimuscarinics
LCQ	Leicester Cough Questionnaire
LFA-1	Lymphocyte function-associated antigen-1
LLN	Lower limit of normal
LLOD	Lower limit of detection

LLOQ	Lower limit of quantification
LOA	Limit of agreement
LPS	Lipopolysaccharide
LRTI	Lower respiratory tract infection
LSOA	Lower-layer super output area
LTB4	Leukotriene B4
LVRS	Lung volume reduction surgery
MAPK	p38 mitogen-activated protein kinase
MCID	Minimal clinically important difference
MESA	Multi-Ethnic Study of Atherosclerosis
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MMEF	Maximal mid-expiratory flow
MMP	Matrix metalloproteinase
mMRC	Modified Medical Research Council
NE	Neutrophil elastase
NELSON	Dutch-Belgian Randomised Lung Cancer Screening
NET	Neutrophil extracellular trap
NETT	National Emphysema Treatment Trial
NHS	National Health Service
NIHR	National Institute for Health Research
NSHD	National Survey of Health and Development
NSP	Neutrophil serine proteinases
ONS	Office for National Statistics
OR	Odds ratio
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PDE4	Phosphodiesterase-4
PD-L1	Programmed death-ligand 1
PE	Phycoerythrin

PHQ-9	Patient Health Questionnaire
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PLATINO	Latin American Project for the Investigation of Obstructive Lung Disease
POPE	Phenotypes of COPD in Central and Eastern Europe
PR3	Proteinase 3
PRM	Parametric response mapping
PSGL-1	P-selectin glycoprotein ligand-1
PTEN	Phosphatase and tensin homolog
QEHB	Queen Elizabeth Hospital Birmingham
R20	Respiratory resistance at 20Hz
R5	Respiratory resistance at 5Hz
R5-20	Difference between R5 and R20
RAGE	Receptor for advanced glycation end-products
RB-ILD	Respiratory-bronchiolitis interstitial lung disease
REC	Research Ethics Committee
RETHINC	Redefining Therapy in Early COPD
ROC	Receiver operator characteristics curve
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute (in relation to cell culture media)
RR	Relative risk
Rrs	Respiratory resistance
SABA	Short-acting beta2-agonist
SAD	Small airway dysfunction
SAMA	Short-acting antimuscarinics
SD	Standard deviation
SES	Socioeconomic status
SF-36	Short-Form Health Survey
SGRQ	St George’s Respiratory Questionnaire

SLPI	Secretory leukocyte proteinase inhibitor
SP-D	Surfactant protein D
SPIROMICS	Subpopulation and Intermediate Outcome Measures in COPD
sRAGE	soluble RAGE
STOIC	Steroids in COVID-19
TBST	Tris-buffered saline with Tween
TESRA	Treatment of Emphysema with a Selective Retinoid Agonist
TLCO	Transfer capacity for carbon monoxide
TNF-α	tumour necrosis factor-α
t-SNE	t-Distributed Stochastic Neighbour Embedding
UHB	University Hospitals Birmingham
UK	United Kingdom
UOB	University of Birmingham
USA	United States of America
WHO	World Health Organisation
X5	Respiratory reactance at 5Hz
Xrs	Respiratory reactance
Zrs	Respiratory impedance
ΔX5	Difference between inspiratory and expiratory X5

CHAPTER 1 – INTRODUCTION

1.1 Chronic obstructive pulmonary disease (COPD)

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) has defined COPD as a 'common, preventable and treatable disease that is characterised by persistent respiratory symptoms and airflow limitation'.[1] It is usually a progressive disease associated with a chronic inflammatory response in the airways and lungs to noxious particles and gases.[2]

1.1.1 Risk factors

In the western world, the leading risk factor remains to be cigarette smoking and exposure to cigarette smoke. However, there is increasing recognition of other factors in COPD pathogenesis, such as genetic factors,[3] exposure to other particles[4 5] and poor lung growth in early life. A significant familial risk of COPD has been observed in smokers who are siblings of COPD patients,[3] suggests that genetics and environmental factors could influence COPD susceptibility. However, α_1-antitrypsin deficiency (AATD) is the only monogenetic variant clearly shown to have a causative role in COPD[6] and will be discussed later in section 1.1.7.1.

Processes that occur during gestation birth (e.g. prematurity, low birth weight and maternal smoking during pregnancy)[7 8] and during childhood and adolescence (e.g. childhood respiratory illnesses and early-life exposure to cigarette smoke)[7 9] may affect lung growth. This leads to reduced maximal attained lung function and may identify individuals at risk for COPD development.[10] Occupational exposures,[5] high levels of urban air pollution[4] and exposure to indoor pollutants due to using biomass fuel for cooking and heating[11] are also underappreciated risk factors for COPD.

1.1.2 COPD epidemiology and disease burden

COPD is a common respiratory condition, with a global prevalence estimated at approximately 9-10% in adults aged ≥40.[12] There is a significant variation in COPD prevalence depending on the geographical location. Part of the variation is due to differences in survey methods among epidemiological studies, but this does not fully account for the differences. For example, The Latin American Project for the Investigation of Obstructive Lung Disease (PLATINO) study has estimated that the prevalence of moderate or more severe COPD is from 2.6% to 7.1% among the five major cities in the South American continent.[13] This is compared to data from the Burden of Obstructive Lung Disease (BOLD) study that showed a prevalence of 14.3% in Lexington, United States of America (USA) and 19.1% in Cape Town, South Africa, for COPD of similar severity.[14] Both studies use an identical methodology to obtain estimates of disease burden.

COPD is associated with significant morbidity and mortality. It is poised to become the third leading cause of death worldwide after ischaemic heart disease and cerebrovascular disease.[15] According to the British Lung Foundation (BLF), the age-standardised mortality rate attributed to COPD in the United Kingdom (UK) and the USA is 210.7 and 248.2/million, respectively.[16] COPD is associated with the high utilisation of healthcare systems. It accounts for 115,000 emergency admissions annually in the UK.[17] COPD patients are also at higher risk of comorbidities such as cardiovascular disease,[18 19] diabetes[20] and musculoskeletal disorders[21], contributing to the disease burden. COPD also results in a significant economic burden in most countries. For example, National Health Service (NHS) costs in the UK are estimated at £1.9

billion annually.[22] In 2010, total national medical costs attributed to COPD in the USA were estimated at $32.1 billion.[23]

It is also likely that COPD will remain a significant health issue for many years due to several reasons. Firstly, although smoking prevalence has fallen in the UK and the USA,[24] [25] a substantial proportion of adolescents still smoke. It is estimated that 16.0% of UK children aged 13 to 14 have tried tobacco, and 2.8% of this same group are regular smokers.[26] In this population, there will likely be a time lag of many years before COPD becomes apparent. Secondly, it has been observed that COPD hospitalisation has not reduced despite a decline in smoking prevalence,[27] and this trend may continue for years to come. Thirdly, age is often a risk factor for COPD, although it is unclear whether ageing leads to COPD or whether this reflects long-term cumulative exposure to risk factors.[28] As more of the world's population is living longer, more people would be at risk for chronic medical conditions such as COPD.

1.1.3 Symptoms

Chronic cough, breathlessness and sputum production are the top three prevalent symptoms of COPD.[29] [30] Chronic and progressive breathlessness is a significant cause of disability and psychological distress associated with the disease.[29] In fact, many COPD patients had to alter their daily routine to accommodate their reduced ability to perform everyday activities, such as personal hygiene and dressing, due to breathlessness.[29] Chronic cough is frequently attributed as an expected consequence of cigarette smoking and/or environmental exposures.[1] The cough may initially be intermittent but can subsequently be present throughout the day. It may be productive or non-productive.[1] [31] A productive cough results

from increased sputum, and it is common for COPD patients to expectorate sputum with coughing.[1] Regular cough with sputum expectoration for three months or more in two consecutive years is the classical definition of chronic bronchitis (CB).[32]

Wheezing in COPD may be audibly heard by the patient or present as an abnormality during chest auscultation and is the result of expiration through narrowed airways.[1] COPD patients also frequently experience muscular chest tightness following physical exertion.[1] Apart from physical symptoms, psychological symptoms of depression and/or anxiety are also common among COPD patients and merit specific enquiry.[33 34] The prevalence rates of depression and anxiety in COPD vary widely between studies, with depression rates reported at 10-42%[33 35] and anxiety rates at 13-46%.[34] As COPD progresses, fatigue, weight loss and anorexia become increasingly prevalent.[36-38] In particular, weight loss as measured by loss of fat-free mass has prognostic importance in COPD patients.[36] Cachexia was not only more prevalent in those with advanced COPD according to GOLD staging (described in section 1.1.5) but was also associated with a greater mortality risk than COPD patients with no impediment in body composition over five years (relative risk (RR) 1.91, 95% confidence interval (CI) 1.37-2.67, p=0.006).[36]

There is also significant variability in symptom prevalence across 24 hours in COPD patients. Tsiligianni et al. concluded from a systematic review that COPD symptoms were generally more prevalent in the daytime than at night, reducing the patients' ability to perform daily activities. Breathlessness, cough, and increased sputum are most prevalent upon waking and taper off through the remainder of the day while wheezing and chest tightness become more commonplace in the evening and night.[29]

1.1.4 Assessment of symptoms

1.1.4.1 Modified Medical Research Council (mMRC) dyspnoea scale

The mMRC dyspnoea scale is one of the most widely used measures of breathlessness in lung disease.[39] It is a simple self-rating five-point scale that assesses the degree of disability that breathlessness imposes on daily life. The scale ranges from 0 to 4, with a higher grade indicating a higher severity of breathlessness in everyday living. In COPD patients, mMRC grade relates well with the St George's Respiratory Questionnaire (SGRQ) and Chronic Respiratory Questionnaire (CRQ)[40], while also predicting future mortality risk.[41]

1.1.4.2 COPD Assessment Test (CAT)

As mentioned in section 1.1.3, COPD impacts patients beyond just dyspnoea and a more comprehensive assessment of symptoms are recommended. The CAT is a short and simple measure to use in routine clinical practice.[42] The questionnaire consists of eight items on a 6-point scale (from 0-5) with a maximum total score of 40. A higher CAT score indicates a more significant health status impairment due to COPD. As part of the validation process, Jones et al. have shown that the CAT has an excellent internal consistency with a Cronbach's α of 0.88 and an excellent test-retest reproducibility with an intra-class correlation coefficient (ICC) of 0.8.[42] CAT score has been shown to relate well to SGRQ (Spearman's rho= 0.84, $p<0.001$)[43] in a European cross-sectional study of 1817 COPD patients from primary care. A systematic review also found the predictive value of CAT for disease exacerbation and mortality in COPD patients.[44]

1.1.4.3 Leicester Cough Questionnaire (LCQ)

The LCQ is a self-completed health-related quality of life measure of chronic cough.[45] It consists of 19 questions that assess the impact of cough on one of three health domains: physical, psychological and social. Each question is presented on a 7-point scale (from 1 to 7), and each domain score is calculated as the mean score of the related questions. The total score is obtained by adding all three domain scores, giving a minimum score of 3 and a maximum total score of 21. A lower score indicates a higher adverse impact of cough on quality of life. LCQ correlates highly with the cough visual analogue score (rho= -0.72, $p<0.001$) and moderately with SGRQ (rho= -0.54, $p<0.001$) and Short-Form Health Survey (SF-36) questionnaire.[45] LCQ has also been validated in COPD patients with a high internal consistency (Cronbach's α of 0.86) and an excellent test-retest reproducibility with an ICC of 0.92.[46]

1.1.4.4 Hospital Anxiety and Depression Scale (HADS)

The HADS was developed in 1983 by Zigmond and Snaith to identify clinical cases of anxiety disorders and depression for use among patients in a nonpsychiatric hospital setting.[47] It is divided into an anxiety subscale (HADS-A) and a depression subscale (HADS-D) containing seven items. Each item has a score from 0-3, with a possible score of 0-21 for each subscale. A higher score on either subscale suggests more severe symptoms, with a score of 0 to 7 considered normal, 8 to 10 borderline abnormal and 11 to 21 abnormal.

In an extensive systematic review by Bjelland et al., the reported mean Cronbach's α of internal consistency was 0.83 (range 0.68-0.93) for the anxiety subscale and 0.82 (range 0.67-

0.90) for the depression subscale.[48] HADS-A related well with the Clinical Anxiety Scale (*r*=0.69-0.75) and the General Health Questionnaire (GHQ-28; *r*=0.50-0.68), and HADS-D correlated well with the Beck's Depression Inventory (*r*=0.62-0.73) and GHQ-28 (*r*=0.50-0.66).[48] The discriminant validity of the HADS was investigated in COPD patients by comparing it to a gold standard of the Mini Neuropsychiatric Interview. The optimal cut-off in COPD patients reported by Phan et al. for HADS-A was ≥9 (area under curve (AUC) 0.78), and HADS-D was ≥7 (AUC 0.95), which was a close approximation to the recommendation of ≥8 for both subscales in the general population.[49]

1.1.5 Diagnosis and disease severity

The clinical diagnosis of COPD depends on the presence of symptoms (described in section 1.1.3), history of exposure to known risk factors, and evidence of airflow limitation. Post-bronchodilator spirometry is the conventional diagnostic tool used to demonstrate airflow limitation. This is determined by the ratio of the forced expiratory volume in the first second (FEV_1) to the forced vital capacity (FVC) being less than 0.7.[1]

Expected spirometric results for FEV_1 and FVC are calculated using Global Lung Initiative (GLI) equations based on the patient's age, sex, height and race.[50] The spirometric results for the patients are compared to normal predicted values to calculate the %predicted values for FEV_1 and FVC. Using these %predicted values, GOLD has classified COPD severity on post-bronchodilator spirometry (see Table 1.1). This classification is crucial in COPD patient assessment as it helps determine their prognosis[51] and future risk of exacerbations.[52]

It is increasingly recognised that there is a poor correlation between patient symptoms and COPD severity as defined by spirometry. There exists wide variation in symptom burden and exercise capacity within patients with similar GOLD staging[53] and thus highlights the fact that FEV_1 alone does not capture the COPD complexity. In recognition of this, GOLD released an updated strategy for diagnosing and managing COPD, including an 'ABCD' assessment tool to aid the management of COPD patients.[1] Patients are grouped according to their symptom burden (using the mMRC dyspnoea scale and the CAT) and exacerbation history. This grouping is separate from the GOLD spirometric staging and helps facilitate individualised patient care according to the patient's health status (Table 1.2).

In patients with FEV_1/FVC ratio <0.7:		
Stage	**Severity**	**FEV_1 value**
GOLD 1	Mild	≥80% predicted
GOLD 2	Moderate	50-79% predicted
GOLD 3	Severe	30-49% predicted
GOLD 4	Very severe	<30% predicted

Table 1.1 – GOLD classification of airflow limitation severity in COPD

Legend: Airflow limitation is assessed using spirometry after administering a short-acting bronchodilator. %predicted FEV_1 value is calculated using Global Lung Initiative equations and is used to stage COPD severity. FEV_1: forced expiratory volume in the first second; FVC: forced vital capacity

	mMRC 0-1 CAT <10	mMRC ≥2 CAT ≥10
≥2 or ≥1 leading to hospital admission	**C**	**D**
0 or 1 (not leading to hospital admission)	**A**	**B**

Table 1.2 – The GOLD 'ABCD' assessment tool for COPD patients

Legend: COPD patients are grouped into four groups according to their exacerbation history of symptom burden (as assessed by the CAT or mMRC dyspnoea scale. This assessment approach highlights the importance of symptoms and exacerbation risk in making treatment decisions for individualised patient care. CAT: COPD Assessment Test; mMRC: modified Medical Research Council

1.1.6 Treatment options for stable COPD

1.1.6.1 Non-pharmacologic management

Non-pharmacologic treatment for COPD is complementary to pharmacologic treatment (described later) and forms part of comprehensive COPD management. Initial management should address reducing exposure to risk factors, including smoking cessation, as this has the greatest capacity to influence the natural history of COPD. As part of the Lung Health Study.[54] Other non-pharmacologic interventions, such as influenza vaccination and pulmonary rehabilitation, play a substantial role in managing COPD patients. Vaccinations reduce exacerbation rate[55], and pulmonary rehabilitation has been shown to improve dyspnoea, health status and exercise capacity in COPD patients in meta-analyses.[56]

1.1.6.2 Pharmacologic management

The three main classes of pharmacological therapy used to treat COPD are beta$_2$-agonists, antimuscarinics and inhaled corticosteroids (ICS). These inhaled medications are given alone (apart from ICS) or combined by considering the severity of the patient's symptoms, airflow limitation and exacerbation frequency.[1] Beta$_2$-agonists stimulate beta$_2$-adrenergic receptors, which relaxes airway smooth muscle. Two types of beta$_2$-agonists exist – short-acting (SABA) and long-acting (LABA). The use of SABAs, such as salbutamol, improves FEV_1 and breathlessness[57] but has an effect lasting only 4 to 6 hours[58] and is thus used as rescue medication for rapid relief of breathlessness. The same therapeutic effect can be seen with LABAs, such as salmeterol, over 12 or more hours.[59] COPD patients treated with LABAs also have better long-term health outcomes, with better symptom control as well as reduced exacerbation rate and associated hospitalisations.[60]

Antimuscarinics block the bronchoconstrictor effects of acetylcholine of M3 muscarinic receptors on airway smooth muscle.[61] Long-acting antimuscarinics (LAMAs), such as tiotropium, have high selectivity for these M3 receptors and longer dissociation half-life than the short-acting antimuscarinics (SAMAs), such as ipratropium.[61] Like LABAs, COPD patients who receive regular inhaled tiotropium report better symptom control, reduced exacerbations and associated hospitalisations.[62] ICS are anti-inflammatory agents that suppress activated inflammatory genes by reversing histone acetylation via histone deacetylase 2 (HDAC2) recruitment.[63] Most studies have found that ICS monotherapy does not modify FEV_1 decline in COPD patients. Furthermore, they were associated with an increased risk of oropharyngeal candidiasis and pneumonia.[64] However, combined inhaled

therapy such as ICS/LABA is more effective than either component alone in reducing exacerbations in patients with moderate to very severe COPD, although the increased risk of pneumonia is still evident.[65 66] Using the 'ABCD' assessment tool, GOLD has proposed a model for initiating pharmacological management according to an individualised assessment of symptoms and exacerbation risk[1] (see Table 1.3).

	mMRC 0-1 CAT <10	**mMRC ≥2 CAT ≥10**
≥2 or ≥1 leading to hospital admission	Group C **LAMA**	Group D **LAMA or LAMA + LABA* or ICS + LABA****
0 or 1 (not leading to hospital admission)	Group A **A bronchodilator**	Group B **LABA or LAMA**

Table 1.3 – A proposed model for initiation of pharmacological treatment of COPD patients

Legend: The proposed initial treatment options for COPD patient groups using the 'ABCD' assessment tool. The patient response is assessed after treatment initiation, and adjustments to pharmacological treatment can be made if necessary. *Consider if highly symptomatic (e.g. CAT>20) **Consider if eosinophils ≥300cells/μL. LABA: long-acting beta$_2$-agonists; LAMA: long-acting antimuscarinics; ICS: inhaled corticosteroids.

1.1.7 Clinical phenotypes in COPD

Significant heterogeneity exists in clinical presentation and disease progression within COPD. However, in a push towards precision medicine in disease management, there have been efforts to classify COPD patients beyond GOLD staging and 'ABCD' grouping. In 2010, Han et al. proposed the following definition as a concept of a clinical phenotype in COPD: *'a single or combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes (symptoms, exacerbations, response to therapy, rate of disease progression, or death)'.*[67] In essence, COPD phenotypes should be able to classify COPD patients into distinct groups with prognostic values and to determine appropriate therapy that achieves the maximal clinical benefit. The Spanish Guidelines for Management of COPD (GesEPOC) have recognised four common COPD phenotypes: non-exacerbators, frequent exacerbators with CB, frequent exacerbators with predominant emphysema, and asthma-COPD overlap (ACO).[68]

1.1.7.1 COPD phenotypes: health and prognostic implications

The non-exacerbators are defined by COPD patients who present a maximum of one episode of moderate exacerbation (not requiring hospitalisation) in the previous year, synonymous with groups A and B of the 'ABCD' assessment tool.[1 68] This is the most common COPD phenotype in large COPD cohorts and is supported by findings in the Participation in the Phenotypes of COPD in Central and Eastern Europe (POPE) study involving 3362 COPD patients, which found that non-exacerbators made up 63% of the cohort.[69] COPD patients

with this phenotype have a lower risk of a deterioration in health status, lung function decline and mortality than the frequent exacerbator phenotypes.[68]

The frequent exacerbators are defined by COPD patients with two or more moderate exacerbations in the previous year or at least one severe exacerbation (requiring hospital admission), synonymous with groups C and D of the 'ABCD' assessment tool.[1 68] Frequent exacerbators with CB are the second most common COPD phenotype, with 20.4% of the POPE cohort classified with this phenotype.[69] Patients with CB are at a higher risk of moderate and severe exacerbations and worse respiratory symptoms than those without CB.[70] Emphysema is a pathological term defined by the destruction of the lung alveoli and represents one of the structural abnormalities present in COPD patients. It is usually diagnosed radiologically using chest computed tomography (CT) scanning.[1] Frequent exacerbators with emphysema represent a smaller proportion of COPD patients, with a 9.5% prevalence in the POPE cohort.[69] COPD patients with this phenotype also have the highest mortality rate compared with other COPD phenotypes.[71] In a retrospective study involving 891 Spanish COPD patients, exacerbators with emphysema had the shortest mean survival time of 69.9 months (95% CI 62.4-72.4) compared to those with ACO at 94.5 months (95% CI 87.9-101.1), exacerbators with CB at 80.7 months (95% CI 75.3-86.2) and non-exacerbators at 85.2 months (95% CI 81.9-87.6)[71]

GesEPOC proposes the ACO phenotype for COPD patients who meet the diagnostic criteria for asthma, such as a positive bronchodilator test (15% increase in FEV_1 and by >400mls after SABA administration), a history of atopy and/or peripheral blood eosinophilia >300 cells/µL.[68] However, this phenotype is not universally recognised as GOLD emphasises that asthma and

COPD are different disorders, although they may coexist in an individual patient.[1] The prevalence rate of ACO among COPD patients reported in studies showed considerable variability. A meta-analysis of 22 studies showed that ACO prevalence among COPD patients ranged from 13.0 to 55.7%, with a pooled prevalence of 29.6% (95% CI 19.3-39.9%).[72] ACO patients have a female predominance with a younger age compared to general COPD patients.[73]

Apart from the four phenotypes mentioned above, COPD patients with AATD also represent an important phenotype. AATD is the most well-established genetic risk factor for COPD and is characterised by low circulating levels of α_1-antitrypsin (AAT), the most abundant serine protease inhibitor.[6 74] AAT is predominantly synthesised in the liver and partially protects against damage by proteolytic enzymes by activated and migrating neutrophils. Thus, reduced circulating levels of functional AAT are associated with a high risk of developing early-onset emphysema and subsequent COPD.[6] Severe AATD genotypes have been found in 0.12% (range 0.08-0.24%) of COPD patients, with a prevalence ranging from 1 in 408 in Northern Europe to 1 in 1274 in Eastern Europe.[75]

1.1.7.2 COPD phenotypes: treatment implications

Apart from prognostic purposes, identifying COPD phenotypes has the added benefit of predicting the response of a particular patient to different pharmacological treatments. Thus clinicians can initiate the most appropriate treatment. For example, roflumilast is an oral phosphodiesterase-4 (PDE4) inhibitor that reduces inflammation by inhibiting the breakdown of intracellular cyclic adenosine monophosphate (cAMP).[76] Reduction in exacerbations with

roflumilast was most pronounced in patients who had a severe exacerbation in the previous year and those who had more than two moderate exacerbations in the last year.[77] Roflumilast is recommended by both GOLD and GesEPOC to be initiated for COPD patients with an FEV_1 <50% predicted and CB if they have experienced at least one severe exacerbation in the previous year despite treatment with LABA/LAMA/ICS.[1 68]

In COPD patients with the emphysema phenotype, lung hyperinflation is the main driving component of symptoms and exercise limitation. Lung volume reduction surgery (LVRS) is a surgical procedure in which the least functional parts of the lungs are resected to reduce hyperinflation.[78] Findings from the National Emphysema Treatment Trial (NETT) have demonstrated that in those with predominantly upper lobe emphysema and low exercise capacity, LVRS resulted in a lower risk of death, improved exercise capacity and better symptom control compared to those treated with medical therapy alone.[79] Less invasive approaches to lung reduction, such as endobronchial valve treatment, have also been examined due to the high morbidity and mortality associated with LVRS.[80] In patients with severe emphysema and an absence of interlobar collateral ventilation, those treated with endobronchial valves had significantly increased FEV1 and exercise capacity more than those with standard medical care. However, greater benefits were shown in patients with heterogeneous emphysema compared to those with homogenous emphysema.[81]

ACO is associated with a higher degree of airway eosinophilic inflammation, accounting for its greater clinical and spirometric response to ICS.[82] Thus, GesEPOC has recommended using ICS/LABA as a first option to improve lung function and respiratory symptoms and reduce exacerbations (if any).[68] Blood eosinophil count has been used as a surrogate measure of

airway eosinophilia. Several studies have shown that blood eosinophil counts predict the magnitude of the effect of ICS in preventing future exacerbations.[83-85] Due to this, GOLD has recommended the threshold of a blood eosinophil count >300 cells/µL for consideration for the addition of ICS to regular bronchodilator treatment for COPD patients with frequent exacerbations (see Table 1.2).[1]

For COPD patients with AATD, an approach to minimise the development and progression of lung disease is AAT augmentation therapy, which is the infusion of AAT to increase circulating levels.[1] Studies have suggested a reduction in FEV_1 decline[86] and emphysema progression as determined by CT scans in those who received augmentation therapy.[87] However, the main limitation of augmentation therapy is the high cost, leading to a lack of availability in many countries. The annual medical costs among AATD patients in the USA have been calculated at $127537 among patients receiving augmentation therapy compared to $15874 among non-users, with 75.3% of the difference in costs attributed to the therapy itself.[88]

1.1.8 Barriers to COPD care

Several factors have hampered advancements in COPD care. Firstly, there is a high burden of diagnostic delay with many missed opportunities to diagnose COPD among the symptomatic population. Assessment of data for 38859 patients in the UK had shown that 85% of patients attended primary care for respiratory symptoms in the five years before a diagnosis of COPD was made, which may represent lost opportunities for earlier diagnosis. Furthermore, among patients where FEV_1 data were available, 42% of patients had GOLD 3 or GOLD 4 COPD at

diagnosis.[89] This impacts our understanding of COPD pathology, as most of what we know about the mechanisms of COPD comes from studying patients with more severe disease.

Secondly, there are significant health inequalities associated with COPD and its treatment. People from low socioeconomic status (SES) suffer a disproportionate COPD burden. Areas with lower SES have greater adverse results on COPD prevalence, risk, health outcomes and availability of healthcare both nationally and globally.[90] The issue of SES on COPD outcomes will be described in further detail in the next section. Thirdly, there is a lack of consensus on what constitutes early disease in COPD. In other non-communicable diseases, early disease or 'predisease' has been adopted, such as in diabetes,[91] hypertension,[92] or eclampsia.[93] Such a definition allows the identification of an at-risk population for closer monitoring and risk management. However, this classification has not been defined in COPD and hampers study into the early disease state.

1.1.9 Effect of SES on COPD outcomes

In both the initial Marmot report in 2010[94] and the follow-up report released in 2020,[95] regional deprivation has a strong relationship with healthy life expectancy, with worse health and life expectancy noted in poorer areas. Similar associations have been observed specifically in smoking habits in the general population, with lower SES being associated with smoking prevalence and reduced chances of smoking cessation.[96] [97] With COPD, lower SES is also associated with both increased COPD prevalence and worse health outcomes[98] [99]

An international survey among 12 European countries found a higher smoking prevalence in the lower educated group (used as a measure of SES). In the UK, lower education was associated with an OR of 2.26 (95% CI 1.94-2.64) with current smoking among 20–44 year-olds and an OR of 1.74 (95% CI 1.47-2.05) among 45-74 year-olds.[96] A separate study among smokers in Canada, the USA, the UK, and Australia also found that lower education levels were less likely to quit or make a quit attempt than smokers with higher education.[97] Data from these studies suggest that tobacco smoking disproportionately affects populations with lower SES. A data analysis among the general population from low- and middle-income countries showed a positive association between low SES and odds of COPD. Even after controlling for environmental exposures, the odds of having COPD were more significant with lower SES (interquartile odds ratio (OR) 1.23, 95% CI 1.05-1.43).[99] Lower SES among COPD patients was also consistently related to greater disease severity, lower exercise capacity and a greater longitudinal risk of exacerbations.[98]

1.2 'Early' COPD

1.2.1 Importance of early diagnosis

There have been efforts to study early disease in other chronic non-communicable diseases. For example, the Birmingham Early Inflammatory Arthritis Cohort was set up in 2000 to discover and improve diagnostic testing and predict the disease course from the onset of symptoms.[100] However, studies on early disease in COPD are lacking, and recommendations of care have mainly been derived from data from studies involving older individuals (mean age >60 years) with established disease.[101]

To improve long-term clinical outcomes and mitigate subsequent health economic impact, the goal of interventions must shift from reducing symptoms and exacerbations in advanced disease to halting progression in the early stages before the disease process becomes irreversible. For example, if smoking cessation was achieved and sustained early in the disease process, the rate of FEV_1 decline returned to normal ageing and established COPD may not develop.[54] Apart from an opportunity for earlier intervention, earlier diagnosis of COPD also has added benefits in COPD research. Unlike most other common non-communicable diseases, COPD has not seen significant advancements in therapeutic options. Treatment options, as described earlier, are aimed mainly at symptomatic relief of breathlessness and reducing exacerbation frequency, with none being shown to alter the natural history of the disease.

The complexity of disease phenotypes and the fact that several components of the disease can become self-perpetuating hampers the search for new therapeutic agents. It is not known whether these components can influence the initiating pathological processes or physiological

progression in early disease. For these reasons, studying the initial phases of the disease is critical. It requires identifying individuals exhibiting early changes in symptoms, physiology, and pathology before the complexities of the disease phenotypes become established.

1.2.2 Definition of 'early' COPD

The terms 'mild COPD' and 'early COPD' have been used interchangeably. However, these terms refer to different concepts. The term 'early' implies a time in the natural history of COPD when the disease may not have progressed to full clinical effect, whereas the term 'mild' is used as an established marker of disease severity. For example, an 80-year-old individual with a 60-pack-year smoking history and an FEV_1 of approximately 85% predicted with an FEV_1/FVC ratio of 0.67 for the last five years may be classified as having 'mild COPD' but not necessarily 'early COPD' as the disease has likely developed slowly over many years. On the other hand, a 40-year-old individual with a ten-pack-year history and the same lung function parameters may have rapidly deteriorated from the normal range. This latter individual is also classified as having 'mild COPD' but may also have a highly active 'early COPD' disease process which has only been present for a short time.

Several longitudinal studies have provided insight into the difficulty in developing a definition for 'early COPD'. Most studies concentrate on the prevalent FEV_1 and its decline as the surrogate for the underlying disease process. The rate of change in FEV_1 over time is variable in COPD, with observational studies describing FEV_1 decline rates in established COPD ranging from 25-79mls/year[102-104] compared to 24-32mls/year[105 106] in non-smokers without COPD. If

these decline rates were consistent throughout the disease course, the time it would take for an individual to reach the spirometric threshold for COPD would vary (as shown in Figure 1.1).

The ideal patient cohort to study and those who would benefit most from early interventions would be smokers with rapid lung function decline, suggestive of highly active disease, as demonstrated in trajectory 3 of Figure 1.1. However, on initial assessment, it can be difficult to differentiate these smokers from those with a slower lung function decline (Trajectory 2) or those with a decline only due to age-related changes (Trajectory 1). Based purely on FEV_1, this differentiation may require years of longitudinal follow-up. Furthermore, specific individuals can have super-normal lung function with a peak FEV_1 starting above 100% predicted. There is often COPD under-diagnosis in this population as they take longer to reach the COPD diagnostic threshold. Thus, other methods apart from FEV_1 should be employed to detect at-risk populations.

Despite the lack of clinical consensus defining early disease in COPD, there has been an attempt to describe the 'at-risk' population for research purposes. Martinez et al.[107] proposed that early changes leading to COPD should be studied in those <50 years old with ≥10 pack-year smoking history with any one of the below:

- early airflow obstruction (post-bronchodilator FEV_1/FVC ratio of less than the lower limit of normal)
- presence of compatible CT abnormalities such as visual emphysema, air trapping or bronchial thickening
- evidence of accelerated FEV_1 decline (≥60mls/year) even when in the 'normal' range

These criteria will need validation and acceptance but represent a crucial step in moving beyond FEV_1 to identify individuals with high disease activity at a relatively young age. It is hoped that studies using these criteria can help reveal early key pathophysiological processes to predict future COPD risk. Currently, apart from AATD, we cannot reliably predict which smokers are more likely to develop COPD. However, some pathological studies are guiding where to gain further insight.

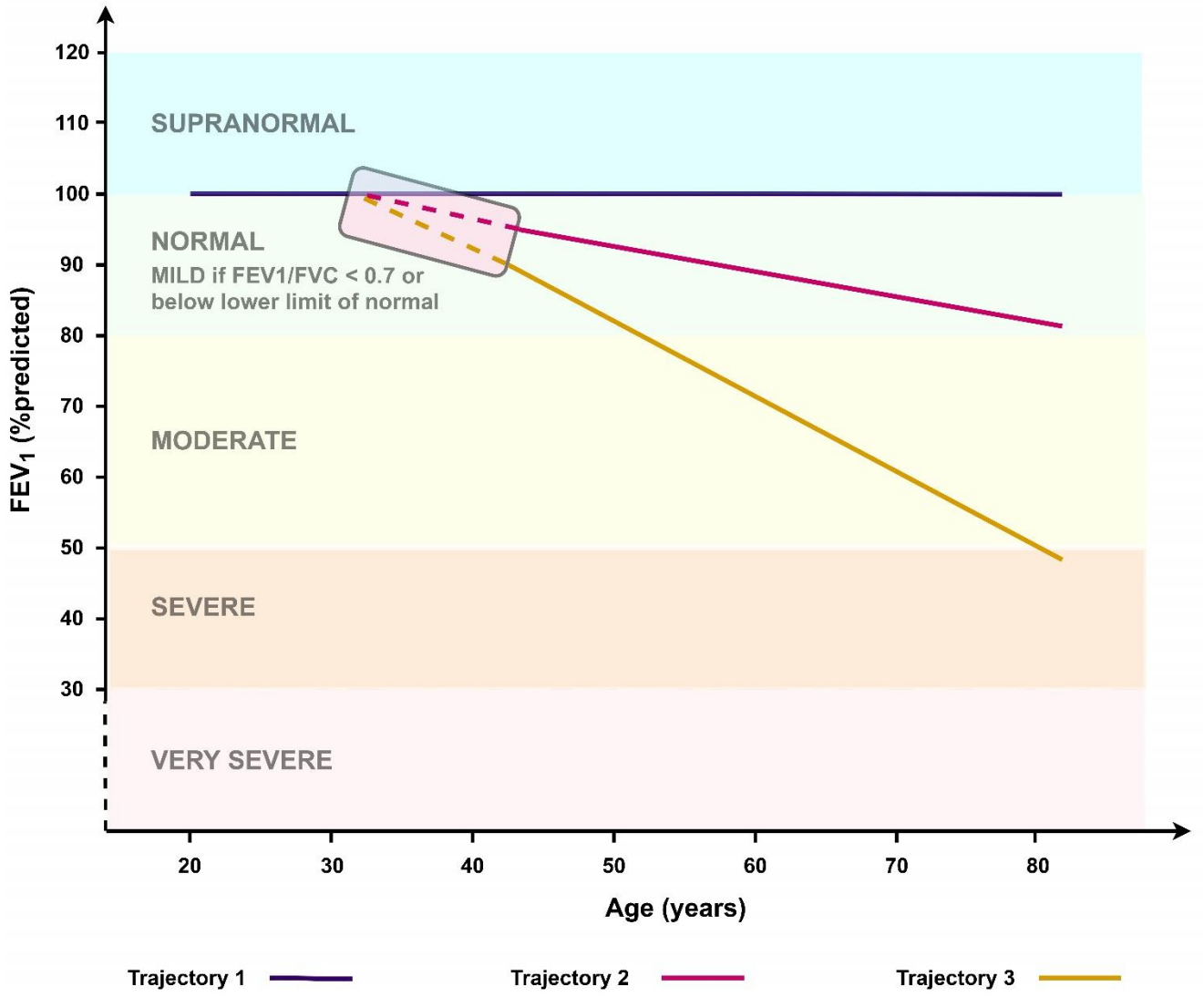


Figure 1.1 – Hypothetical trajectories of lung function (FEV_1 %predicted) that may be seen in the general population of smokers

Legend: Horizontal coloured areas defined by the vertical axis represent COPD severity according to GOLD staging. Trajectory 1 refers to the FEV_1 trajectory of smokers due to age-related changes. They may be asymptomatic and may not develop COPD. Trajectory 2 refers to smokers with mild FEV_1 decline greater than age-related changes. They may develop cross the spirometric threshold for COPD but may only develop mild disease or respiratory symptoms. Trajectory 3 represents smokers with rapid FEV_1 decline who will develop severe disease later in life with the associated high morbidity burden. Early disease (marked by the shaded rectangle) is rarely identified but should obtain clues regarding the initiating processes of COPD development. 'International Journal of Chronic Obstructive Pulmonary Disease' 2021: 16 957 968[108] published initially by and used with permission from Dove Medical Press Ltd

1.2.3 Small airways in health

The human airways consist of approximately 23 generations of branching airways from the trachea to the alveoli. Its principal function is to ventilate the alveoli, which serve as the gas-exchanging units of the lung.[109] The conducting airways, which constitute the anatomical dead space, make up the first 15 generations of airways.[110] Beyond this lie the respiratory bronchioles that continue to divide until they reach the alveolar sacs. These airways constitute the acinar airways and take part in gas exchange.[111]

The small airways are defined as airways less than 2mm in diameter, occurring from approximately generation 8 onwards. These airways include a portion of the conducting airways and the acinar airways.[112] The small airways lack the cartilaginous support seen in larger airways and lack mucous glands. They are lined with a surfactant to prevent closure on expiration, which reduces surface tension.[113] In health, the small airways are low-resistance pathways, contributing only approximately 10% of the total airway resistance.[114]

1.2.4 Small airways disease as an early feature of COPD

The non-proportional COPD Venn diagram (Figure 1.2), first proposed by Snider[115], describes the clinical and pathological features which are significant components of COPD. There are currently diagnostic methods to reliably detect these features (e.g., detection of emphysema by CT scans and airflow obstruction by spirometry), but these features reflect late or severe disease. Several pathological studies have shown that small airway disease can be an early feature of pending COPD.

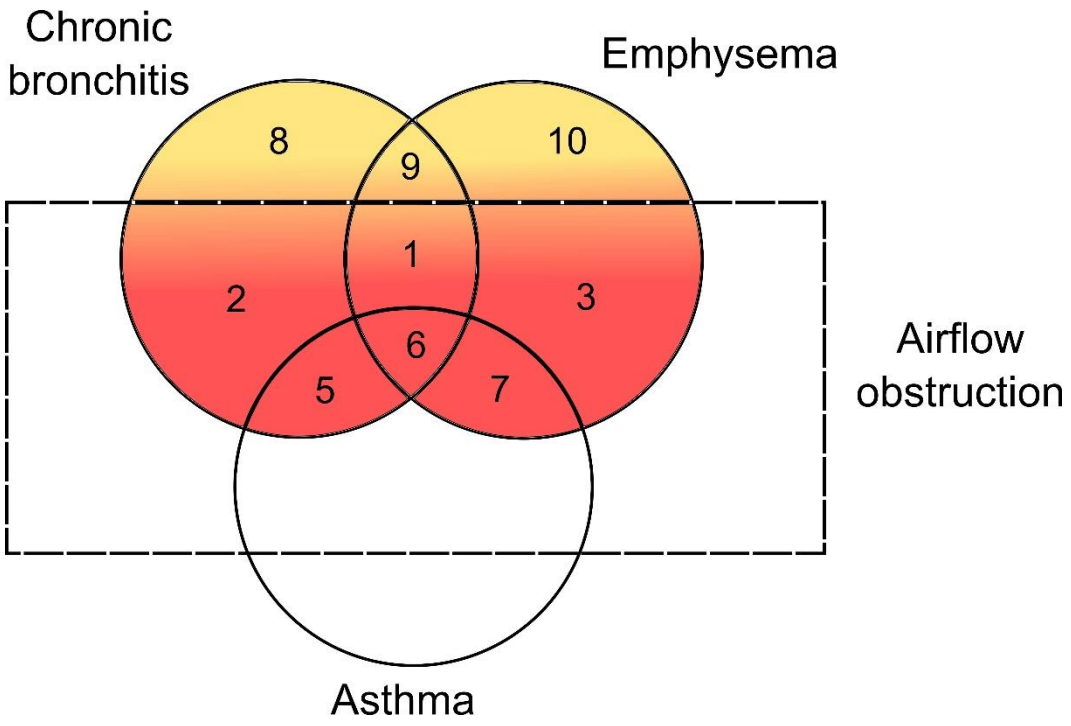


Figure 1.2 – Non-proportional Venn diagram of COPD

Legend: This diagram illustrates patients with CB, emphysema, and asthma subsets. Subsets 1-7 consist of COPD patients with different clinical and pathological COPD phenotypes. Those without airflow obstruction (subsets 8-10) are not classified as having COPD but may have pathophysiological features such as CB (subset 8), emphysema (subset 10) or both (subset 9) that, if detected and treated early, may prevent progression to established disease. 'International Journal of Chronic Obstructive Pulmonary Disease' 2021: 16 957 968[108] published initially by and used with permission from Dove Medical Press Ltd

In 1968, Hogg et al. used a retrograde catheter technique in excised human lungs to examine the site and nature of airway obstruction in COPD. A catheter was wedged in small airways of 2mm diameter to measure peripheral airway resistance (from airways of 2mm diameter to alveoli).[116] Peripheral airway resistance was increased up to 40 times in excised emphysematous lungs compared to healthy lungs due to the narrowing and destruction of small airways.[116] Other studies have expanded on this concept since then.

Volumetric CT scanning has shown that small airway numbers were reduced in COPD patients compared to healthy controls ($p=0.001$ for GOLD 1, $p=0.02$ for GOLD 2 and $p<0.001$ for GOLD 3 or GOLD 4 disease). When micro-CT was used to analyse lung specimens from GOLD 4 COPD patients, a 72-89% reduction ($p<0.001$) in the number of terminal bronchioles was observed compared to lungs from deceased patients without COPD.[117] Further work by Koo et al. has also demonstrated these micro-CT findings in patients with GOLD 1 and GOLD 2 COPD. Compared to lung samples from smokers with normal lung function, there was a reduction in terminal bronchioles in GOLD 1 COPD patients and GOLD 2 COPD patients. This reduction was also noted in COPD lungs without evidence of emphysema which supports the hypothesis that small airway disease precedes the appearance of emphysema.[118] Due to the growing evidence of the importance of small airway disease, there have been efforts to employ various clinical and diagnostic measures to quantify this in smokers.

1.2.5 Symptoms to identify early COPD

The use of symptoms to help identify individuals at risk of COPD has been debated over the last few decades. In 2001, GOLD released a report introducing the GOLD 0 stage, defined by risk factors and CB symptoms in the absence of airflow limitation on spirometry. However, retrospective data analysis from the Copenhagen City Heart study showed that the GOLD 0 stage was not a stable feature and not all GOLD 0 smokers eventually progressed to established COPD. Although there was a significant difference between progression to COPD in GOLD 0 subjects and asymptomatic smokers at 15 years ($p=0.02$), the difference was slight

(20.5% in GOLD 0 subjects vs 18.5% of asymptomatic smokers).[119] Due to these findings, the GOLD 0 concept was removed from the 2007 GOLD report.

Further studies have since suggested that individuals with persistent symptoms have a greater risk of developing COPD than asymptomatic individuals or those whose symptoms resolve. Data analyses were performed in an international cohort of 5002 participants with normal lung function as part of the European Community Respiratory Health Survey (ECRHS). Subjects with persistent CB symptoms had an increased risk of developing COPD than asymptomatic subjects (incidence rate ratio (IRR) 2.88, 95% CI 1.44-5.79). The risk of developing COPD was similar to asymptomatic subjects in those whose symptoms remit at follow-up and those asymptomatic at baseline but developed symptoms at follow-up.[120]

The National Survey of Health and Development (NSHD), which involved a prospective cohort enrolled at birth within the UK, had shown that CB symptoms between ages 36 to 43 were associated with a higher risk of incident airflow obstruction in later life, with OR 3.70 (95% CI 1.62-8.45) and 4.11 (1.85-9.13) respectively.[121] There has thus been compelling evidence for the relationship between persistent CB symptoms and the subsequent development of COPD. However, there needs to be a refinement of the term 'persistent symptoms' and confounding effects of comorbidities before establishing an at-risk population among smokers.

1.2.6 Lung physiology testing to identify early COPD

1.2.6.1 Quality of diagnostic tests

A diagnostic test is expected to accurately indicate the risk of having a specific condition. In assessing the quality of a test, two matters are essential to address. Firstly, whether the process produces consistent results if repeated under similar circumstances tells us about the diagnostic test's reliability. Secondly, whether the measurement reflects what it intends to measure tells us about the test's validity.[122 123]

The reliability of a diagnostic test can be measured by assessing both the inter-rater and intra-rater reliability. The inter-rater reliability is the agreement on a result between two or more assessors. In contrast, intra-rater reliability is the agreement between results obtained by the same assessor at two different time points.[122 123] ICC and Bland-Altman plots have been used to evaluate the reliability of diagnostic tests.[124 125] ICC is a reliability index reflecting both degrees of correlation and agreement between measurements. Calculated ICC values range between 0 and 1, with values closer to 1 representing more robust reliability.[124] Koo et al. have previously recommended that values <0.50 indicate poor reliability, 0.50-0.75 indicate moderate reliability, 0.75-0.90 indicate good reliability, and >0.90 indicate excellent reliability.[124] The Bland-Altman plot evaluates the agreement between two different measurements. The plots allow the identification of any systematic difference between measurements (fixed bias) and are also used to investigate any possible relationship between the discrepancies between measurements and the actual value (proportional bias).[126]

The validity of a diagnostic test refers to the ability of the test to achieve a correct diagnosis. It can be measured by assessing the test's sensitivity and specificity. The sensitivity of a

diagnostic test is the ability to detect pathology when there is pathology present. In contrast, the specificity of a diagnostic test is the ability to produce a negative finding in the absence of pathology.[122] [123] The validity of a test is usually determined by comparing a test method with a 'gold standard' previously validated.[122]

Both the reliability and validity of a diagnostic test are essential. No matter how sophisticated the diagnostic test is, it will not be applicable unless the variables are measured accurately and reliably. For example, suppose a diagnostic test to measure airway obstruction is inaccurate. The test may thus fail to distinguish individuals with actual airway obstruction from those without. In this regard, diagnostic measures have been used to measure lung function to determine the presence or absence of respiratory health.

1.2.6.2 Spirometry

Spirometry has thus far been the most reproducible and objective measurement of airflow limitation.[1] It is both non-invasive and a readily available test. Recent evidence has shown the predictive value of FEV_1 measured sequentially within cohorts of children. Data from the Tasmanian Longitudinal Health Study have shown that sequential spirometry to model lung function trajectories may be of use to predict those at risk of developing COPD.[127] The role of sequential spirometry has also been elucidated in the Lovelace Smokers Cohort, a prospective cohort of ever-smokers aged 40 to 75. Within the cohort, incident COPD was significantly higher in subjects with low baseline lung function with rapid decline compared to subjects with high lung function without rapid decline (hazard ratio 36.6, 95% CI 4.1-320.9).[128]

Apart from FEV_1 and FVC, small airway physiology can be examined using the mid-portion of the expiratory flow. The flow between 25 and 75% of the FVC (FEF_{25-75}), also known as the maximal mid-expiratory flow, is one of the most commonly cited measures of small airway physiology.[129] It was postulated that the latter part of the FVC is affected by increased small airway resistance as lung volume falls. Pathology in the small airways causes airway narrowing and collapse closer to the alveoli earlier during exhalation.[129] There are currently no longitudinal studies looking at the role of MMEF in predicting COPD risk in smokers. Still, a study in AATD patients has shown that a reduction in MMEF is associated with subsequent more significant FEV_1 decline.[130]

1.2.6.3 Gas transfer testing

The lung's ability to transfer gas from inspired air to the bloodstream is measured by the transfer capacity of the lung for the uptake of carbon monoxide (TLCO), also known as the transfer factor. The carbon monoxide transfer coefficient (KCO) is the transfer capacity per litre of lung volume and reflects the efficiency of carbon monoxide transfer by alveoli.[131] Inhaled carbon monoxide is used for this test due to its high affinity for haemoglobin, and it follows the same pathway as oxygen to bind with haemoglobin. Gas transfer testing can evaluate the severity of parenchymal lung disease and pulmonary vascular disease.[132]

TLCO and KCO decrease with increasing disease severity in COPD due to emphysema. As emphysema progresses with COPD severity, a lower surface area is available for diffusion.[133] Gas transfer testing can provide information on the functional impact of emphysema and helps assess breathlessness that may seem out of proportion to the degree of airflow

obstruction.[1] Importantly, decreased TLCO in non-COPD smokers can identify individuals at risk of subsequent spirometric obstruction.[134]

1.2.6.4 Forced oscillometry technique (FOT)

FOT propagates a train of oscillating sound waves along the bronchial tree to determine the mechanical properties of the lung. Multiple frequencies (between 5 and 37 Hz) are applied over tidal breathing from a loudspeaker for 30-40 seconds.[135-137] The resulting pressure and flow changes are measured to determine the impedance of the respiratory system (Z_{rs}). Z_{rs} is composed of the in-phase component, also known as the resistance (R_{rs}) and the out-of-phase element, called reactance (X_{rs}).[135-137] Three technically acceptable manoeuvres are used, as in spirometry.[136 137] FOT has the advantage of being simple to use and is also effort-independent due to measurements being taken at tidal breathing.[135-137]

R_{rs} is typically measured at 5Hz, and 20Hz.[136 137] Higher frequencies (>20Hz) are absorbed by the respiratory system before reaching the small airways and thus reflect the resistance of large central airways (R_{20}). Low frequencies (5Hz) can penetrate deeper into the lung and therefore represent the resistance of the whole lung (R_5).[135-137] Resistance of the peripheral airways can be determined by the difference between the resistance at 5Hz and 20Hz (R_{5-20}) and can give an insight into small airway physiology (see Figure 1.3).[135-137] In healthy adults, R_{rs} is independent of oscillation frequency – meaning that resistance is similar at frequencies between 5 and 20Hz. In obstructive airway diseases such as COPD and asthma, R_5 increases to a greater degree than R_{20}, resulting in increased R_{5-20} values.[135-137]

X_{rs} is typically measured at 5Hz (X_5) and primarily represents the elastic forces within the peripheral airways. At low frequencies (such as at 5Hz), X_{rs} is typically negative. At high frequencies, X_{rs} is positive and is determined by inertial forces within the lung.[135-137] The frequency where opposing inertial and elastic components cancel out is the resonant frequency (F_{res}).[135-137] The reactance area (Ax) represents low-frequency reactance in smaller airways where elastance exceeds inertance and is measured as the area under the reactance curve between 5Hz and F_{res} (see figure 1.4).[135-137] Unlike asthma patients, dysfunction in pulmonary physiology in COPD patients, is better seen in X_{rs} than R_{rs}. In the Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) trial, a proportional increase in R_{5-20} was observed in R_{5-20} and Ax with increasing COPD severity compared to non-COPD smokers or ex-smokers.[138] However, the increase in Ax was higher with a 136% increase when comparing GOLD 4 vs GOLD 2 patients in comparison with $R_{5-20,}$ where the increase was only 60%.[138]

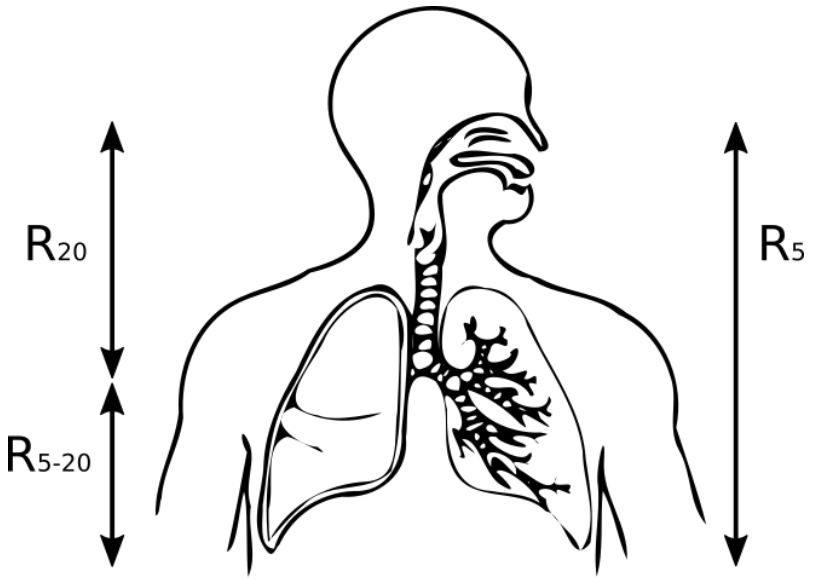


Figure 1.3 – Distance travelled by sound waves of different frequencies

Legend: Airway resistance is measured by FOT at 5Hz and 20Hz. The 5Hz frequency can penetrate deeper into the lungs and, therefore, measures the whole lung's resistance, represented by R_5. The 20Hz frequency only manages to penetrate the larger airways before being absorbed and therefore measures the resistance of the larger central airways, characterised by R_{20}. The difference between R_5 and R_{20}, represented by $R_{5\text{-}20}$, reflects the resistance of the smaller airways, which the 20Hz frequency could not measure.

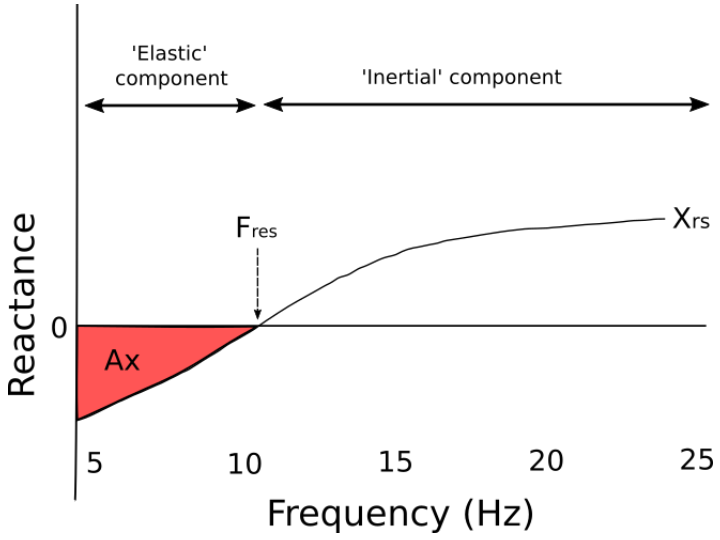


Figure 1.4 – Measurement outputs of respiratory reactance (X_{rs}) at different frequencies

Legend: Reactance values in a healthy subject show the 'elastic' and inertial components of reactance. At lower frequencies, the effect of tissue elastance of the small airways dominates. At higher frequencies, the inertial properties of the large airways dominate. Ax: reactance area; F_{res}: resonant frequency

FOT also allows for discrimination between inspiratory and expiratory impedance. The difference in inspiratory and expiratory reactance at 5Hz (ΔX_5) is a sensitive and non-invasive method of detecting expiratory flow limitation in patients with COPD.[135 136] When expiratory flow limitation is present, pressure signals cannot pass the choke point within the airway and cause a decrease in expiratory reactance.[139] Within-breath analyses have shown that a difference exists between inspiratory and expiratory X_5 in COPD patients, whereas these changes are not present in asthma patients.[140]

Currently, no longitudinal studies study the use of FOT in predicting COPD incidence among smokers. Whether FOT might be more suitable than spirometry for detecting early damage in COPD remains to be seen. However, as the role of small airways in COPD is increasingly understood, FOT may offer important physiological information that may drive our understanding of early COPD features.

1.2.7 Lung imaging to identify early COPD

1.2.7.1 Chest X-ray (CXR)

CXR images are inexpensive, easily obtained and involve minimal radiation exposure. Several features have been proposed for detecting emphysema on CXR, such as increased radiolucency of the lung fields and flattening of the diaphragms.[141] However, such features are only seen in patients with moderate to severe emphysema and rarely in early disease. Furthermore, applying such criteria for detecting emphysema has had mixed success in

correlation to histopathologic examination.[142] Instead, it is valuable in excluding alternative diagnoses and detecting the presence of significant comorbidities.[1]

1.2.7.2 Computed tomography (CT)

Chest CT, like CXR, is not currently the standard of care for COPD.[1] However, it is widely available and routinely used in lung cancer screening programs. Furthermore, quantitative CT imaging has provided valuable information regarding imaging abnormalities and their relationship to disease progression. The use of CT densitometry to quantify emphysema before it becomes macroscopically obvious may enable an understanding of early disease mechanisms whilst subjects still retain normal spirometry.[143] Emphysema presence and severity were assessed using CT densitometry using two methods. The first is by determining the Hounsfield unit (HU) that represents the lowest 15th percentile lung density value (Perc15), whereby the lower the Perc15, the lower the lung density distribution for an individual patient. The second method is calculating the percentage of the lung with low density on CT imaging, whereby -910HU and -950HU have both been used as thresholds.[144] Figure 1.5 illustrates how these parameters are obtained.

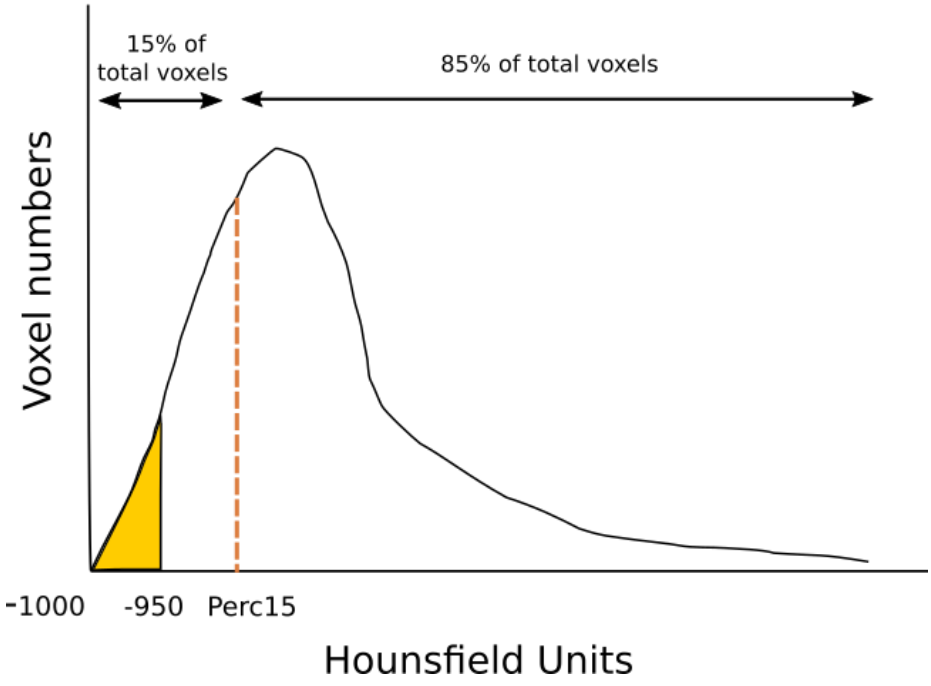


Figure 1.5 – Quantitative CT outputs from a density histogram

Legend: Example of a density histogram and how the percent emphysema and Perc15 are calculated. Voxels from CT chest scans are assigned a HU depending on their attenuation and plotted on a density histogram. In this example, a threshold of -950HU is used to calculate the percentage of the lung with low density. Perc15 is the HU value, with the lowest 15% of the total voxels found below this HU value. Perc15: HU: Hounsfield unit; 15th percentile point

1.2.7.3 CT quantification variability

A potential pitfall of quantitative CT analysis is that it can be confounded by changes in inspiration levels between scans, limiting its sensitivity to detect changes over time. CT densitometry requires CT scans following inhaled bronchodilator therapy during a breath-hold manoeuvre close to total lung capacity.[145] The importance of standardised lung volumes is shown in a repeatability study by Stolk et al. Patients were scanned twice within two weeks.

Differences in lung volume between the two scans were significantly correlated with the resulting difference in Perc15 (R^2=0.512, p=0.02). Standardising lung volume with patient coaching for deep inhalation would allow data to be compared longitudinally to measure disease progression or treatment effect.

1.2.7.4 Role of quantitative CT analysis

The utility of both methods in predicting the risk of future airflow obstruction has been previously studied. For example, Perc15 has been studied in the Dutch-Belgian Randomised Lung Cancer Screening (NELSON) trial, a population-based CT screening program for lung cancer in men. Those without baseline airway obstruction who developed COPD after three years had lower Perc15 values at baseline than those who did not develop obstruction (-934.2 ± 17.1 vs -930.2 ± 19.7HU, p<0.001).[146] The Multi-Ethnic Study of Atherosclerosis (MESA) trial also examined the relationship between percent emphysema (using the threshold of ≤-950HU) and incident spirometric airflow obstruction. Within the MESA trial, emphysematous lung percentage greater than the upper limit of normal at baseline was associated with increased odds of incident airflow obstruction (OR 4.38, 95% CI 1.63 to 11.74) after five years.[147]

Directly quantifying small airways disease has been challenging as they are beyond the resolution of CT scanners.[135] However, methods assessing air trapping have been used as a functional measure of small airway abnormality. Parametric response mapping (PRM) combines data from paired inspiratory and expiratory scans to quantify regional changes in lung density. By applying separate density thresholds to these paired scans, the normal lung

can be distinguished from regions of 'functional small airways disease' (fSAD; >-950HU on inspiration and <-856HU on expiration) and emphysema (<-950HU on inspiration and <-856HU on expiration).[148] Assessing fSAD provides a radiological equivalent of the dynamic airway collapse seen spirometrically.

PRM has been used to analyse CT images of patients in the Genetic Epidemiology of COPD (COPDGene) study.[104 149] Areas of functional small airway disease (PRM^{fSAD}) and emphysema (PRM^{emph}) were calculated from participants' paired inspiratory and expiratory chest CTs. Individuals with chronic respiratory symptoms but no airflow limitation (GOLD 0) at or above the 75th percentile PRM^{fSAD} level (≥16%) had a higher mean FEV_1 decline than those below the 75th percentile point (49.2 ± 50.2 vs 39.0 ± 46.4mls/year, p=0.009).[104] Furthermore, longitudinal CT analyses have also suggested that areas with PRM^{fSAD} among at-risk smokers progress to voxels with emphysema over time.[149]

As demonstrated, physiological and imaging techniques have shown promise in detecting small airway disease, which likely reflects damage and loss of small airways seen pathologically in early disease. Currently, there is a lack of robust prospective studies looking at using such techniques to identify smokers with subsequent excess lung function decline. Thus, whether any of the above methods might be a more sensitive marker than FEV1 to study the early disease processes in COPD remains to be seen.

1.3 Biomarkers

1.3.1 Role of biomarkers

A biomarker is an indicator of the severity or presence of a disease state, which can be measured accurately and reproducibly. International groups have proposed more precise definitions of biomarkers. The International Programme on Chemical Safety has defined a biomarker as 'any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease'.[150] In clinical practice, biomarkers have been used as essential laboratory parameters that help physicians make decisions in making a diagnosis. A classic example is detecting rheumatoid factor as an important diagnostic marker for rheumatoid arthritis for over 50 years.[151]

In clinical research, biomarkers are commonly used as surrogate endpoints in clinical trials, where they act as surrogates for clinically meaningful endpoints. To be considered, robust evidence must show that a biomarker reliably and accurately predicts a clinical outcome.[152] There are several advantages to using biomarkers as surrogate endpoints in clinical trials. Firstly, some clinical endpoints may occur so infrequently that their use can be considered impractical. For example, clear adverse events such as cancer recurrence may occur after many years of treatment. Secondly, biomarkers can provide interim evidence on the safety and efficacy of experimental therapies while more definitive clinical data is collected. Using established biomarkers as surrogate endpoints can allow researchers to stop interventions potentially harmful to participants. Thirdly, using biomarkers enables the design of smaller, more efficient studies that speed up the overall drug development process. Effective

treatments can thus reach target populations sooner, while resources can be conserved for use in other clinical trials.[152]

1.3.2 Types of biomarkers

Biomarkers can be classified based on their application or characteristics based on different criteria. A single biomarker may meet various criteria for other purposes based on its use.[153] Diagnostic biomarkers detect the presence of a disease and can be measured with sufficient precision and reliability. For example, cardiac troponins have been used in clinical practice as diagnostic biomarkers of myocardial injury in acute coronary syndrome.[154] A monitoring biomarker can be measured serially to assess the status of a disease or to detect clinical response to an intervention.

An example would be the serial use of haemoglobin A1c in clinical practice to assess response to antidiabetic treatment.[155] The likelihood of a clinical event or prediction of survival in patients with a disease can be supported using a prognostic biomarker. In this regard, the TNM staging system based on a combination of tumour size, lymph node spread, and absence or presence of metastases provides a basis for the prediction of survival in cancer patients.[156]

Biomarkers are commonly classified as imaging or molecular biomarkers in terms of characteristics. An imaging biomarker is a measurement derived from medical imaging, and many are used in clinical practice. An example of this is in guidelines from the American College of Cardiology that recommend initiating pharmacological treatment upon detection of low ejection fraction on echocardiography.[157] Molecular biomarkers refer to biomarkers

that allow measurements in biological samples such as serum, plasma or sputum samples and have seen significant advances in developing new methods for diagnosing and monitoring disease.[158]

1.3.3. Issues in COPD biomarker interpretation

There has been an extensive array of molecular biomarker studies in COPD recently. Some studies have investigated lung media, such as sputum, bronchoalveolar lavage and exhaled breath condensate.[159] However, most studies have focused on blood biomarkers instead of lung media due to ease of access and reproducibility.[160] There are several issues with using blood biomarkers in clinical practice, particularly in early disease.

Firstly, many studies find statistically significant differences in biomarkers between healthy control subjects and COPD patients. However, a considerable overlap exists between groups, with similarities in other lung diseases, rendering them ineffective as diagnostic tools. An analysis of plasma molecular biomarkers in the COPDGene (n=2123) and the Subpopulation and Intermediate Outcome Measures in COPD (SPIROMICS; n=1117) study highlights these issues. Significant p-values were obtained when COPD patients with differing severity and healthy never-smokers were compared, but data ranges were broad, with a substantial overlap between the groups.[161] Secondly, there exists significant intra-patient variability of these biomarkers in COPD patients and patients with AATD, which is unexplained but likely reflects sampling issues. Such variability can be mitigated to a certain degree by taking sequential samples and using a rolling mean.[162 163]

Thirdly, most biomarker studies focus on COPD populations with established disease and likely varying clinical phenotypes. Most patients at this stage would have extensive lung damage with raised biomarker levels that may reflect a physiological response to the damage. It is thus difficult to establish 'cause or effect' as the biomarker may reflect disease severity rather than underlying disease activity.[160] Thus, biomarker studies in younger smokers will be needed to focus on disease activity before developing significant lung damage, coupled with subsequent disease monitoring to assess progression. This will help identify early-stage differences in disease processes from those caused by established COPD, which may reflect future progression.

1.3.4 COPD biomarkers and disease progression

Table 1.4 illustrates some frequently studied blood biomarkers in COPD patients. The referenced studies highlight a disproportionate focus on the older population (both with and without disease) and patients with more severe stages of COPD. Therefore, these biomarkers will unlikely be able to capture the critical processes in the early disease state and may likely reflect physiological responses to established disease.

Surfactant protein D (SP-D) has a role in pulmonary innate immune defence, while club cell protein 16 (CC16) is a protein secreted by club cells that protect against excessive lung injury.[164] Increased blood SP-D and decreased CC16 concentrations were observed in COPD patients in the ECLIPSE cohort compared with healthy controls.[165] [166] However, there was considerable data overlap between the groups in both cases. In the study by Park et al., a statistically significant correlation was observed between CC16 and FEV_1 decline ($p=0.001$). It

was concluded that the measurement of CC16 could be used to identify those with rapid FEV_1 decline.[167] However, a wide range of values was observed for the patient group. The low r^2 value (0.0043) indicates that CC16 accounts for only a tiny proportion of the variability in FEV_1 decline, and therefore the use of CC16 to identify rapid FEV_1 decliners would be limited.[167]

The receptor for advanced glycation end-products (RAGE) is a cell-surface receptor that binds multiple ligands highly expressed in lung tissues and is believed to have a homeostatic function.[168] RAGE cleavage by metalloproteinases releases soluble RAGE (sRAGE), which acts as a decoy receptor and prevents signalling at the cell surface receptor.[169] Iwamoto et al. observed that sRAGE concentrations were lower in COPD patients than in healthy non-smokers, with a relationship between baseline sRAGE and subsequent FEV_1/FVC decline.[170] Cheng et al. also demonstrated using data from the Treatment of Emphysema with a Selective Retinoid Agonist (TESRA; n=410) and ECLIPSE (n=1847) studies that sRAGE was associated with emphysema severity as measured by CT densitometry.[171] However, as with the SP-D and CC16 studies mentioned, there was a significant overlap between COPD patients and healthy controls. Furthermore, in the study by Iwamoto et al.,[170] sRAGE concentrations for COPD patients and non-COPD smokers were similar, suggesting that sRAGE is modulated by smoking and is not COPD specific.

Biomarker	Sample size	Mean age (years)	Mean FEV_1 (% pred)	GOLD staging	Associations	Ref
CC16	2385	63.4 (COPD) 54.7 (HS) 53.2 (HNS)	48.7 (COPD) 108.6 (HS) 114.8 (HNS)	GOLD 2 – 846 GOLD 3 – 811 GOLD 4 – 229	Smoking status COPD severity	165
	4724	52.1 – 54.9	N/A	N/A	Smoking status FEV_1 decline	167
Fibrinogen	2163	63	48	GOLD 2 – 954 GOLD 3 – 911 GOLD 4 – 296	Baseline FEV_1 FEV_1 decline	172
	5011	72.7	N/A	N/A	Baseline FEV_1 Baseline FEV_1/FVC FEV_1 decline	173
sRAGE	295	58.9 (COPD) 52.1 (HS) 56.0 (HNS)	70.4 (COPD) 94.6 (HS) 108.1 (HNS)	N/A	Baseline FEV_1 Baseline FVC FEV_1/FVC decline	170
	2759	63.6 – 66.7 (COPD) 55.0 (HS) 53.8 (HNS)	48.9 – 49.1 (COPD) 108.4 (HS) 116 (HNS)	GOLD 2 – 1027 GOLD 3 – 989 GOLD 4 - 241	Emphysema COPD severity	171
SP-D	2385	63.4 (COPD) 54.7 (HS) 53.2 (HNS)	48.7 (COPD) 108.6 (HS) 114.8 (HNS)	GOLD 2 – 846 GOLD 3 – 811 GOLD 4 – 229	Smoking status Exacerbation risk	166
CRP	6574	67	80	GOLD 1/2 - 6109 GOLD 3/4 – 465	Exacerbation risk	174
IL-6	2553	63.7 (COPD) 60 (non-COPD)	66.1 (COPD) 99 (non-COPD)	N/A	Baseline FEV_1	175
Blood eosinophils	7428	64 – 72	50 – 78	GOLD 1 – 3344 GOLD 2 – 3332 GOLD 3/4 – 752	Exacerbation risk	176
	3448	63.3 – 68.3	48.0 – 53.1	GOLD 2 – 1722 GOLD 3 – 1292 GOLD 4 - 434	Exacerbation risk	177

Table 1.4 – Studies of commonly researched blood biomarkers in COPD

Legend: N/A is listed where data is not available. Although there is extensive research assessing biomarkers in COPD, most either do not include patients with mild COPD or do not distinguish them from those with more severe COPD. Furthermore, none of the studies has younger smokers (<50 years old) who may be at risk of developing COPD. CC16: club cell protein 16; sRAGE: soluble receptor for advanced glycation end products; SP-D: surfactant protein D; CRP: C-reactive protein; IL-6: interleukin 6; HS: healthy smokers/non-COPD smokers; HNS: healthy non-smokers.

1.4 Neutrophils in health

Neutrophils are the dominant innate immune effector cell in the human circulatory system, accounting for 60-70% of leucocytes. Average concentrations of neutrophils in the blood range between 2.5-6.0 x 10^6 cells/ml, which can change significantly in many diseases and infections. With a relatively short half-life of 6-10 hours, the neutrophils are part of the first response to inflammation.[178 179] In response to pathogens, neutrophils leave the circulation and migrate to affected sites, employing mechanisms that include phagocytosis, degranulation, and release of neutrophil extracellular traps.

1.4.1 Neutrophil development

Neutrophil development is tightly regulated by cytokines such as granulocyte-colony stimulating factor (G-CSF). The importance of G-CSF on neutrophil maturation has been demonstrated using mice lacking the G-CSF receptor. These mice had decreased numbers of mature neutrophils, and haematopoietic progenitors are reduced in the bone marrow with impaired terminal differentiation compared to mice with the receptor.[180]

Neutrophil development occurs in the bone marrow (Figure 1.6). Myeloblasts, relatively undifferentiated cells, initially proliferate and differentiate into promyelocytes. These cells then mature into myelocytes, limited to the production of neutrophils, monocytes and macrophages, depending on the condition within the bone marrow.[181] Myelocytes then proceed through the steps of neutrophilic metamyelocyte, band cell and mature neutrophil.

As they mature, cells cease mitotic activity and continue to develop the characteristic features of neutrophils, such as granules and a segmented nucleus.[182]

1.4.2 Neutrophil release into the circulation

When neutrophils are fully mature, they are released into the bloodstream at $5\text{-}10 \times 10^{10}$ cells/day. However, production can increase ten-fold during periods of infection.[179] Neutrophil release from the bone marrow is influenced by the neutrophil expression of C-X-C containing receptor (CXCR)2 and CXCR4. Interactions between neutrophil CXCR4 and C-X-C containing ligand (CXCL)12, expressed in high numbers in the bone marrow, cause neutrophil retention. In contrast, increasing neutrophil expression of CXCR2 promotes mature neutrophil release.[183] Immature neutrophils express higher levels of CXCR4, which supports retention in the bone marrow.[184] As neutrophils mature, CXCR4 expression decreases with a concurrent increase in CXCR2 expression, and neutrophils no longer respond to the retention signal and allow migration into the bloodstream.[183 185]

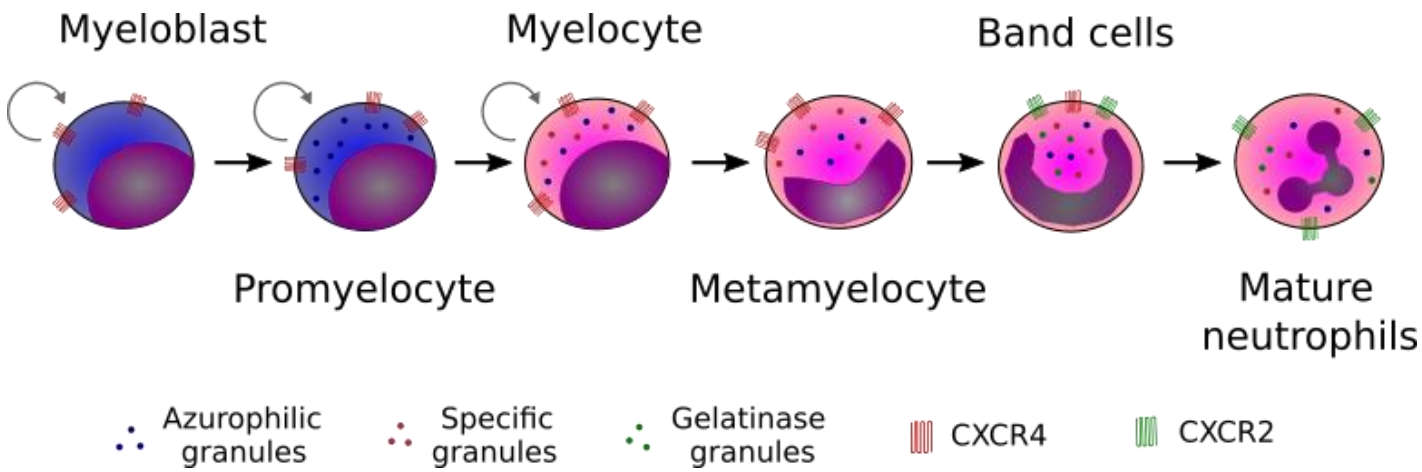


Figure 1.6 – Overview of the neutrophil development stages

Legend: Several phenotypic changes occur as neutrophils mature. Myeloblasts have a high expression of CXCR4 and high proliferative potential. Granule production starts at the promyelocyte stage, beginning with the azurophilic or primary granules. Myelocyte is the last stage where cells retain replicative potential and is also the stage where specific or secondary granule production begins. The replicative potential is lost with metamyelocytes and is accompanied by increased granule production. The band cells (immature neutrophils) are characterised by the production of gelatinase or tertiary granules. At this stage, cells also begin to express CXCR2 and start to lose CXCR4 surface expression. Mature neutrophils have high levels of CXCR2, which readies cells for release into the circulation.

1.4.3 Recruitment and transmigration

To be recruited from the circulation to sites of infection or inflammation, neutrophils must first penetrate the vascular endothelium. This process of neutrophil transmigration is complex and tightly regulated by neutrophils and vascular endothelium. A few distinct stages are involved in the transmigration of neutrophils – rolling, adhesion and diapedesis.[186] [187] Endothelial cells are initially activated by inflammatory signals such as histamine or cytokines or by directly detecting lipopolysaccharide (LPS), a bacterial product.[188] Upon activation, endothelial cells upregulate adhesion molecules (E-selectin and P-selectin), which can bind with their associated ligands (P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1

(ESL-1) and L-selectin) which are expressed on the neutrophil cell surface. The binding of selectins is reversible, and circulating neutrophils repeatedly 'bind and release' to these selectins, which slows the rate of flow of the neutrophil and causes 'rolling' along the endothelium.[187]

Once rolling has been initiated, firm adhesion to the endothelium must occur to allow diapedesis. Two integrins vital for firm adhesion – the lymphocyte function-associated antigen-1 (LFA-1) and the macrophage-1 antigen (Mac-1) are expressed by neutrophils and upregulated upon activation.[187] [189] These integrins bind to the intracellular adhesion molecules (ICAM-1 and ICAM-2), which are upregulated on activated endothelial cells.[187] [190] Chemokines such as C5a and leukotriene B4 (LTB4) released from the endothelium or other inflammatory cells are presented on the endothelial cell surface and contribute to integrin activation.[191] This leads to a conformational change of these integrins, increasing the affinity towards their binding partners and leading to firm adhesion.[192]

Upon firm adhesion, two processes occur: 'crawling' along the vascular luminal surface and diapedesis across the vascular endothelium. Diapedesis predominantly occurs paracellularly between endothelial cell junctions but also may occur by movement through the endothelial cytoplasm.[182] [193] Neutrophil crawling enables the location of ICAMs expressed at endothelial cell junctions to facilitate neutrophils to exit from blood vessels and stabilises their binding to the endothelium.[187] Migration through the basement membrane is a slow process (about 5-15 minutes) compared to penetrating the endothelium (about 2-5 minutes) and involves the use of neutrophil proteases stored within their granules.[182] [187] Proteases involved included neutrophil elastase (NE), matrix metalloproteinase (MMP)-8 and MMP-9.[194]

1.4.4 Migration and chemotaxis

After diapedesis, neutrophils must be able to migrate accurately to the sites of infection or inflammation. Accurate chemotaxis depends on the appropriate response of neutrophils to a complex milieu of different chemoattractants. These chemoattractants may be derived from host inflammatory cytokines such as CXCL8 or bacteria-derived proteins, such as N-Formylmethionine-leucyl-phenylalanine (fMLP) or LPS. Neutrophils can sense the chemokine gradient allowing migration towards the source of the chemoattractant and thus towards sites of inflammation or invading pathogens.[195]

A hierarchy of chemoattractants has been proposed in which neutrophils preferentially migrate towards certain chemoattractants more than others. Heit et al. demonstrated in a competing migrating system in agarose gel that neutrophils responded with lower priority 'intermediate signals' such as CXCL8 and LTB4, which are often host-derived compared with 'endpoint' chemoattractants such as fMLP, which are bacterial-derived.[196] This same hierarchy was observed in a complex microfluidic system with neutrophils *in vitro*.[197] Both studies highlight how complex signalling networks allow neutrophils to migrate accurately within tissues. Two major signalling pathways regulate neutrophil chemotaxis: the phosphoinositide 3-kinase (PI3K) and the p38 mitogen-activated protein kinase (MAPK).[196] The dual action of PI3K and phosphatase and tensin homolog (PTEN) mediate neutrophil chemotaxis to intermediate signals,[198] while responses to endpoint chemoattractants rely on the p38 MAPK pathway.[199] (Figure 1.7)

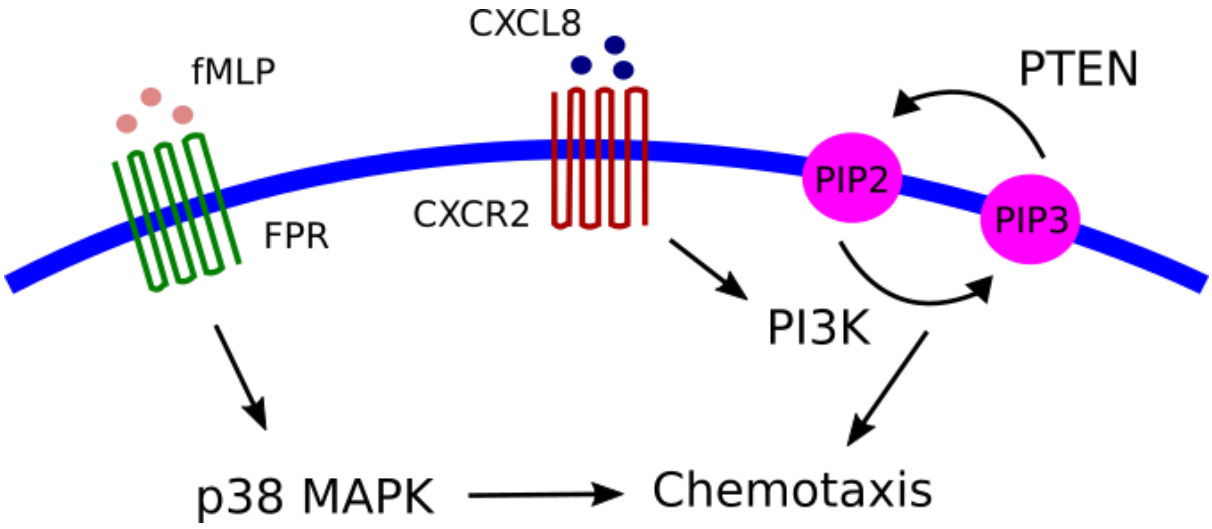


Figure 1.7 – Simplified overview of chemotaxis signalling pathways

Legend: fMLP binds to the formyl peptide receptor (FPR), which leads to the G-protein coupled receptor activation. The MAPK pathway is activated through several signalling intermediates, activating chemotaxis pathways. The binding of the CXCL8 to the CXCR2 leads to the activation of phosphoinositide 3-kinase that catalyses the addition of a phosphate group to phosphatidylinositol (PI) 4,5 biphosphate (PIP2), forming PI 3,4,5 triphosphate (PIP3). This leads to cell polarisation and activation of chemotaxis pathways. PTEN can dephosphorylate PIP3.

1.4.5 Phagocytosis

Upon reaching the site of injury or infection, pathogen clearing is achieved mainly by phagocytosis, a process where they are engulfed and internalised into a neutrophil and subsequently eliminated. It is a complex process involving interaction between the pathogen surface and cell membrane receptors, causing rearrangement in the neutrophil cytoskeleton.[200] Bacterial phagocytosis beings with direct interaction of specific cell membrane receptors on the neutrophil with bacterial surface or immunoglobulins, predominantly immunoglobulin G (IgG), bound to opsonised pathogens.[200] [201] Once this

occurs, rearrangement of the neutrophil cytoskeleton allows engulfment and internalisation of the bacteria to form a phagosome.[200 202] Phagosomes then fuse with lysosomes containing bactericidal compounds and reactive oxygen species (ROS) which creates a cytotoxic environment leading to the killing of the pathogen.[200]

1.4.6 Neutrophil granules

Neutrophils appear to have a 'grainy' cytoplasm due to different granules. These granules are formed during cell development and contain various bactericidal compounds, pro-inflammatory cytokines, additional surface receptors, and membrane proteins. Three different granule subsets exist with distinct proteins essential for neutrophil immune function – azurophilic, specific and gelatinase. These granules are also formed at various stages of cell development (see Figure 1.6).[194] Granule release is tightly regulated and dependent on receiving appropriate stimuli. Different granule subsets are released selectively in a process regulated by calcium, with different calcium signalling thresholds required to release each granule type.[203]

Azurophilic (primary) granules are first detected in the cytoplasm in the promyelocyte stage. They are the major bactericidal granules and are identified by the presence of antimicrobial and cytotoxic proteins such as NE, proteinase 3 (PR3) and cathepsin G.[194 204] Specific (secondary) granules and gelatinase (tertiary) granules are produced from myelocytes onward and share similar contents with overlapping functions.[205] Both granules include proteins involved in neutrophil adhesion, such as LFA-1 and Mac-1 and enzymes used to digest the extracellular matrix (ECM) during transmigration.[205 206]

The release of neutrophil serine proteinases (NSPs), such as NE and PR3, from azurophilic granules, is crucial in aiding neutrophil migration through the ECM. While most NSPs are released into the extracellular space with neutrophil migration, some are also retained on the plasma membrane.[207] Released proteinases can potentially cause direct lung damage while anti-proteinases, such as AAT and secretory leukocyte proteinase inhibitor (SLPI), protect to limit this process, especially in the lungs. However, higher concentrations of NSPs are released from granules compared to the immediate concentration of physiological inhibitors, meaning that NSPs are only partly inhibited at the point of release.[208] As NSPs diffuse rapidly away from the cell, the concentration decreases until it equals that of the surrounding inhibitors, at which point activity ceases. As this inhibition is not immediate, an obligate area of local tissue damage due to proteinase occurs – a phenomenon known as 'quantum proteolysis'.[209]

1.4.7 Neutrophil extracellular traps (NETs)

Another mechanism that neutrophils can deploy is the ability to form extracellular traps. These are composed of released nuclear and mitochondrial deoxyribonucleic acid (DNA) complexed with attached bactericidal proteins to trap and kill bacteria, viruses, fungi and parasites extracellularly.[210] It is thought that NET formation occurs in the presence of larger microbes such as fungal hyphae[211] or once phagocytic capacity is overwhelmed.[212] As a relatively new area of neutrophil function, the mechanisms underlying NET formation are not yet fully understood. There is currently no consensus on how best to study their formation and function *in vivo* or *in vitro*.[213]

1.5 Neutrophils in COPD

Over the years, many potential mechanisms have been implicated in COPD pathophysiology. However, it has been recognised that multiple facets of neutrophil biology have been linked to COPD.[214] Patients with COPD demonstrate airway neutrophilia, which correlates with disease severity and FEV_1 decline and airway bacterial colonisation, which heavily suggests the role of neutrophils in COPD pathogenesis.[2 215] Bacterial colonisation among COPD patients is also associated with neutrophilic airway inflammation[216] which means that neutrophil function is impaired, leading to reduced antimicrobial function and lung damage.

1.5.1 Neutrophil serine proteases (NSPs)

The role of neutrophils and proteolytic enzymes in COPD pathogenesis has been widely accepted. Cell and animal studies have demonstrated the ability of neutrophils to damage lung tissues by releasing NSPs such as NE and PR3. These proteinases degrade all ECM components, leading to the development of emphysema in animal models and, by implication, in humans as well.[204 217] These enzymes are pro-inflammatory and cause hyperplasia of submucosal glands and goblet cells, leading to excess mucus secretion and impairment of mucociliary clearance.[218 219] Subsequently, symptoms of chronic bronchitis and bacterial colonisation develop, which further amplifies inflammation.

1.5.1.1 Neutrophil elastase

As demonstrated in animal studies, NE has been thought to be central to emphysema development. Intratracheal instillation of purified NE in hamsters has been shown to produce emphysema.[220] Furthermore, NE-deficient mice were significantly protected from the development of emphysema compared to wild-type control mice.[221] However, studies have yet to link NE activity conclusively with COPD pathogenesis as assessment of NE activity in humans has proven challenging. Detection of free NE is not usually possible as its physiological inhibitors, especially AAT, rapidly inactivate it.

Detection of NE using immunoassays can quantify both free and bound enzymes but does not provide evidence of the destructive potential of NE at the point of degranulation. Thus, assay procedures have recently been developed to quantify footprints of lung NE activity systemically by specific cleavage products of lung elastin[222] or the accompanying fibrinogen.[223] Aa-Val^{360} is a NE-specific fibrinogen degradation product reflecting lung elastolytic activity. Plasma levels of Aa-Val^{360} are raised in stable COPD and increase further during exacerbations.[224] It remains to be seen whether NE activity relates to future outcomes, but the elastin-specific cleavage product does reflect long-term mortality,[225] and the fibrinogen footprint does reflect subsequent FEV_1 decline early in subjects with AATD.[226]

1.5.1.2 Proteinase 3

It was previously believed that NE is the key neutrophilic enzyme that causes tissue damage leading to emphysema. However, recent studies have challenged this concept and support a role for PR3.[204] There is an estimated mean PR3 concentration which is 3-4 fold higher than

NE in each azurophilic granule. Thus more PR3 is expected to be released than NE at degranulation.[227] Also, lung antiproteases cannot inhibit NE to the same extent as PR3, and persistent PR3 activity is detectable in sputum samples from COPD patients when NE is not.[228] This implies that emphysematous changes in the lung attributed to NE may also be produced by PR3 and possibly to a greater extent.

This theory is supported in animal studies by the development of emphysema in hamsters receiving intratracheal administration of PR3.[229] In addition, NSP-knockout mice are protected against developing emphysema after long-term exposure to cigarette smoke. In contrast, mice that are only deficient in NE are more susceptible, implying that either cathepsin G or PR3.[230] Taken together, these models suggest that apart from NE, PR3 also contribute to the development of emphysema in humans. Like NE, there have been efforts to quantify PR3 lung elastolytic activity in human plasma samples. Quantification of Aa-Val^{541}, which is a PR3-specific fibrinogen degradation product, has been used as a marker of this activity.[231] PR3 activity has already been shown to be increased in patients with AATD compared with healthy controls.[231] However, studies have not yet assessed the relationship of PR3 activity with longitudinal risk of established disease or COPD outcomes.

1.5.2 Matrix metalloproteinases (MMPs)

MMPs are proteolytic enzymes that can degrade ECM components such as collagen and elastin in physiological and abnormal pathological processes. MMP-8 and MMP-9 are stored within the neutrophil specific and gelatinase granules, respectively.[194] There is growing evidence to support the role of both MMP-8 and MMP-9 in emphysema development and

small airway disease. Evidence of MMP-8 in animal models of COPD is scarce, but such evidence exists for MMP-9. Pre-treatment of guinea pigs with MMP-9/MMP-12 inhibitors has significantly ameliorated structural emphysema and small airways remodelling in a cigarette smoke exposure model.[232] Horio et al. demonstrated that pre-treatment with galectin-9 suppressed emphysema development in mice instilled with porcine pancreatic elastase. Furthermore, pre-treatment of neutrophils *in vitro* with galectin-9 inhibited neutrophil MMP-9 production.[233] These findings suggest that MMP-9 released from neutrophils has a role to play in COPD pathogenesis in humans.

Despite the lack of MMP-8 data in COPD animal models, more data in observational studies involving COPD patients have been published. Ilumets et al. looked at MMP-8 levels in a cohort of non-smokers (n=32), GOLD 0 smokers (n=23) and asymptomatic smokers (n=23). Higher levels of MMP-8 were found in GOLD 0 smokers, differentiating them from asymptomatic smokers ($p=0.02$).[234] MMP-8 and MMP-9 concentrations in bronchoalveolar lavage have also been directly related to the extent of small airway disease identified on CT scanning and spirometry (as measured by MMEF).[235] This suggests that both MMPs play a role in small airway remodelling, which is thought to be an early feature of COPD.

1.5.3 Neutrophil chemotaxis in COPD

Previous work has suggested neutrophils from patients with established emphysema had an increased migratory response to chemoattractants with a more destructive proteinase response than patients with other neutrophilic lung diseases.[236] More recent work has confirmed these findings, demonstrating a migratory defect of peripheral neutrophils in COPD

patients. These neutrophils migrated with increased speed in response to chemoattractants such as fMLP and CXCL8 but with reduced accuracy.[237]

These findings could represent an insight into the cause of the COPD disease process. Neutrophils with dysfunctional migratory dynamics may take a more prolonged and convoluted route toward sites of infection or inflammation. Thus, bystander tissue damage increases as they migrate within the lung architecture by creating a trail of obligate proteinase activity. The exact mechanism affecting the neutrophil response has yet to be elucidated. However, this dysfunctional migratory response is not secondary to the environment. It can be normalized to that of neutrophils of healthy volunteers by specific PI3K inhibitors, which suggests this pathway is central.[237]

1.5.4 Other neutrophil functions in COPD

Neutrophils can also perpetuate damage to lung tissues via enzyme-independent functions discussed in section 1.4, such as phagocytosis and NET formation. Data so far on the phagocytic ability of neutrophils have yielded conflicting results. It has been shown that neutrophils isolated from COPD patients maintain the mechanisms to carry out phagocytic functions as they were able to phagocytose non-physiological targets such as latex particles or polystyrene beads.[238 239] Some studies have shown a change in the ability to sense bacteria and fungi as phagocytosis of *Candida, H. influenzae* and *S. pneumoniae* by neutrophils from COPD patients were reduced compared to neutrophils from non-COPD smokers and healthy non-smokers.[239 240] However, not all studies agree with these findings, with studies by Muns

et al.[241] and Walton et al.[242] finding no significant differences in phagocytosis by neutrophils from COPD patients and age-matched healthy controls.

As mentioned in section 1.4.7, NET formation is believed to be an integral part of the immune system in combating infections. However, a putative role for NETs in COPD is beginning to emerge. *In vitro* studies have shown the reduced ability of circulating neutrophils from patients with COPD exacerbations to produce NETs compared to those in stable state or healthy controls.[243] Despite this, increased NET production in COPD patients has been demonstrated that may contribute to collateral lung damage.[244 245] Both studies postulate that altered NET function is linked to a reduction in effective bacterial clearance and a mechanism of damage in COPD. NETs have also been proposed as a source of self-antigen leading to autoimmunity. They have been thought to be the bridge between innate and adaptive immunity in the lung, where they activate local plasmacytoid dendritic cells, which are involved in T-cell activation and subsequent inflammation.[210 246]

1.6 Neutrophil phenotypes in disease

Cellular phenotypes have been described as distinct groups within a cell population with differing morphology, surface receptor expression and functions due to multiple cellular processes.[247] The differing roles of the neutrophil demonstrate the plasticity of their responses which might represent different phenotypes or represent an ability to alter their function depending on the cell environment. The neutrophil changes discussed in section 1.5 are fundamental mechanisms capable of causing lung tissue damage but do not explain susceptibility to COPD. Studying neutrophils with a different phenotype in early COPD could

identify differences between healthy and susceptible smokers to help bridge this knowledge gap.

1.6.1 Activation and adhesion

1.6.1.1 CD11b

Activation markers such as CD11b may play a role in identifying potentially aberrant neutrophil responses. Studies assessing CD11b demonstrate the delicate balance that exists in neutrophil responses. For example, neutrophils from patients lacking leucocyte adhesion molecules such as CD11b have a severely impaired phagocytic response.[248] On the other end of the spectrum, neutrophils from COPD patients with higher expression of CD11b may be linked to airflow limitation.[249]

1.6.1.2 CD62L

Murine studies have demonstrated that CD62L expression requires temporal expression and shedding for normal neutrophil function. Mice with mutant CD62L molecules resistant to cleavage have an increased number and prolonged presence of neutrophils migrating into inflamed peritoneum compared to wild-type mice. However, the same study also showed that neutrophils lacking cleavable CD62L could not migrate as far as wild-type neutrophils following stimulation with a murine CXCL8 homologue.[250] In human studies, blood neutrophils from long-term smokers and COPD patients have been linked with lower CD62L expression than

healthy controls.[251] This phenotype has also been demonstrated compared to asthma patients and is linked with reduced lung function.[252 253]

1.6.2 Chemokine sensing and migration

1.6.2.1 CXCR2

A model of severe sepsis in mice has shown a reduction in the neutrophil numbers in the peritoneal cavity (the site of bacterial insult) and CXCR2 expression compared to non-severe sepsis. Furthermore, the same study showed that the neutrophil phenotype observed in mice with severe sepsis was replicated in those with non-severe sepsis with CXCR2 blockade.[254] The role of CXCR2 in neutrophil migration is also supported by observations that pharmacological blockade of CXCR1 and CXCR2 exhibited altered migration.[255] In COPD, the complexities of neutrophil CXCR2 expression are illustrated in a study by Pignatti et al.[256] Compared to peripheral neutrophils from healthy controls, CXCR2 expression was reduced in peripheral neutrophils from COPD patients and even further in neutrophils isolated from sputum samples from these patients.[256]

The above studies demonstrate that CXCR2 expression and migration accuracy are key neutrophil phenotypes. However, A recent phase 2b trial to assess the safety and efficacy of an oral CXCR2 antagonist (danirixin) in mild-to-moderate COPD patients (n=614) found not only a lack of improvement in symptoms but also an increased incidence of exacerbation and cases of pneumonia among patients in the treatment arm.[257] This shows that simply

modulating CXCR2 is not the answer, as it likely mediates complex pathways in mediating neutrophil function.

1.6.2.2 CD54

Human neutrophils have been shown *in vitro* to reverse migrate, maintaining a pro-inflammatory and apoptotic-resistant phenotype with increased CD54 expression. These neutrophils moved through endothelium monolayers, modelling movement back into circulation.[258] Other scenarios where neutrophils display increased CD54 expression also exist. An activated phenotype comprising of increased CD54 and CD11b expression and a reduction of CD62L occurs when neutrophils enter the lung tissue both in patients with lung disease (sarcoidosis in this instance) and healthy individuals. However, the same study showed that peripheral neutrophils from sarcoidosis patients had a higher expression of CD54 than healthy individuals.[259] Together, these studies highlight that CD54 provides an avenue to identify transmigrated neutrophils and could provide a link between chronic lung inflammation and systemic inflammation.

1.6.3 Senescence and apoptosis

CXCR4 is the primary receptor for CXCL12 and a potential marker of neutrophil senescence. Studies in mice showed that neutrophils increase CXCR4 expression with cellular age, allowing homing of neutrophils back to the bone marrow.[183 260] Evidence of this process occurring in humans also exists as human neutrophils cultured for 12 to 18 hours showed reduced CXCR2

and increased CXCR4 expression with an increase in migration towards CXCL12.[261] In health, the impact of CXCR4 functionality is partly revealed by a genetic condition where CXCR4 signalling is enhanced, leading to neutrophil retention in the bone marrow and resultant peripheral neutropenia.[262]

1.6.4 Maturity

CD10 is a useful marker to identify neutrophil maturation status. As neutrophils mature, neutrophils lose not only CXCR4 expression but also gain CD10 expression.[263] Studies with peripheral blood neutrophils have shown that CD10 expression reliably identified mature neutrophils.[264] CD10 may also participate in intracellular signalling events involved in regulating chemotaxis. Two studies report that the use of CD10 inhibitors resulted in enhanced chemotaxis across an acellular membrane towards fMLP.[238 265] CD10 expression may be an important marker of both maturity and neutrophil bacterial responses in humans.

1.6.5 Inflammatory phenotypes

1.6.5.1 CD11c

Neutrophils expressing higher levels of CD11c *in vivo* have been linked to an immunosuppressive phenotype. Healthy subjects who received an intravenous injection of LPS resulted in neutrophils with higher expression of CD11c that *in vitro* suppressed T-cell activation.[266] Furthermore, peripheral neutrophils from patients with type 2 diabetes have also been reported to have increased surface expression of CD11c. These neutrophils showed

blunted upregulation of CD11b expression in response to fMLP, which supports the notion that CD11c expression may indicate an immunosuppressive phenotype.[267]

1.6.5.2 Programmed death-ligand 1 (PD-L1)

Recently, PD-L1 has received attention because of its role in cancer. Expression of PD-L1 by tumour cells inhibits T-cell mediated killing through engagement with programmed cell death protein 1 (PD-1).[268] PD-1 is predominantly expressed in T-cells, and PD-1 engagement inhibits T-cell proliferation and activation, maintaining immune tolerance in health.[269] Thus, there has been extensive research aiming to target this PD-1/PD-L1 interaction as a basis for cancer therapy with great success.[270]

Neutrophils can also express PD-L1, which is implicated in some disease settings. Patients with systemic lupus erythematosus showed an increased proportion of PD-L1 expressing neutrophils in the circulation that correlated with disease severity.[271] *In vitro* exposure of human neutrophils to cancer-associated fibroblast conditioned media also upregulated PD-L1 expression, leading to inhibition of T-cell proliferation and reduced neutrophil apoptosis.[272] The PD-1/PD-L1 axis in COPD has also been observed as T-cells isolated from lung sections of COPD patients have increased PD-1 expression, which has been linked to reduced antiviral responses.[273] However, it is not yet well understood whether altering this axis will have a meaningful impact on COPD patients.

1.6.5.3 Human Leucocyte Antigen (HLA)-DR

HLA-DR is a class II major histocompatibility complex (MHC) molecule that presents antigens to $CD4^+$ T-cells. It is commonly linked to the functions of dendritic cells, macrophages and B-cells.[274] In neutrophils, HLA-DR expression may be linked to activation. Stimulation of synovial neutrophils *in vitro* with LPS and fMLP induced HLA-DR expression,[275] supported by HLA-DR expression by synovial neutrophils in patients with rheumatoid arthritis.[276] Peripheral neutrophils have also been shown to express HLA-DR *in vitro*. Meinderts et al. demonstrated that isolated neutrophils incubated with IgG-opsonised red blood cells express HLA-DR.[277] Furthermore, these neutrophils can also present antigens to T-cells and induce T-cell proliferation.[277] The role of HLA-DR expression on human neutrophils in the context of COPD still requires further investigation.

1.7 Aims and hypothesis

In other non-communicable diseases, there has been extensive research on early disease, leading to the systematic screening of at-risk populations and the development of new therapies. This concept remains the most critical target for future COPD research prevention and treatment. Only a proportion of smokers develop COPD and pathology is likely to progress over many years before airflow obstruction can be detected spirometrically. Furthermore, a wealth of data shows that neutrophils play a wide-ranging role in COPD pathogenesis.

It was hypothesised that smokers at risk of developing COPD would have clinical or pathophysiological changes that can be revealed by symptom presence or abnormalities on lung function testing or CT densitometry. Peripheral neutrophils from these at-risk smokers

would also have impaired neutrophil function with changes in surface expression consistent with an activated and pro-inflammatory phenotype.

As such, the main aims of the current thesis were originally as follows:

1. To assess whether smokers with chronic respiratory symptoms such as CB have worse clinical outcomes such as more significant FEV_1 decline or higher rate of chest infections (Chapter 3)
2. To assess the repeatability of FOT and CT densitometry as tools to investigate changes in lung physiological and imaging that may be seen in smokers (Chapters 4 and 5)
3. To assess whether smokers with these changes in lung physiology and imaging have worse clinical outcomes as described in aim 1 (Chapter 4 and 5)
4. To determine neutrophil migratory dynamics and neutrophil phenotypes from smokers with chronic respiratory symptoms, asymptomatic smokers, and healthy non-smokers (Chapter 6)
5. To assess NE and PR3 activity as well as plasma MMPs among the groups mentioned above and describe their relationship to clinical outcomes in smokers (Chapter 6)

Due to the global COVID-19 pandemic, not all outcomes, such as longitudinal FEV_1 decline and CT densitometry, could be assessed in all participants. The impact of the COVID-19 pandemic on this thesis has been described in chapters 4 and 5.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Early COPD Cohort Study

The UK Early COPD cohort study is a national multi-centre longitudinal study to research the early stages of COPD development. This was achieved by recruiting a novel cohort of young smokers with either normal lung function or mild lung function abnormalities (described later in the chapter) to identify prospectively those at risk of excess lung function decline. Eight UK centres are based in Royal Victoria Hospital (Belfast), Edinburgh Royal Infirmary, Royal Brompton Hospital (London), Royal Free Hospital (London), Manchester Royal Infirmary, Queen's Medical Centre (Nottingham), Southampton General Hospital and Queen Elizabeth Hospital (Birmingham) were involved in recruitment into this study.

The principal investigator was Professor Wisia Wedzicha, with 13 co-investigators based in one of the eight recruiting centres. The study was funded by five commercial companies – GlaxoSmithKline, AstraZeneca, Boehringer Ingelheim, Novartis and Chiesi. Imperial College London was the primary research sponsor for the study and acted as the study coordination centre. Eligible participants were invited for a screening/baseline visit with subsequent follow-up visits every six months for four years. Ancillary studies using data from the Early COPD cohort (such as the study of neutrophil function detailed in chapter 6) underwent prior approval by the coordinating centre in London. Apart from the ancillary studies mentioned, study protocols were developed by the Early COPD Consortium. Table 2.1 summarises the investigations and assessments carried out during study visits.

	Screening/Baseline Visit	Six months follow-up (for four years)	Performed at Birmingham site
Informed consent	**X**		**X**
Post-BD spirometry	**X**	**X**	**X**
Physical examination, including height, weight, and blood pressure	**X**	**X**	**X**
Medical history for eligibility screening	**X**		**X**
Body plethysmography (gas transfer and lung volumes)	**X**	**X**	**X**
Quality of life questionnaires	**X**	**X**	**X**
Sputum sample processing and storage	**X**	**X**	**X**
Blood sample processing and storage (serum and plasma)	**X**	**X**	**X**
Blood pellet frozen for genetic analysis	**X**	**X**	**X**
Chest CT scan	**X**		**X**
FOT	**X**	**X**	**X**
Neutrophil functions			**X**

Table 2.1 – Summary of assessments done in the Early COPD cohort study

Legend: List of evaluations performed by Early COPD cohort participants during each visit. Those listed in bold were core assessments done at all sites. The study on neutrophil function was an ancillary study involving local patients recruited at the Birmingham site. Post-BD: post-bronchodilator; CT: computed tomography; FOT: forced oscillometry technique

2.2 Ethical approval

All participants included in the study provided written consent for all study activities. The Early COPD cohort study was approved by the Research and Development (R&D) Department of University Hospitals Birmingham (UHB) NHS Foundation Trust and also had appropriate ethical approvals from the London Riverside Research Ethics Committee (REC 16/LO/2041). Healthy adult volunteers were recruited as part of a separate study with appropriate approval from the West Midlands – Solihull Research Ethics Committee (REC 18/WM/0097). The latter study was sponsored by the University of Birmingham (UOB) and approved by the R&D Department of UHB NHS Foundation Trust.

2.3 Participant Recruitment

2.3.1 Recruitment of healthy adult volunteers

Healthy volunteers were recruited from staff members at the UOB or the UHB NHS Foundation Trust using approved advertisements and acted as controls for the Early COPD cohort participants. All healthy volunteers were seen at the Queen Elizabeth Hospital Birmingham (QEHB), where blood samples were obtained. Volunteers were aged between 30 to 45 years with no significant chronic diseases and were lifelong non-smokers.

2.3.2 Recruitment of Early COPD cohort

Participants in the Early COPD cohort were recruited from the public and staff and students from the UOB and the UHB NHS Foundation Trust. Recruitment of staff and students was facilitated by advertising posters around the hospital and university grounds and in weekly online bulletins. Participants from the public were also recruited using social media advertising (Facebook and Instagram) and print advertising via a local newspaper publication (Birmingham Mail). All study advertising materials used had obtained the appropriate ethical approvals. The inclusion and exclusion criteria for the Early COPD cohort study are in Table 2.2, and the local timeline for the study is shown in Figure 2.1.

Inclusion criteria	Exclusion criteria
1) Age 30 to 45 years old at the time of screening 2) Smoker with at least ten pack-year history 3) Able to provide informed consent 4) Have either normal lung function or mild spirometric abnormalities (FEV_1/FVC <0.7, FEV_1 ≥80% predicted)	1) Current diagnosis of asthma 2) Other known chronic respiratory disease 3) Predominant cannabis or shisha user 4) Known diagnosis of autoimmune disease, diabetes, or significant cardio-renal disease 5) Known diagnosis of malignancy 6) Currently enrolled in an interventional clinical trial 7) BMI>35.9 8) Female participants who were pregnant or breastfeeding

Table 2.2 – Inclusion and exclusion criteria for recruitment into the Early COPD study

Legend: Potential participants were screened against the requirements listed above during the initial visit to ensure eligibility to be recruited into the Early COPD study. FEV_1: forced expiratory volume in the first second; FVC: forced vital capacity, BMI: body mass index

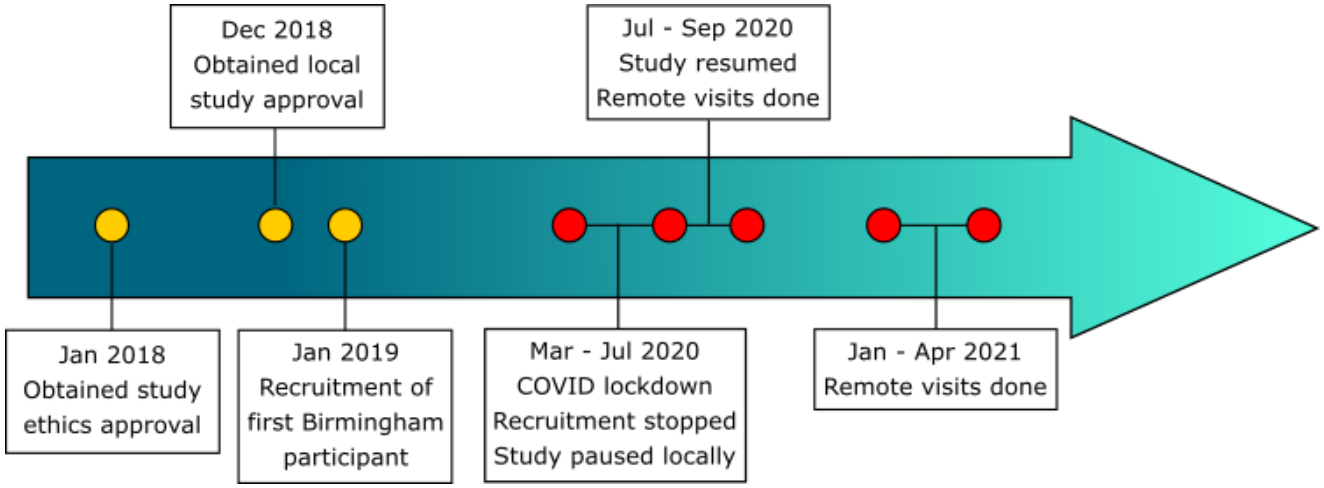


Figure 2.1 – Local timeline for the Early COPD study

Legend: The timeline shows important events that occurred nationally and locally during the study. The yellow circles denote study approvals and participant recruitment, whereas the red circles denote events during the COVID-19 pandemic that impacted the study. Remote visits were done at specific periods due to national lock-down restrictions and hospital visiting policies.

2.4 Clinical Data Collection

2.4.1 Screening and baseline visit

Participants who expressed interest in the study were initially pre-screened via telephone, and those eligible at this stage were invited to attend a baseline/screening visit at QEHB. Figure 2.2 illustrates a typical journey for a participant enrolled in the Early COPD cohort study. Participants who attended were screened and asked to undertake spirometry testing described in section 2.6.1 to ensure that they met the inclusion criteria.

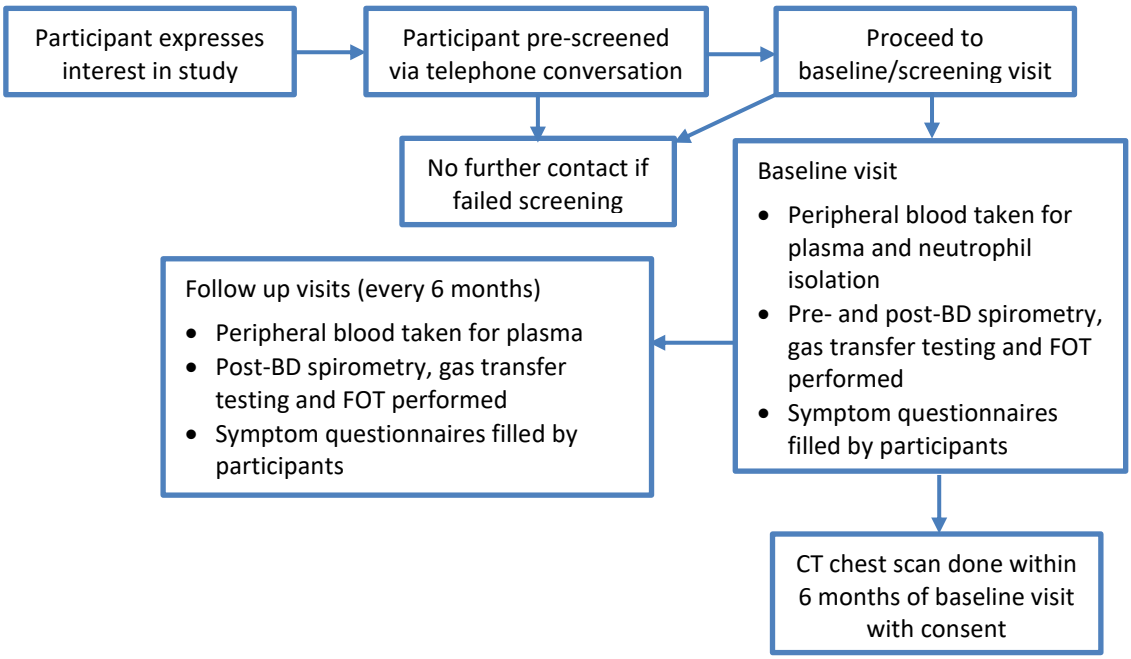


Figure 2.2 – Involvement process of a potential participant through the Early COPD study

Legend: Participants were screened and underwent further baseline investigations if eligible. They were then invited for follow-up visits every six months for up to 4 years (a total of 9 visits) for repeat investigations. Participants were also invited for a CT chest scan within six months of the baseline visit with their consent.

Eligible participants were asked to complete symptom questionnaires described in section 2.5, and blood samples were obtained from these participants. Participants were also asked about their history of lower respiratory tract infections (if any) over the last 12 months and screened for symptoms of chronic bronchitis (defined by the presence of chronic cough and sputum expectoration on most days for at least three months on at least two consecutive years).[32]

The postcode of their current residence was used to obtain deprivation data. An online tool by the Ministry of Housing, Communities and Local Government[278] was used for this purpose. All postcodes were entered, and results were generated in an Excel file that listed the English

index of multiple deprivation (IMD) decile according to their lower-layer super output area (LSOA). LSOAs are small geographical regions designed to be of similar population sizes. They are used by the Office for National Statistics (ONS) to improve the reporting of small-area statistics in England and Wales.[279] The IMD is an overall relative measure of deprivation derived from the combination of seven domains of deprivation – namely income, employment, education, health, crime, living environment and barriers to housing and services.[280] The IMD was ranked and grouped into deciles, with decile 1 falling within the 10% most deprived areas nationally and decile 10 falling within the 10% least deprived areas nationally.[280]

During their baseline visit, eligible participants underwent gas transfer measurements described in section 2.6.1 and the FOT described in section 2.6.2. Participants were also offered a chest CT scan within six months of recruitment, which was undertaken with consent. The protocol for the scan is described in section 2.7.1.

2.4.2 Follow-up visits

Recruited participants were invited for follow-up visits every six months for a total period of up to four years. However, for this thesis, only longitudinal data from baseline to 12 months were used for analysis as all recruited participants had completed 12 months of follow-up during the time of write-up.

During these visits, participants were asked about their smoking habits (if they continued smoking tobacco). Participants were asked to complete symptom questionnaires described in

section 2.5, and blood samples were obtained. In addition, participants were screened for a history of lower respiratory tract infections (if any) and development or remission of chronic cough and sputum expectoration since their last visit. During these visits, participants also underwent post-bronchodilator spirometry, gas transfer, and FOT measurement, as described in section 2.6.

2.4.3 Effect of the COVID-19 pandemic on data collection

Participant recruitment was stopped during the COVID-19 pandemic in March 2020 due to national lock-down restrictions. Since April 2020, post-bronchodilator spirometry, gas transfer measurements and chest CT scans could not be performed on participants due to reduced capacity and increased demands on hospital resources. However, FOT measurements were still performed during face-to-face visits during the COVID-19 pandemic.

Face-to-face visits were paused between March and September 2020 and January to May 2021. Remote visits using telephone calls were utilised between July to August 2020 and January to May 2021 to replace face-to-face visits. Symptom questionnaires (described in section 2.5) were sent either by email or post, and participants were asked to return the questionnaires upon completion.

2.5 Symptom questionnaires

The questionnaires were intended to assess the impact of long-term cigarette smoking across multiple aspects of a participant's quality of life. Copies of all symptom questionnaires can be found in the appendix section.

2.5.1 mMRC Dyspnoea Scale

Previously described in section 1.1.4.1, the mMRC dyspnoea scale[39] is a self-rating five-point scale to assess the degree of disability that breathlessness imposes on daily activity. The scale ranges from 0 to 4 and reflects the degree of various physical activities that precipitate breathlessness. A higher grade on the scale indicates higher severity of breathlessness in daily living. An example of this scale can be found in appendix 1.

2.5.2 COPD Assessment Test

The CAT[42] is a self-administered questionnaire developed to quantify the specific symptom burden of patients with COPD, as described in section 1.1.4.2. It consists of eight items, presented on a 6-point scale (from 0-5), and each item is added, providing a score out of 40. A higher score indicates a higher respiratory symptom burden. An example of this questionnaire can be found in appendix 2.

2.5.3 Leicester Cough Questionnaire

As described in section 1.1.4.3, the LCQ[45] is a self-reported quality-of-life measure of chronic cough. It consists of 19 questions with a 7-point response scale, and each assessed symptoms and impact of cough on one of three domains: physical, psychological, and social. Scores were calculated as a mean of each domain, and the total score was calculated by adding all three domains with a higher score indicating a better quality of life. An example of this questionnaire can be found in appendix 3.

2.5.4 Hospital Anxiety and Depression Scale

As described in section 1.1.4.4, the HADS was developed as a self-assessment tool to screen for anxiety and depression in an outpatient setting.[281] It is a fourteen-item scale with seven items assessing for anxiety and depression symptoms. Each item was scored from 0-3, with a possible score of 0-21 for both domains. A higher score in either domain denotes more severe symptoms of anxiety or depression. An example of this questionnaire can be found in appendix 4.

2.6 Pulmonary Function Tests

2.6.1 Spirometry and gas transfer measurement

Spirometry and gas transfer measurements were performed on participants in the Lung Investigation Unit, UHB NHS Foundation Trust. Reported values generated by spirometry and gas transfer measurements are shown in Table 2.3. Trained respiratory physiologists performed testing to the standard set by the Association for Respiratory Technology and Physiology (ARTP).[282] During the initial visit, potential participants were screened by initial post-bronchodilator spirometry, and eligible participants underwent gas transfer measurement. Bronchodilation was achieved before all testing by administering 400µg of salbutamol using a metered-dose inhaler via a Volumatic spacer device (GlaxoSmithKline, Uxbridge, UK). Before testing, a delay of 15 minutes was taken post-salbutamol administration to allow for maximum bronchodilator effect.

Post-bronchodilator spirometry	
Reported value	**Definition**
FEV_1	Maximal volume of gas that can be expired from the lungs in the first second of a forced expiration from full inspiration
FVC	Maximal volume of gas that can be expired from the lungs during a complete forced expiration from full inspiration
$FEF_{25\%}$	Maximum flow achievable during maximum forced expiration when 25% of the FVC has been exhaled
$FEF_{75\%}$	Maximum flow achievable during maximum forced expiration when 75% of the FVC has been exhaled
MMEF ($FEF_{25\%-75\%}$)	Mean expiratory flow generated between 25% and 75% of the FVC during maximum forced expiration
Gas transfer measurement	
Reported value	**Definition**
TLCO	Measure of conductance of gas transfer from inspired gas to the red blood cells in the alveolar capillaries
KCO	The rate of gas transfer per unit volume of lung

Table 2.3 – Definition of reported values obtained during spirometry and gas transfer testing

Legend: Reported values obtained during post-bronchodilator spirometry and gas transfer testing, as well as the definition of each value. FEF: forced expiratory flow; MMEF: maximal mid-expiratory flow; TLCO: transfer capacity for carbon monoxide; KCO: carbon monoxide transfer coefficient

2.6.2 Forced oscillometry technique (FOT)

FOT measurements were obtained using the THORASYS® tremoFlo® C-100 Airwave Oscillometry System™ (Montreal, Canada). An illustration of the device is shown in Figure 2.3. The device utilises a breath-through vibrating mesh technology to generate oscillations between 5Hz to 37 Hz to measure respiratory resistance, reactance, and Ax, which can be plotted on a line chart as illustrated in Figure 2.4. R_5, R_{20}, X_5 and Ax were analysed for this thesis. A laptop or desktop computer with the tremoFlo® c-100 software installed (v1.0.43; Nowus Healthcare A/S, Denmark) was used during measurements to generate readings and subsequent reports. The device was calibrated every 24 hours using a reference load of 2 $cmH_2O/L/s$ (Nowus Healthcare A/S, Denmark).

Figure 2.3 – Illustration of a tremoFlo® C-100 Airwave Oscillometry System™

Legend: The tremoFlo® device was used in the Early COPD cohort study to obtain forced oscillometry measurements during baseline and follow-up visits. Image reproduced with permission from Thorasys Inc.

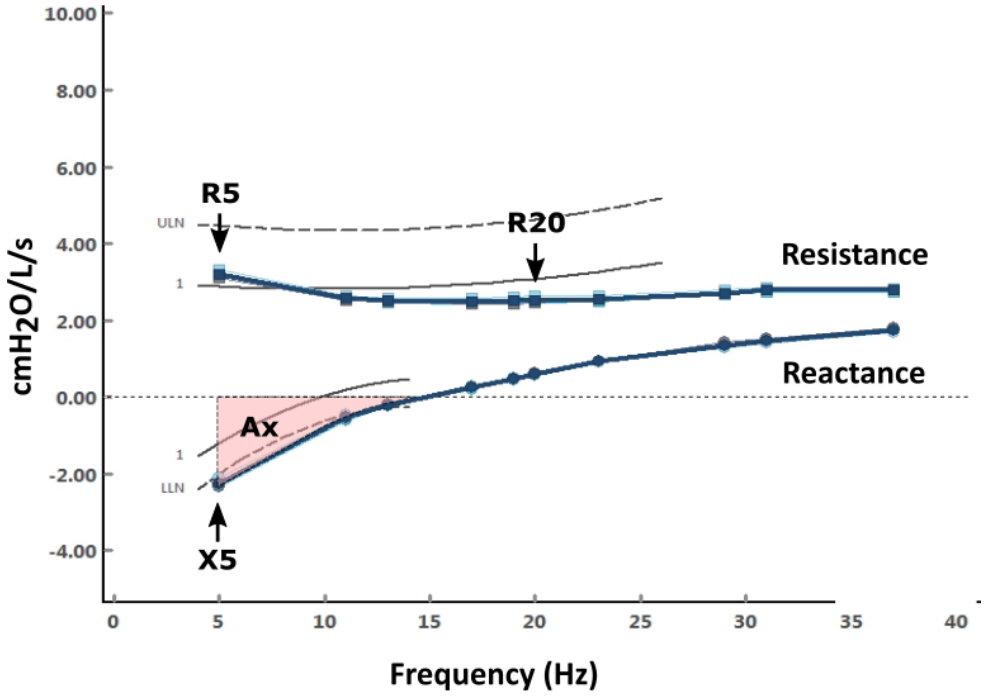


Figure 2.4 – Example of an FOT trace and where data of interest are located on the trace.

Legend: The top line represents respiratory resistance. Conventionally, this was obtained at 5Hz and 20Hz. The bottom line represents the respiratory reactance, typically measured at 5Hz. Ax was calculated from 5Hz to where the reactance trace crosses the 0cmH_2O/L/s line.

Bronchodilation was performed as described in section 2.6.1 before all FOT measurements. Measurements were performed in the sitting position with the head in a neutral or slightly extended position and legs uncrossed. The participant was connected to the device via a disposable mouthpiece containing a bacterial/viral filter. A tight seal was ensured between the mouthpiece and lips to prevent air leaks. A nose clip was worn during the procedure, and the participant was asked to support their cheeks using both hands firmly. During testing, the participant was asked to perform normal tidal breathing in a relaxed state. Figure 2.5 depicts the participant position during measurements.

Figure 2.5 – Participant position during FOT measurement

Legend: Illustration of the ideal participant position while undergoing FOT measurement. Participants were asked to firmly hold their cheeks while the technician held the Tremoflo device in front of them. A nose clip was worn throughout each measurement, and the participant was asked to perform normal tidal breathing through a disposable mouthpiece containing a bacterial/viral filter. Image reproduced with permission from Thorasys Inc.

Measurements were carried out over 30 seconds with a minimum of three technically acceptable measurements. The average values were used to determine if the coefficient of variation (CV) between tests was <15%, as recommended by the European Respiratory Society task force.[283] The reference study published by Oostveen[284] was used to generate average predicted FOT values depending on the participant's age, sex, height and weight.

2.6.3 FOT observer variability

FOT measurements were performed on two separate occasions not more than two weeks apart for ten healthy volunteers to assess intra-observer variability. R_5, R_{20}, X_5 and Ax were measured on each occasion, and the correlation coefficient between the two measurements was calculated. A further eleven healthy volunteers were selected to assess the inter-observer variability. Measurements were taken in succession between KPY and a trained respiratory physiologist (JS), and the same parameters were used to calculate the agreement between the two measurements.

2.7 Chest CT scan

2.7.1 CT scanning protocol

Chest CT scans were performed within six months of recruitment using a protocol-defined technique which obtained CT images of the entire lung at full inspiration. 400µg of salbutamol was administered using a metered-dose inhaler via a Volumatic spacer device (GlaxoSmithKline, Uxbridge, UK) 15 minutes before CT scanning. Scanning was performed by trained CT certified radiographers to the standard set by the Society of Radiographers (SoR)[285], and the SIEMENS Definition AS scanner was used throughout the study.

Standardised breathing instructions based on the COPDGene study[286] were given to the participants to obtain appropriate CT images at total lung capacity. In summary, participants were instructed to inhale and exhale twice as practice before holding their breath at full inspiration. CT scan images could be reconstructed with different slice thickness and

reconstruction kernels after scanning. Slice thickness refers to the axial resolution of each scan image, and reconstruction kernel refers to the algorithm or 'filter' used to process and generate images. Both parameters determines the trade-off between spatial resolution and noise in scan images and were known to cause systematic variation during emphysema quantification.[287] Therefore, CT scan images in the Early COPD Cohort Study were standardised to result in a slice thickness of 0.75mm and image data were reconstructed using a 'smooth' (B35) kernel. A phantom model containing manufactured rods of material containing identical CT density to air and water was scanned every month to ensure consistent calibration of the CT scanner.[288]

2.7.2 CT densitometry analysis

The PULMO CMS software (v2.2.0; Leiden University Medical Centre, Netherlands) was used to analyse CT scan images. Before analysis, CT images were initially converted into Digital Imaging and Communications in Medicine (DICOM) format. Stoel and Stolk have previously described the analysis process using the same software.[145] As a brief description, blood and air density measurements were performed before each analysis session as part of the calibration procedure. The density of blood in the descending aorta was used for measurements and was carried out semi-automatically. Two separate points were marked: at the proximal part of the descending aorta and close to the diaphragm (Figure 2.6a). The software automatically calculated the blood density in slices between the two marked points. A similar calibration process was repeated for air density in all scan images (Figure 2.6b). The air density calibration process was performed automatically, where an area was defined

outside the patient above the sternum in each slice. Image areas were taken with care to exclude any clothing.

The lung parenchyma was detected automatically by automatic lung segmentation using a method known as a region-growing algorithm.[289] The starting point of this algorithm was determined by the 'seed point', automatically placed by the software within the trachea. This 'seed point' then expands caudally through each lung slice image until the border of the lungs has been reached. During the running of this algorithm, a calibrated threshold value of -380HU was used to differentiate lung parenchyma and extrapulmonary tissue. This enables lung contours (boundaries where the lungs were predicted to lie) to be delineated among the CT images. After completion, the trachea was subtracted from the initial lung segments.

A manual check was undertaken to ensure correct lung segmentation before the final analysis. A histogram of voxel density, including low attenuation areas less than a threshold of -950HU (LAA-950HU%) and 15th percentile point (Perc15) values (Figure 2.6c and 2.6d) was then generated by the software. The software also separated lung boundaries into partitions, which allowed separate analysis and comparison of apical and basal lung slices. This process is illustrated in Figure 2.7.

The craniocaudal locality was also calculated for each scan using the partitioning features described by Bakker et al.[290] In their calculation, the most apical (partition 1) and the most basal partition (partition 12) were omitted to exclude partial volume effects. The craniocaudal locality was calculated as the slope of the regression line of the Perc15 values over several partitions multiplied by the number of partitions included (typically ten). The locality was expressed in g/L; a negative value indicates predominant basal emphysema, while a positive

value represents predominant apical emphysema. Figure 2.8 show examples of the regression line among the CT scans in the cohort.

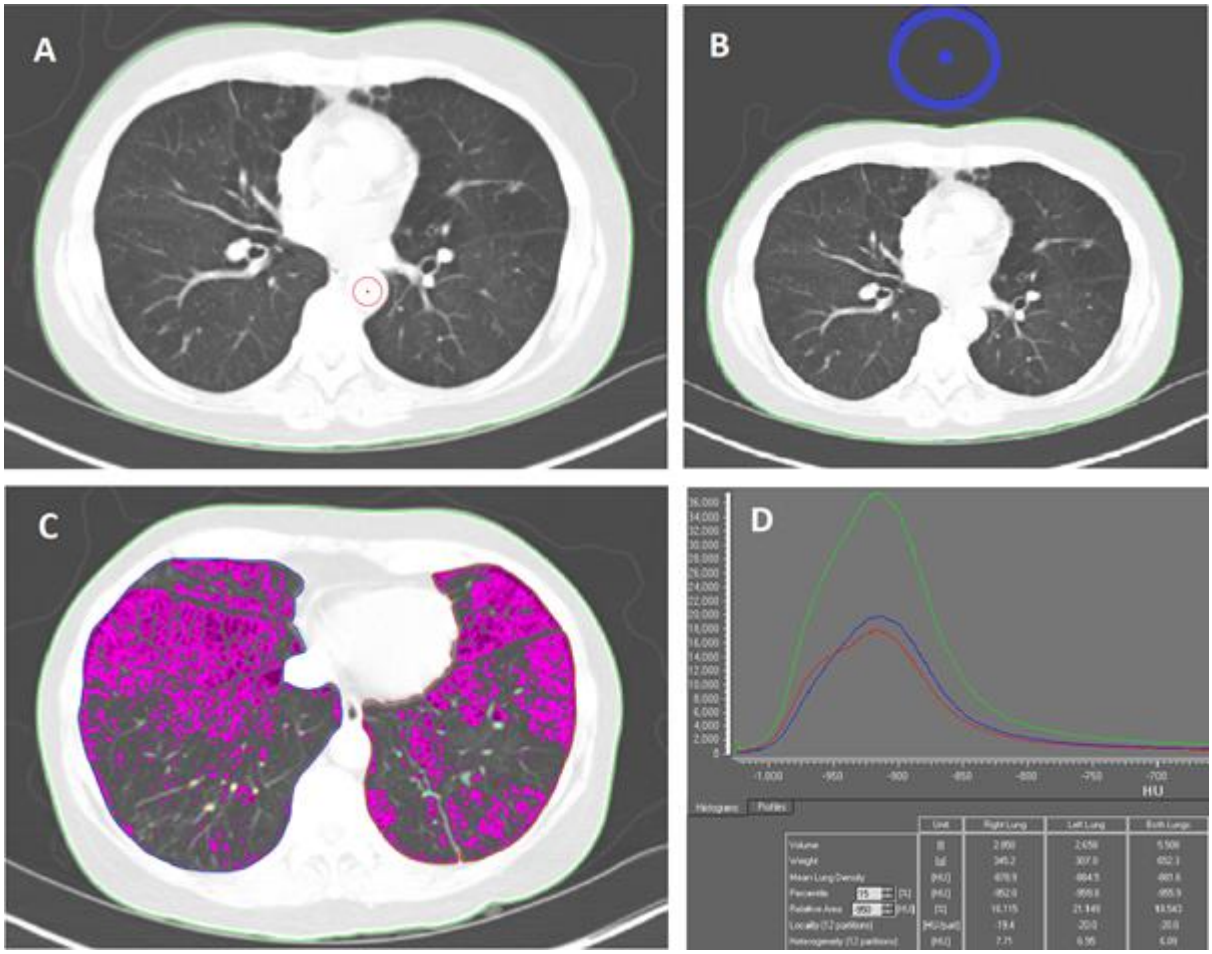


Figure 2.6 – Demonstration of CT densitometry analysis using the PULMO CMS software.

Legend: Density calibration was performed before each scan analysis by determining the density of blood (A) and air (B) in each set of CT scans. The blood density was calculated semi-automatically between two selected points in the descending aorta, and the air density was calculated using a defined area outside the patient in each slice. The lung contours were identified, and the lung parenchyma was segmented using a region-growing algorithm. The software and voxels then calculated the density of all voxels delineated within the lung contours within a pre-specified range (usually <-950HU) can be highlighted (C). These voxels were then plotted on a density histogram (D) to determine LAA-950HU% and Perc15 values. HU: Hounsfield unit; LAA-950HU%: proportion of low attenuation areas less than the threshold of -950HU; Perc15: 15th percentile point

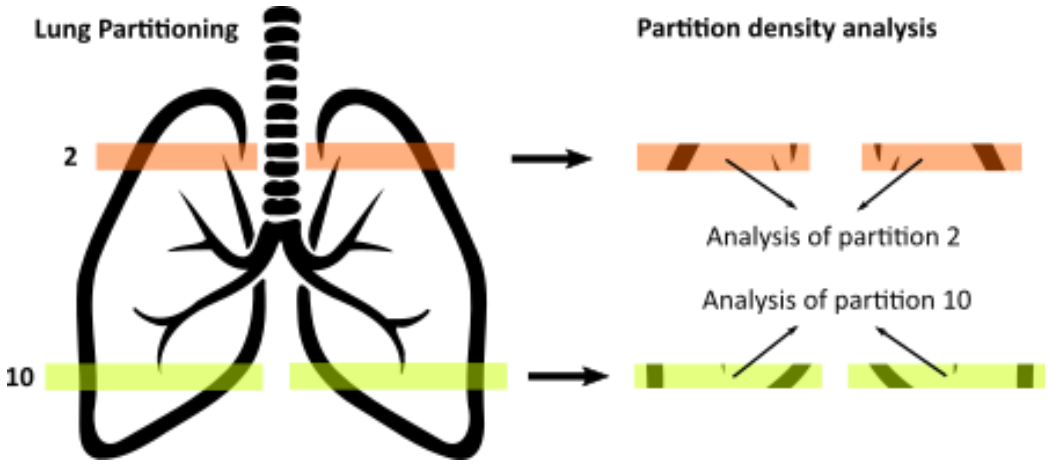


Figure 2.7 – Partitioning of CT slices for separate analysis

Legend: Each lung was divided into 12 partitions of equal volume. Partitions were numbered 1 to 12 from lung apex to base. In this example, partition 2 and partition 12 can be analysed separately, generating partition-specific LAA-950HU% and Perc15 to compare apical and basal partitions.

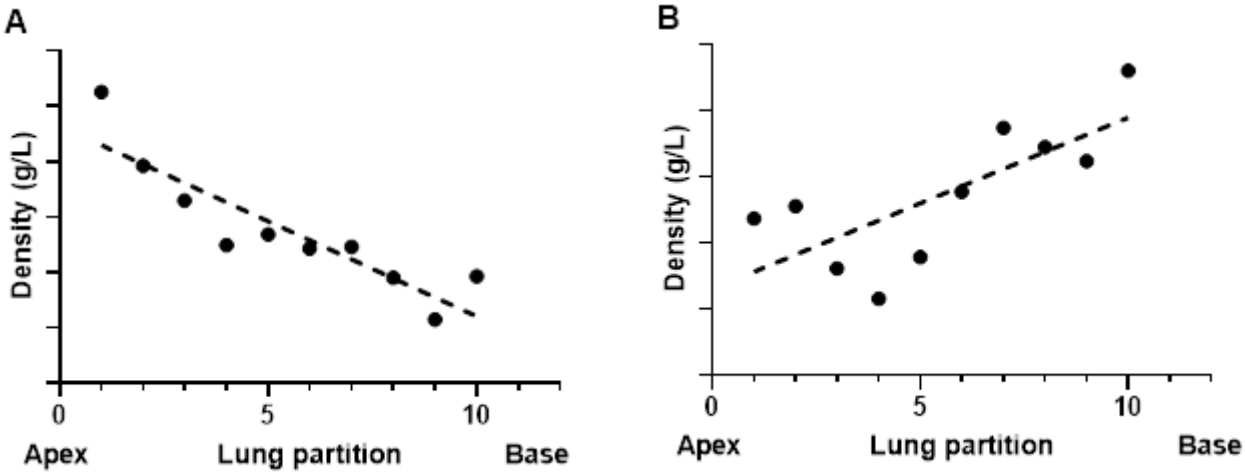


Figure 2.8 – Example of Perc15 regression lines over the CT lung partitions

Legend: The slope of the regression line of measured Perc15 values vs the included lung partitions was calculated. The craniocaudal locality is then obtained by multiplying the slope value by the number of included partitions (typically ten). A negative value indicates that emphysematous features are predominantly located in the lung bases (as in figure A), and a positive value suggests predominantly apical emphysema (as in figure B). Graphs are based on patient data from the Early COPD cohort.

2.7.3 CT analysis observer variability

Chest CT densitometry analyses were repeated twice three months apart for eleven patients to assess for intra-observer variability. The CT scans were obtained from patients with COPD and AATD who provided informed consent as part of the National Institute for Health Research (NIHR) Rare Diseases Translational Research Collaboration study in AATD.[291] This study was approved by the R&D Department of University UHB NHS Foundation Trust and had appropriate ethical approvals from the South Birmingham Research Ethics Committee (REC 3359a).

The LAA-950HU% and Perc15 values were measured for each scan, and the correlation between the two scans was assessed. Chest CT scans for another eleven patients were analysed to ascertain the level of inter-observer agreement. These scan images were analysed by KPY and subsequently re-analysed independently by a previous clinical lecturer (DC). The same parameters were used to calculate the correlation between the two measurements.

2.8 Blood sample collection

Blood was collected during baseline, and subsequent follow-up visits (if face-to-face visits were undertaken) from participants enrolled on the Early COPD cohort and healthy non-smoking volunteers. Blood collection was achieved via peripheral venepuncture into 6ml lithium heparin-containing tubes (BD Vacutainer® Systems, Plymouth, UK), and a total of 30mls were taken for plasma samples and neutrophil isolation.

2.9 Isolation of human neutrophils from whole blood

Neutrophils were isolated from peripheral blood as previously described.[292] In summary, red cells from blood collected in lithium heparin vacutainer tubes were sedimented with 2% dextran (Sigma-Aldrich, UK) in 0.9% saline with a ratio of 1ml 2% dextran to every 6mls of whole blood and left for 30 to 40 minutes. Isotonic Percoll (GE Healthcare) was made by diluting 100% pure Percoll with 9% sterile saline solution in a 9:1 ratio. This isotonic Percoll solution was then diluted with 0.9% saline solution in a 4:1 and 14:11 ratio to generate 80% Percoll and 56% Percoll solutions, respectively. A discontinuous density gradient was prepared by underlaying 2.5mls of 80% Percoll beneath 5mls of 56% Percoll in a 15ml sterile Falcon™ tube (BD Biosciences, UK) using an extended fine-tipped sterile Pasteur pipette (Alpha Laboratories).

The leucocyte-rich plasma obtained after the sedimentation of red cells was carefully layered on the discontinuous Percoll gradient and centrifuged at 470g for 20 minutes at room temperature with no brake or acceleration (Figure 2.9). After centrifugation, the plasma and peripheral blood mononuclear cell (PBMC) layer was removed. The neutrophils were extracted from the interface between the two Percoll densities using a fine-tipped Pasteur pipette. Extracted neutrophils were diluted in sterile phosphate-buffered saline (PBS; Sigma-Aldrich) and re-centrifuged at 250g for 10 minutes at room temperature.

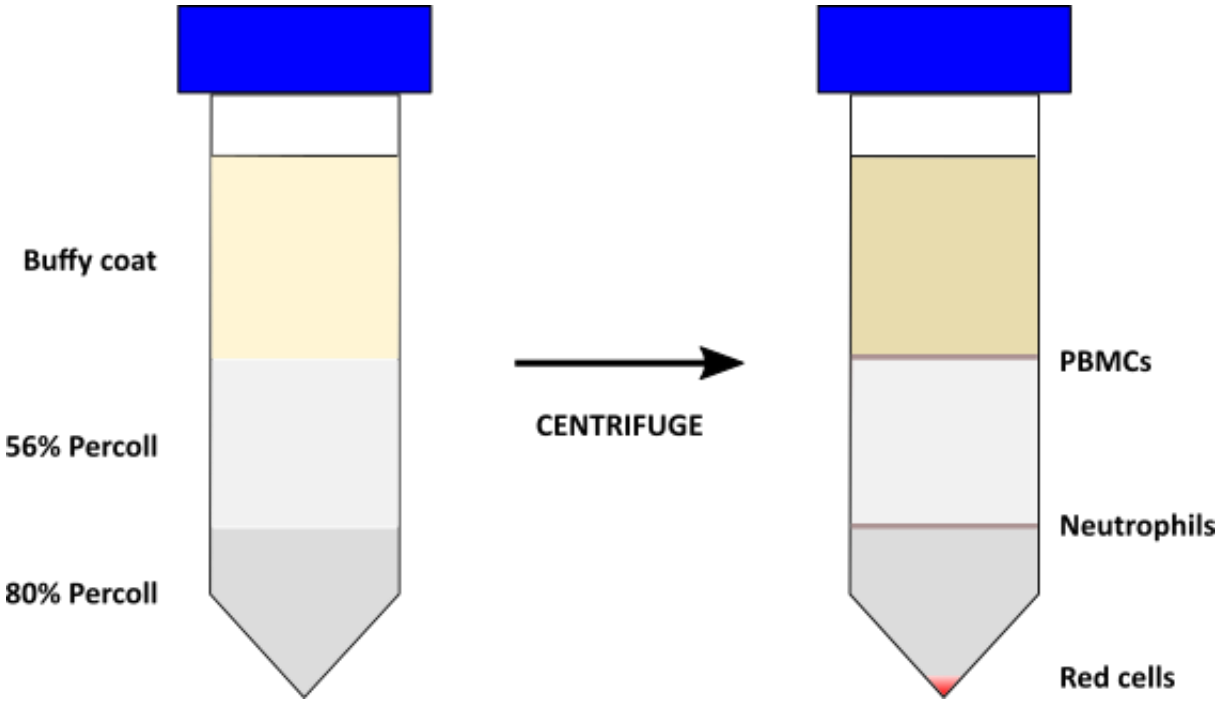


Figure 2.9 – Percoll density gradient for neutrophil isolation before and after centrifugation.

Legend: The figure on the left shows how the discontinuous Percoll gradient and buffy coat were layered before centrifugation. After a 20-minute centrifugation process with no acceleration or brake, the resulting layers are shown in the figure on the right. The neutrophils were extracted from the layer between the 56% and 80% Percoll solution.

The PBS supernatant was discarded following centrifugation. The resultant neutrophil pellet was re-suspended in sterile RPMI-1640, which contained 2mM L-glutamine and was supplemented with 1% Penicillin/Streptomycin (all Sigma-Aldrich media). The neutrophils were then counted with a Neubauer haemocytometer and, if needed, diluted further to achieve the required concentration. Neutrophil purity was accessed by cytospin, and slides were stained using a differential Giemsa staining kit (Diff-Quik; Gentaur Europe, Brussels, Belgium). Viable samples with neutrophil purity of >95% were used for further assays.

2.10 Neutrophil migration

2.10.1 Migration assay process

Bovine serum albumin (BSA; Sigma-Aldrich: 1.125% v/v) was added to the neutrophil suspension at a $2x10^6$ cells/ml concentration previously isolated, as described in section 2.9. Glass coverslips (22x22mm, Leica Biosystems) were sterilised in 0.4M sulphuric acid and then rinsed with double distilled water. The sterilised coverslip was left to be dried and then coated on one side with 400µL of 7.5% BSA, and the excess BSA was discarded. After 30 seconds, 400µL of the neutrophil suspension was added to the coated coverslip and incubated for 20 minutes at room temperature to allow the neutrophils to adhere to the coverslip.

An Insall chemotaxis chamber (Weber Scientific, Teddington, UK) was used to assess neutrophil migration as previously described[237] and shown in Figure 2.10. The chamber channels were initially rinsed and left filled level with RPMI-1640. Once excess neutrophils were discarded, the coverslip was inverted onto the Insall chemotaxis chamber. Excess

medium in the channels was drained out using filter paper and subsequently filled with either 70µL of RPMI-1640, 100nM CXCL8 (R&D Systems, UK) or 10nM fMLP (Sigma-Aldrich). The concentrations of CXCL8 and fMLP used were determined by a series of dose-response experiments performed before the initiation of this study, as used in previous studies.[293]

The chamber was left for a minute to allow a mediator gradient to form. Neutrophil migration was then assessed in real-time using a Leica DMI6000 Microscope with Leica Application Suite X software (v 3.3.0; Leica Microsystems) set to record a brightfield image at 20x magnification. Images were captured at baseline and every 20 seconds for 12 minutes, creating a stack of 37 frames.

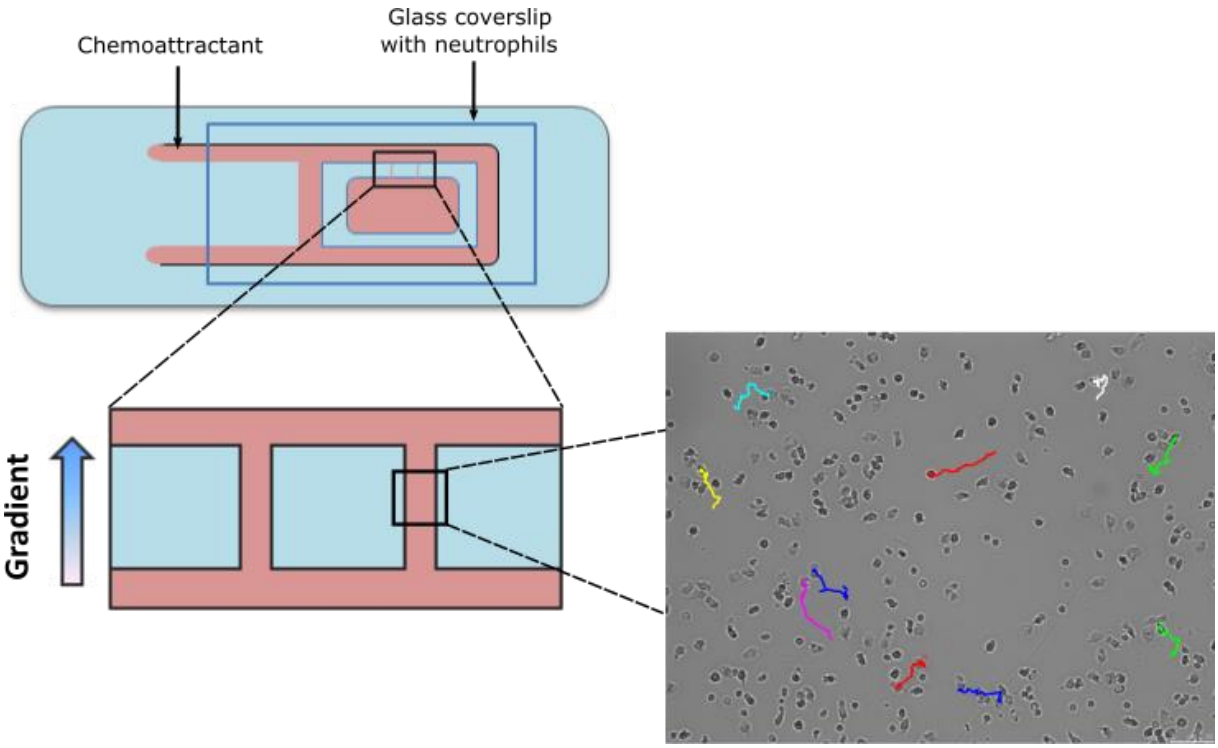


Figure 2.10 – Assessment of neutrophil migration using an Insall chemotaxis chamber

Legend: A prepared glass coverslip with adherent neutrophils was inverted onto the Insall chemotaxis chamber. After the excess medium was drained using filter paper, the chamber wells were filled with chemoattractant or RPMI as the negative control. A gradient was then allowed to form, with neutrophil migration assessed in real-time. The picture on the lower right shows a typical brightfield image of adherent neutrophils with ten tracked cells using the 'manual tracking' plugin within the ImageJ software.

2.10.2 Migration analysis

Exported images were analysed using ImageJ software (v1.52i; National Institute of Health, USA) to determine cell movement. Ten cells were randomly selected and digitally tracked between stacked images to determine neutrophil migration parameters in each experiment. Power calculation based on intercell variability within individuals was performed before the initiation of this study and confirmed that the random selection of ten cells was sufficient for the assessment of neutrophil migration. Cells were randomly selected using the ImageJ software grid function with a random grid reference generator.[293] Three neutrophil migration parameters were assessed in each experiment: speed, velocity, and chemotactic index.

Migration speed, or chemokinesis, was defined as the distance a neutrophil travelled in any direction over time and expressed in μm/minute. Migration velocity, or chemotaxis, was defined as the distance a neutrophil travelled towards (or away from) the chemotactic source over time. This was calculated as movement along the vertical axis and expressed in μm/minute. The chemotactic index was a measure of neutrophil movement along this axis towards the chemotactic gradient and was calculated using the cosine transformation of the angle between the direction of the cell movement and the orientation of the chemotactic gradient. The chemotactic index ranged from -1 to 1, with -1 representing cell chemotaxis directly away from the chemotactic source and 1 representing cell chemotaxis directly towards the chemotactic source.

2.11 Neutrophil phenotyping by flow cytometry

2.11.1 Sample preparation and staining

100µL of neutrophils that were isolated as described in section 2.9 at a concentration of $1x10^6$ cells/ml were added to wells of a polyvinyl chloride 96-well U-bottomed plate (Costar, Loughborough, UK) and incubated for 20 minutes on ice with relevant antibodies (Table 2.4). Cells were then washed and centrifuged at 300g for 5 mins with 2% BSA in PBS and subsequently with 100µL of Annexin V binding buffer (Biolegend UK Ltd).

Either 100µL of PE-conjugated Annexin V (1:40 in Annexin V binding buffer; Biolegend UK Ltd)[294] or 100µL of Annexin V binding buffer were used to resuspend cell pellets and then incubated for 15 minutes in the dark at room temperature. Each well was washed and centrifuged with Annexin V binding buffer. Following the final centrifugation step, cell pellets were resuspended with either 100µL of Annexin V binding buffer or 100µL of 7-aminoactinomycin D (7AAD; 1:20 in Annexin V binding buffer; Biolegend UK Ltd) and transferred to 12x75mm polystyrene tubes (BD Biosciences, UK) containing 150µL of annexin V binding buffer. Analysis by flow cytometry was performed using a BD LSR Fortessa X20. Another PhD student had previously validated these antibody panels to assess the phenotype of isolated human neutrophils from peripheral blood.[295]

PRIMARY ANTIBODIES							ISOTYPE CONTROL				
Target	**Alternative names**	**Conjugate**	**Manufacturer**	**Catalogue number**	**Concentration (μg/ml)**	**Dilution**	**Isoform**	**Manufacturer**	**Catalogue number**	**Concentration (μg/ml)**	**Dilution**
PANEL 1											
CD184	CXCR4	BV421	Biolegend	306518	100	1:20	IgG2a, κ	Biolegend	400260	100	1:40
CD10	Neprilysin	BV510	Biolegend	312220	100	1:20	IgG1, κ	Biolegend	400172	100	1:20
CD62L	L-selectin	BV605	Biolegend	304834	50	1:100	IgG1, κ	Biolegend	400162	100	1:20
CD11b	Mac-1	BV785	Biolegend	301346	100	1:40	IgG1, κ	Biolegend	400170	100	1:20
CD182	CXCR2	FITC	Biolegend	320704	400	1:40	IgG1, κ	Biolegend	400108	500	3:40
CD54	ICAM-1	APC	Biolegend	322712	100	1:100	IgG1, κ	Biolegend	400122	200	1:20
CD16	FcγRIII	AF700	eBiosciences	56-0168-42	25	1:100	IgG1, k	Biolegend	400144	200	1:40
PANEL 2											
HLA-DR	MHCII	BV421	Biolegend	307636	50	1:40	IgG2a, κ	Biolegend	400260	100	1:20
CD10	Neprilysin	BV510	Biolegend	312220	100	1:20	IgG1, κ	Biolegend	400172	100	1:20
CD274	PD-L1	BV605	Biolegend	329724	150	1:100	IgG2b, κ	Biolegend	400350	100	1:40
CD11b	Mac-1	BV785	Biolegend	301346	100	1:40	IgG1, κ	Biolegend	400170	100	1:20
CD11c	CR4	FITC	Biolegend	371516	100	1:40	IgG2b, κ	Biolegend	400310	200	1:40
CD66b	CEACAM8	APC	Biolegend	305118	200	1:100	IgM, κ	Biolegend	401616	200	1:25

Table 2.4 – Primary and isotype control antibodies used for the neutrophil phenotyping panel.

Legend: The table details the primary and isotype control antibodies and the conjugated fluorophores. The concentration listed was the stock concentration of the supplied antibody, and the dilution was the standard dilution used during sample preparation. All neutrophil phenotyping experiments were performed using antibodies from the same lot number.

2.11.2 Flow cytometry data analysis

Gates were set up on the FACSDiva software (v7; BD Biosciences, USA) to exclude doublets and gate for neutrophils based on forward scatter and side scatter profiles. Live cells were also gated where viability dyes were included. Figure 2.11 shows the standard gating strategy utilised. Data were exported from FACSDiva as Flow Cytometry Standard (FCS) files and analysed using FlowJo (v10.6, BD Biosciences, USA). The raw median fluorescence intensity (MFI) based on 5000 gated live events was exported into a table for each channel. MFI values of the samples were used after subtracting MFI values for isotype controls.

Investigating cell surface marker expression using MFI and manual gating strategies provides useful knowledge-driven analyses. However, there are pitfalls to such approaches, especially with complex data sets. For example, it is impossible to investigate every possible expression profile, and assessments of its reproducibility have recognised it as a significant contributor to study variation.[296] In this respect, dimension-reduction algorithms have been developed that enable the analysis of multi-parameter datasets, such as the expression of multiple surface markers on individual cells. Algorithms such as the Rphenograph method[297] allow these complex datasets to be clustered according to all these markers. These clustered datasets can be visualised in a 2-D plot using a dimensionality reduction algorithm, such as t-Distributed Stochastic Neighbour Embedding (t-SNE).[298] Using the cytofkit package,[299] exported FCS files for each sample were clustered using Rphenograph, and the resulting clusters were visualised using t-SNE to identify neutrophil phenotypes. The expression profiles of markers illustrated in Table 2.4 can then be investigated in each cluster.

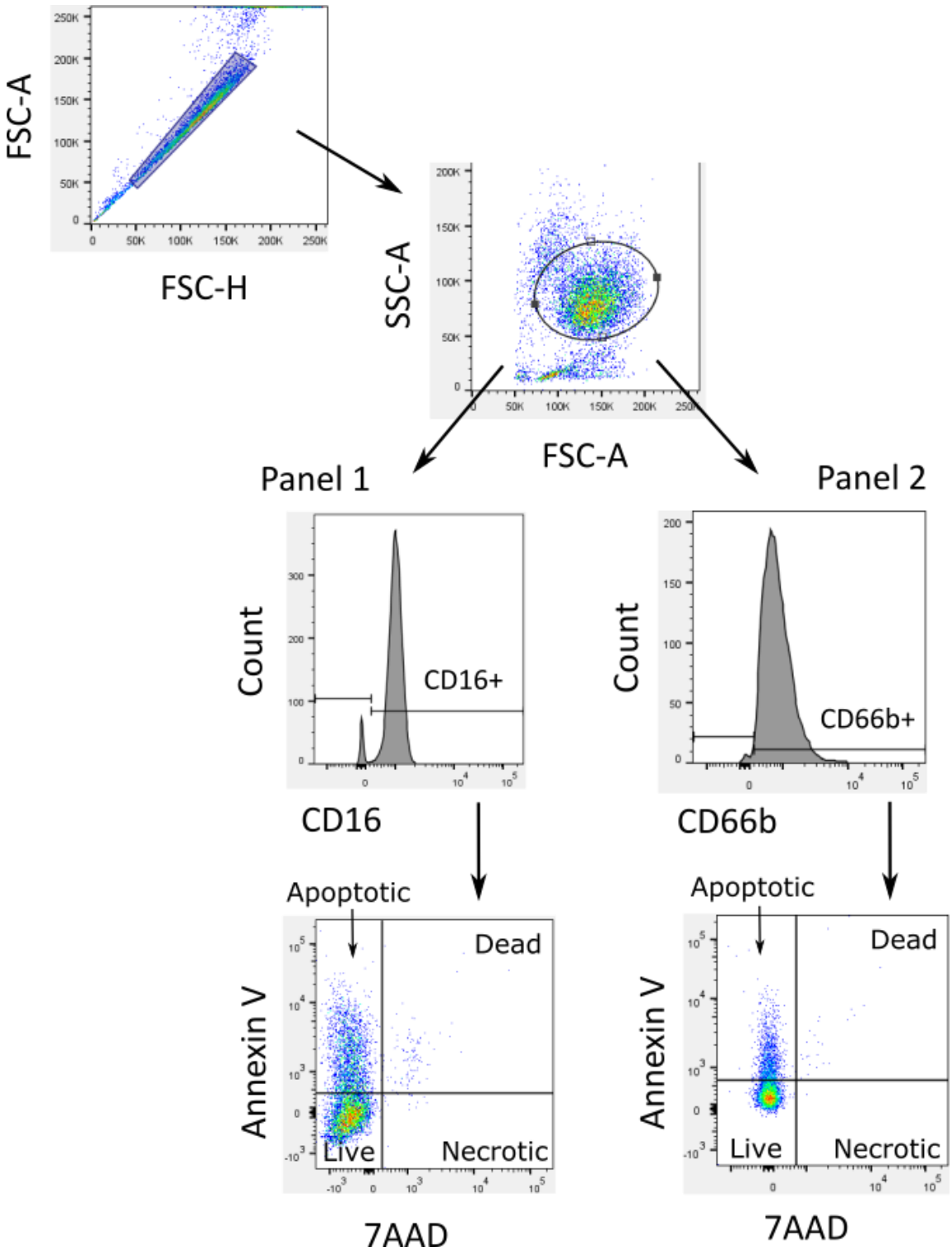


Figure 2.11 – Flow cytometry gating strategy for neutrophil phenotyping

Legend: Doublets were excluded based on cell circularity, and neutrophils were subsequently identified. Cells positive for CD16 on Panel 1 or CD66b+ on Panel 2 were selected. Viability was assessed using Annexin V and 7AAD to identify live cells (double negative). Live cells were then evaluated for the surface expression of each subsequent marker by percentage positive and MFI.

2.12 Collection of plasma from whole blood

Plasma samples were obtained by centrifuging blood collected in lithium heparin vacutainer tubes at 1700g for 10 minutes at 4^{o}C. Aliquots of plasma (500µL) were pipetted from the top of the centrifuged blood samples and stored at -80^{o}C until subsequent analysis.

2.13 Quantification of NE and PR3 activity

The NE and PR3 activity footprints were determined by quantifying the amount of NE-specific and PR3-specific fibrinogen cleavage products, Aα-Val360 and Aα-Val541, respectively, in previously-stored plasma samples using an indirect enzyme-linked immunosorbent assay (ELISA). Both these assays were developed and validated in-house.[223 231] Briefly, two 96-well black high-binding flat-bottom plates were coated with 50µL of NE or PR3-cleaved fibrinogen, diluted in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and incubated overnight at 4^{o}C.

The peptide CJTSESSV (for NE activity) or COMLGEFV (for PR3 activity assay) was serially diluted 1:2 in block buffer (Tris-buffered saline with 1% BSA and 0.05% Tween 20) to serve as standards for the assay. Two separate 96-well polypropylene U-bottomed plates (Nunc A/S, Denmark) were used to incubate 75µL of Aα-Val^{360} or Aα-Val^{541} antibody (both rabbit anti-serum) with 75 µL of samples (plasma diluted in block buffer) or each standard overnight at 4^{o}C. Plasma samples and standards were run as duplicates on each plate, and the mean value was obtained as a sample result. Control plasma samples were run on each plate, and if replicates were outside the CV expected for the control, each plate was repeated.

On the following day, the NE or PR3 cleaved-fibrinogen coated plates were washed three times with wash buffer (TBST; Tris-buffered saline with 0.05% Tween 20) and incubated with 300µL of block buffer in each well at 37 ^{o}C for an hour to prevent non-specific binding. 100µL of the peptide/sample/antibody mixture was then transferred from the polypropylene plates to the coated plates and incubated for two hours. The plates were washed with TBST as described, and 100µL of europium-conjugated anti-rabbit IgG antibody (concentration of 806ng/ml; PerkinElmer, Seer Green, UK) was added to each well.

After an incubation period of an hour and a further plate washing process, 100µL of dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) enhancement solution (PerkinElmer, Seer Green, UK) was added to each well and allowed to incubate for 20 minutes in the dark to develop a fluorescent signal. Fluorescence was then read at 340nm excitation and 620nm emission using a multi-detection plate reader (Biotek Synergy 2; Northstar Scientific, USA).

The difference in signal between the application of pure anti-serum and that incubated with varying peptide concentrations was used to derive a 3-order polynomial standard curve. The concentration of Aα-Val^{360} or Aα-Val^{541} from plasma samples was then determined by direct interpolation. The average results for readings in both plates were used for further analysis.

2.14 Determination of concentration of MMPs from plasma

MMP-8 and MMP-9 concentrations were quantified in stored plasma samples using the human MMP-8 Quantikine ELISA kit and the MMP-9 Quantikine ELISA kit (Bio-techne, Minnesota, USA). Protocols were completed according to the manufacturer's instructions. The

human MMP-8 Quantikine ELISA kit was reported to have a lower limit of detection (LLOD) of 13pg/ml with a lower limit of quantification (LLOQ) of 156pg/ml. The human MMP-9 Quantikine ELISA kit was reported to have an LLOD of 156pg/ml with an LLOQ of 313pg/ml.

Supplied reagents were brought up to room temperature, and frozen plasma samples were thawed. Lyophilised MMP-8 or MMP-9 were reconstituted in distilled water and subsequently serially diluted 1:2 in the supplied assay diluent to serve as standards for the assay, with a diluent blank used as a control. Thawed plasma samples were spun at 12000g for 10 minutes in a microcentrifuge (Prism™ R; Labnet, USA) before loading onto the supplied antibody-coated 96-well plate. A separate assay diluent was added to the provided antibody-coated 96-well plate, followed by either standard or plasma samples in duplicates. The plate was incubated for 2 hours at room temperature on an orbital plate shaker (ThermoFisher Scientific, UK) at 500rpm.

The plate was washed three times using the supplied wash buffer after incubation. The plate was blotted against clean paper towels to remove liquid from the plate completely. 200µL of supplied conjugated antibody was then added to each well, and the plate was incubated for an hour (for MMP-9) or two hours (for MMP-8) on an orbital plate shaker (ThermoFisher Scientific, UK) at 500rpm. The plate washing process was repeated, and 200µL of a 1:1 solution of supplied Substrate A and Substrate B was added to each well.

The plate was incubated for 30 minutes in the dark, and 50µL of supplied Stop solution was added to each well. The optical density of each well was then read within 30 minutes using an absorbance plate reader (BioTek Synergy HT; Northstar Scientific, USA) set at 450nm, together

with a reading at 570nm to correct for optical plate imperfections. The final absorbance value was obtained as follows:

$Abs_{final} = Abs_{450} - Abs_{570} - Abs_{blank}$

The standard curve was created by reducing the absorbance value in the standard values by generating a four-parameter logistic curve fit. The concentration of MMP-8 or MMP-9 was then obtained by directly interpolating the generated standard curve.

2.15 Statistical analysis

The statistical analyses and graph generations were done using Graphpad Prism (v8.4.3; Graphpad Software, California, USA). Data were assessed for a normal distribution using the Shapiro-Wilk test or the Kolmogorov-Smirnov test, where a p-value>0.05 was considered normally distributed.

Normally distributed data were analysed using a Student's t-test for two independent datasets or a paired t-test for matched datasets. A one-way analysis of variance (ANOVA) was performed to analyse more than two independent groups. A one-way repeated measures ANOVA was performed to analyse more than two matched datasets. Multiple comparisons were then carried out using Tukey's comparison test. Pearson's rank correlation test was used to determine associations between two variables.

Non-parametric data, or where at least one comparison group was not normally distributed, were analysed using a Mann-Whitney U test for two independent datasets or a Wilcoxon matched-paired signed-rank test for matched datasets. For analysis between more than two

independent groups, a Kruskal-Wallis analysis was performed. A Friedman test was used for analysis between more than two matched datasets. Multiple comparisons were then carried out using Dunn's multiple comparisons. Spearman's rank correlation test was used to determine associations between two variables.

Categorical data were analysed using Fisher's exact test. The reliability of CT densitometry analysis and FOT measurements was tested with the intraclass correlation coefficient. Values <0.5 indicated poor reliability, 0.5-0.75 indicated moderate reliability, 0.75-0.9 indicated good reliability, and values >0.90 indicated excellent reliability.[124] In all cases, all tests were two-tailed with a p-value<0.05 considered statistically significant.

CHAPTER 3 – SYMPTOM BURDEN OF THE EARLY COPD COHORT

3.1 Introduction

Smokers commonly experience chronic respiratory symptoms even at a young age. Evidence for this can be found in an analysis using ECRHS-1.[300] In this analysis, Isabel et al. included data from 2647 participants with a mean age of 32 years from five Spanish areas.[300] It was found that smoking was associated with a greater risk of chronic bronchitis (CB), with the risk increasing with the number of cigarettes smoked per day, even after adjustment for geographical area, total IgE, age, sex and FEV_1.[300] CB symptoms which consist of chronic cough and sputum production are significant features of COPD and GOLD has recommended that the diagnosis of COPD be considered in individuals with these symptoms and a history of exposure to risk factors (such as cigarette smoking).[1]

There is a poor correlation between the symptoms of patients diagnosed with COPD and the severity of COPD as defined by the GOLD stages.[301] Even in the absence of spirometric evidence of airflow obstruction, it is recognised that certain smokers report severe symptom burden or suffer from respiratory exacerbations.[10] [302] In these studies, respiratory exacerbations were defined as worsening of symptoms requiring the use of antibiotics, systemic glucocorticoids, or a combination of both. Exacerbation severity was determined using healthcare resources with severe exacerbations requiring hospital admission or an emergency department visit.[302] In particular, smokers with CB have a worse quality of life, reduced exercise capacity and higher risk of respiratory exacerbations than 'healthy smokers' irrespective of spirometry results.[303] [304]

As detailed in section 1.1.3, the detrimental effects of cigarette smoking are not limited to physical health. Many studies have also shown a relationship between cigarette smoking and

psychological disorders.[305] Data from the National Comorbidity Survey show more individuals with a lifetime history of depression and generalised anxiety disorder were current or past smokers than those without this history.[306] The relationship between cigarette smoking on mental health is also supported by the finding that smoking cessation is associated with reduced depression and anxiety compared with those who continue to smoke.[307]

SES is also strongly related to COPD prevalence and outcomes. This was discussed in section 1.1.9. SES is associated with a higher respiratory symptom burden and worse COPD outcomes, including higher rates of severe exacerbation and higher disease progression (measured by emphysema progression and FEV_1 decline).[308] SES is also related to COPD prevalence in the community, with higher COPD prevalence found among communities with lower income and education level.[99 309]

3.1.1 Chapter hypotheses

It was hypothesised that a proportion of smokers in the Early COPD cohort would have CB symptoms. It was also hypothesised that smokers with CB will have worse quality of life, more episodes of chest infections, and live in more deprived neighbourhoods than their asymptomatic counterparts. Given that this study took place during the COVID-19 pandemic, it was further hypothesised that there would be an improvement in respiratory symptoms but worsening psychological symptoms over the UK national lock-down period among the cohort participants.

To test these hypothesises, this chapter had the following aims:

- identify the proportion of smokers who have symptoms of CB as defined previously[32] (see section 2.4.1) within the Early COPD cohort
- compare demographics and quality-of-life scores (as measured by questionnaires detailed in section 2.5) among smokers with CB and asymptomatic smokers
- identify and compare the prevalence of self-reported chest infection retrospectively (12 months before recruitment) and prospectively (12 months after recruitment) among smokers with CB and asymptomatic smokers
- compare quality-of-life measurement scores (as measured by questionnaires detailed in section 2.5) before and after implementation of the COVID-19 national lock-down measures

All methods relevant to this chapter were described in sections 2.4 and 2.5.

3.2 Results

3.2.1 Baseline demographics

Seventy-four volunteers consented and attended the initial screening/baseline visit for the Early COPD study, and 70 participants were eligible to participate. Figure 3.1 illustrates the number of participants who contacted the study site and those found to be ineligible to participate in the study both during telephone and face-to-face screening, together with the reasons for screen failure.

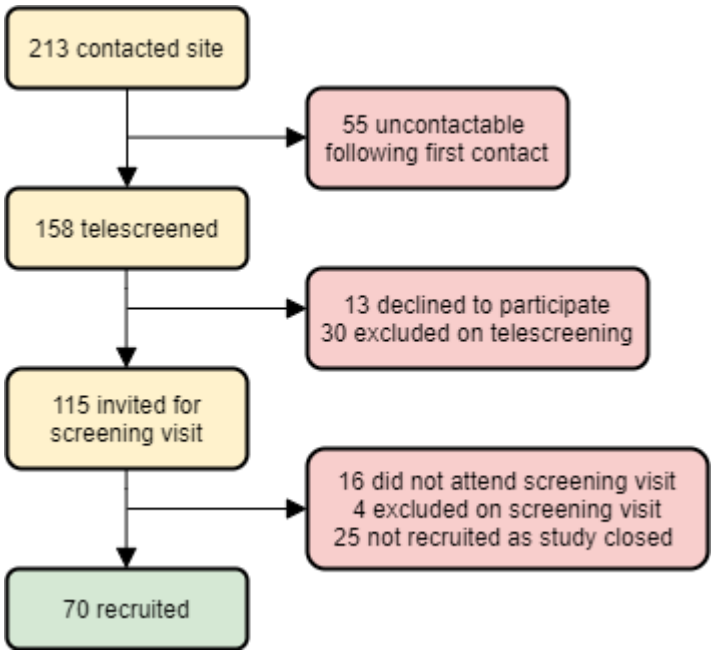


Figure 3.1 – A modified consort diagram showing the screening and recruitment of participants into the Early COPD cohort

Legend: Participants who contacted the site via telephone or email were provided with a participant information sheet and underwent telephone pre-screening before being invited for a screening/baseline visit. Although 25 participants had passed the telephone pre-screening process, they were not recruited as the study was closed after implementing the UK national lock-down measures.

The demographic details of enrolled participants with basic lung function parameters are shown in Table 3.1. The median age of the total cohort was 35.5 years (interquartile range; IQR 32-40) with a median smoking history of 14 pack years (IQR 11.0-17.3). A majority (68.6%) of participants were reported to be of white ethnicity. A chi-square test for goodness of fit test was performed, comparing the cohort ethnicity data to the 2011 census data for West Midlands County.[310] No significant differences were found between the Early COPD and West Midlands census datasets (p=0.81).

Twenty-one (30.0%) participants were found to have features of CB during their baseline visit, as defined using standard criteria[32] described in section 2.4.1. The demographic details and basic lung function parameters of participants with CB symptoms and asymptomatic smokers (AS) are shown in Table 3.2. Participants in the CB group were found to have a higher body mass index (BMI; median 29.1 (IQR 25.8-31.7) vs 25.0 (IQR 21.2-28.5), p=0.0025) than the AS group. Participants in the CB group were also found to live in postcodes with a lower IMD (median 2.0 (IQR 1.0-4.0) vs 3.0 (IQR 2.0-5.5), p=0.04) which indicated that they live in more deprived areas than participants in the AS group.

Age (years)	35.5 (32.0-40.0)
Sex, n (% female)	43 (61.4)
Smoking history (pack-years)	14.0 (11.0-17.3)
BMI (kg/m^2)	25.8 (22.5-29.4)
IMD Decile	3.0 (1.0-5.0)
Ethnicity, n (%)	
White	48 (68.6)
Asian/Asian British	18 (25.7)
Black/African/Caribbean	4 (5.7)
Co-morbidity, n (%)	
None	40 (57.1)
Psychological disorders (depression/anxiety)	12 (17.1)
Gastro-oesophageal reflux disease	6 (8.6)
Musculoskeletal disorders	6 (8.6)
Hypothyroidism	3 (4.3)
Hypertension	1 (1.4)
Other	4 (5.7)
Occupation, n (%)	
Professional	35 (50.0)
Clerical support worker	10 (14.3)
Elementary occupation	7 (10.0)
Unemployed	7 (10.0)
In full-time education	3 (4.3)
Craft and related trades worker	3 (4.3)
Technician and associate professional	2 (2.9)
Service and sales worker	1 (1.4)
Other	2 (2.9)
Lung function	
Post-BD FEV_1 (L)	3.57 ± 0.64
Post-BD FEV_1 (%predicted)	102.9 ± 10.4
FEV_1/FVC ratio	0.83 ± 0.06

Table 3.1 – Baseline demographics and spirometric parameters of the Early COPD cohort

Legend: Ethnicity categories were recommended by the Office of National Statistics,[311] and occupation categories were recommended by the International Standard Classification of Occupations (ISCO).[312] Continuous data are displayed as median (IQR) apart from lung function data which is shown as mean ± standard deviation (SD). Comorbidities listed as 'Others' include hereditary angioedema, renal stones, psoriasis and attention deficit hyperactivity disorder. BMI: body mass index; IMD: index of multiple deprivation; Post-BD: post-bronchodilator; FEV_1: forced expiratory volume in the first second; FVC: forced vital capacity

	CB (n=21)	AS (n=49)	p-value
Age (years)	36.0 (32.5-40.0)	35.0 (32.0-40.5)	0.45[#]
Sex, n (% female)	13 (61.9)	30 (61.2)	>0.99[+]
Smoking history (pack-years)	14.0 (11.5-21.5)	13.5 (11.0-16.1)	0.36[#]
BMI (kg/m^2)	29.1 (25.8-31.6)	25.0 (21.2-28.5)	0.003[#]
IMD Decile	2.0 (1.0-4.0)	3.0 (2.0-5.5)	0.04[#]
Ethnicity, n (%)			
White	15 (71.4)	33 (67.3)	0.554[+]
Asian/Asian British	6 (28.6)	12 (24.5)	
Black/African/Caribbean	0 (0)	4 (8.1)	
Co-morbidity, n (%)			
None	11 (52.4)	29 (59.2)	0.64[+]
Psychological disorders (depression/anxiety)	4 (19.0)	8 (16.3)	
Gastro-oesophageal reflux disease	2 (9.5)	4 (8.2)	
Musculoskeletal disorders	4 (19.0)	2 (4.1)	
Hypothyroidism	1 (4.8)	2 (4.1)	
Hypertension	0 (0)	1 (2.0)	
Other	1 (4.8)	3 (6.1)	
Occupation, n (%)			
Professional	8 (38.1)	27 (55.1)	0.38[+]
Clerical support worker	2 (9.5)	8 (16.3)	
Elementary occupation	4 (19.0)	3 (6.1)	
Unemployed	3 (14.3)	4 (8.2)	
In full-time education	1 (4.8)	2 (4.1)	
Craft and related trades worker	1 (4.8)	2 (4.1)	
Technician and associate professional	0 (0)	2 (4.1)	
Service and sales worker	1 (4.8)	0 (0)	
Other	1 (4.8)	1 (2.0)	
Lung function			
Post-BD FEV_1 (L)	3.43 ± 0.68	3.64 ± 0.63	0.20*
Post-BD FEV_1 (%predicted)	100.4 ± 10.1	104.0 ± 10.4	0.19*
FEV_1/FVC ratio	0.84 ± 0.06	0.83 ± 0.06	0.63*

Table 3.2 – Comparison of baseline demographics and basic lung function parameters between smokers with chronic bronchitis (CB) and asymptomatic smokers (AS)

Continuous data are displayed as median (IQR) apart from lung function data which are expressed as mean ± SD. Statistical differences between the two groups were analysed with the [#]Mann-Whitney U test, *independent t-test or the [+]Fisher's exact test. All statistically significant p-values are in bold.

3.2.2 Symptom scores at baseline

3.2.2.1 mMRC dyspnoea scale

Using the mMRC dyspnoea scale, 40 (57.1%) participants reported an mMRC grade of 0 (no breathlessness except on strenuous exercise) and 28 (40%) reported an mMRC grade of 1 (breathless when hurrying or walking up a hill) at their baseline visit. Two (2.9%) reported an mMRC grade of 2 or higher. The CB group were found to have a higher mMRC grade than the AS group at baseline (median 1 (IQR 0-1) vs 0 (IQR 0-1), p=0.021). Figure 3.2 shows the comparison in mMRC grades between the two groups.

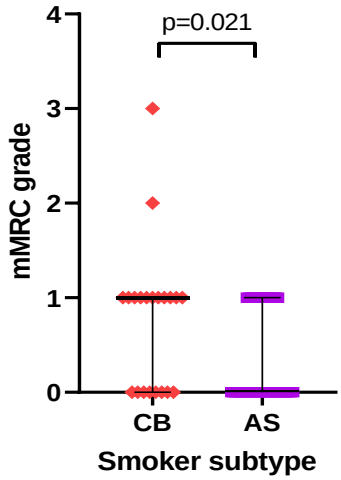


Figure 3.2 – mMRC grading in the CB and AS group

Legend: The severity of breathlessness experienced was documented using the mMRC dyspnoea scale. Error bars in the plots represent the median (IQR) of the data. n=21 for the CB group and n=49 for AS group. Comparisons were made using the Mann-Whitney U test.

3.2.2.2 COPD Assessment Test

When assessed using the CAT, the Early COPD cohort reported a median score of 10 (IQR 6-16) at their baseline visit. A CAT score of 5 was considered the upper limit of normal in healthy non-smokers.[313] However, a score of ≥10 was determined to be the threshold where COPD symptoms would have a moderate impact on daily living[314] and GOLD has recommended that this threshold be used to consider the initiation of regular treatment for COPD patients[1]. Thirty-six (51.4%) cohort participants reported a CAT score of ≥10 at baseline. The CB group reported a higher CAT score than the AS group (mean 18.6 ± 6.8 vs 8.3 ± 4.7, p<0.0001). Figure 3.3 shows the comparison in CAT scores between the two groups.

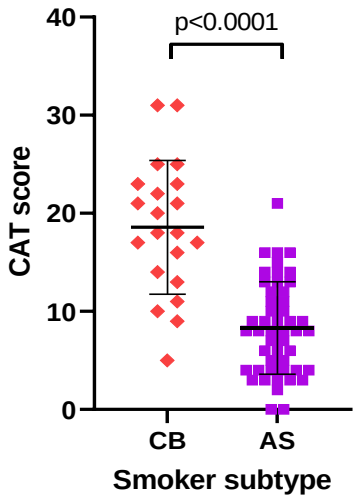


Figure 3.3 – CAT scores in the CB and AS group

Legend: General health status in the cohort was assessed using the CAT. Error bars in the plots represent the median (IQR) of the data. n=21 for the CB group and n=49 for AS group. Comparisons were made using the independent t-test.

3.2.2.3 Leicester Cough Questionnaire

The cohort reported a median score of 6.1 (IQR 5.3-6.4) in the physical domain of the LCQ, a median score of 6.7 (IQR 5.6-7.0) in the psychological domain and a median score of 6.9 (IQR 5.6-7.0) on the social domain. The median total score reported from the cohort was 19.5 (IQR 16.8-20.6) at the baseline visit. The CB group reported a higher score on all domains of the LCQ, including the total score than the AS group ($p<0.0001$ in all comparisons). Figure 3.4 compares LCQ domain scores and total LCQ scores between the two groups.

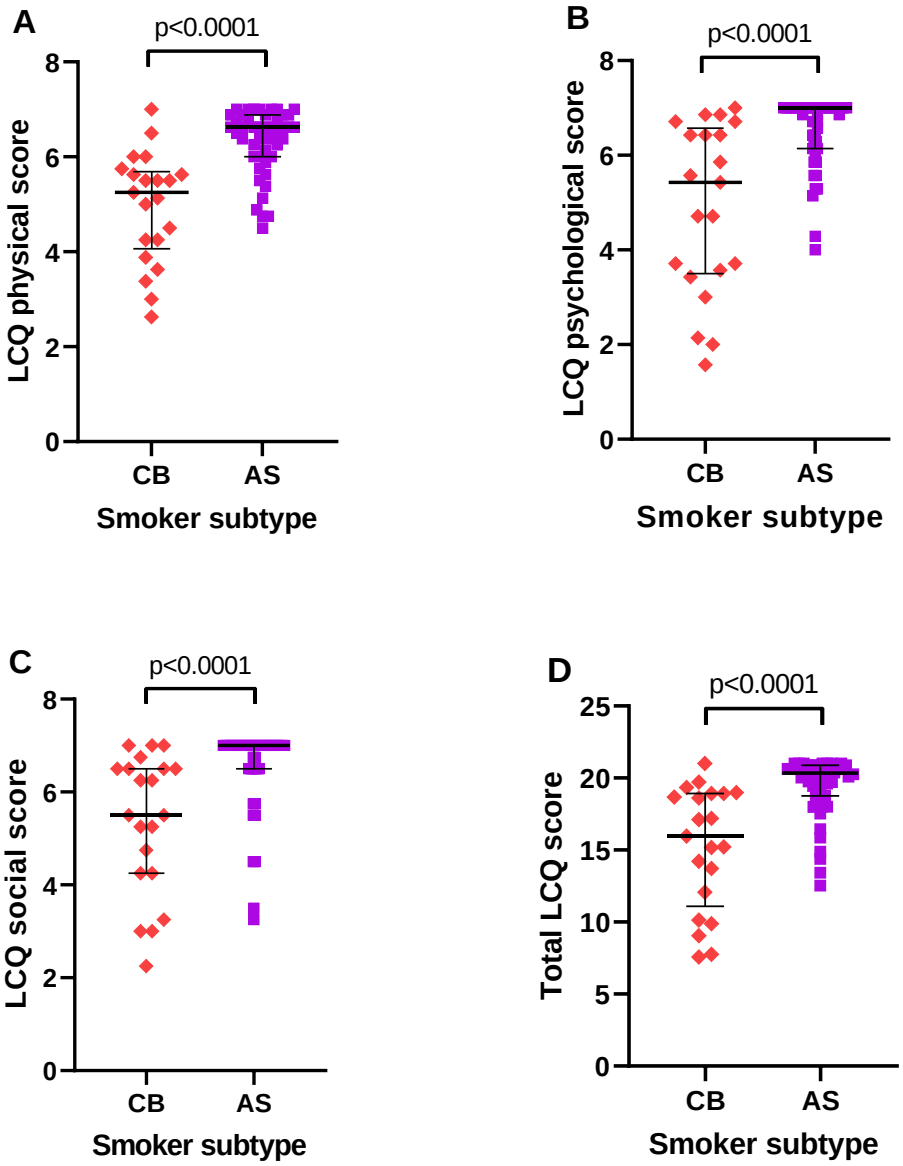


Figure 3.4 – LCQ scores in the CB and AS group

Legend: Cough-specific health status in the cohort was assessed using the LCQ. The LCQ physical domain score (figure A), psychological score (figure B), social score (figure C) and total LCQ score (figure D) are shown. Error bars in the plots represent the median (IQR) of the data. n=21 for the CB group and n=47 for the AS group. Comparisons in all domains and total scores were made using the Mann-Whitney U test.

3.2.2.4 Hospital Anxiety and Depression Scale

The Early COPD cohort reported a median anxiety score of 8 (IQR 5.0-10.5) and a median depression score of 4 (IQR 1-8) when assessed using the HADS at their baseline visit. Both were higher than reported in the study published by Crawford et al. involving 1792 healthy UK adults with a median anxiety score of 6 and the median depression score of 3.[315] A score of ≥8 on either scale indicates significant anxiety and/or depression.[48 281] Using this threshold, 35 (50.0%) of the cohort participants had significant anxiety, and 19 (27.1%) had significant depression. Seventeen (24.3%) participants had significant anxiety and depression symptoms.

The CB group had a higher depression score (median 6.5 (IQR 3.0-9.0) vs 3.0 (IQR 1.0-8.0), p=0.042) and anxiety score (median 9.5 (IQR 7.3-12.0) vs 7.0 (IQR 4.0-9.5), p=0.004) than the AS group at baseline. The CB group had a higher proportion of smokers with significant anxiety (n=15, 71.4%) compared to the AS group (n=20, 40.8%; p=0.04). However, no differences were found in the proportion of smokers with significant depression among both groups (CB: n=6, 28.6% vs AS: n=13, 26.5%; p>0.99). There were no differences in the proportion of participants with significant anxiety and depression in both groups (CB: n=6, 28.6% vs AS: n=11, 22.4%; p=0.76). Figure 3.5 compares anxiety and depression scores for both groups. The comparison of symptom scores between the CB and AS groups are shown below in Table 3.3.

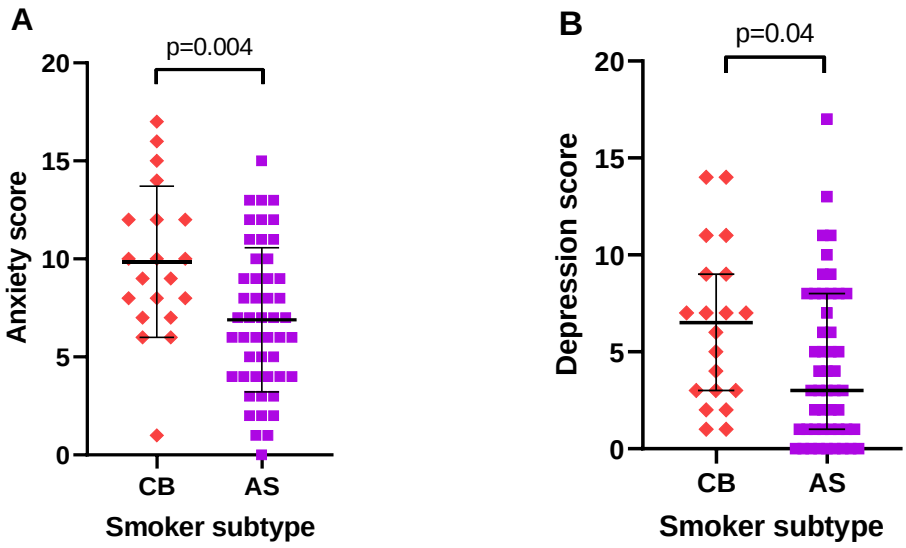


Figure 3.5 – HADS scores in the CB and AS group

Legend: The severity of anxiety and depression symptoms in the cohort was assessed using the HADS. The individual LCQ anxiety (figure A) and depression scores (figure B) for each patient are shown. Error bars in the plots represent the median (IQR) of the data. n=20 for the CB group and n=49 for AS group. Comparisons were made using the independent t-test for anxiety scores and the Mann-Whitney U test for depression scores.

	CB (n=21)	**AS (n=49)**	**p-value**
mMRC	1 (0-1)	0 (0-1)	0.021#
CAT score	18.0 (13.5-23.0)	8.0 (4.0-12.0)	<0.0001*
HADS	n=20	n=49	
Anxiety score	9.5 (7.3-12.0)	7.0 (4.0-9.5)	0.004*
Depression score	6.5 (3.0-9.0)	3.0 (1.0-8.0)	0.042#
LCQ score	n=21	n=47	
Physical score	5.3 (4.1-7.0)	6.6 (6.0-6.9)	<0.0001#
Psychological score	5.4 (3.5-6.6)	7.0 (6.1-7.0)	<0.0001#
Social score	5.5 (4.3-6.5)	7.0 (6.5-7.0)	<0.0001#
Total score	16.0 (11.1-18.9)	20.3 (18.8-20.9)	<0.0001#

Table 3.3 – Comparison of baseline symptom scores between the CB and AS group

Participant symptom scores were assessed using the mMRC dyspnoea scale, CAT, HADS and LCQ. All values are displayed as median (IQR). Statistical differences between the two groups were assessed using the #Mann-Whitney U test or the *independent t-test. All statistically significant p-values are in bold.

3.2.2.5 History of chest infections

Twenty-three (32.8%) participants reported a chest infection during the 12 months preceding enrolment. Among the CB group, 9 (42.9%) reported a chest infection during the 12 months preceding enrolment, compared to 14 (28.6%) in the AS group. This difference was not found to be statistically significant (p=0.28).

3.2.3 Symptom-specific question scores

3.2.3.1 Cough and sputum production

All relationships assessed in this section were performed using Spearman's correlation coefficient. Question 1 (I never cough/cough all the time) and question 2 (I have no phlegm in my chest at all/My chest is completely full of phlegm) of the CAT and LCQ physical domain scores were used to assess the severity of cough and sputum production (if any). The cohort reported a median score of 2.0 (1.0-3.0) for question 1 and 1.5 (1.0-3.0) for question 2 of the CAT. The CB group were found to have a higher score on both CAT question 1 (median 3.0 (IQR 2.0-4.5) vs 1.0 (IQR 1.0-2.0), p<0.0001) and question 2 (median 3.0 (IQR 3.0-4.0) vs 1.0 (IQR 0-2.0), p<0.0001) compared to the AS group. The LCQ physical domain scores were negatively correlated with question 1 (Spearman's rho= -0.62, 95% confidence interval (CI) -0.75 to -0.45; p<0.0001) and question 2 (rho= -0.67, 95% CI -0.79 to -0.51; p<0.0001) CAT scores. Figure 3.6 compares CAT question 1 and 2 scores between the two groups, and figure 3.7 shows the scatterplots of the above correlations.

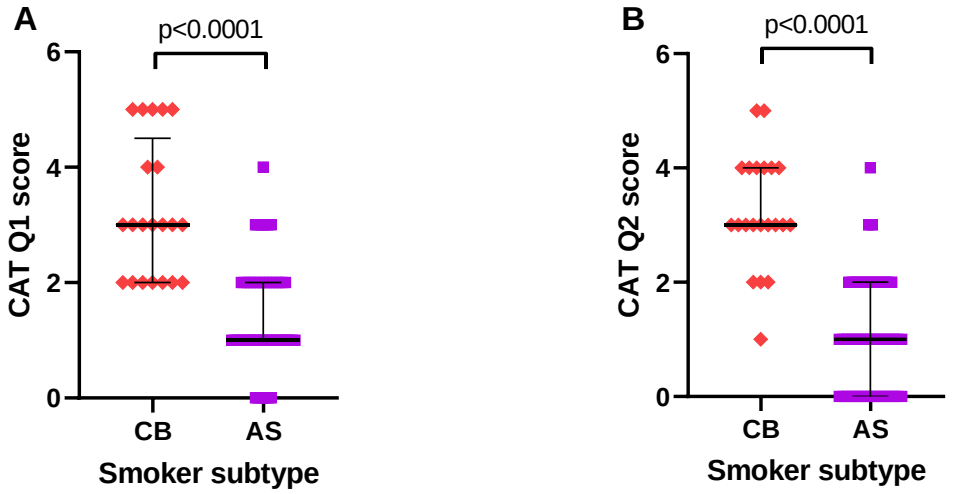


Figure 3.6 – Question 1 (Q1) and question 2 (Q2) CAT scores in the CB and AS group

Legend: The severity of cough and sputum production was self-assessed using Q1 (figure A) and Q2 (figure B) CAT. Error bars in the plots represent the median (IQR) of the data. n=21 for the CB group and n=49 for AS group. Comparisons were made using the Mann-Whitney U test.

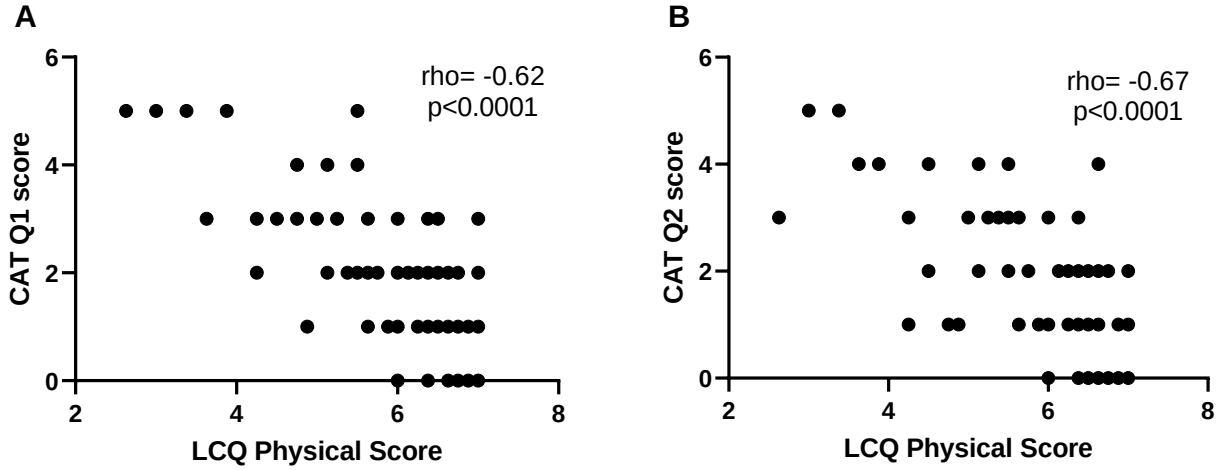


Figure 3.7 – Correlation of CAT Q1 scores and Q2 scores with LCQ physical domain scores

Legend: The severity of cough and sputum production symptoms were from Q1 (figure A) and Q2 (figure B) CAT scores compared to the LCQ physical domain scores. Correlations were assessed using Spearman's correlation coefficient test. Sixty-eight pairs of data were available for both correlations.

3.2.3.2 Breathlessness

Question 3 (My chest does not feel tight at all/feels very tight) and question 4 (When I walk up a hill or one flight of stairs, I am not/very breathless) from the CAT and mMRC dyspnoea scale were used to assess the severity of breathlessness. The cohort reported a median score of 1.5 (0-3.0) for question 3 and 2.0 (1.0-3.0) for question 4 of the CAT. The CB group had a higher score on both CAT question 3 (median 3.0 (IQR 1.5-3.0) vs 1.0 (IQR 0-2.0), p=0.0007) and question 4 (median 3.0 (IQR 3.0-4.0) vs 1.0 (IQR 1.0-3.0), p<0.0001) than the AS group. The mMRC grading had a significant positive correlation with questions 3 (rho=0.54, 95% CI 0.34 to 0.69; p<0.0001) and 4 (rho=0.53, 95% CI 0.33 to 0.68; p<0.0001) CAT scores. Figure 3.8 shows the comparison in CAT questions 3 and 4 individual scores for the two groups, and figure 3.9 shows the scatterplots of the correlations.

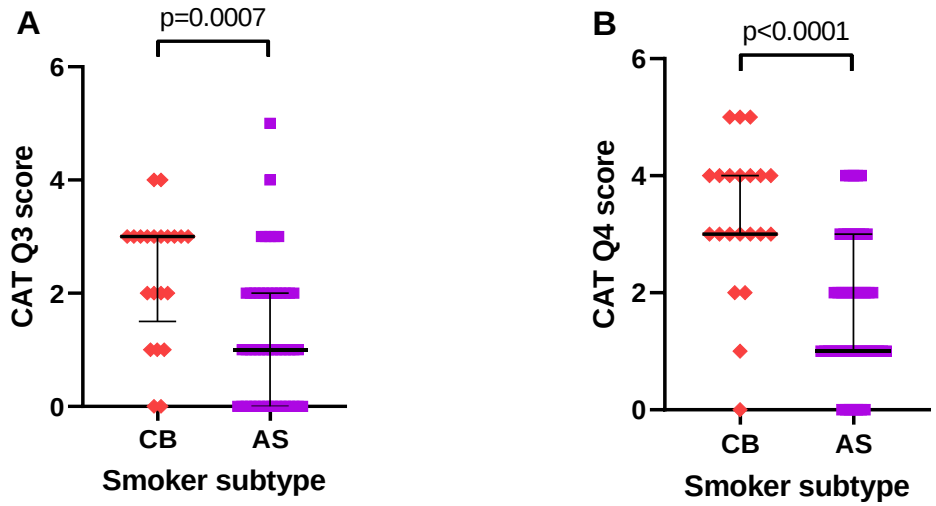


Figure 3.8 – Question 3 (Q3) and question 4 (Q4) individual CAT scores for the CB and AS group

Legend: The severity of breathlessness was assessed using Q3 (figure A) and Q4 (figure B) scores of the CAT. Error bars in the plots represent the median (IQR) of the data. n=21 for the CB group and n=49 for AS group. Comparisons were assessed with the Mann-Whitney U test.

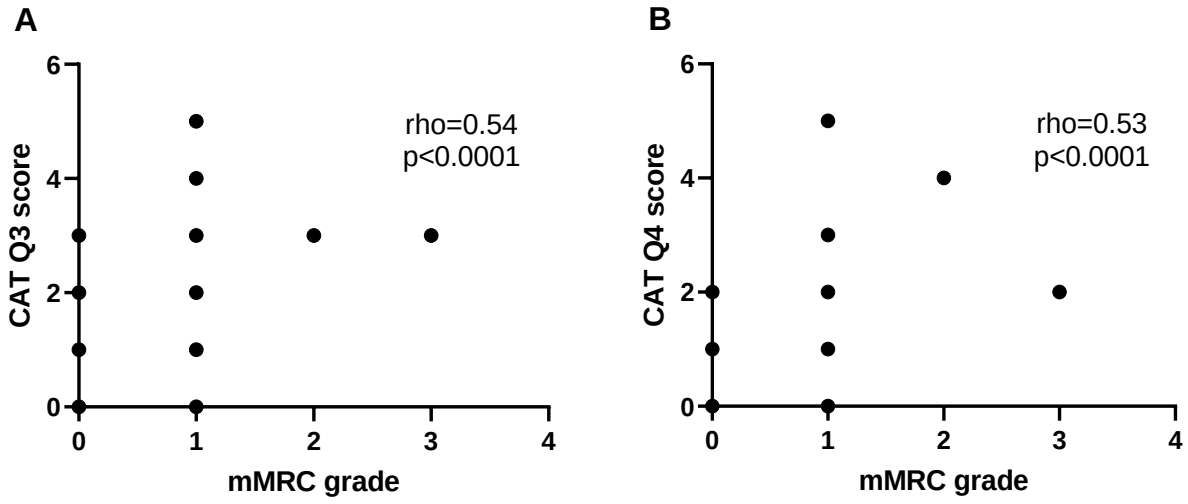


Figure 3.9 – Correlation of CAT Q3 scores and Q4 scores with mMRC grades

Legend: CAT severity of breathlessness scores for Q3 (figure A) and Q4 (figure B) are shown on the vertical axis, and the mMRC dyspnoea scale is shown on the horizontal axis. Correlations were assessed using Spearman's correlation coefficient. Seventy pairs of data were available for both correlations. Figure A: rho=0.54, p<0.0001; Figure B: rho=0.53, p<0.0001.

3.2.3.3 Psychological symptoms (anxiety/depression)

The LCQ psychological domain scores and the HADS score were used to assess the severity of anxiety and depressive symptoms in the cohort. Participants who had a significant HADS anxiety score (≥8) were found to have lower LCQ psychological domain scores (median 5.9 (IQR 4.5-6.9) vs 7.0 (IQR 6.4-7.0), p=0.001) than those with HADS score <8. However, no difference in LCQ psychological domain score was found between those who had a significant HADS depression score and those who did not (median 6.4 (IQR 5.5-7.0) vs 6.7 (IQR 5.5-7.0), p=0.45). The LCQ psychological domain scores had a significant negative correlation with the HADS anxiety score (rho= -0.37, 95% CI -0.57 to -0.14; p=0.002) and HADS depression score (rho = -0.38, 95% CI -0.57 to -0.15, p=0.001). Figure 3.10 compares LCQ psychological domain scores between those with significant psychological symptoms (as assessed by HADS) and those without significant psychological symptoms. Figure 3.11 shows the scatterplots of the correlations mentioned.

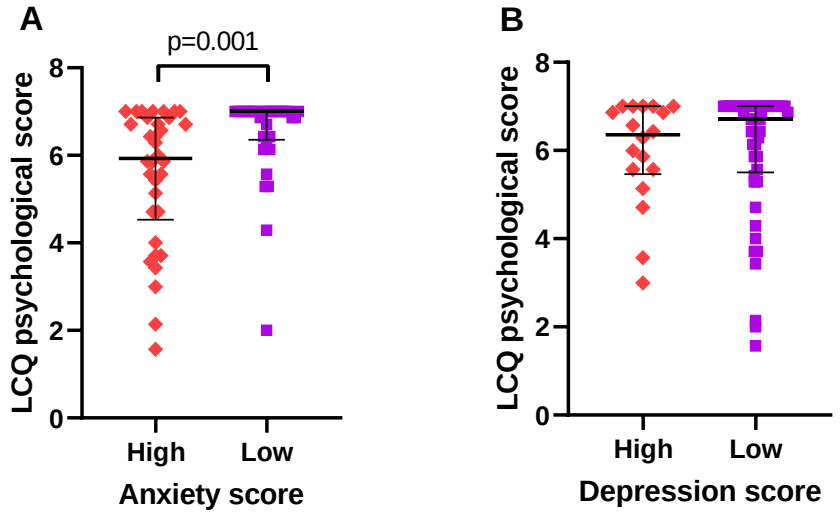


Figure 3.10 – LCQ psychological scores in those with high anxiety or depression scores (as assessed by HADS) compared to those who did not

Legend: The cohort was stratified into a high (≥8) or low (<8) anxiety or depression group based on the HADS score. The LCQ psychological scores are shown for participants in anxiety groups (A) and depression groups (B). Error bars in the plots represent the median (IQR) of the data. n=34 for the high anxiety group and n=33 for the low anxiety group. n=18 for the high depression group and n=49 for the low depression group. Comparisons were assessed using the Mann-Whitney U test.

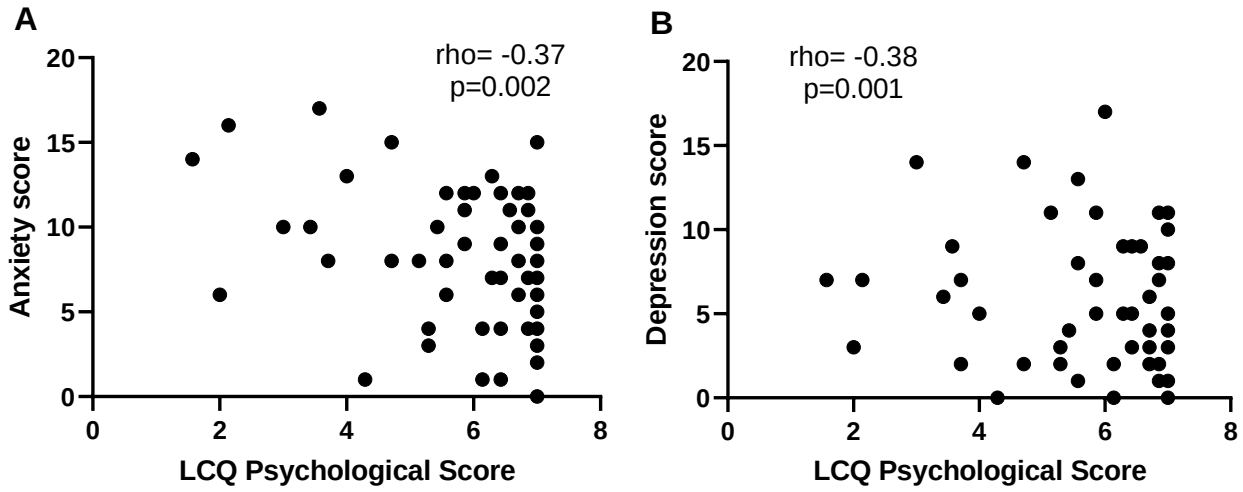


Figure 3.11 – Correlation of HADS anxiety and depression score with LCQ psychological domain scores

Legend: The severity of psychological symptoms was self-assessed using the anxiety (figure A) and depression (figure B) scale of HADS (vertical axis) and the LCQ psychological domain scores (horizontal axis). Correlations were assessed using Spearman's correlation coefficient. Sixty-seven pairs of data were available for both correlations.

3.2.4 Correlation of symptoms at baseline

All relationships of symptom scores at the baseline visit assessed in this section were performed using Spearman's correlation coefficient. CAT scores were found to correlate positively with both the anxiety (rho=0.55, 95% CI 0.36 to 0.70; p<0.0001) and depression scores (rho=0.53, 95% CI 0.32 to 0.68; p<0.0001) assessed using HADS. CAT scores also correlated negatively with the total LCQ score (rho= -0.75, 95% CI -0.84 to -0.62; p<0.0001) and positively with the mMRC dyspnoea scale (rho=0.54, 95% CI 0.34 to 0.69; p<0.0001). Figure 3.12 shows the scatterplots of these correlations.

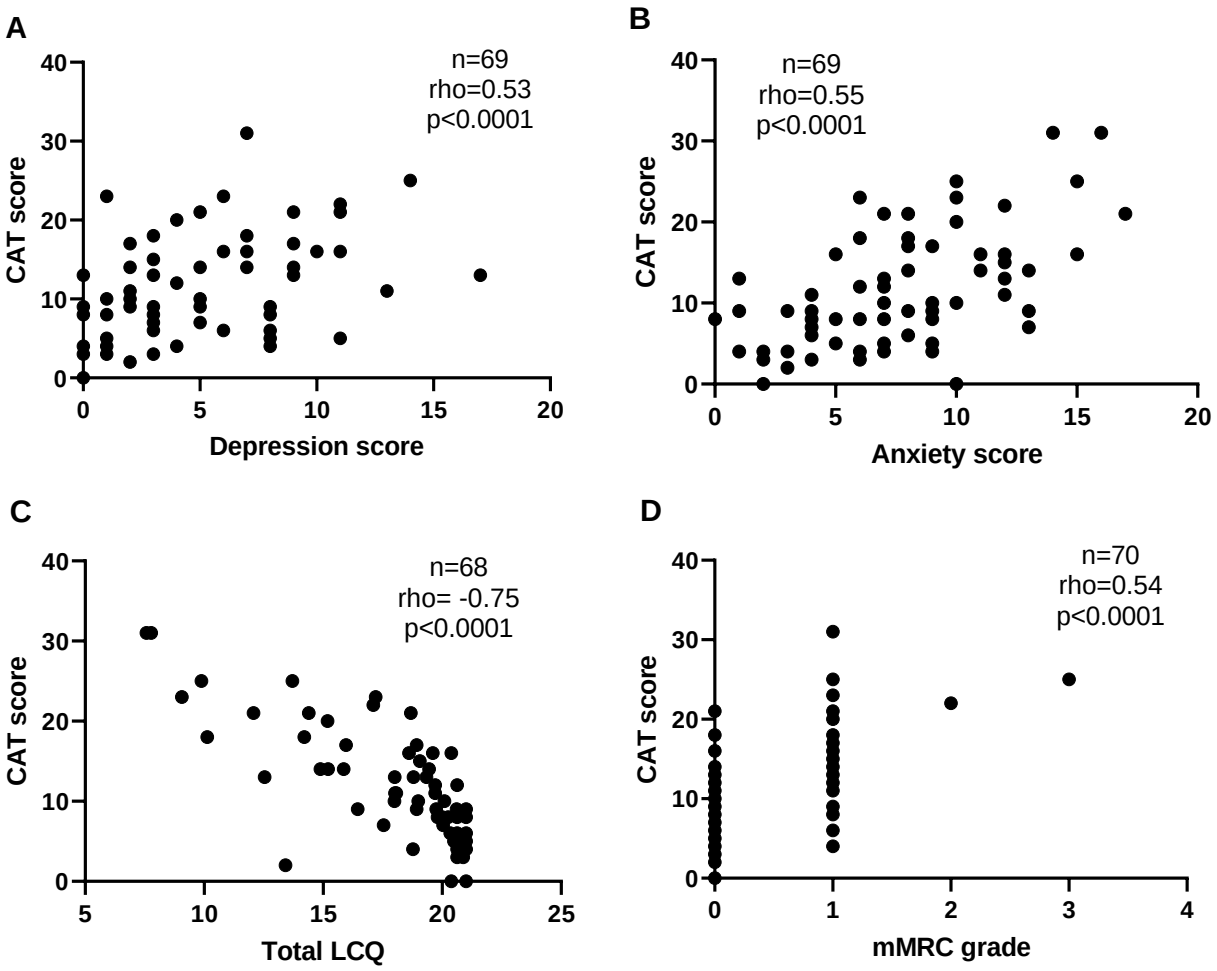


Figure 3.12 – Correlation of symptom questionnaire scores at baseline visit

Legend: Symptom burden among the Early COPD cohort was assessed using the mMRC dyspnoea scale, CAT, LCQ and HADS. Correlations between CAT scores with HADS depression (figure A) and anxiety scores (figure B), total LCQ scores (figure C) and mMRC grades (figure D) are shown. Correlations were assessed using Spearman's correlation coefficient test.

Participants who had a self-reported chest infection 12 months before enrolment had a lower total LCQ score than those who did not (median 18.8 (IQR 15.9-19.7) vs 20.1 (IQR 16.8-20.8), p=0.025). There was also a trend towards a higher CAT score, depression score and anxiety score in those with a previous self-reported chest infection, but this did not reach statistical significance. Table 3.4 shows the comparison of symptom scores between the two groups.

	Previous LRTI (n=23)	**No previous LRTI (n=47)**	**p-value**
mMRC	0 (0-1)	0 (0-1)	0.64[#]
CAT score	13.0 (8.0-18.0)	9.0 (4.0-15.0)	0.051[#]
HADS			
Anxiety score	8.0 (6.0-11.0)	7.0 (4.0-10.0)	0.20*
Depression score	5.0 (3.0-8.0)	3.5 (1.0-8.0)	0.29[#]
LCQ score			
Physical score	5.6 (4.8-6.4)	6.5 (5.6-6.8)	0.012[#]
Psychological score	6.4 (5.3-6.9)	6.9 (5.6-7.0)	0.07[#]
Social score	6.5 (5.5-7.0)	7.0 (6.0-7.0)	0.09[#]
Total score	18.8 (15.9-19.7)	20.1 (16.8-20.8)	0.025[#]

Table 3.4 – Comparison of baseline symptom scores between those who had a self-reported chest infection 12 months before enrolment and those who did not

Legend: All data are displayed as median (IQR). Statistical differences between the two groups were analysed using the [#]Mann-Whitney U test or the *independent t-test. All significant p-values are in bold. LRTI: lower respiratory tract infection

3.2.5 Change in the Early COPD cohort over time

Complete symptom score data were available for two consecutive visits (baseline and six months) for 51 (72.9%) participants and three consecutive visits (baseline, six months, and 12 months) for 36 (51.4%) participants. Figure 3.13 illustrate the reasons for the missing symptom score data at both six months and 12 months.

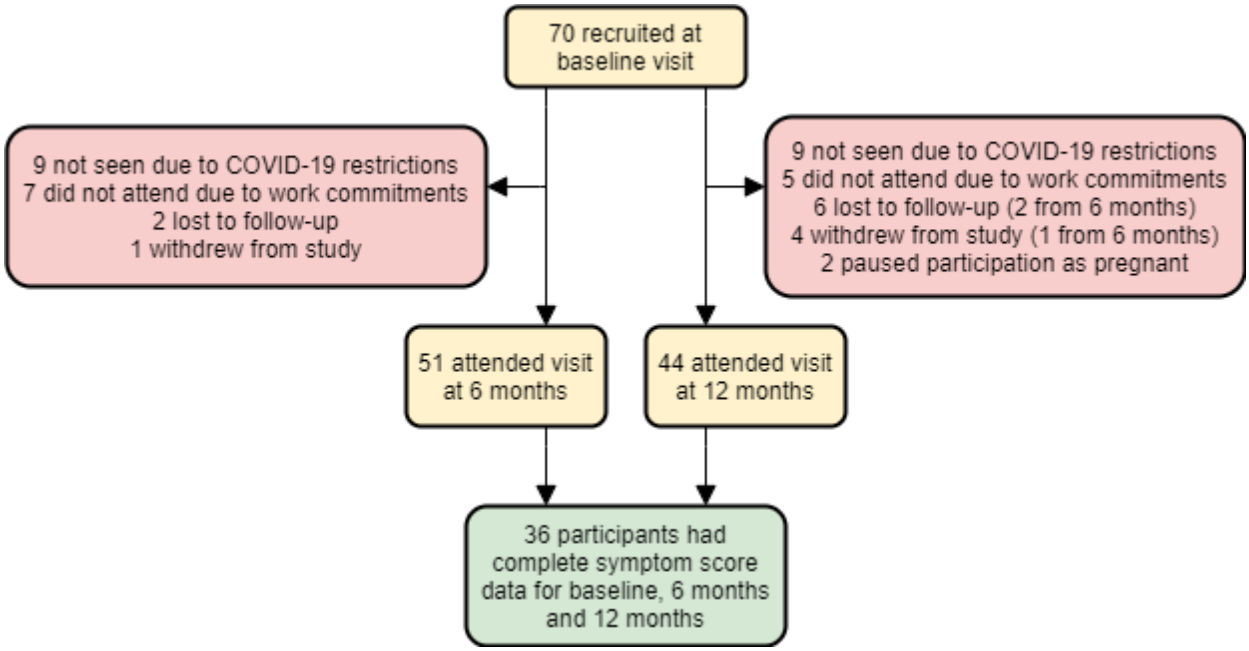


Figure 3.13 – A modified consort diagram showing the reasons for missing symptom score data at six months and 12 months

Legend: Recruited participants were invited for follow-up visits every six months. Follow-up visits are conducted face-to-face or via telephone (over the COVID-19 restriction period). Data from the first three visits (baseline, six months, and 12 months) were analysed for this thesis. Reasons for non-attendance at both follow-up visits were shown.

3.2.5.1 Change in smoking habit over time

Four (5.7%) participants reported that they had stopped smoking cigarettes at six months follow-up. At 12 months follow-up, three (4.3%) participants had quit smoking, and two (2.9%) had remained ex-smokers from the previous visit. Two participants were found to have relapsed with cigarette smoking at 12 months follow-up. Of the two participants who successfully stopped cigarette smoking over both follow-up visits, one was from the CB group, while the other was from the AS group.

3.2.5.2 Changes in symptoms over time

Changes in symptom burden in the Early COPD cohort were assessed using symptom scores available over two consecutive visits (baseline and six months) and three successive visits (baseline, six months, and 12 months). Table 3.5 compares demographic details between those included in the 6-month longitudinal analysis and those not, while table 3.6 shows a similar comparison between those included in the 12-month longitudinal analysis and those not. No significant differences were found in baseline demographics between those included in the 6-month and 12-month longitudinal analysis and those not.

	Included (n=51)	Not included (n=19)	p-value
Age (years)	36.0 (32.0-40.0)	35.0 (32.0-40.0)	0.71[#]
Sex, n (% female)	32 (62.7)	11 (57.9)	0.79[+]
Smoking history (pack-years)	13.5 (11.0-15.8)	14.0 (11.0-19.5)	0.45[#]
BMI (kg/m^2)	25.7 (22.5-29.0)	28.7 (21.5-30.0)	0.70*
IMD Decile	3.0 (2.0-6.0)	2.0 (1.0-4.0)	0.07[#]
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 34 (66.7) 15 (29.4) 2 (3.9)	 14 (73.7) 3 (15.8) 2 (10.5)	 0.29[+]
Lung function Post-BD FEV_1 (L) Post-BD FEV_1 (%predicted) FEV_1/FVC ratio	 3.48 (3.13-4.08) 102.0 (96.0-111.0) 0.84 (0.79-0.87)	 3.37 (3.08-4.15) 106.0 (98.0-111.0) 0.85 (0.81-0.89)	 0.81* 0.32[#] 0.38*

Table 3.5 – Comparison of baseline demographics and basic lung function parameters between participants included in the 6-month longitudinal symptom analysis and those who were not

Legend: Participants with symptoms score data available over two consecutive visits (baseline and six months) were included in the 6-month longitudinal symptom analysis. Continuous data are displayed as median (IQR). Statistical differences between the two groups were analysed using the [#]Mann-Whitney U test, *****independent t-test or the [+]Fisher's exact test.

	Included (n=36)	Not included (n=34)	p-value
Age (years)	36.0 (32.3-40.0)	34.5 (32.0-40.3)	0.48[#]
Sex, n (% female)	23 (63.9)	20 (58.8)	0.81[+]
Smoking history (pack-years)	13.0 (11.0-15.6)	14.0 (11.0-20.4)	0.21[#]
BMI (kg/m^2)	25.8 (22.6-29.0)	26.4 (21.4-29.9)	0.94*
IMD Decile	3.0 (1.0-5.0)	3.0 (1.8-4.3)	0.98[#]
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 21 (58.3) 13 (36.1) 2 (5.6)	 27 (79.4) 5 (14.7) 2 (5.9)	 0.12[+]
Lung function Post-BD FEV_1 (L) Post-BD FEV_1 (%predicted) FEV_1/FVC ratio	 3.44 (3.06-4.04) 101.5 (93.0-110.8) 0.84 (0.78-0.87)	 3.60 (3.12-4.15) 106.0 (97.0-111.3) 0.85 (0.81-0.87)	 0.43* 0.35[#] 0.39*

Table 3.6 – Comparison of baseline demographics and basic lung function parameters between participants included in the 12-month longitudinal symptom analysis and those who were not

Legend: Participants with symptoms score data available over three consecutive visits (baseline, six months, and 12 months) were included in the 12-month longitudinal symptom analysis. Continuous data are displayed as median (IQR). Statistical differences between the two groups were analysed using the [#]Mann-Whitney U test, *****independent t-test or the [+]Fisher's exact test.

Among the participants included in the 6-month longitudinal analysis, there was a statistically significant increase in HADS depression score at baseline compared to that at six months (median 6.0 (IQR 2.0-10.0) vs 3.0 (IQR 1.5-7.0), p=0.04). There was also a trend towards a lower CAT score, but this did not reach statistical significance (p=0.10). There were no statistical differences in all other symptom scores in the cohort in the 6-month longitudinal analysis. Table 3.7 shows the available consecutive symptom scores of the cohort over six months from baseline.

Among the participants included in the 12-month longitudinal analysis, there was a trend towards a lower CAT score with time, but this did not reach statistical significance (p=0.09). There were no statistical differences in all other symptom scores in the cohort in the 12-month longitudinal analysis. Table 3.8 shows the available consecutive symptom scores of the cohort over 12 months from baseline.

	Baseline	6 months	p-value
mMRC	0 (0-1)	1 (0-1)	0.12[#]
CAT score	12.0 (8.0-16.0)	10.0 (5.0-16.0)	0.10[#]
HADS			
Anxiety score	7.0 (4.0-11.0)	8.0 (5.0-12.0)	0.15*
Depression score	3.0 (1.5-7.0)	6.0 (2.0-10.0)	0.04[#]
LCQ score			
Physical score	6.0 (5.4-6.6)	6.3 (5.6-6.8)	0.36[#]
Psychological score	6.4 (5.4-7.0)	6.9 (5.3-7.0)	0.62[#]
Social score	6.8 (5.5-7.0)	7.0 (6.0-7.0)	0.64[#]
Total score	19.1 (16.0-20.4)	19.9 (16.9-20.8)	0.62[#]

Table 3.7 – Symptom scores in the cohort over two consecutive visits over six months

Legend: Symptom score trend over six months was available for 51 participants of the Early COPD cohort. All values are displayed as median (IQR). Differences in scores between each visit were assessed using the [#]Wilcoxon signed-rank test or the *****paired t-test. All significant p-values are in bold.

	Baseline	6 months	12 months	p-value
mMRC	1 (0-1)	1 (0-1)	1 (0-1)	0.34*
CAT score	13.5 (8.3-18.0)	12.0 (6.0-16.8)	10.5 (7.0-15.5)	0.09*
HADS				
Anxiety score	8.0 (6.0-11.0)	9.0 (6.0-12.0)	8.0 (4.0-13.0)	0.62[#]
Depression score	4.0 (2.0-8.0)	6.0 (2.0-10.0)	5.0 (3.0-8.0)	0.34*
LCQ score				
Physical score	6.0 (5.1-6.5)	6.3 (5.6-6.6)	6.1 (5.4-6.8)	0.19*
Psychological score	6.4 (5.1-7.0)	6.7 (5.3-7.0)	6.7 (6.0-7.0)	0.85*
Social score	6.5 (5.3-7.0)	7.0 (6.0-7.0)	7.0 (6.0-7.0)	0.53*
Total score	18.9 (15.2-20.3)	19.6 (16.9-20.6)	19.7 (16.6-20.6)	0.39*

Table 3.8 – Symptom scores in the cohort over three consecutive visits over 12 months

Legend: Symptom score trend over 12 months was available for 36 participants of the Early COPD cohort. All values are displayed as median (IQR). Differences in scores between each visit were assessed using either the *Friedman test or the [#]one-way repeated measures ANOVA test.

3.2.5.3 Changes to symptom burden over time between smoker subtypes

Consecutive symptom score data over six months was available for 16 (76.2%) CB subjects and 35 (71.4%) AS subjects. CB participants included in the 6-month longitudinal analysis were found to have a lower BMI (median 28.0 (IQR 23.6-29.9) vs 31.2 (IQR 29.7-34.6), p=0.04) and higher IMD decile (median 3.0 (IQR 1.3-4.0) vs 1.0 (IQR 1.0-1.5), p=0.03) than those excluded from the analysis (see Table 3.9). No differences in symptom scores were seen at 6-month follow-up compared to baseline in both groups.

Consecutive symptom score data over 12 months was available for 15 (71.4%) CB subjects and 21 (42.9%) AS subjects. CB participants included in the 12-month longitudinal analysis were found to have a lower BMI (median 28.0 (IQR 22.9-29.6) vs 31.6 (IQR 29.8-34.1), p=0.018) than those excluded from the analysis (see Table 3.10). There was a decrease in CAT score in the CB group at the 12-month follow-up (median 13.0, IQR 9.0-19.0) compared to baseline (median 18.0, IQR 16.0-22.0; p=0.014). There were no differences in other symptom scores in the CB group with time. No differences in symptom scores were seen in the AS group at 12-month follow-up compared to baseline (see table 3.11). Figure 3.14 illustrates the trend of the CAT score over time points.

	CB			AS		
	Included (n=16)	**Not included (n=5)**	**p-value**	**Included (n=35)**	**Not included (n=14)**	**p-value**
Age (years)	36.0 (33.3-39.8)	39.0 (32.0-40.5)	0.91[#]	35.0 (32.0-41.0)	34.5 (32.0-38.8)	0.86[#]
Sex, n (% female)	10 (62.5)	3 (60.0)	>0.99[+]	23 (65.7)	8 (57.1)	0.75[+]
Smoking history (pack-years)	13.5 (11.0-18.3)	14.0 (13.0-87.0)	0.32[#]	13.0 (11.0-15.6)	14.0 (11.0-20.4)	0.21[#]
BMI (kg/m^2)	28.0 (23.6-29.9)	31.2 (29.7-34.6)	0.04*	25.8 (22.6-29.0)	26.3 (21.4-29.9)	0.91*
IMD Decile	3.0 (1.3-4.0)	1.0 (1.0-1.5)	0.03[#]	4.0 (2.0-6.0)	3.0 (1.8-4.0)	0.21[#]
Ethnicity, n (%)						
White	11 (68.8)	4 (80.0)	>0.99[+]	23 (65.7)	10 (58.8)	0.36[+]
Asian/Asian British	5 (31.2)	1 (20.0)		10 (28.6)	2 (14.3)	
Black/African/Caribbean	0 (0)	0 (0)		2 (5.7)	2 (14.3)	
Lung function						
Post-BD FEV_1 (L)	3.26 (2.89-3.93)	3.29 (2.81-4.23)	0.88*	3.53 (3.14-4.14)	3.51 (3.09-4.15)	0.64*
Post-BD FEV_1 (%predicted)	100.5 (92.3-108.8)	106.0 (96.0-112.0)	0.40*	103.0 (97.0-112.0)	106.5 (99.5-111.8)	0.57[#]
FEV_1/FVC ratio	0.84 (0.80-0.87)	0.87 (0.79-0.92)	0.38*	0.84 (0.78-0.87)	0.83 (0.81-0.87)	0.80*

Table 3.9 – Comparison of baseline demographics and spirometric parameters between CB and AS participants included in the 6-month longitudinal symptom analysis and those who were not included

Legend: Symptoms score data available for CB and AS participants over two consecutive visits (baseline and six months) were included in the 6-month longitudinal symptom analysis. Continuous data are displayed as median (IQR). Statistical differences between those included and those not in both groups were assessed using the [#]Mann-Whitney U test, *independent t-test or the [+]Fisher's exact test. All significant p-values are in bold.

	CB			AS		
	Included (n=15)	**Not included (n=6)**	**p-value**	**Included (n=21)**	**Not included (n=28)**	**p-value**
Age (years)	36.0 (33.0-39.0)	39.5 (32.0-40.3)	0.67[#]	38.0 (32.0-40.5)	34.0 (32.0-40.8)	0.54[#]
Sex, n (% female)	9 (60.0)	4 (66.7)	>0.99[+]	14 (66.7)	16 (57.1)	0.56[+]
Smoking history (pack-years)	13.0 (11.0-16.0)	19.0 (13.5-58.5)	0.12[#]	12.4 (11.0-15.0)	13.8 (11.0-18.8)	0.41[#]
BMI (kg/m^2)	28.0 (22.9-29.6)	31.6 (29.8-34.1)	0.018*	25.1 (22.1-28.0)	24.8 (20.7-28.8)	0.66*
IMD Decile	3.0 (1.0-4.0)	1.0 (1.0-2.5)	0.14[#]	3.0 (1.0-6.0)	3.5 (2.0-5.0)	0.97[#]
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 10 (66.7) 5 (33.3) 0 (0)	 5 (83.3) 1 (16.7) 0 (0)	 0.62[+]	 11 (52.3) 8 (38.1) 2 (9.5)	 22 (78.6) 4 (14.3) 2 (7.1)	 0.11[+]
Lung function Post-BD FEV_1 (L) Post-BD FEV_1 (%predicted) FEV_1/FVC ratio	 3.44 ± 0.66 99.2 ± 10.9 0.83 ± 0.05	 3.39 ± 0.79 105.2 ± 7.6 0.85 ± 0.07	 0.89* 0.24* 0.51*	 3.58 ± 0.65 104.6 ± 10.6 0.82 ± 0.07	 3.70 ± 0.62 103.5 ± 10.5 0.84 ± 0.06	 0.52* 0.71* 0.43*

Table 3.10 – Comparison of baseline demographics and spirometric parameters between CB and AS participants included in the 12-month longitudinal symptom analysis and those who were not included

Legend: Symptoms score data available for CB and AS participants over three consecutive visits (baseline, six months and 12 months) were included in the 12-month longitudinal symptom analysis. Continuous data are displayed as median (IQR) apart from lung function data which are expressed as mean ± SD. Statistical differences between those included and those not in both groups were assessed using the [#]Mann-Whitney U test, *****independent t-test or the [+]Fisher's exact test. All significant p-values are in bold.

	CB				AS			
	Baseline	**6 months**	**12 months**	**p-value**	**Baseline**	**6 months**	**12 months**	**p-value**
mMRC	1 (0-1)	1 (1-1)	1 (0-1)	0.31*	1 (0-1)	1 (0-1)	1 (0-1)	0.72*
CAT score	18.0 (16.0-22.0)	16.0 (12.0-21.0)	13.0 (9.0-19.0)	0.014#	9.0 (6.5-13.0)	9.0 (4.0-14.0)	8.0 (3.5-13.0)	0.89#
HADS								
Anxiety score	9.0 (6.8-12.0)	12.0 (7.0-13.0)	11.0 (7.8-13.3)	0.58*	7.0 (4.0-10.5)	8.0 (5.0-11.0)	7.0 (4.0-12.0)	0.38*
Depression score	4.5 (2.8-7.5)	9.5 (3.5-11.0)	6.0 (3.8-9.8)	0.20*	4.0 (2.0-8.5)	6.0 (2.0-8.5)	4.0 (2.0-9.0)	0.43*
LCQ score								
Physical score	5.2 (4.3-5.5)	5.8 (4.5-6.6)	5.7 (4.3-6.3)	0.06*	6.4 (6.0-6.6)	6.5 (6.0-6.6)	6.6 (5.9-6.8)	0.18*
Psychological score	5.1 (3.7-6.4)	5.4 (3.6-6.4)	6.2 (3.6-7.0)	0.31*	6.9 (6.0-7.0)	7.0 (6.5-7.0)	6.9 (6.4-7.0)	0.33*
Social score	5.5 (4.6-6.6)	6.0 (5.1-7.0)	6.6 (4.4-7.0)	0.09*	7.0 (6.5-7.0)	7.0 (6.5-7.0)	7.0 (6.4-7.0)	0.67*
Total score	15.6 (13.3-18.7)	17.1 (14.1-19.7)	18.4 (11.5-20.2)	0.17*	20.0 (18.8-20.6)	20.5 (19.0-20.6)	20.1 (19.0-20.8)	0.95*

Table 3.11 – Symptom scores in the CB and AS groups over three consecutive visits over 12 months

Legend: Symptom score for 15 CB and 21 AS subjects over 12 months. All values are displayed as median (IQR). Differences in scores between each visit in both groups were assessed using either the *Friedman test or the #one-way repeated measures ANOVA test. All significant p-values are in bold.

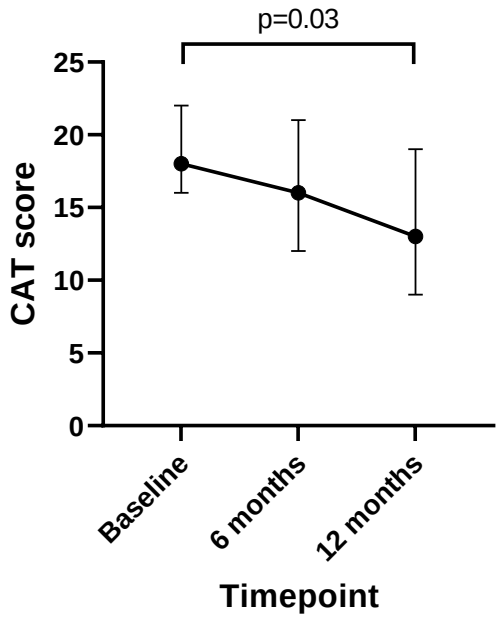


Figure 3.14 – CAT score changes in the CB group over three consecutive visits over 12 months

Legend: CAT score data were collected over three consecutive visits (baseline, six months, and 12 months) and were available for 15 participants within the CB group. Error bars represent the median (IQR) of the data. A one-way repeated-measures ANOVA was performed with comparisons between different time points assessed using Tukey's multiple comparisons test.

3.2.5.4 Subsequent occurrence of chest infections

Over 12 months, five participants in the Early COPD cohort reported at least one episode of chest infection. Three (60%) participants had reported at least one chest infection in the 12 months before the study enrollment. Four (80%) participants also reported CB symptoms at baseline. However, when analysed using Fisher's exact test, there were no differences between the groups (see Figure 3.15).

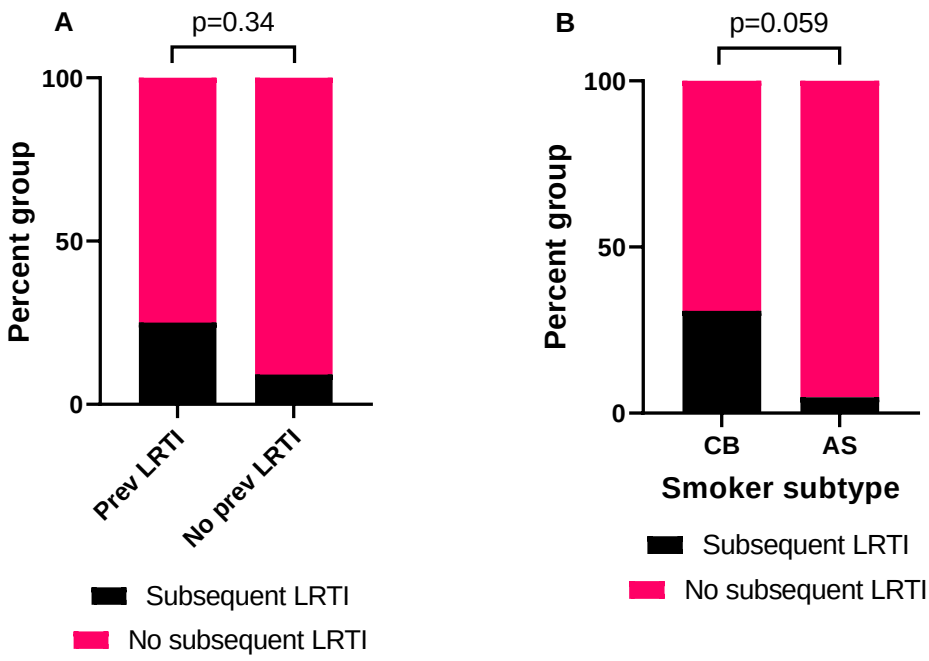


Figure 3.15 – Percentage of participants who reported at least a chest infection in the 12 months after enrolment according to whether they reported an episode before study enrolment (A) and smoker subtype (B)

Legend: There was a trend toward a higher prevalence of participants who reported at least one chest infection 12 months after enrolment in those who said a previous LRTI (n=12, 25.0%) compared to those who did not (n=22, 9.1%). This trend was also found in the CB group (n=13, 30.8%) compared to the AS group (n=21, 4.8%). However, these trends were not statistically significant (analysed using Fisher's exact test).

3.2.5.5 Change in CB symptoms over time

Figure 3.16 shows the longitudinal pattern of CB symptoms, defined by the presence of chronic cough and sputum expectoration. Longitudinal data regarding CB symptoms were available for 36 (51.4%) participants in the cohort. Twenty-two (61.1%) reported CB symptoms during the first 12 months of enrolment in the study. Five (13.9%) have reported remission of their CB symptoms by 12 months, and 14 (38.9%) reported incident or ongoing CB.

Table 3.12 compares demographic details between those who had reported CB symptoms during the first 12 months from enrolment ('ever CB') and those who did not ('never CB'). The 'ever CB' group had a higher BMI (median 27.9 (IQR 24.6-29.7) vs 24.5 (IQR 20.9-25.6) kg/m^2, p=0.02) and lower post-bronchodilator FEV_1 %predicted (mean 99.4 ± 11.1 vs 107.0 ± 9.1% predicted, p=0.04) at baseline compared to the 'never CB' group.

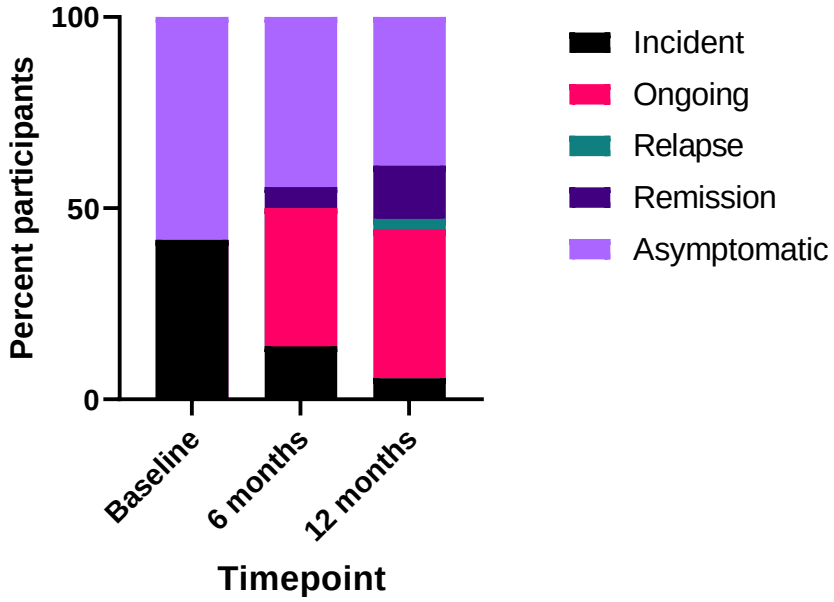


Figure 3.16 – The change in CB symptoms over 12 months

Legend: Thirty-six participants provided data for three consecutive time points (baseline, six months, and 12 months). At each time point, participants were classified according to their previous and current presence of CB symptoms (if any) as: **Asymptomatic** – no present/previous CB symptoms, **Incident** – first report of CB symptoms, **Ongoing** – persistent CB symptoms since the last report, **Remission** – CB symptoms reported previously but currently absent and **Relapse** – CB symptoms currently reported following the previous remission.

	Ever CB (n=22)	Never CB (n=14)	p-value
Age (years)	36.0 (32.0-40.8)	38.5 (33.8-42.0)	0.61[#]
Sex, n (% female)	13 (59.1)	10 (71.4)	0.50[+]
Smoking history (pack-years)	14.0 (11.0-18.3)	11.8 (10.4-14.3)	0.11[#]
BMI (kg/m^2)	27.9 (24.6-29.7)	24.5 (20.9-25.6)	0.02*
IMD Decile	3.0 (1.0-4.0)	4.0 (1.0-6.5)	0.41[#]
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 15 (68.2) 7 (31.8) 0 (0)	 6 (42.9) 6 (42.9) 2 (14.2)	 0.13[+]
Lung function Post-BD FEV_1 (L) Post-BD FEV_1 (%predicted) FEV_1/FVC ratio	 3.53 ± 0.69 99.4 ± 11.1 0.82 ± 0.06	 3.51 ± 0.59 107.0 ± 9.1 0.84 ± 0.06	 0.95* 0.04* 0.45*

Table 3.12 – Comparison of baseline demographics and spirometric parameters between the 'ever CB' group and the 'never CB' group

Legend: 'Ever CB' group: participants who have reported CB symptoms during the first 12 months from enrolment. 'Never CB' group: participants who did not report CB symptoms over the first 12 months from enrolment. Continuous data are displayed as median (IQR) apart from lung function data which are expressed as mean ± SD. Statistical differences between the two groups were analysed with the [#]Mann-Whitney U test, *****independent t-test or the [+]Fisher's exact test. All significant p-values are in bold.

3.2.6 Effects of UK COVID-19 lock-down on symptoms

The UK government implemented a national lockdown on the 26th of March 2020 as a public health measure to reduce the transmission of COVID-19.[316] To assess how this affected the Early COPD cohort, symptom scores that were available up to six months before the UK national lock-down was implemented were compared to symptom scores that were available up to six months after the UK national lock-down started. Symptoms scores before and after the start of the national lock-down were available for comparison for 54 (77.1%) participants. Table 3.13 compares demographic details between those included in this longitudinal analysis and those not. There was no difference in the baseline demographics and basic lung function parameters between participants included in this analysis and those not.

	Included (n=54)	Not included (n=16)	p-value
Age (years)	35.0 (32.0-40.3)	38.5 (32.0-40.0)	0.86
Sex, n (% female)	34 (63.0)	9 (56.3)	0.77
Smoking history (pack-years)	13.3 (11.0-15.4)	14.5 (11.0-24.0)	0.21
BMI (kg/m^2)	25.3 (22.5-28.8)	29.1 (21.3-31.9)	0.19
IMD Decile	3.0 (1.8-5.0)	2.5 (1.0-4.0)	0.20
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 36 (66.7) 15 (27.8) 3 (5.5)	 12 (75.0) 3 (18.8) 1 (6.2)	 0.89
Lung function Post-BD FEV_1 (L) Post-BD FEV_1 (%predicted) FEV_1/FVC ratio	 3.57 ± 0.63 102.7 ± 10.9 0.83 ± 0.06	 3.61 ± 0.73 103.1 ± 10.4 0.84 ± 0.06	 0.82 0.90 0.90

Table 3.13 – Comparison of baseline demographics and spirometric parameters between those included in the national lock-down longitudinal symptom analysis and those who were not included

Legend: Symptoms score data was available for participants up to six months before the start of the UK national lock-down, and scores that were available up to six months after the beginning of the UK national lock-down were included. Continuous data are displayed as median (IQR) apart from lung function data which are expressed as mean ± SD. Statistical differences between the two groups were analysed with the #Mann-Whitney U test, *independent t-test or the +Fisher's exact test.

3.2.6.1 Changes in mMRC score

There were no significant changes seen in mMRC grade in the cohort before and after the start of the national lock-down (median 0.5 (IQR 0-1) vs 1.0 (IQR 0-1), p=0.55). Table 3.14 shows the mMRC grade comparison pre- and post- lock-down, and figure 3.17 shows the plot of grade differences in each participant.

3.2.6.2 Changes in HADS score

1.5 to 2 units have been identified as the validated minimal clinically important difference (MCID) of the HADS questionnaire in COPD patients for depression and anxiety.[317 318] Overall depression score was increased after lock-down (median 4.0 (IQR 1.0-8.3) vs 5.5 (IQR 2.0-10.0), p=0.007) but not the anxiety score (median 8.5 (IQR 5.0-11.3) vs 7.5 (IQR 4.0-13.0), p=0.40). Depression scores increased in 21 (38.9%) participants and decreased in 13 (24.1%) participants by two or more units suggesting a clinical change in mood. Anxiety scores increased in 19 (35.2%) participants and decreased in 6 participants (11.1%) by two or more units (see figure 3.17), suggesting a noticeable effect. Table 3.14 shows the comparison of HADS scores pre- and post- lock-down.

3.2.6.3 Changes in LCQ score

Total LCQ score improved from a median of 19.0 (IQR 15.6-20.6) to 20.5 (IQR 18.9-20.8, p=0.0001) after lock-down. 1.3 units were deemed to be the MCID of the total LCQ score in COPD patients.[319] Total LCQ scores increased in 19 (35.2%) participants and decreased in 5 (9.3%) participants by 1.3 or more units (see figure 3.17). Table 3.14 compares each domain of the LCQ and the total LCQ score pre- and post- lock-down.

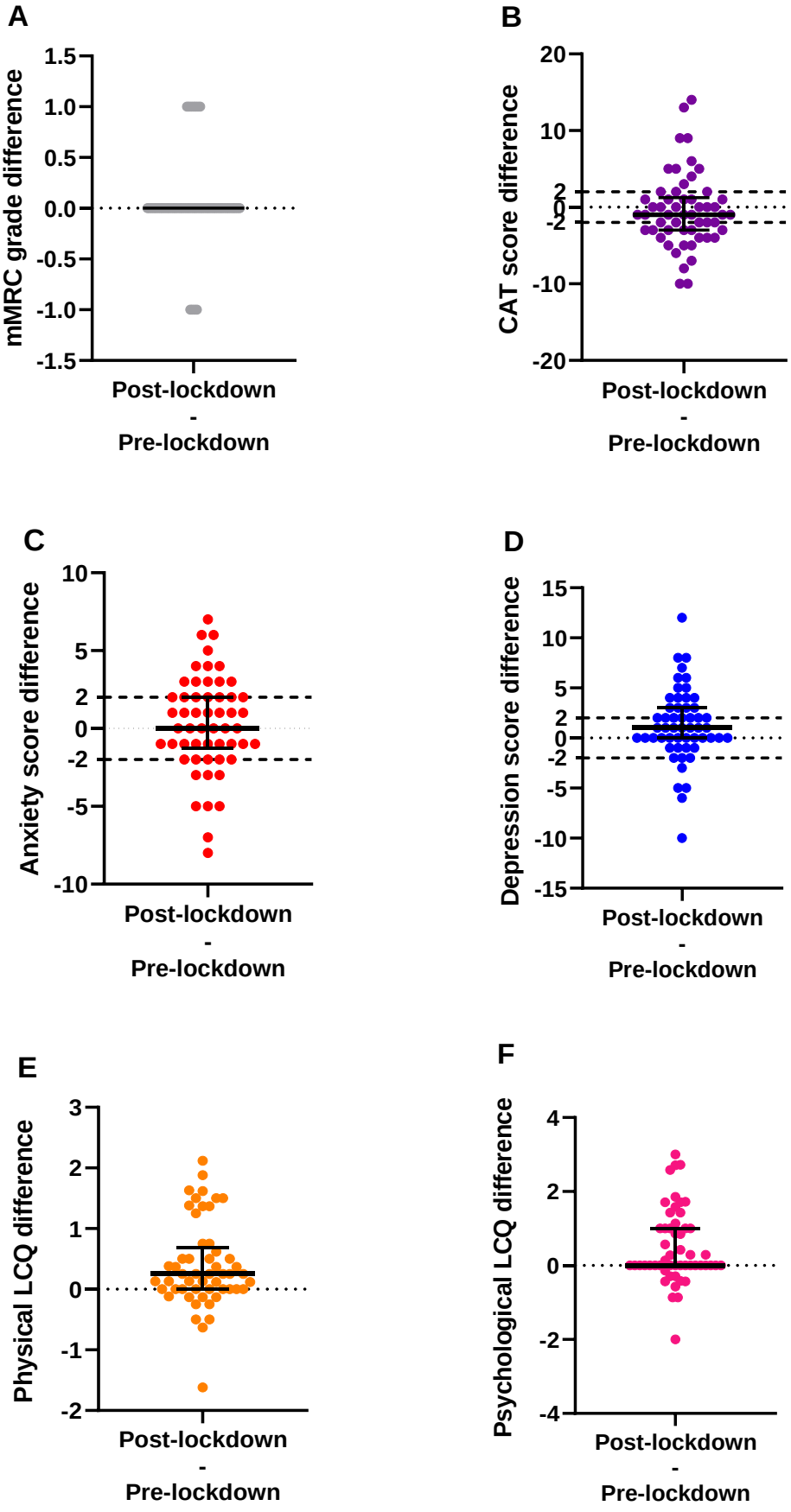
3.2.6.4 Changes in CAT score

There was no difference in overall CAT scores before and after the start of lock-down (p=0.20). Two units were considered the MCID for the CAT questionnaire.[320] The CAT score increased by two units or more in 13 (24.1) participants and decreased by two units or more in 23 (42.6%) participants (see figure 3.17). Table 3.14 compares CAT scores before and after the start of lock-down.

	Pre-lockdown	**Post-lockdown**	**p-value**
Table	0.5 (0-1.0)	1.0 (0-1.0)	0.55
CAT score	9.0 (5.0-15.5)	9.0 (4.8-16.3)	0.20
HADS			
Anxiety score	8.5 (5.0-11.3)	7.5 (4.0-13.0)	0.40
Depression score	4.0 (1.0-8.3)	5.5 (2.0-10.0)	0.007
LCQ score			
Physical score	6.3 (5.1-6.6)	6.6 (6.0-6.8)	<0.0001
Psychological score	6.4 (5.1-7.0)	7.0 (6.4-7.0)	0.0008
Social score	6.8 (5.5-7.0)	7.0 (6.5-7.0)	0.005
Total score	19.0 (15.6-20.6)	20.5 (18.9-20.8)	0.0001

Table 3.14 – Symptom scores in the Early COPD cohort before and after the start of the UK national lock-down measure

Legend: Symptom scores before and after the national lock-down were available for 54 participants. All values are displayed as median (IQR). Differences in scores between visits were analysed using the Wilcoxon signed-rank test. All significant p-values are in bold.


A
mMRC grade difference
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Post-lockdown
-
Pre-lockdown
B
CAT score difference
20
10
2
0
-2
-10
-20
Post-lockdown
-
Pre-lockdown
C
Anxiety score difference
10
5
2
0
-2
-5
-10
Post-lockdown
-
Pre-lockdown
D
Depression score difference
15
10
5
2
0
-2
-5
-10
-15
Post-lockdown
-
Pre-lockdown
E
Physical LCQ difference
3
2
1
0
-1
-2
Post-lockdown
-
Pre-lockdown
F
Psychological LCQ difference
4
2
0
-2
-4
Post-lockdown
-
Pre-lockdown

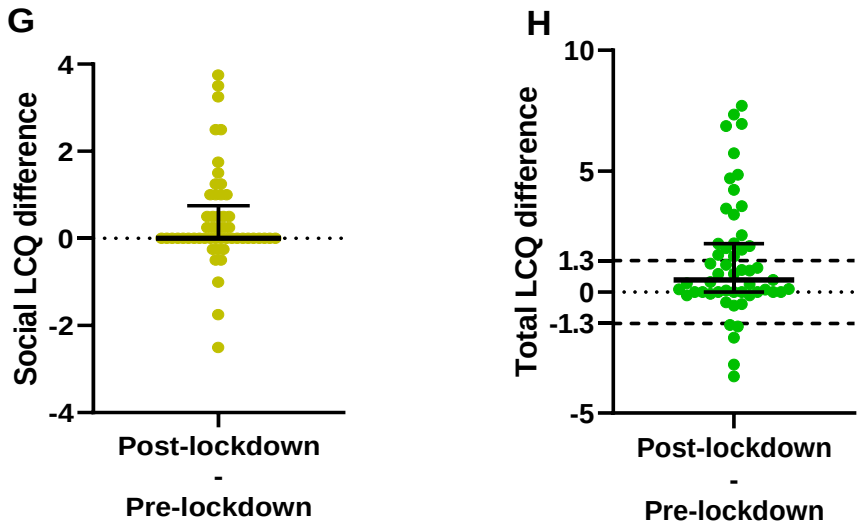


Figure 3.17 – Differences in symptom scores before and after UK lockdown

Legend: Plots of individual differences in symptom scores between the two time points for mMRC grade (figure A), CAT score (figure B), HADS anxiety score (figure C), HADS depression score (figure D), LCQ physical domain score (figure E), LCQ psychological domain score (figure F), LCQ social domain score (figure G) and total LCQ score (figure H). Statistically significant differences were found in the depression score, total LCQ score, and each domain of the LCQ. Extra gridlines were added to denote the minimal clinically important difference in anxiety, depression, CAT and total LCQ score.

3.3 Discussion

This chapter has reviewed the symptomatology of the Early COPD cohort, consisting of current smokers between 30 to 45 years of age and with at least ten-pack-year smoking history. The Early COPD study is an ongoing UK-wide study to recruit a cohort of 550 smokers with either normal lung function or mild lung function abnormalities from eight UK centres. Birmingham is one of the major recruiting sites for the study, accounting for 12.7% of the total cohort.

Apart from London, the West Midlands region is the most ethnically diverse in England and Wales.[321] This represents an opportunity for early COPD research among ethnic minority groups. The recruited cohort is a good representation of the population in West Midlands County, with no significant differences found between the cohort ethnicity demographics compared to the West Midlands County ethnicity data in the 2011 census. According to the census, 70.1% of the population in West Midlands County were of White ethnicity, 18.9% were Asian or Asian British, and 6% were Black African, Caribbean or Black British.[310]

The relationship between respiratory symptoms (such as CB) in smokers without COPD and the subsequent development of COPD remains controversial.[119-121] However, such individuals have been shown to have distinct pathological abnormalities with goblet cell hyperplasia, increased mucin production[322] and airway wall thickening radiologically.[10] Data in sections 3.2.1 to 3.2.3 support the hypothesis that a significant proportion of smokers (30.0%) have CB symptoms and have a worse quality of life than asymptomatic smokers. Data indicate that smokers with CB live in areas with lower IMD deciles and have worse symptom questionnaire scores than asymptomatic smokers. There was no evidence that smokers with CB have more

episodes of chest infections compared to asymptomatic smokers. However, this lack of evidence may reflect the lack of power in the Birmingham cohort alone.

3.3.1 Prevalence of CB among smokers

In this cohort, the prevalence of CB at baseline visit was 30%, which is higher than the prevalence estimates reported in other population-based studies.[323-325] Ferre et al. analysed data from 9050 French individuals aged ≥45 years and found that the CB prevalence was 7.4% among active smokers.[324] An analysis using data from the PLATINO (Proyecto Latinoamericano de Investigación en Obstrucción Pulmonar) study involving 5312 individuals from five Latin American cities showed that the proportion of individuals with CB symptoms in those without spirometric evidence of COPD was only 2.5%.[323] A further analysis involving 17,966 individuals aged 20 to 44 throughout 16 European countries (ECRHS-2) found that 3.2% reported CB symptoms.[325] The prevalence of CB found in the PLATINO and the ECRHS-2 studies did not differentiate between current smokers and non-smokers. However, current smokers were a substantial proportion of individuals with CB symptoms in both studies (36.3% and 73.3%, respectively).

3.3.2 Relationship of CB to other socio-demographic variables

The symptoms of CB among participants in the current cohort were not influenced by smoking history or lung function parameters. However, those with CB in this cohort had a higher BMI than those who were asymptomatic. This was similar to the Tucson Epidemiologic Study of

Airways Obstructive Disease, which compared 299 incident cases of physician-diagnosed CB with 1475 control subjects and found an increased prevalence (23.8%) of obesity (defined as BMI ≥28 kg/m^2) in CB patients compared to that in control subjects (16.3%).[326] In addition, in the Taiwan Children Health Study, which involved 3634 Taiwanese adolescents between 12 to 13 years, obesity was found to be associated with an increased risk of incident bronchitis.[327] The above studies were consistent with data from the Early COPD cohort recruited from Birmingham. However, as per previous studies, the link between obesity and CB is still unexplained and may reflect other factors related to differences in patient demographics, such as diet, work, exercise, or SES.

Smokers with CB symptoms in this cohort resided in areas with lower IMD deciles (more deprived neighbourhoods) than asymptomatic smokers. However, no differences in occupation classes were seen between the CB and AS groups within the cohort. However, the low number of participants renders any in-depth modelling impractical. Analyses of larger datasets in ECRHS-1 and 2 assessed the risk between SES (occupation class and education level in the study) and incident CB in a large European community involving 9023 individuals.[328] The study found that CB prevalence and incidence were related to low education level (OR 1.7, 95% CI 1.1 to 2.5 for prevalence; risk ratio 2.5; 95% CI 1.2 to 5.1 for incidence) but not occupation class (OR 1.2, 95% CI 0.8 to 1.9 for prevalence; risk ratio 1.4; 95% CI 0.5 to 3.4 for incidence) in a fully adjusted model.[328] BMI was taken into account in the fully adjusted model but the study did not collect data on factors which may potentiate obesity.[328] Factors such as diet and physical activity will likely differ in a disadvantaged population.

3.3.3 Physical and psychological symptom burden among smokers

Analysis of the symptom scores in the Early COPD cohort has revealed that smokers without spirometric evidence of airway obstruction have a significant symptom burden, especially those with CB features. More than half of the participants in the cohort had a CAT score of the threshold burden score ≥10 suggested by GOLD[1] at the baseline visit, indicating that their symptoms have at least a moderate effect on their daily living.[314] Baseline data showed that smokers with CB in the current cohort had significantly worse scores on all symptom questionnaires compared to the AS group. This is consistent with previous studies where patients without spirometric evidence of airflow obstruction but with CB symptoms have a poorer quality of life.[303 304]

The COPDGene study was established to identify genetic factors associated with COPD.[329] Using data from this study of 4880 individuals aged 45 to 80 years with a post-BD FEV_1/FVC ratio ≥ 0.7, Martinez et al. found that those with CB symptoms had a worse SGRQ score than those without CB symptoms.[304] Meek et al. analysed a cohort of current or former smokers aged 40 to 75 years from New Mexico (Lovelace Smokers Cohort).[303] Those with an FEV_1/FVC ratio ≥ 0.7 and CB symptoms (n=341) had worse SGRQ scores (all domains and total score) and SF-36 scores (all domains and total score) compared to those without CB (n=1069). Data in the Early COPD cohort from Birmingham reported similar results.

CB in smokers does not only negatively impact their physical health but also their psychological health. This was reflected by higher anxiety and depression scores and lower scores in the psychological domain of the LCQ in the CB group of our cohort than in asymptomatic smokers. Similar findings have also been reported by Meek et al. involving the

Lovelace Smoker's Cohort.[303] Individuals with CB and no airflow obstruction (n=341) had worse mental health, role emotional, social function and depression domain of the SF-36 compared to those with airflow obstruction but no CB (n=302).[303]

Further analysis of the anxiety and depression scores in the Birmingham cohort positively correlated with CAT scores which may suggest a relationship. However, the relationship between psychological issues (such as anxiety and depression) and respiratory symptoms is complex. Patients with anxiety and depression perceive their health as poorer than the general population[330], possibly overestimating perceived physical symptoms such as CB experienced by smokers. Conversely, respiratory symptoms from COPD also increase the risk of anxiety and depression, demonstrating a bidirectional relationship.[331]

3.3.4 Variability of respiratory symptoms in smokers

This chapter also attempted to analyse the trend of symptoms longitudinally for our cohort after six months and 12 months. Despite COPD being perceived as a progressive and unremitting disease with symptoms associated with worsening lung function, symptoms in COPD patients can also vary daily or weekly.[332] Kessler et al. conducted a pan-European cross-sectional study involving 2441 patients with severe COPD (based on GOLD staging). These patients underwent a telephone interview to enquire about daily, weekly or seasonal variability in their respiratory symptoms.[332] The authors found that 44.7% and 54.4% of patients perceived variability in one or more of their respiratory symptoms throughout the day and week, respectively.[332] In our cohort, symptoms were stable over 12 months. However, differences in symptom trends were seen between the CB group and AS group. Physical

symptoms in the CB group improved with time, as demonstrated by an improvement in the CAT score and LCQ physical score. It is mainly unclear why the CB group had an improvement in physical symptoms with time. Still, isolation measures imposed over the UK national lock-down period may partially explain this. A BLF press release reported that in a survey of over 14000 individuals with diagnosed lung conditions, 16.2% noticed an improvement in their symptoms over lock-down,[333] which was consistent with our findings.

Several studies have shown that the pattern of chronic bronchitis[32] is variable among smokers. In a data analysis of the British Medical Research Council 1946 Birth Cohort, the longitudinal pattern of CB presence was studied for 156 individuals. These individuals reported CB presence on at least one occasion and provided data at six time points over 40 years, with 5-11 years separating each time point.[121] CB presence followed a relapsing-remitting course over several decades, with at least 50% reporting CB remission by age 60-64.[121] This was supported by data in ECRHS-1 and 2 described earlier involving 4854 individuals aged 20-44 years.[120] In the study, de Marco et al. demonstrated that only 38% of subjects had persistent chronic cough and phlegm after nine years. Conversely, 7.4% of subjects who were asymptomatic at baseline were found to have CB symptoms after nine years.[120]

In our cohort, variability was found over a shorter time. Most participants in the CB group had ongoing symptoms of chronic cough and phlegm after 12 months, but some had remission. Conversely, some participants who initially had no cough and phlegm developed these symptoms within 12 months. The data above demonstrates the dynamic nature of symptoms that define CB among smokers, even at a young age. Using the classical definition of CB (chronic cough and sputum production for three months for two consecutive years) has been

helpful in epidemiological studies.[32] However, applying this definition in clinical practice fails to include individuals with relapsing-remitting symptoms. For example, Kim et al. showed a greater prevalence of CB when the SGRQ definition (39.9%) was applied compared to the classical definition (26.1%) in an analysis of 4513 COPD patients within the COPDGene cohort.[334] The individuals defined using SGRQ still display a similar clinical and radiological phenotype to those defined by the classical definition and may also be at risk of similar clinical outcomes.[334] As such, it may be better to use the SGRQ definition for long-term prognosis and risk assessment, but clearly, such longitudinal studies need to include both definitions for validation and acceptance.

3.3.5 CB symptoms and their utility in informing exacerbation risk

Although baseline data in our CB patient group suggested a higher risk of chest infection than the AS group, this comparison did not achieve statistical significance. The COPDGene study reported that CB presence increases the likelihood of recurrent exacerbations among non-COPD smokers or ex-smokers.[304] Those with CB had a higher annual frequency of exacerbations requiring antibiotic or steroid use compared to those without CB in the year before enrolment (0.30 ± 0.8 vs 0.10 ± 0.4 annual events/subject, $p<0.001$) and during follow-up (0.30 ± 0.7 vs 0.16 ± 0.7 annual events/subject, $p<0.001$).[304] In the study, the difference in annual exacerbation rate was small with a wide CI, suggesting that the lack of a significant finding in the Early COPD cohort was due to underpowered analyses from a low sample size.

Interestingly, only five participants reported an episode of chest infection 12 months after enrolment as opposed to 22 participants before. The reason for the low rate of chest

infections in the cohort longitudinally remains unclear. Still, it may partially be explained by the UK national lock-down period due to the ongoing COVID-19 pandemic, which was consistent with reports of decreased incidence rates of respiratory diseases[335] and COPD exacerbations[336] in the UK during this period.

3.3.6 Physical isolation on health among smokers

The World Health Organisation (WHO) declared the novel COVID-19 outbreak a global pandemic on 11th March 2020.[337] As part of efforts to combat a rise in the infection rate, the UK government implemented a national lock-down on the 26th March 2021. A stay-at-home order was imposed nationwide, banning non-essential travel and activity, and all individuals with certain illnesses were asked to self-isolate. The national lock-down measures had stimulated observations on physical and mental well-being in the general population, and fewer influenza-like illnesses and respiratory diseases were recorded over the lock-down period compared to previous years.[335]

There was also a significant reduction in emergency hospital admissions due to COPD exacerbations over the lock-down period compared to previous years, with no corresponding increase in COPD mortality.[336] These observations were attributed to a mixture of physical distancing and hygiene measures leading to reduced transmission of respiratory pathogens and a fall in outdoor air pollution levels.[333 335 336] Inhaled glucocorticoids that many COPD patients use regularly have also been postulated as a possible factor. In the Steroids in COVID-19 (STOIC) trial, 146 adults in Oxfordshire were randomly assigned to receive inhaled budesonide or usual care within seven days of the onset of COVID-19 symptoms.[338] It was

found that early administration reduced the likelihood of needing urgent medical care (difference in proportions 0.12, 95% CI 0.03 to 0.21) and a reduced time to recovery (median 7 days for inhaled budesonide group vs 8 days for usual care group).[338]

The national lockdown, however, also adversely impacted the mental well-being of the general population. Studies of the UK national datasets found the prevalence of depressive and anxiety symptoms had increased in the general population over the lockdown period compared to pre-pandemic periods. Jia et al. recruited 3097 UK adults and assessed anxiety and depression symptoms using the 7-item Generalised Anxiety Disorder Scale (GAD-7) and the 9-item Patient Health Questionnaire (PHQ-9).[339] It was found that anxiety and depression scores in the cohort were worse than pre-pandemic normative values, with GAD-7 and PHQ-9 increasing from a mean of 2.95 ± 3.4 and 2.91 ± 3.5 respectively, pre-pandemic to a mean of 6.59 ± 5.6 and 7.69 ± 6.0 (both $p<0.0001$) in the cohort.[339]

Data from section 3.2.4 of the current thesis confirmed an improvement in respiratory symptoms but worsening psychological symptoms in our early COPD cohort studied during the UK national lock-down period. There were improved scores in each LCQ domain and total LCQ scores, as well as worsening depression scores compared to data before the lockdown period. No differences were noted in CAT scores and anxiety scores in the cohort over the same period. However, this reflected the small sample size and lack of power to detect differences in scores before and during lock-down. This is supported by data from the national Early COPD cohort (including the current Birmingham dataset), which shows the findings above but also an improvement in CAT scores and increased anxiety scores.[340] Overall, the

data indicate that self-isolation and hygiene improve physical health for patients with 'early' symptoms and signs of COPD.

3.3.7 Data limitations

The inclusion and exclusion criteria stated in section 2.3.2 were set to recruit and study the COPD disease process in cigarette smokers before frank airflow obstruction is detectable via spirometry. However, the specified criteria have several limitations. The exclusion of individuals with other known chronic respiratory diseases, such as asthma, may mean that some individuals with true early COPD may be excluded, as diagnostic confusion between asthma and COPD is common, leading to misdiagnosis.[341] Furthermore, the exclusion of regular shisha or cannabis users from the study also limits the study of the early disease process among these individuals. However, whether they have differing pathological processes leading to COPD is unknown.

The recruitment methods for this study, described in section 2.3.2, also introduce the likelihood of a self-selection bias. Using advertising in the university and hospital grounds to recruit eligible smokers in this study may mean that cohort participants have different characteristics than the general smoking population. This is supported by the fact that a large proportion (50%) of the study cohort consists of professional workers. Therefore, smokers with early COPD from more disadvantaged populations may be under-represented in this study.

3.3.8 Summary

In summary, this chapter has analysed the symptom burden reported by the Birmingham site participants of the Early COPD cohort. Data in this chapter has supported the original hypothesis detailed in the introduction. Although these individuals do not conform to the classical definition of COPD based on spirometry results, a significant proportion of these individuals (particularly those with CB symptoms) experience substantial adverse effects on their physical and mental health. Furthermore, the dynamic nature of CB symptoms, defined by the presence of chronic cough and sputum expectoration, was demonstrated longitudinally. However, the analysis to date only covers 12 months which is a limited timeframe to inform on a clinical picture that progresses over decades. In addition, this chapter has also provided insight into the beneficial and adverse effects of isolation and health measures instigated as part of the UK national lock-down. These results suggest that any pandemic health measures need to balance deterioration in mental health in specific age groups with improvements in respiratory health.

CHAPTER 4 – PULMONARY FUNCTION TESTS OF THE EARLY COPD COHORT

4.1 Introduction

There is an increasing interest in understanding the early disease mechanisms leading to COPD and identifying those at risk as early as possible. Understanding the early pathophysiology before COPD becomes established would not only enable preventative steps such as smoking cessation to be introduced but would lead to the development of new therapeutic strategies to modulate disease progression. Although cigarette smoking remains the most critical risk factor,[342] only 30-40% of long-term smokers develop COPD of any severity.[343] Therefore, the challenge would be to identify those most likely to develop COPD, particularly among regular smokers, before their level of airflow obstruction passes the COPD diagnostic threshold.

The gold standard for COPD diagnosis is the FEV_1/FVC ratio <0.7 obtained using post-bronchodilator (BD) spirometry.[1] However, the use of standard spirometry has its limitations. Firstly, it is effort-dependant and valid measurements that may require several rigorous and repeatable manoeuvres,[344] which can prove challenging for some patients, especially those with breathing difficulties. Secondly, extensive lung damage can occur before the standard parameters (FEV1 and FVC) deteriorate below the normal range, mainly involving the small airways where much airflow resistance occurs. Hence, standard spirometry parameters lack the sensitivity to detect early and progressive small airway dysfunction (SAD).[136 345] Ideally, it is vital to utilise tests that can detect SAD early and accurately to identify these early stages of disease.

Maximal mid-expiratory flow (MMEF) is the mean forced expiratory flow between 25% and 75% of the FVC ($FEF_{25-75\%}$), and this parameter can be obtained via spirometry. It is commonly cited as a method to detect SAD[135] and is readily accessible from spirometry records. However,

MMEF has several limitations that preclude them from its use in routine clinical care. As MMEF is dependent on the FVC, it can be lower in patients where lung capacity is smaller than average for their age, sex, height and race.[346] Another disadvantage is the sensitivity of MMEF, where it is frequently normal if the FEV_1/FVC ratio is >0.75.[347] Therefore, there may be a false negative result when MMEF is used to detect SAD in individuals with normal FEV_1/FVC ratio.

The FOT has also been suggested to detect SAD. A detailed explanation of the measurement outputs is mentioned in section 1.2.6.4. In brief, FOT captures information about airway function through external impulses that travel superimposed upon normal tidal breathing. These oscillatory Impulses travel through the large and small airways and assess respiratory impedance, including respiratory resistance and reactance, over various frequencies. The main advantage of FOT is that measurements are collected during normal tidal breathing and are effort-independent. However, FOT has some limitations, such as the lack of a standard reference range to identify abnormalities, the lack of standardisation between oscillometry devices and consensus on what defines an abnormal result.

The use of FOT has been more extensively described in children, particularly in asthma management.[348] [349] Less is known about the potential role of FOT in detecting early pathophysiological changes in COPD. However, studies suggest that R_{5-20} and ΔX_5 may reflect small airway function and are elevated in established COPD.[136] [137] If SAD is an early feature of COPD, tests such as FOT may identify those at risk before the development of spirometric abnormality diagnostic of COPD.

4.1.1 Chapter hypotheses

It was hypothesised that a subset of smokers would have decreased MMEF on spirometry, indicating SAD. It was also hypothesised that FOT would help identify smokers with SAD, characterised by an elevated R_{5-20} and/or ΔX_5. Finally, it was predicted that smokers with SAD but without evidence of airway obstruction on spirometry would have worse clinical outcomes and a higher symptom burden than smokers without SAD.

To test these hypotheses, this chapter had the following aims:

- determine FOT validity as a diagnostic technique in the identification of early COPD pathophysiological changes by considering intra-rater and inter-rater reliability
- comprehensively describe the baseline lung function among the Early COPD cohort
- identify the proportion of smokers with evidence of SAD among the cohort using MMEF on spirometry and FOT and compare demographic and other physiological features of those without evidence of SAD
- assess correlations between lung function parameters with symptom burden
- compare symptom burden (assessed using CAT scores) and history of chest infections among smokers with SAD and those without

Due to the global COVID-19 pandemic, there was reduced access to spirometry and gas transfer testing among the Early COPD cohort. Thus, only baseline lung function data was used in this chapter. All methods in this chapter are described in sections 2.5 and 2.6.

4.2. Results

4.2.1 Forced oscillometry validation

4.2.1.1 Intra-observer variability

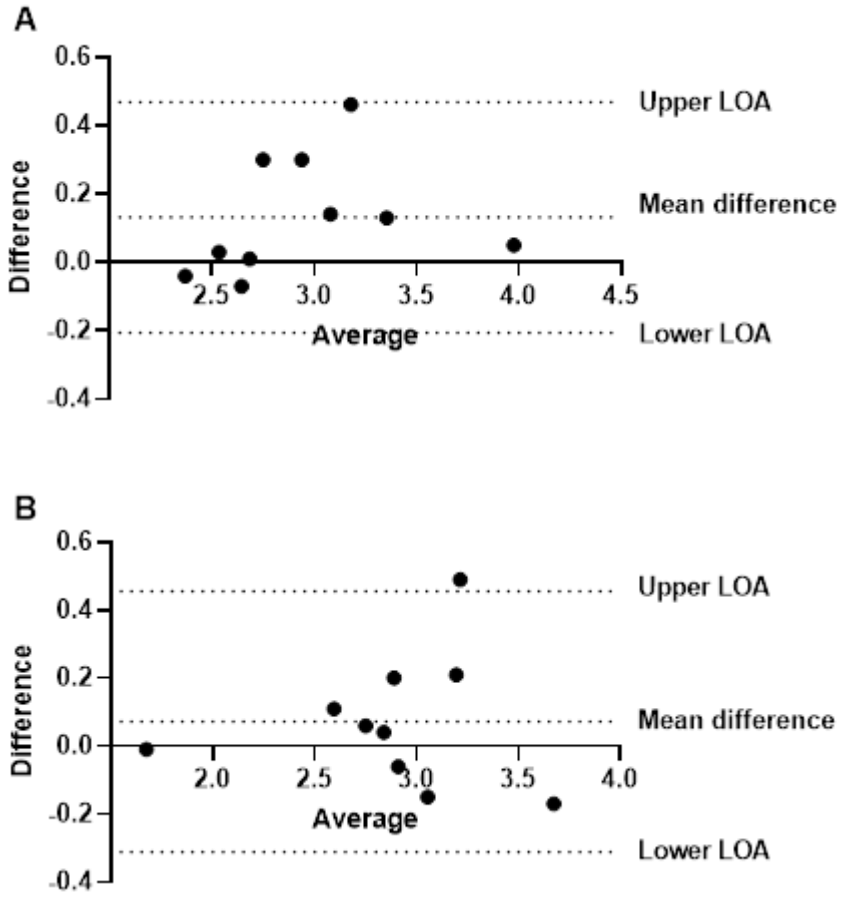
FOT measurements were obtained for ten healthy volunteers and repeated within two weeks as part of this analysis described in section 2.6.3. The median age (IQR) of the volunteers was 28 (25-34) years, with eight (80%) being females. All participants were free of respiratory diagnoses and were 'never' smokers. All ICC results in this section were interpreted using Koo and Li's recommended criteria.[124] Table 4.1 shows the ICC for the FOT parameters between two separate readings, and figure 4.1 shows the Bland-Altman plots for the same parameters.

The expected ICC values of the different FOT parameters show good to excellent intra-rater reliability. However, a wide range was noted on the 95% confidence interval (CI) of the X_5 and Ax parameter. The Bland-Altman plots of the different FOT parameters show no evidence of proportional bias. Apart from one outlier on the R_{20} and Ax plot, all plotted values fall within the 95% limits.

FOT parameter	ICC value	95% CI	Reliability grade
R_5	0.951	0.722-0.989	Moderate to excellent
R_{20}	0.963	0.861-0.991	Good to excellent
X_5	0.827	0.272-0.958	Poor to excellent
Ax	0.862	0.475-0.965	Poor to excellent

Table 4.1 – Intra-rater variability of the different FOT parameters

Legend: The ICC value and the 95% confidence interval (CI) of the ICC estimate for the different FOT parameters are displayed. The reliability grades were based on recommendations from Koo and Li.[124] The ICC was calculated for all parameters using a two-way mixed-effects model looking for absolute agreement. Ten healthy volunteers were used for these analyses. ICC: intra-class correlation; R_5: resistance at 5Hz; R_{20}: resistance at 20Hz; X_5: reactance at 5Hz; Ax: reactance area



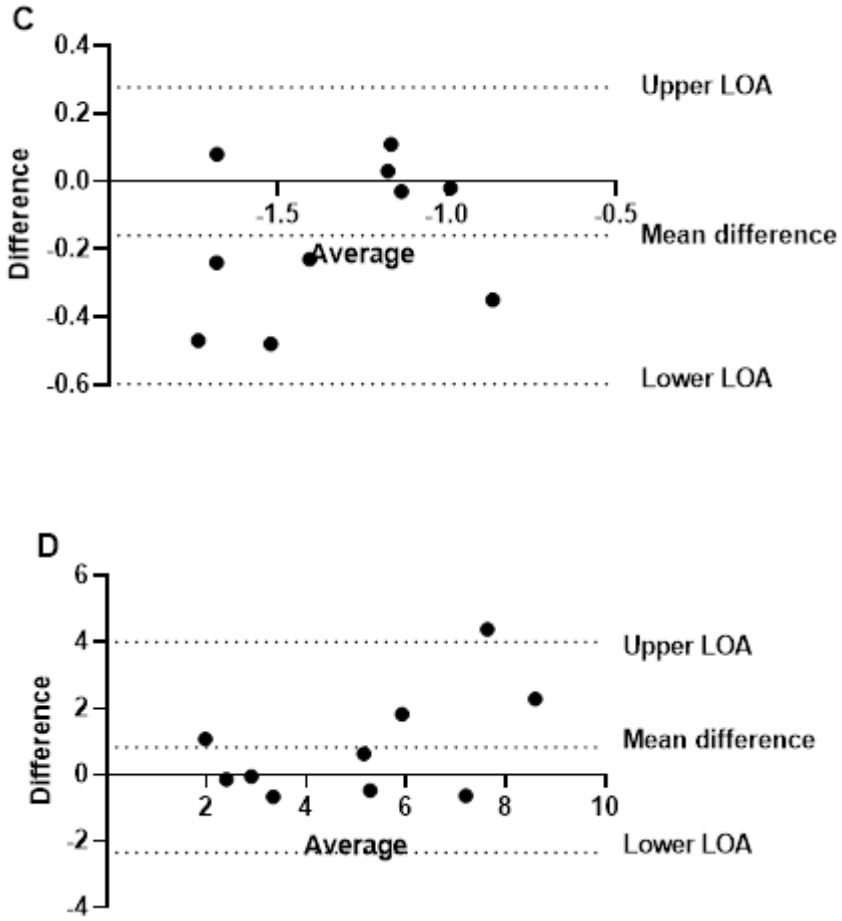


Figure 4.1 – Bland-Altman plots showing intra-rater variability of the different FOT parameters

Legend: Bland-Altman plots for the different FOT parameters, namely R_5 (A), R_{20} (B), X_5 (C) and Ax (D). The differences between the two readings are plotted against the average of the two readings. The mean difference, upper and lower limit of agreement (LOA), are shown as dotted lines parallel to the x-axis. The upper and lower LOA are calculated as +/-1.96 SD of the difference between the two readings.

4.2.1.2 Inter-observer variability

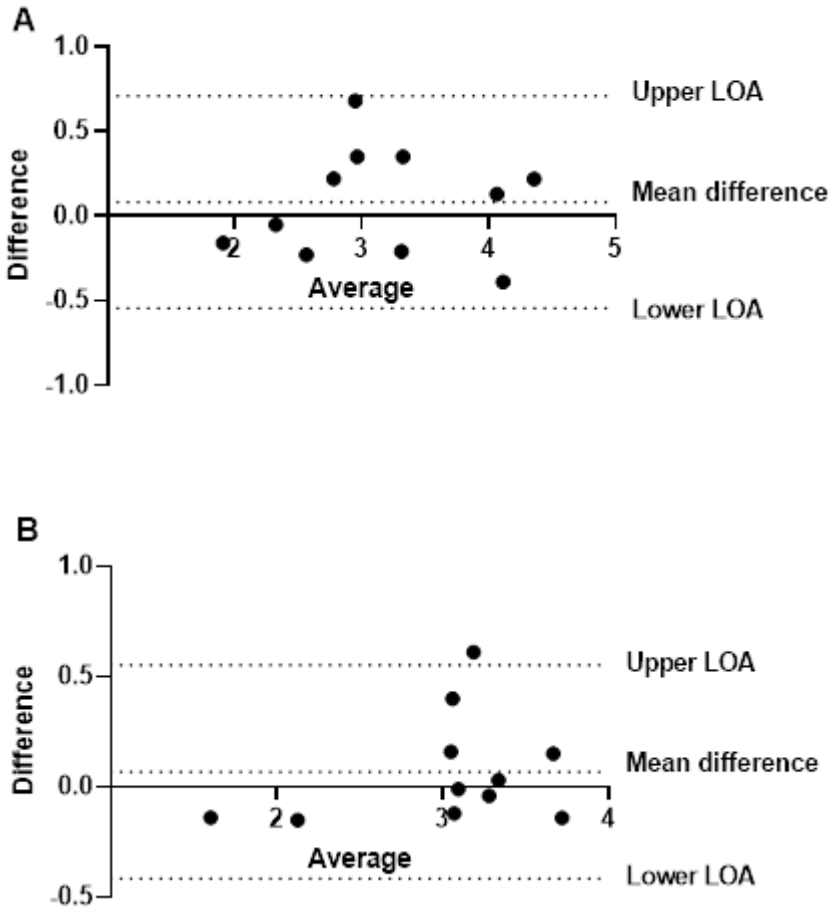
FOT measurements were performed on eleven healthy volunteers sequentially by KPY and a trained respiratory physiologist (JS) for this analysis, as described in section 2.6.3. The median age (IQR) of the volunteers was 29 (25-34) years, with nine (81.8%) being females. All participants were free of respiratory diagnoses and were 'never' smokers. As in section 4.2.1.1, all ICC results in this section were interpreted using Koo and Li's recommended criteria.[124] Table 4.2 shows the ICC for the FOT parameters between the two readings, and figure 4.2 shows the Bland-Altman plots for the same parameters.

The expected ICC values showed excellent inter-rater reliability on all FOT parameters. The Bland-Altman plots of the different FOT parameters show no evidence of proportional bias. Apart from one outlier on the R_{20} and Ax plot, all plotted values fall within the 95% limits.

FOT parameter	ICC value	95% CI	Reliability grade
R_5	0.959	0.854-0.989	Good to excellent
R_{20}	0.962	0.865-0.990	Good to excellent
X_5	0.943	0.800-0.985	Good to excellent
Ax	0.965	0.872-0.991	Good to excellent

Table 4.2 – Inter-rater variability of the different FOT parameters

Legend: The ICC value and the 95% CI of the ICC estimate for the other FOT parameters are displayed. The reliability grades were based on recommendations from Koo and Li.[124] The ICC was calculated for all parameters using a two-way random effects model looking for absolute agreement. Eleven healthy volunteers were used for these analyses.



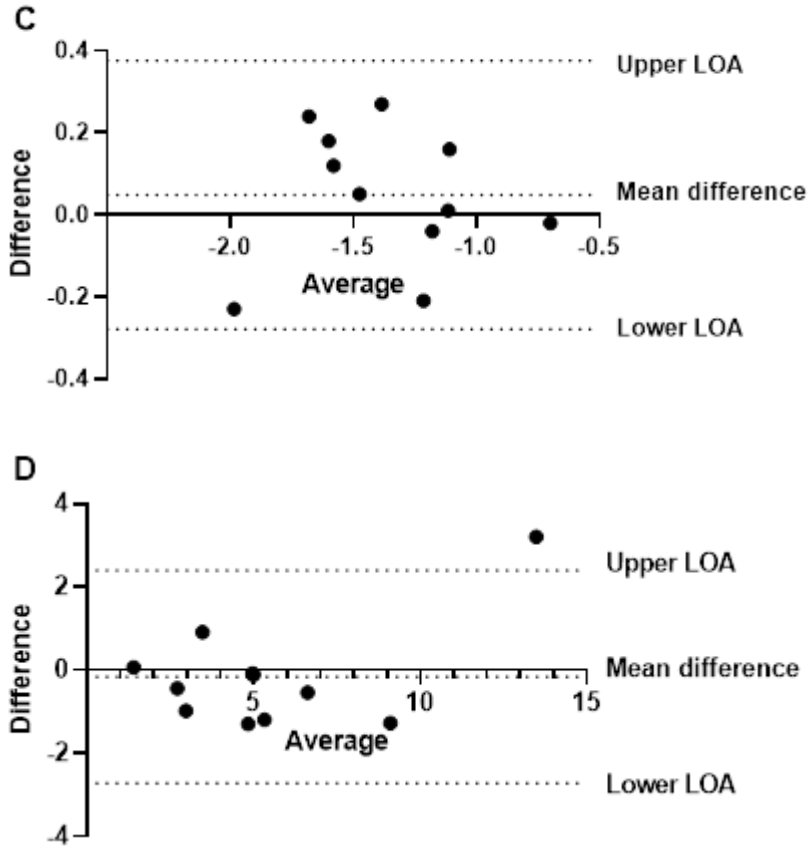


Figure 4.2 – Bland-Altman plots showing inter-rater variability of the different FOT parameters

Legend: Bland-Altman plots for the different FOT parameters, namely R_5 (A), R_{20} (B), X_5 (C) and Ax (D). The differences between the two readings are plotted against the average of the two readings. The mean difference, upper and LOA, are shown as dotted lines parallel to the x-axis. The upper and lower LOA are calculated as +/- 1.96 SD of the difference between the two readings.

4.2.2 Baseline spirometry and gas transfer of the Early COPD cohort

Seventy participants were enrolled on the Early COPD cohort, and baseline demographic features were described in section 3.2.1. Baseline post-BD spirometry and gas transfer tests were performed on all participants. All post-BD spirometry and gas transfer data are presented as median (IQR) values unless stated otherwise.

The median post-BD FEV_1 of the cohort was 3.48L (3.12-4.10), and 103.5% predicted (96.0-111.0) using GLI reference values.[50] The median FEV_1/FVC ratio was 0.84 (0.80-0.87). Both TLCO and KCO were mildly reduced in the cohort. Only one participant showed airflow limitation on post-BD spirometry (FEV_1/FVC ratio <0.7). Table 4.3 summarises the post-BD spirometry and gas transfer results of the cohort.

Spirometry results	
Post-BD FEV_1 (L)	3.48 (3.12-4.10)
Post-BD FEV1 (%predicted)	103.5 (96.0-111.0)
Post-BD FVC (L)	4.20 (3.62-4.98)
Post-BD FVC (%predicted)	102.5 (95.8-109.3)
FEV_1/FVC ratio	0.84 (0.80-0.87)
MMEF (L)	3.86 (3.27-4.41)
MMEF (%predicted)	101.0 (86.0-126.0)
Gas transfer	
TLCO (mmol/min/kPa)	7.83 (6.66-8.94)
TLCO (%predicted)	80.5 (72.0-90.0)
KCO (mmol/min/kPa/L)	1.45 (1.32-1.63)
KCO (%predicted)	81.5 (73.0-91.0)

Table 4.3 – Baseline spirometry and gas transfer results of the Early COPD cohort

Legend: All data are displayed as median (IQR). Demographic details of the cohort (n=70) have been previously described in section 3.2.1. Post-BD: post-bronchodilator; FEV_1: forced expiratory volume in the first second; FVC: forced vital capacity; MMEF: maximal mid-expiratory flow; TLCO: transfer capacity for carbon monoxide; KCO: carbon monoxide transfer coefficient

4.2.3 Exclusion of gas transfer results among 'recent smokers'

Cigarette smoking is known to raise carboxyhaemoglobin levels in smokers, which causes an acute reversible decrease in gas transfer results.[350 351] Participants were asked not to smoke cigarettes on their test day. However, this was not achieved by 37 (52.9%) participants. A cut-off time of an hour post-smoking was recommended by Cotes et al.[352] before results were considered reliable. Eight (11.4%) participants smoked ≤1 hour before lung function testing at baseline and are referred to as 'recent smokers', while those who did not smoke ≤1 hour before baseline lung function testing are referred to as 'non-recent smokers' in this section.

Comparisons of gas transfer results at baseline and six months were undertaken. This was available for 33 (47.1%) participants, of whom five were 'recent smokers'. At the six-month time point, no participants smoked ≤1 hour before testing. TLCO and KCO were increased at follow-up compared to baseline in 'recent smokers' (p=0.02 and p=0.01, respectively). However, there were no differences in TLCO and KCO values at baseline and six months in the 'non-recent smokers'. As gas transfer results of 'recent smokers' may be artificially low, these were excluded from further analyses. Figure 4.3 shows the difference in TLCO and KCO at baseline and six months where available.

'Recent smokers' comprised a higher proportion of males than 'non-recent smokers'. 'Recent smokers' were also found to have a higher FEV_1 absolute value (median 4.29 (IQR 3.41-4.29) vs 3.43L (IQR 3.08-3.95), p=0.005), but this was not statistically significant when adjusted for age, sex, height, and race (p=0.74). Table 4.4 compares demographic details between 'recent smokers' and 'non-recent smokers'. The median TLCO value in the cohort at baseline after excluding gas transfer results from 'recent smokers' was 7.67mmol/min/kPa (IQR 6.64-8.77),

and 80.0% predicted (IQR 72.0-89.3). The median KCO value at baseline after result exclusion was 1.45mmol/min/kPa/L (IQR 1.32-1.67) and 81.5% predicted (IQR 73.0-94.0).

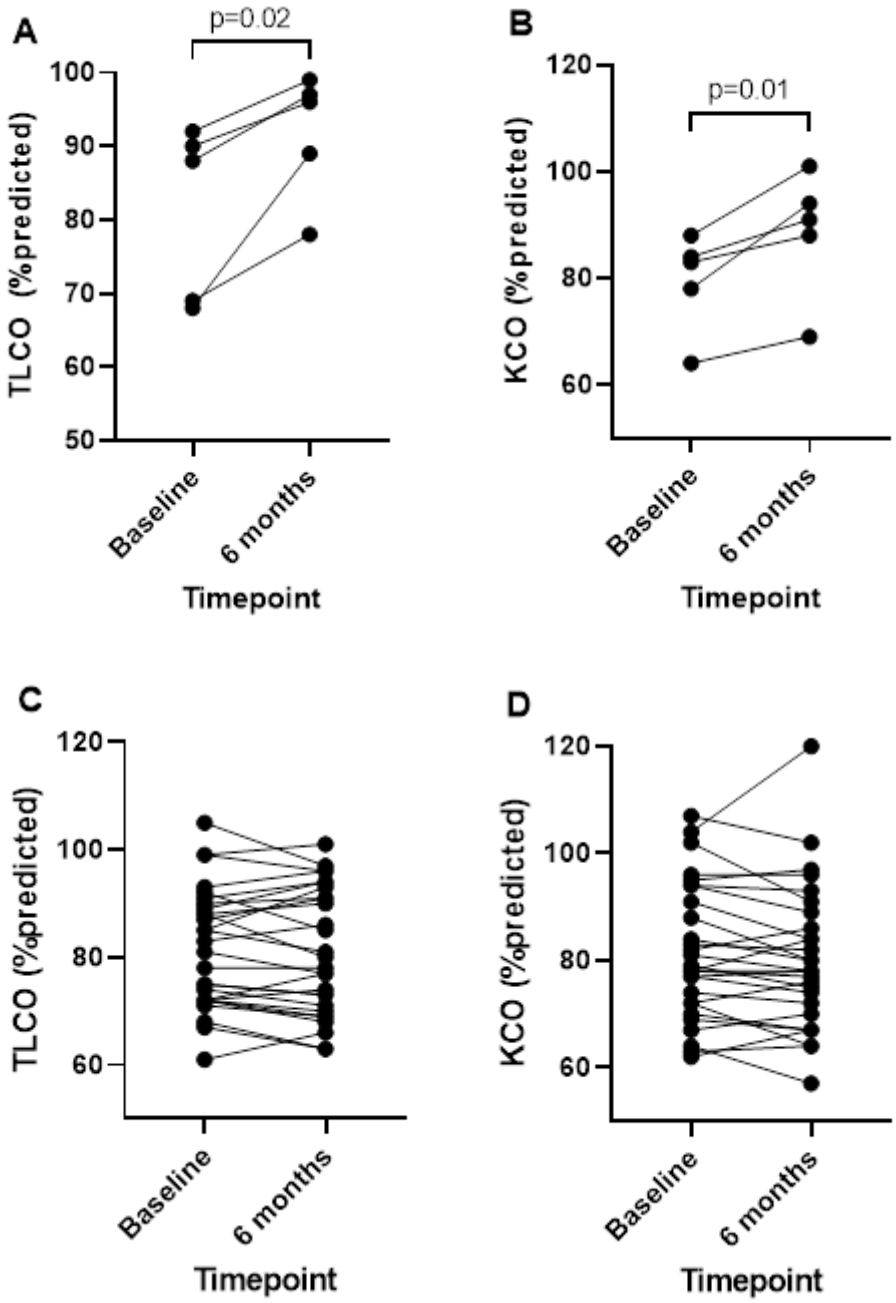


Figure 4.3 – Changes in gas transfer results in 'recent smokers' and 'non-recent smokers'

Legend: 'Recent smokers' (n=5) are those who smoked ≤1 hour before gas transfer testing at the baseline visit, and 'non-recent smokers' (n=28) are those who did not. Figure A compares TLCO at baseline and six months in 'recent smokers', and figure C shows a similar comparison in 'non-recent smokers'. Figure B shows a comparison of KCO at baseline and six months in 'recent smokers', and figure D shows a similar comparison in 'non-recent smokers'. At six months, all participants did not smoke ≤1 hour before gas transfer testing. Comparisons between the two timepoints were analysed using the paired t-test.

	Non-recent smokers (n=62)	Recent smokers (n=8)	p-value
Age (years)	35.5 (32.0-40.3)	35.5 (32.3-38.3)	0.63[#]
Sex, n (% female)	41 (66.1)	2 (25.0)	**0.048**[+]
Smoking history (pack-years)	14.0 (11.0-17.3)	13.5 (11.6-21.8)	0.51[#]
BMI (kg/m^2)	25.7 (22.3-29.4)	28.7 (23.7-33.7)	0.13*****
IMD Decile	3.0 (1.0-4.3)	2.5 (1.0-8.0)	0.95[#]
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 42 (67.7) 16 (25.8) 4 (6.5)	 6 (75.0) 2 (25.0) 0 (0)	>0.99[+]
Lung function Post-BD FEV_1 (L) Post-BD FEV_1 (%predicted) FEV_1/FVC ratio	 3.43 (3.08-3.95) 104.0 (95.5-111.3) 0.85 (0.80-0.87)	 4.29 (3.41-4.79) 102.5 (96.3-106.5) 0.80 (0.77-0.86)	 **0.005*** 0.74***** 0.20*****
Gas transfer TLCO (mmol/min/kPa) TLCO (%predicted) KCO (mmol/min/kPa/L) KCO (%predicted)	 7.67 (6.64-8.77) 80.0 (72.0-89.3) 1.45 (1.32-1.67) 81.5 (73.0-94.0)	 9.40 (7.67-10.85) 89.0 (70.5-91.8) 1.42 (1.25-1.56) 81.5 (73.5-87.0)	 **0.053**[#] 0.48[#] 0.35***** 0.50*****

Table 4.4 – Comparison of baseline demographics and basic lung function parameters between 'recent smokers' and 'non-recent smokers'

Legend: 'Recent smokers' are those who smoked ≤1 hour before gas transfer testing at the baseline visit, and 'non-recent smokers' are those who did not. The final analyses excluded gas transfer results of 'recent smokers'. Continuous data are displayed as median (IQR). Statistical differences between the two groups were analysed using the [#]Mann-Whitney U test, *****independent t-test or the [+]Fisher's exact test. All significant p-values are in bold.

4.2.4 Spirometry and gas transfer results comparison between smoker subtypes

4.2.4.1 Comparison according to the presence of chronic bronchitis

Participants were stratified according to chronic bronchitis (CB) features described in section 2.4.1. Post-BD spirometry and gas transfer results of participants with CB symptoms and asymptomatic smokers (AS) are shown in table 4.5. The CB group was found to have a lower TLCO compared to the AS group at baseline when using both absolute values (mean 6.85 ± 1.11 vs 8.32 ± 1.65 mmol/min/kPa, p=0.001) and when adjusted for sex, age, and height (mean 73.6 ± 10.4 vs 84.1 ± 11.8 % predicted, p=0.002). There was a similar trend for reduced KCO in the CB group compared to the AS group, although this did not reach statistical significance.

	CB (n=21)	**AS (n=49)**	**p-value**
Spirometry results			
Post-BD FEV_1 (L)	3.28 (2.93-3.90)	3.53 (3.14-4.15)	0.20*
Post-BD FEV_1 (%predicted)	100.0 (93.5-108.5)	105.0 (97.0-112.0)	0.19*
Post-BD FVC (L)	3.76 (3.46-4.69)	4.23 (3.68-5.05)	0.09#
Post-BD FVC (%predicted)	97.0 (90.5-107.0)	104.0 (98.0-110.5)	0.12*
FEV_1/FVC ratio	0.85 (0.81-0.87)	0.84 (0.79-0.87)	0.63*
MMEF (L)	3.65 (3.00-4.33)	3.88 (3.29-4.53)	0.77*
MMEF (%predicted)	101.0 (87.0-125.5)	101.0 (86.0-126.0)	0.98*
Gas transfer	**n=18**	**n=44**	
TLCO (mmol/min/kPa)	6.85 ± 1.11	8.32 ± 1.65	**0.001***
TLCO (%predicted)	73.6 ± 10.4	84.1 ± 11.7	**0.002***
KCO (mmol/min/kPa/L)	1.41 ± 0.19	1.51 ± 0.23	0.09*
KCO (%predicted)	78.3 ± 9.6	84.8 ± 13.5	0.072*

Table 4.5 – Comparison of baseline spirometry and gas transfer results between smokers with chronic bronchitis (CB) and asymptomatic smokers (AS)

Legend: All data are displayed as median (IQR) apart from gas transfer parameters expressed as mean ± SD. Statistical differences between the two groups were analysed using *independent t-test or #Mann-Whitney U test. All significant p-values are in bold.

4.2.4.2 Comparison according to the history of chest infection

Participants were stratified according to the history of self-reported chest infections during the preceding 12 months before study enrollment. Post-BD spirometry and gas transfer results between participants who reported a previous episode of chest infection and those who did not are shown in table 4.6. There were no differences in post-BD spirometry or gas transfer results between the two groups.

	Prev LRTI (n=23)	**No prev LRTI (n=47)**	**p-value**
Spirometry results			
Post-BD FEV_1 (L)	3.64 (3.16-4.16)	3.39 (3.09-3.86)	0.29*
Post-BD FEV_1 (%predicted)	106.0 (97.0-114.0)	102.0 (94.0-109.0)	0.13*
Post-BD FVC (L)	4.32 (3.55-5.35)	4.14 (3.62-4.93)	0.40#
Post-BD FVC (%predicted)	107.0 (96.0-112.0)	102.0 (93.0-107.0)	0.12*
FEV_1/FVC ratio	0.85 (0.80-0.89)	0.84 (0.78-0.87)	0.76*
MMEF (L)	3.92 (3.43-4.54)	3.48 (3.25-4.32)	0.47*
MMEF (%predicted)	109.0 (88.0-124.0)	100.0 (86.0-126.0)	0.62#
Gas transfer	**n=20**	**n=42**	
TLCO (mmol/min/kPa)	7.78 ± 1.39	7.95 ± 1.77	0.71*
TLCO (%predicted)	80.4 ± 11.7	81.4 ± 12.6	0.76*
KCO (mmol/min/kPa/L)	1.42 ± 0.18	1.50 ± 0.24	0.24*
KCO (%predicted)	79.2 ± 9.2	84.7 ± 13.9	0.11*

Table 4.6 – Comparison of baseline spirometry and gas transfer results between participants who reported a previous history of chest infection and those who did not

Legend: All data are shown as median (IQR) apart from gas transfer parameters (mean ± SD). Statistical differences between the two groups were analysed using the *independent t-test or the #Mann-Whitney U test. Prev: previous; LRTI: lower respiratory tract infection

4.2.5 Correlation of spirometry and gas transfer at baseline

All correlations in this section were assessed using Spearman's correlation coefficient. Apart from FEV_1/FVC ratio, adjusted values (%predicted) for spirometry and gas transfer results were used for correlation analysis. There were no significant correlations between MMEF and KCO (n=62, p=0.74) as well as between all post-BD spirometry parameters and CAT scores at baseline (n=70; Spearman's rho= -0.15, p=0.21; rho=0.03, p=0.80; rho= -0.006, p=0.96 for FEV_1, FEV_1/FVC ratio and MMEF respectively). However, there was a statistically significant negative correlation between KCO and CAT scores at baseline (n=62; rho= -0.34, p=0.007). Figure 4.4 shows the scatterplot of the latter relationships. On the other hand, MMEF positively correlated with both FEV_1 (rho=0.58, p<0.0001) and the FEV_1/FVC ratio (rho=0.86, p<0.0001). Figure 4.5 shows the scatterplot of these two correlations.

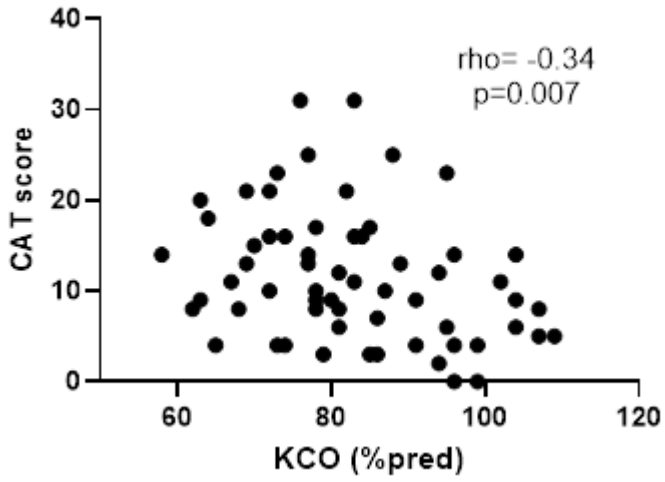


Figure 4.4 – Correlation of KCO (%predicted) with CAT score

Legend: Correlation was assessed using Spearman's correlation coefficient. Sixty-two pairs of data were available for this correlation.

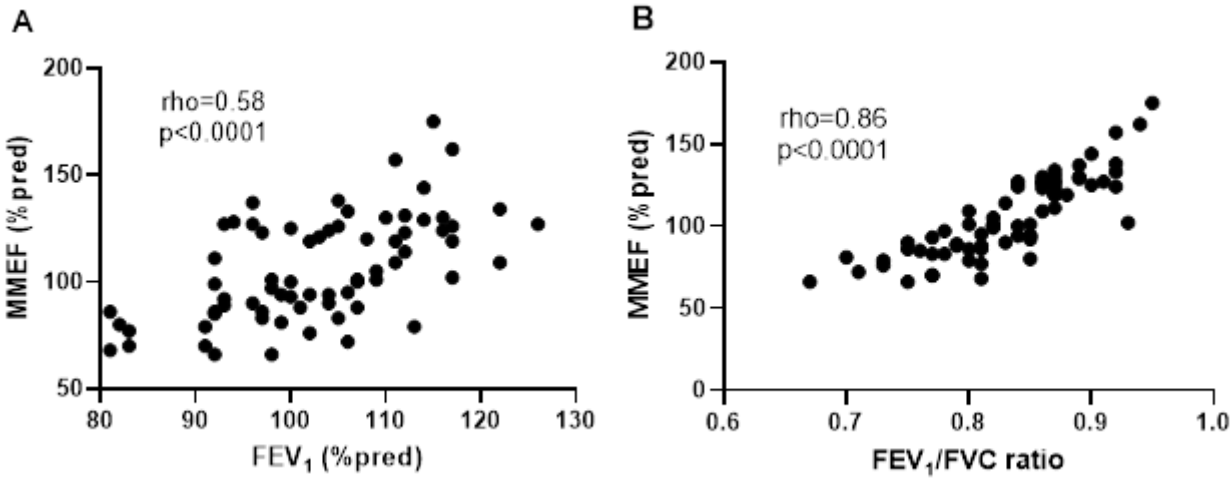


Figure 4.5 – Correlation of MMEF with other post-BD spirometry parameters

Legend: Correlations of MMEF were shown with FEV_1 (figure A) and FEV_1/FVC ratio (figure B). Correlations were assessed using Spearman's correlation coefficient. Seventy pairs of data were available for both correlations.

4.2.6 Comparison between groups with 'normal MMEF' and those with 'low MMEF'

The cohort was stratified into a 'normal MMEF' and a 'low MMEF' group (indicating possible SAD) according to their MMEF results. A cut-off of 80% predicted was used to delineate the normal range as reported previously[130 353] and hence less likely to include subjects with true SAD. Figure 4.6 shows the scatterplot as in figure 4.5B but with the groups indicated on the graph.

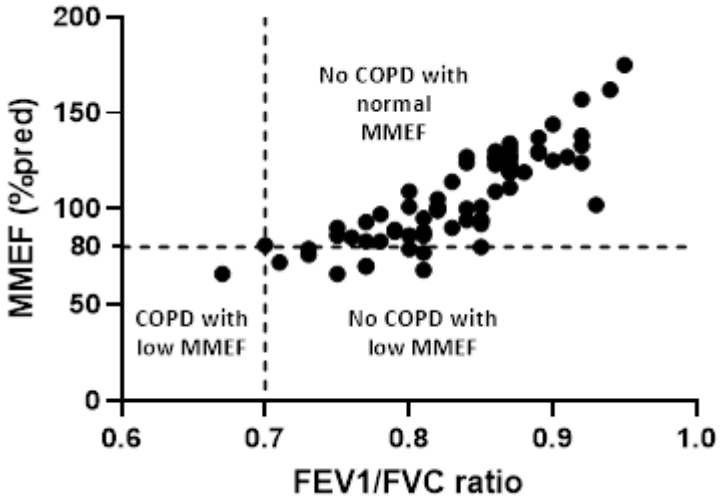


Figure 4.6 – FEV_1/FVC ratio plotted against MMEF

Legend: Separate gridlines were added to stratify the cohort into groups. Those with an FEV1/FVC ratio <0.7 met the diagnostic criteria for COPD. MMEF below 80% predicted was used as a cut-off to stratify the cohort into a 'normal MMEF' and a 'low MMEF' group.

The majority (n=60, 85.7%) of the cohort were found to have MMEF values ≥80% predicted. Out of those with MMEF values <80% predicted, one met the spirometric criteria for COPD (FEV_1/FVC ratio of <0.7). No participants in the 'normal MMEF' group met the COPD spirometric criterion.

There was no difference in absolute post-BD FEV_1 (p=0.33) between the 'normal MMEF' and the 'low MMEF' group. However, a significant difference was found when values were adjusted for age, sex, height, and race, with lower values found in the 'low MMEF' group (median 91.5 (IQR 83.0-103.0) vs 104.5 (IQR 97.0-111.8) % predicted, p=0.003). The 'low MMEF' group were also found to have a lower FEV_1/FVC ratio compared to the 'normal MMEF' group (median 0.76 (IQR 0.73-0.80) vs 0.85 (IQR 0.81-0.87), p<0.0001). There were no differences in the two groups' demographic features, gas transfer results and CAT scores. Table 4.7 summarises these data. Among the 'low MMEF' group, three (30.0%) reported at least one episode of chest infection in the 12 months before enrolment, but this was similar to the 'normal MMEF' group, where 20 (33.3%) also had at least one episode of chest infection (p>0.99).

	Normal MMEF (n=60)	Low MMEF (n=10)	p-value
Age (years)	35 (32-40)	39 (34-42.5)	0.11[#]
Sex, n (% female)	35 (65.0)	4 (40.0%)	0.17[+]
Smoking history (pack-years)	13.8 (11.0-15.9)	14.5 (11.0-20.3)	0.77[#]
BMI (kg/m^2)	25.8 (22.5-29.4)	26.5 (20.8-30.9)	0.98*****
IMD decile	3.0 (1.0-4.8)	3.5 (2.5-6.5)	0.334
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 42 (70.0) 14 (23.3) 4 (6.7)	 6 (60.0) 4 (40.0) 0 (0)	 0.49[+]
Spirometry results Post-BD FEV_1 (L) Post-BD FEV_1 (%predicted) Post-BD FVC (L) Post-BD FVC (%predicted) FEV_1/FVC ratio MMEF (L) MMEF (%predicted)	 3.50 (3.14-4.08) 104.5 (97.0-111.8) 4.20 (3.64-4.95) 102.5 (96.3-108.0) 0.85 (0.81-0.87) 3.92 (3.42-4.54) 110.0 (93.3-127.0)	 3.02 (2.74-4.33) 91.5 (83.0-103.0) 4.11 (3.48-6.03) 103.5 (87.5-113.8) 0.76 (0.73-0.80) 2.74 (2.32-3.23) 71.0 (67.5-77.5)	 0.33[#] **0.003*** 0.69[#] 0.99***** **<0.0001*** **<0.0001*** **<0.0001[#]**
Gas transfer TLCO (mmol/min/kPa) TLCO (%predicted) KCO (mmol/min/kPa/L) KCO (%predicted)	**n=53** 7.58 (6.65-8.66) 79.0 (72.0-87.5) 1.44 (1.32-1.65) 81.0 (73.5-92.5)	**n=9** 8.75 (6.24-11.13) 88 (72.5-96.5) 1.47 (1.28-1.74) 83 (71.0-96.5)	 0.32[#] 0.27***** 0.97***** 0.65*****
CAT score	10.5 (6.3-16.0)	6.5 (3.8-17.3)	0.24[#]

Table 4.7 – Comparison of baseline demographics, lung function parameters and CAT scores between the 'normal MMEF' group and the 'low MMEF' group

Legend: Continuous data are displayed as median (IQR). Statistical differences between the two groups were analysed with the [#]Mann-Whitney U test, *****independent t-test or the [+]Fisher's exact test. All significant p-values are in bold.

4.2.7 Baseline FOT results

FOT measurements were done at baseline for 45 (64.3%) participants and were included in the analysis. Table 4.8 compares demographic details and lung function parameters between participants who had FOT measurements at baseline and those who did not. There were no differences between the two groups.

	Included (n=45)	**Not included (n=25)**	**p-value**
Age (years)	35.0 (32.0-40.5)	37.0 (32.0-40.0)	0.90#
Sex, n (% female)	28 (62.2)	15 (60.0)	>0.99+
Smoking history (pack-years)	13 (10.9-15.6)	14 (11.1-21.0)	0.15#
BMI (kg/m^2)	25.3 (22.4-28.9)	27.4 (22.6-31.7)	0.25*
IMD Decile	3.0 (1.5-5.0)	3.0 (1.0-4.5)	0.93#
Ethnicity, n (%)			
White	27 (60.0)	21 (84.0)	0.09+
Asian/Asian British	15 (33.3)	3 (12.0)	
Black/African/Caribbean	3 (6.7)	1 (4.0)	
Spirometry results			
Post-BD FEV_1 (L)	3.52 (3.07-4.12)	3.37 (3.14-4.00)	0.97#
Post-BD FEV_1 (%predicted)	103.0 (96.0-111.5)	104.0 (93.5-111.0)	0.71*
Post-BD FVC (L)	4.20 (3.50-4.97)	4.19 (3.67-5.02)	0.72#
Post-BD FVC (%predicted)	103.0 (95.0-110.0)	101.0 (95.5-108.0)	0.73*
FEV_1/FVC ratio	0.84 (0.79-0.87)	0.85 (0.80-0.87)	0.94*
MMEF (L)	3.68 (3.27-4.53)	3.90 (3.24-4.23)	0.84*
MMEF (%predicted)	101.0 (86.0-126.5)	101.0 (87.5-122.5)	0.86#
Gas transfer	**n=42**	**n=20**	
TLCO (mmol/min/kPa)	8.11 (6.70-8.82)	7.08 (6.48-8.79)	0.33#
TLCO (%predicted)	83.5 (75.0-89.3)	76.5 (71.3-90.8)	0.30*
KCO (mmol/min/kPa/L)	1.51 (1.34-1.73)	1.39 (1.29-1.54)	0.13*
KCO (%predicted)	84.0 (75.0-94.5)	77.0 (72.5-86.0)	0.11*

Table 4.8 – Comparison of baseline demographics and lung function parameters between participants who had FOT measurements and those who did not

Legend: Continuous data are displayed as median (IQR). Statistical differences between the two groups were analysed using the #Mann-Whitney U test, *independent t-test or the +Fisher's exact test.

All FOT measurement parameters in this section were listed as median (IQR) unless stated otherwise. R_{5-20} and ΔX_5 have been used in some studies as a marker of SAD. Cut-off values of R_{5-20} >0.7cmH2O/L/s[354] and ΔX_5 >0.7cmH2O/L/s[355] were considered abnormal, and thus a similar threshold was used for this analysis. When this threshold was applied, 12 (26.7%) participants in the cohort had at least one abnormal parameter (either abnormal R_{5-20} or ΔX_5), and 5 (11.1%) participants had abnormal readings in both parameters. Table 4.9 shows the FOT results of the cohort.

Resistance parameters	
R_5 (cmH2O/L/s)	2.71 (2.34-3.43)
R_5 (%predicted)	90.0 (78.5-105.5)
R_{20} (cmH2O/L/s)	2.70 (2.44-3.04)
R_{20} (%predicted)	84.0 (75.0-103.0)
R_{5-20} (cmH2O/L/s)	0.07 (-0.19-0.46)
Reactance parameters	
X_5 (cmH2O/L/s)	-1.13 (-1.46-(-0.77)
X_5 (%predicted)	98.0 (73.0-131.0)
ΔX_5 (cmH2O/L/s)	0.47 (0.33-0.66)
Ax (cmH2O/L)	4.24 (2.48-7.06)

Table 4.9 – Baseline FOT parameters of the early COPD cohort

Legend: FOT measurement data were available for 45 participants of the Early COPD cohort. All values are displayed as median (IQR). R_5, R_{20} and X_5 are reported in both absolute and adjusted values, while R_{5-20}, ΔX_5 and Ax are reported in absolute values only. $R5_{-20}$: difference between R_5 and R_{20}; ΔX5: difference between inspiratory and expiratory X_5

4.2.8 FOT parameter comparison between smoker subtypes

Twenty-one volunteers were recruited as age-matched healthy controls for this analysis. None of the healthy controls had respiratory symptoms or diagnoses and was 'never' smokers. The basic demographic details of the healthy non-smoker (HNS) group are displayed in the sections below. Post-BD spirometry and gas transfer results were not available for the HNS group.

4.2.8.1 Comparison according to the presence of chronic bronchitis

Participants in the early COPD cohort were stratified into the CB and AS groups as in section 4.2.4.1 and compared to the HNS group described earlier. The CB group had a higher median BMI than the HNS group (median 28.1 (IQR 22.9-29.6) vs 23.9 (IQR 20.9-26.2) kg/m^2, p=0.029) but not compared to the AS group (median 25.0 (IQR 21.5-27.8) kg/m^2, p=0.13). There were no differences in the other demographic details (Table 4.10).

The comparison of the FOT results between these three groups is shown in table 4.11, indicating no differences in the resistance parameters between them. The CB group had a higher ΔX_5 than the HNS group, just achieving statistical significance (median 0.55 (IQR 0.49-1.15) vs 0.38 (IQR 0.25-0.64) cmH2O/L/s, p=0.04) but this was not significantly higher compared to the AS group (median 0.41 (IQR 0.32-0.62) cmH2O/L/s, p=0.12).

	CB (n=15)	AS (n=30)	HNS (n=21)	p-value
Age (years)	36.0 (32.0-39.0)	34.5 (32.0-41.0)	34.0 (33.0-37.5)	0.96*
Sex, n (% female)	9 (60)	19 (63.3)	13 (61.9)	>0.99^
Smoking history (pack-years)	13.0 (11.0-16.0)	13.4 (10.8-15.7)	0	0.86+
BMI (kg/m^2)	28.1 (22.9-29.6)	25 (21.5-27.8)	23.9 (20.9-26.2)	**0.04#**
IMD Decile	2.0 (1.0-4.0)	3.0 (1.8-5.3)	N/A	0.18+
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 10 (66.7) 5 (33.3) 0 (0)	 17 (56.7) 10 (33.3) 3 (10)	 15 (71.4) 5 (23.8) 1 (4.8)	0.75^

Table 4.10 – Comparison of demographic features between the CB, AS and HNS groups

Legend: Continuous data are displayed as median (IQR). Statistical differences between groups were analysed using the *Kruskal-Wallis test with Dunn's multiple comparisons between groups, #one-way ANOVA test with Tukey's comparison test between groups, +Mann-Whitney U test or the ^Fisher's exact test. In the smoking history and IMD decile, only the CB group and AS group were compared. All significant p-values are in bold.

	CB (n=15)	AS (n=30)	HNS (n=21)	p-value
Resistance parameters				
R_5 (cmH2O/L/s)	2.85 (2.31-3.64)	2.68 (2.34-3.26)	2.43 (1.92-3.08)	0.19*
R_5 (%predicted)	85.0 (76.0-105.0)	91.0 (79.3-106.5)	87.0 (73.0-100.0)	0.47*
R_{20} (cmH2O/L/s)	2.80 (2.22-3.13)	2.69 (2.47-2.99)	2.52 (2.08-2.71)	0.24*
R_{20} (%predicted)	84.0 (73.0-95.0)	85.0 (75.0-109.3)	82.0 (71.5-96.5)	0.39*
R_{5-20} (cmH2O/L/s)	0.31 (0.04-0.66)	-0.02 (-0.21-0.34)	-0.04 (-0.19-0.23)	0.12*
Reactance parameters				
X_5 (cmH2O/L/s)	-1.19 (-1.65 to -0.78)	-1.09 (1.45 to -0.75)	-1.11 (-1.19 to -0.92)	0.86*
X_5 (%predicted)	102.0 (73.0-121.0)	94.5 (73.8-133.3)	93.0 (78.5-121.5)	0.93#
ΔX_5 (cmH2O/L/s)	0.55 (0.49-1.15)	0.41 (0.32-0.62)	0.38 (0.25-0.64)	**0.042***
Ax (cmH2O/L)	5.5 (2.8-8.2)	3.5 (2.0-6.2)	3.4 (2.5-4.6)	0.12*

Table 4.11 – Comparison of baseline FOT parameters between the CB, AS and HNS group

Legend: All values are displayed as median (IQR). Statistical differences between groups were analysed using the *Kruskal-Wallis test with Dunn's multiple comparisons between groups or the #one-way ANOVA test with Tukey's comparison test between groups. All significant p-values are in bold.

4.2.8.2 Comparison according to the history of chest infection

Cohort participants were stratified according to the history of self-reported chest infections in the previous 12 months before recruitment as per section 4.2.4.2 and compared to the HNS group. There was no difference in the demographic features between the three groups (see Table 4.12).

The comparison of the FOT results between the three groups is shown in table 4.13. There was a statistically significant difference in R_{20} absolute values between the groups (p=0.03), but this significance was not sustained after correction for multiple comparisons. A higher X_5 %predicted was observed in those who had a history of self-reported chest infection compared to those who did not (median 121.0 (IQR 88.5-144.0) vs 83.5 (IQR 68.8-110.8) % predicted, p=0.031) but this was not significantly higher compared to the HNS group (median 93.0 (IQR 78.5-121.5) % predicted, p=0.19).

	Prev LRTI (n=13)	No prev LRTI (n=32)	HNS (n=21)	p-value
Age (years)	35.0 (32.0-38.0)	32.0 (35.5-41.8)	34.0 (33.0-37.5)	0.77*
Sex, n (% female)	6 (46.2)	22 (68.8)	13 (61.9)	0.38^{+}
Smoking history (pack-years)	14.0 (11.8-15.5)	12.6 (10.8-15.8)	N/A	0.64$^{\#}$
BMI (kg/m^2)	28.1 (22.7-29.2)	25.1 (22.4-28.5)	23.9 (20.9-26.2)	0.13*
IMD Decile	3.0 (1.5-5.0)	3.0 (1.3-5.0)	N/A	0.79$^{\#}$
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 8 (61.5) 5 (38.5) 0 (0)	 19 (59.4) 10 (31.3) 3 (9.3)	 15 (71.4) 5 (23.8) 1 (4.8)	 0.78^{+}

Table 4.12 – Comparison of demographic details between participants who reported a history of chest infection in the 12 months before recruitment, those who did not and the HNS group

Legend: Continuous data are displayed as median (IQR). Statistical differences between groups were analysed using the *Kruskal-Wallis test with Dunn's multiple comparisons between groups, $^{\#}$Mann-Whitney U test or the $^{+}$Fisher's exact test. In the smoking history and IMD decile, the HNS group was not compared.

	Prev LRTI (n=13)	No prev LRTI (n=32)	HNS (n=21)	p-value
Resistance parameters				
R_5 (cmH2O/L/s)	2.31 (1.98-3.53)	2.75 (2.44-3.39)	2.43 (1.92-3.08)	0.054*
R_5 (%predicted)	84.0 (64.0-97.0)	91.0 (82.0-113.3)	87.0 (73.0-100.0)	0.09*
R_{20} (cmH2O/L/s)	2.27 (1.98-2.92)	2.72 (2.53-3.09)	2.52 (2.08-2.71)	(**0.03***)
R_{20} (%predicted)	80.0 (71.0-93.0)	90.5 (76.5-106.5)	82.0 (71.5-96.5)	0.11*
R_{5-20} (cmH2O/L/s)	0.07 (-0.19-0.58)	0.09 (-0.19-0.43)	-0.04 (-0.19-0.23)	0.61*
Reactance parameters				
X_5 (cmH2O/L/s)	-0.87 (-1.23 to -0.62)	-1.20 (-1.56 to -0.91)	-1.11 (-1.19 to 0.92)	0.07*
X_5 (%predicted)	121.0 (88.5-144.0)	83.5 (68.8-110.8)	93.0 (78.5-121.5)	**0.04$^{\#}$**
ΔX_5 (cmH2O/L/s)	0.41 (0.30-0.59)	0.51 (0.35-0.76)	0.38 (0.25-0.64)	0.17*
Ax (cmH2O/L)	3.5 (1.5-6.6)	4.4 (2.5-7.5)	3.4 (2.5-4.6)	0.28*

Table 4.13 – Comparison of baseline FOT parameters between participants who reported a previous history of chest infection, those who did not and the HNS group.

Legend: All values are displayed as median (IQR). Statistical differences between the groups were analysed using the *Kruskal-Wallis test with Dunn's multiple comparisons between groups or the $^{\#}$one-way ANOVA test with Tukey's comparison test between groups. All significant p-values are in bold. P-values in brackets were no longer significant when corrected for multiple testing.

4.2.9 Correlation between FOT parameters and symptoms

All correlations in this section were assessed using Spearman's correlation coefficient. Adjusted values (%predicted) of the FOT parameters (apart from R_{5-20} and ΔX_5) were used for correlation analyses. There were no significant relationships between FOT parameters and CAT score at baseline (all p>0.1). There was a suggestion of a positive relationship between R_{5-20} and CAT score, but this did not reach statistical significance (p=0.054).

4.2.10 Correlation between FOT parameters and spirometric parameters

FEV_1 negatively correlated with R_5 (rho= -0.35, p=0.018), R_{5-20} (rho= -0.36, p=0.014) and Ax (rho= -0.36, p=0.014) but was positively correlated with X_5 (rho=0.51, p=0.0005). Figure 4.7 shows the scatterplots of these correlations. X_5 had a negative correlation of borderline significance with KCO (rho= -0.31, p=0.041), as shown in figure 4.8. There were no significant correlations between MMEF and any FOT parameters (all p>0.2) nor between FEV_1/FVC ratio and any FOT parameters (all p>0.15). Individual results are summarised in Table 4.14.

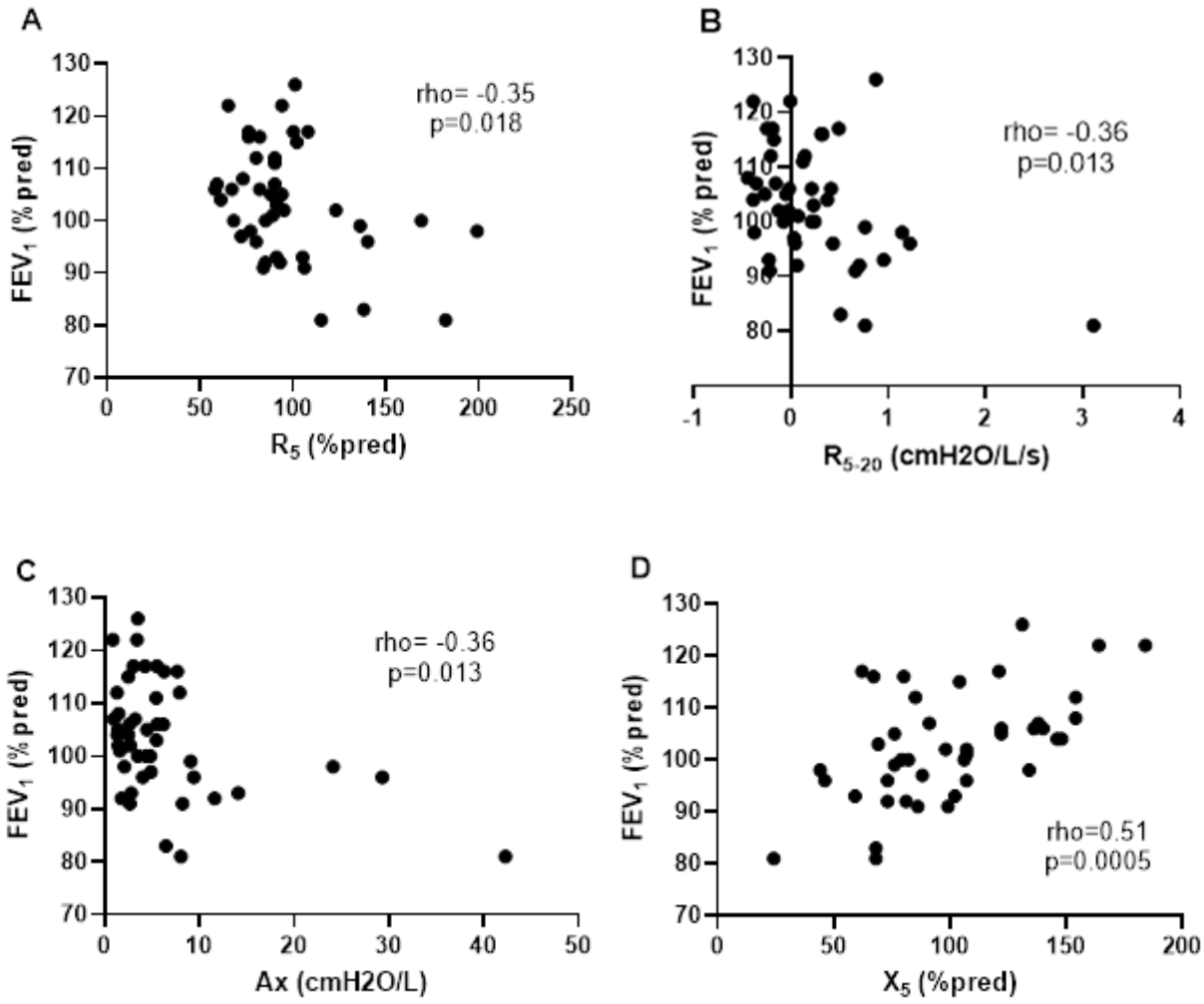


Figure 4.7 – Correlation of FEV_1 with FOT parameters

Legend: Correlations of FEV_1 were shown with R_5 (figure A), R_{5-20} (figure B), Ax (figure C) and X_5 (figure D). Correlations were assessed using Spearman's correlation coefficient. Forty-five pairs of data were available for all correlations.

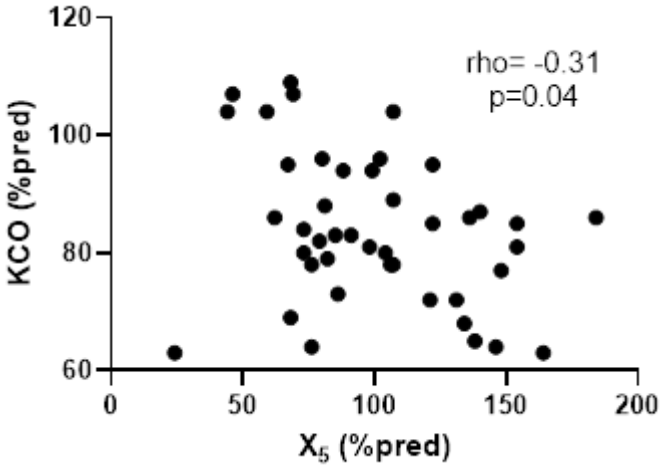


Figure 4.8 – Correlation of KCO (%predicted) with X_5 (%predicted)

Legend: Correlation was assessed using Spearman's correlation coefficient. Forty-five pairs of data were available for this correlation.

	FEV_1 (%predicted)		FEV_1/FVC ratio		KCO (%predicted)		MMEF (%predicted)	
	Correlation, rho	**p-value**	**Correlation, rho**	**p-value**	**Correlation, rho**	**p-value**	**Correlation, rho**	**p-value**
R_5 (% predicted)	-0.35 (-0.59 to -0.06)	**0.018**	-0.17 (-0.45 to 0.13)	0.25	0.13 (-0.18 to 0.42)	0.39	-0.18 (-0.46 to 0.13)	0.24
R_{20} (% predicted)	-0.25 (-0.52 to 0.07)	0.11	-0.22 (-0.50 to 0.09)	0.15	0.13 (-0.19 to 0.42)	0.41	-0.18 (-0.47 to 0.13)	0.24
R_{5-20} (%predicted)	-0.36 (-0.60 to -0.07)	**0.014**	-0.15 (-0.43 to 0.16)	0.32	0.07 (-0.23 to 0.37)	0.63	-0.14 (-0.42 to 0.17)	0.37
X_5 (%predicted)	0.51 (0.23 to 0.70)	**0.0005**	0.12 (-0.19 to 0.42)	0.42	-0.31 (-0.57 to -0.005)	**0.04**	0.16 (-0.15 to 0.45)	0.30
Ax (cmH2O/L)	-0.36 (-0.60 to -0.07)	**0.014**	-0.06 (-0.35 to 0.25)	0.70	0.18 (-0.13 to 0.46)	0.24	-0.04 (-0.33 to 0.27)	0.82
ΔX_5 (cmH2O/L/s)	-0.29 (-0.55 to 0.02)	0.057	-0.02 (-0.33 to 0.29)	0.90	-0.005 (-0.31 to 0.30)	0.97	-0.02 (-0.32 to 0.29)	0.92

Table 4.14 – Correlations between the FOT parameters (shown in rows) with post-BD spirometry and gas transfer parameters (shown in columns)

Legend: Correlations were assessed using Spearman's correlation coefficient. Spearman's rho is shown together with 95% CI. Forty-five pairs of data were available for all correlations. Significant p-values are in bold.

4.2.11 Differences between 'SAD group' and 'non-SAD' group using FOT

Participants who underwent baseline FOT measurement were stratified into either the 'SAD' group or the 'non-SAD' group according to their R_{5-20} and ΔX_5 results. As mentioned in section 4.2.7, a cut-off value of 0.7cmH2O/L/s was used for R_{5-20} and ΔX_5, and those with >0.7cmH2O/L/s on either parameter were placed into the 'SAD' group while the rest that did not meet the set parameters were placed in the 'non-SAD' group.

When demographic details and standard spirometric parameters were compared, the 'SAD' group had a higher BMI (median 29.5 (IQR 28.1-33.2) vs 24.0 (IQR 21.9-27.2) kg/m^2, p<0.0001) and a lower FEV_1/FVC ratio (median 0.80 (IQR 0.75-0.84) vs 0.86 (IQR 0.81-0.89), p=0.03) than the 'non-SAD' group. When FOT parameters were compared between the two groups, the 'SAD' group had a higher absolute R_5 (median 3.62 (IQR 2.98-4.41) vs 2.52 (IQR 2.24-2.87) cmH2O/L/s, p=0.002) and Ax value (median 8.63 (IQR 3.48-21.60) vs 3.01 (IQR 1.69-5.48) cmH2O/L/s, p=0.0004) but a lower X_5 both in absolute value (p=0.0003) and for %predicted values (p=0.03). The difference in R_5 became non-significant when adjusted for sex, age, height, and weight (p=0.41). The 'SAD' group also reported a higher CAT score than the 'non-SAD' group (median 15.5 (IQR 8.0-21.0) vs 9.0 (IQR 5.0-15.5), p=0.04). Table 4.15 compares demographic details between the two groups based on FOT results. Table 4.16 shows the two groups' lung function, FOT, and CAT scores.

In the 'SAD' group, four (33.3%) reported at least one episode of chest infection in the 12 months before enrolment compared to nine (27.3%) in the 'non-SAD' group. This difference was not statistically significant (p=0.72).

	SAD (n=12)	Non-SAD (n=33)	p-value
Age (years)	34.5 (32.0-43.3)	36.0 (32.0-40.0)	0.97[#]
Sex, n (% female)	6 (50.0)	22 (66.7)	0.32[+]
Smoking history (pack-years)	14.0 (11.1-17.3)	13.0 (10.8-15.6)	0.50[#]
BMI (kg/m^2)	29.5 (28.1-33.2)	24.0 (21.9-27.2)	**<0.0001***
IMD decile	2.5 (1.0-6.0)	3.0 (2.0-4.5)	>0.99[#]
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 6 (50.0) 6 (50.0) 0 (0)	 21 (63.6) 9 (27.3) 3 (9.1)	 0.40[+]

Table 4.15 – Comparison of baseline demographics between the 'SAD' group and the 'non-SAD' group

Legend: Continuous data are displayed as median (IQR). Statistical differences between the groups were analysed using the [#]Mann-Whitney U test, *****independent t-test or the [+]Fisher's exact test. All significant p-values are in bold. SAD: small airway dysfunction

	SAD (n=12)	Non-SAD (n=33)	p-value
Post-BD spirometry			
Post-BD FEV_1 (L)	3.44 (2.77-4.16)	3.53 (3.15-4.02)	0.50*
Post-BD FEV_1 (%predicted)	97.0 (91.3-109.3)	105.0 (99.0-112.0)	0.15*
Post-BD FVC (L)	4.19 (3.43-5.32)	4.2 (3.56-4.85)	>0.99#
Post-BD FVC (%predicted)	102.0 (90.0-111.5)	103.0 (95.5-109.5)	0.80#
FEV_1/FVC ratio	0.80 (0.75-0.84)	0.86 (0.81-0.89)	**0.03***
MMEF (L)	3.49 (2.60-4.18)	3.91 (3.32-4.54)	0.14*
MMEF (%predicted)	89.0 (82.0-120.3)	119.0 (89.0-126.5)	0.11*
Gas transfer			
TLCO (mmol/min/kPa)	7.58 (5.99-9.37)	8.15 (6.80-8.79)	0.80*
TLCO (%predicted)	83.0 (68.0-87.0)	83.5 (75.0-89.3)	0.52*
KCO (mmol/min/kPa/L)	1.41 (1.18-1.74)	1.53 (1.35-1.72)	0.33*
KCO (%predicted)	78.0 (69.0-104.0)	85.0 (78.8-94.3)	0.43*
FOT resistance parameters			
R_5 (cmH2O/L/s)	3.62 (2.98-4.41)	2.52 (2.24-2.87)	**0.002#**
R_5 (%predicted)	96.0 (72.0-139.0)	90.0 (78.5-101.0)	0.41#
R_{20} (cmH2O/L/s)	2.87 (2.50-3.29)	2.59 (2.37-2.91)	0.14*
R_{20} (%predicted)	78.5 (70.0-111.8)	86.0 (80.0-103.0)	0.43#
R_{5-20} (cmH2O/L/s)	0.76 (0.35-1.09)	-0.02 (-0.23- 0.22)	**<0.0001#**
FOT reactance parameters			
X_5 (cmH2O/L/s)	-1.73 (-2.31-(-1.11))	-0.92 (-1.27-(-0.65)	**0.0003#**
X_5 (%predicted)	78.5 (49.3-102.3)	104.0 (79.0-136.0)	**0.03***
ΔX_5 (cmH2O/L/s)	0.96 (0.45-1.56)	0.43 (0.33-0.53)	**<0.0001***
Ax (cmH2O/L)	8.63 (3.48-21.6)	3.01 (1.69-5.48)	**0.0004#**
CAT score	15.5 (8.0-21.0)	9.0 (5.0-15.5)	**0.04***

Table 4.16 – Comparison of lung function parameters, FOT parameters and CAT score between the 'SAD' group and the 'non-SAD' group

Legend: Continuous data are displayed as median (IQR). Statistical differences between the groups were analysed using the #Mann-Whitney U test or the *independent t-test. All significant p-values are in bold.

4.2.12 Overlap of SAD identified via MMEF and FOT

Two participants were identified to have SAD on MMEF measured by spirometry and FOT measurement (using both R_{5-20} and ΔX_5). The two participants reported CB symptoms at the baseline visit. Due to the low number of participants identified, comparisons were not made between these two participants and those with SAD identified only with one method (n=18). Figure 4.9 shows a non-proportional Venn diagram with the overlap between the identified SAD groups.

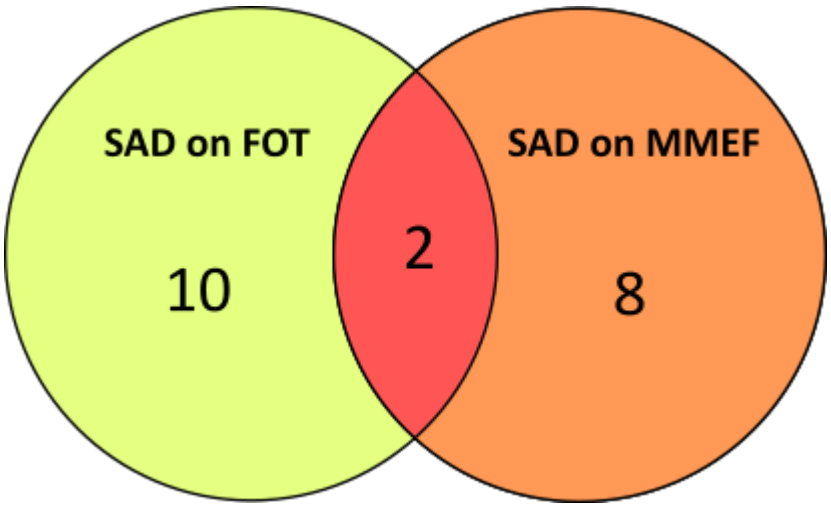


Figure 4.9 – Non-proportional Venn diagram of participants with SAD detected on both spirometry and FOT.

Legend: SAD was identified using an MMEF cut-off of <80% predicted on spirometry and a ΔX_5 and/or R_{5-20} >0.7cmH2O/L/s on FOT. Numbers in the circles show the number of participants found to have SAD with either spirometry or FOT, and the overlapping area shows participants with SAD from both methods.

4.3 Discussion

This chapter has described the baseline lung function data from the Birmingham Early COPD cohort, as assessed by different techniques, including spirometry, gas transfer testing and FOT. Data in sections 4.2.6 and 4.2.11 support the hypothesis that a significant proportion of smokers have evidence of SAD measured both by MMEF (14.3%) and FOT (26.7%). Those with SAD identified via FOT had worse CAT scores but did not have more episodes of chest infections than those without. However, careful interpretation of the data is required due to the low number of participants in the Birmingham Early COPD cohort resulting in the lack of power to detect any significant differences.

There have been efforts to employ diagnostic tests to quantify SAD in smokers, as this has been considered an early feature of COPD. Spirometry measurement outputs, including FEV_1 and FEV_1/FVC ratio, are used to diagnose and determine established COPD and its severity in patients with a history of exposure to the relevant risk factors.[1] However, neither parameters are specific for SAD, and both primarily reflect obstruction of the larger airways.[135] A significant reduction of small airway number and function is required before either FEV_1 or FEV_1/FVC ratio becomes abnormal. To help address this limitation in conventional spirometry outputs using FEV_1 and FEV_1/FVC ratio, both MMEF and FOT have been investigated for identifying SAD.

MMEF can be easily measured as the data is generated during spirometry to obtain FEV_1 and FVC. Leuallen and Folwer introduced MMEF as a sensitive measure of the small airways to expiratory airflow obstruction[356] and has been the most widely studied test to date. However, it should be noted that MMEF is at least partly dependent on the FVC and may be reduced in

the absence of airflow limitation when the lungs are smaller than average for a patient's age, sex, height, and race. To account for this, it has been suggested that MMEF be corrected for the measured FVC.[357] However, we have chosen not to do this as all participants in this study had FVC values within the normal range (>80%).

4.3.1 Reliability of FOT as a diagnostic tool

As mentioned in section 1.2.6.1, several factors, including reproducibility and specificity, need to be considered before FOT can be used as a diagnostic tool or adjunct to standard tests in clinical and research settings. Data presented in section 4.2.1 support the reproducibility of FOT as a potential diagnostic tool in measuring lung respiratory mechanics with minimal training required to perform this test. Bland-Altman plots showed no systematic bias within a single assessor's repeat measurements or between two different assessors. However, this thesis could not assess FOT specificity for detecting SAD due to the absence of a gold standard technique for SAD diagnosis.

There are few previous studies of intra-session variability of FOT measurements. Timmins et al. assessed variability in oscillatory impedance in ten lifelong non-smokers, ten asthmatic subjects and ten COPD subjects using an in-house FOT device.[358] For the resistance parameters, the ICC reported was 0.95, 0.78 and 0.93 for healthy controls, asthma patients and COPD patients, respectively[358] and 0.83, 0.85 and 0.95 for the reactance parameters.[358] Another study also measured within-session variability in a cohort of healthy subjects (n=20), asthmatics (n=22) and COPD patients (n=18) using the Tremoflo device.[359] The median CV of R_5 and X_5 measured over 30 seconds was reported to be both 9% in healthy subjects, 8% and

11% in asthmatics and 6% and 9% in COPD patients,[359] confirming the reproducibility shown here.

Several limitations preclude the widespread use of FOT in clinical practice. Firstly, there have been few attempts to develop normal predicted FOT values among adult populations. In this chapter, we have used the reference study by Oostveen et al.[284] to derive normal predicted FOT values. However, that study only included 368 Caucasian participants, and thus the reference equations derived may not apply to other ethnic groups.[284] Further studies are needed to obtain normative values for multi-ethnic adult populations, similar to the Global Lung Function Initiative values for spirometry.[50] Secondly, there is a lack of consensus on the definition of an abnormal FOT result. For example, we have used the defined cut-off value of 0.7cmH2O/L/s for R_{5-20}. However, a cut-off value of 1.02cmH2O/L/s has also been proposed.[137] Similarly, different ΔX_5 thresholds have been used, with 0.28kPa/L/s (2.86cmH2O/L/s) proposed in one study,[139] whilst another used ΔX_5 ≥0.55kPa/L/s (5.61cmH2O/L/s).[360] However, it is important to note that these studies assessed ΔX_5 in COPD patients rather than in smokers, with no evidence of airflow limitation on spirometry. Similar to the GOLD criteria for COPD diagnosis,[1] a consensus on the definition of an abnormal FOT result is needed and should be validated in large prospective studies.

Thirdly, the possible differences in measurements using different devices present further difficulty. Soares et al. conducted a study evaluating and comparing impedance measurements between two devices (Tremoflo and Jaeger Masterscope).[361] A systematic and proportionate bias in resistance and reactance values was noted when comparing both devices. Resistance values measured with Jaeger Masterscope being higher and reactance

values less negative than Tremoflo values.[361] Further between-device standardisation will be required, and a standard test load with known resistance and reactance will be necessary to compare devices.

4.3.2 Significance of chronic bronchitis on pulmonary physiology

When baseline spirometry results were analysed, no differences were seen when the cohort was stratified according to the presence of CB symptoms or previously reported chest infections. However, a reduction in TLCO and a trend towards reduced KCO were observed in smokers with CB symptoms. These need to be interpreted with caution, as many patients smoked despite being informed not to smoke on the day of lung function testing. Sansores et al. previously demonstrated an acute decrease in the TLCO after cigarette smoking in twelve smokers.[350] TLCO was measured in participants before and after smoking as many cigarettes as possible in one hour, and TLCO decreased after smoking but showed no changes after an hour of sham smoking of an unlit cigarette.[350]

There have been studies that suggest that gas transfer parameters can be chronically impaired in cigarette smokers. A study performed in Tucson, Arizona, showed that the %predicted TLCO in current smokers (n=152) was lower than in ex-smokers (n=172) and non-smokers (n=425).[362] To determine the effects of smoking status alone, all participants included in this study had previously undergone screening spirometry and those with an FEV_1/FVC ratio <0.7 and FEV_1 <75% predicted were excluded, meaning participants included had no spirometric evidence of COPD.[362] This finding is supported by a further study performed in London where spirometry and gas transfer testing was performed at multiple time points up to a year in a

group of 'COPD quitters' (n=11), 'healthy quitters' (n=21), healthy smokers (n=13) and non-smokers (n=19).[363] It was shown that there were abnormalities in gas transfer parameters among COPD and non-COPD smokers. In those who quit smoking on enrolment ('COPD quitters' and 'healthy quitters'), there was an improvement in gas transfer parameters, but a complete resolution was not observed even after a year of quitting smoking, which suggests some residual lung damage affecting bronchoalveolar integrity.[363]

As there were lower gas transfer parameters in the patients reported here who had CB symptoms, this may represent smokers with normal spirometry results but with some underlying lung damage. It remains possible that mucus secretion could cause airway obstruction (at least in part), leading to alveolar airflow mismatch. This may be a significant group of smokers to identify as they may have a higher risk of progression to COPD. A study in the New York metropolitan area compared the risk of progression to COPD among smokers with normal spirometry results.[134] When comparing smokers with normal TLCO (≥80% predicted; n=59) and smokers with low TLCO (<80% predicted; n=46), it was seen that 22% (10/46) of the 'low TLCO' group developed COPD compared to 3% (2/59) of the 'normal TLCO' group after an average follow-up of <4 years.

When FOT results were analysed, no differences were found in resistance parameters when the cohort was stratified according to the presence of CB symptoms or previous self-reported chest infections. A higher R_{5-20} (indicating peripheral airway resistance) was noted among smokers with CB compared to AS and non-smoking controls, but this did not reach statistical significance. However, this group had higher ΔX5 than non-smoking controls, indicating expiratory flow limitation. Large negative swings in reactance during exhalation were due to

the inability of low-frequency oscillatory signals (5Hz) to reach the alveoli during expiration as they are impeded by choke points caused by small airways collapse.[136]

Although the lack of difference in the resistance parameters may be reflective of the small sample size in this Birmingham cohort, it has previously been suggested that FOT reactance parameters are affected to a greater degree than resistance parameters in COPD.[135 136] The reason for this is unclear. Still, a plausible explanation may lie in what the parameters measure physiologically. Resistance parameters represent airflow obstruction, either throughout the entirety of the lung (R_5) or just the large airways (R_{20}).[136 137] In contrast, reactance parameters measure the elastic recoil of the peripheral airways or compliance of the lung periphery.[136 137] In COPD, emphysematous lung destruction causing loss of small airway elastic recoil may lead to a more significant effect on reactance values (more negative) than the resistance values.

The largest known database, including FOT, was the ECLIPSE cohort which studied 2054 patients with GOLD stage 2 to 4 COPD, 322 former smokers without COPD and 233 non-smoking controls.[138] Baseline data in the ECLIPSE cohort showed that COPD patients had worse FOT parameters than former smokers without COPD and non-smoking controls. All FOT parameters showed an incremental worsening across increasing COPD GOLD stages. However, when comparing GOLD stage 2 to 4 COPD patients, there was a more significant change in reactance values (110% decrease in X_5 values reflecting decreased elasticity of the peripheral lung) than the resistance values (60% increase in R_{5-20} values reflecting an increase in peripheral airway obstruction). No differences were found in the FOT parameters between non-smoking controls and former smokers, but the relationship of these parameters to respiratory symptoms was not explored.[138]

Another study by Frantz et al. found that individuals with self-reported symptoms had worse FOT parameters.[364] In their study, individuals were stratified into four groups according to the presence of self-reported respiratory symptoms (Q+/Q-) or airflow obstruction on spirometry (G+/G-), irrespective of smoking status. Those with diagnosed COPD (G+) mostly had mild COPD by GOLD staging (66.1%). R_{5-20} values in the Q+/G- group (n=43) were higher, reflecting increased peripheral airway resistance, than their asymptomatic counterparts (Q-/G-; n=252) but were similar to those with COPD irrespective of symptoms (G+; n=124). X_5 and Ax values in the Q+/G- group were also worse (reflecting decreased elasticity of the peripheral lung) than in the Q-/G- group but were similar to COPD patients (Q-/G+; n=90).[364] This study supported the concept that individuals with respiratory symptoms may have peripheral pathophysiological abnormalities not detectable by FEV_1 or FVC on spirometry and may be similar to those with mild COPD.

4.3.3 Relationship of symptom burden to pulmonary function parameters

No correlation was found between baseline FEV_1 and CAT scores in the Birmingham cohort, although the analysis was underpowered due to the low sample size. It has been shown that FEV_1 has a weak correlation to symptom burden among COPD patients. The Inhaled Steroids in Obstructive Lung Disease in Europe (ISOLDE) study was a double-blind placebo-controlled study looking at the effect of long-term inhaled fluticasone in patients with COPD (n=751).[365] Baseline post-BD FEV_1 had a significant but weak relationship to total SGRQ scores (r=0.23, p=0.0001).[366]

The Birmingham cohort data suggest a weak relationship between KCO and CAT scores, suggesting that alveolar function and integrity play a role in early disease symptomatology. This finding is supported by a study of 57 ex-smokers (19 with GOLD stage 1 COPD and 38 without COPD), where participants underwent spirometry, gas transfer testing and SGRQ questionnaire.[367] Ex-smokers without COPD but with abnormal TLCO results (<75% predicted) had significantly worse SGRQ scores than those with normal TLCO (≥75% predicted) but not when compared to ex-smokers with GOLD stage 1 COPD.[367] Gas transfer parameters represent a functional measure of the distribution and ability of the lung alveolar unit to transfer gas from air to blood and relate to emphysema in smokers.[368] It is plausible that those with reduced TLCO or KCO have early disease activity causing loss and damage to small airways or the early development of emphysema, which manifests as respiratory symptoms.

No relationship was found between FOT parameters and CAT score, which might reflect an underpowered analysis due to the small sample size. However, there was a trend towards higher R_{5-20} values (indicating greater small airway resistance) in CB smokers compared to the AS and HNS groups. Those with SAD were found to have worse CAT scores than those without SAD. In COPD patients (n=202 of varying severity), a positive relationship between R_{5-20} and CAT total score ($r=0.527$, $p<0.001$) has been shown, although less clear in individuals without COPD.[369] The study by Li et al. showed that CAT score had a weak positive relationship with R_5 and Ax but not with R_{20}, R_{5-20} and X_5.[370] However, this study did not discriminate between smokers and non-smokers, which may be a confounding factor.

In the Birmingham cohort, FEV_1 had the strongest correlations to the FOT parameters, as shown by other studies.[371-373] Vukoja et al. performed spirometry and FOT in 100 COPD

patients in a stable state. FEV_1 was moderately correlated with R_5 (r= -0.37, p<0.001) and X_5 (r=0.52, p<0.001).[372] Similarly, another study involving 112 stable COPD patients compared to 15 age-matched controls with normal spirometry showed a negative correlation between FEV_1 and R_5 (r= -0.62, p<0.05) as well as R_{5-20} (r= -0.8, p<0.05) and a positive correlation with X_5 (r=0.75, p<0.05) but not with R_{20}.[373]

4.3.4 Utility of FOT in predicting future exacerbations

Interestingly in the Birmingham cohort reported here, participants with previous self-reported chest infections had a higher X_5 %predicted value (indicating preserved small airway elastic recoil) than those who did not. The reason for this finding is currently unknown. A single-centre study in Tokyo attempted to derive an association between FOT parameters and exacerbations in COPD patients.[374] The study involved 119 COPD patients stratified into exacerbators (one or more exacerbations over the previous two years; n=37) or non-exacerbators (none for the last two years; n=82). Among the GOLD stage 2 COPD patients, the exacerbators had worse FOT parameters (Ax and X_5, which measure peripheral airway elastic recoil) than the non-exacerbators.[374] However, this finding was not seen among the GOLD stage 1, 3 or 4 patients,[374] which made interpretation difficult. Larger prospective datasets will be required to establish the utility of FOT for predicting future exacerbations in COPD patients and smokers with normal spirometry results.

4.3.5 Incidence of SAD in smokers without COPD

In the Birmingham Early COPD cohort, 14.3% and 26.7% of participants had evidence of SAD based on MMEF and FOT, respectively. The incidence of SAD detected by MMEF had been broadly similar to those reported in a large Chinese cross-sectional study (n=50479).[375] In that study, SAD was defined by the presence of at least two out of three indicators (<65% predicted of either MMEF, forced expiratory flow at 50% FVC ($FEF_{50\%}$) or $FEF_{75\%}$). The incidence rate of SAD among current smokers (n=11631) was reported as 11.4% post-bronchodilation[375], which is consistent with the data reported in this thesis.

Direct comparisons of SAD incidence using FOT are difficult due to the different parameters used in the literature to define SAD. In a single-centre study conducted in Spain, FOT was used to assess the incidence of SAD in smokers or ex-smokers with stable ischaemic heart disease (n=118).[376] In that study, the incidence of SAD was found to be 33.0%, and SAD was defined as R_5 and R_{5-20} above the upper limit of normal in a cohort of patients with normal spirometry (n=94).[376] In another study involving 75 asymptomatic smokers with no evidence of airflow obstruction on spirometry and 34 never-smokers, SAD was defined as $R_{5-20,}$ similar to this thesis using a value of >0.07kPa/L/s.[377] 14.6% of asymptomatic smokers in the Spanish study were reported to have SAD using this criterion.[377] However, the study itself only assessed asymptomatic smokers, while the study reported here involved both asymptomatic smokers and smokers with CB, which likely explains the higher incidence of SAD as assessed by FOT found in the Birmingham cohort.

A small number of SAD participants were detected by MMEF and FOT (n=2; 16.7% of SAD participants detected by FOT, and 20% of SAD participants using MMEF). Any meaningful

comparison of demographics between participants with SAD detected by MMEF and FOT with those detected only by MMEF or FOT was not feasible due to the small size. Currently, no studies on COPD patients or non-COPD smokers compare patients diagnosed with SAD using both methods.

Li. et al.'s study involved 209 patients with chronic respiratory symptoms with no evidence of airflow obstruction on spirometry and conducted receiver operator characteristics curve (ROC) analyses of FOT parameters in detecting SAD initially identified by spirometry.[370] In this study, SAD on spirometry was defined as at least two of the three: MMEF, $FEF_{50\%}$, and $FEF_{75\%}$ being less than 65% predicted value, while SAD on FOT was defined as R_{5-20} >0.015kPa/L/s.[370] R_{5-20} was found to have an AUC of 0.646 with a sensitivity of 76.2% and specificity of 47.3%,[370] suggesting poor discrimination between tests.[378] Su et al. adopted another approach by using endobronchial optical coherence tomography (EB-OCT) as the gold standard for small airway disease diagnosis in a cohort of COPD patients (n=59), smokers with preserved lung function (n=26; FEV_1 ≥80% predicted and FEV_1/FVC ≥0.7) and never-smokers (n=21).[379] R_{5-20} had a higher AUC (0.753) compared to MMEF (0.558), but more importantly, a combined approach of spirometry and FOT had the highest AUC of 0.825.[379] This suggests that the combination of FOT and spirometry has better sensitivity and specificity for detecting small airway disease and should be considered for assessment of patients with early COPD.

4.3.6 Significance of SAD in smokers without COPD

Present data show that those with evidence of SAD based on FOT had higher BMI than those without. Studies examining the impact of BMI on lung function have mainly focused on

children and adolescents. In Sweden, a population-based cohort study involving 2889 children between 8 and 16 years old was conducted.[380] In the study, R_{5-20} and Ax were used to assess small airway function, and it was found that overweight people had worse readings on both parameters.[380] A smaller Brazilian study among individuals with obesity (BMI 30.0-39.9kg/m^2; n=13), severe obesity (BMI 40.0-49.9kg/m^2; n=28), morbid obesity (≥50.0kg/m^2) and healthy controls (BMI <30.0kg/m^2; n=31) showed similar results.[381] In that study, the severely or morbidly obese group had higher R_{5-20} values than the control or the obese group despite no significant differences in MMEF.[381] However, this study did not report participants' smoking status, which may be a confounding factor.

No differences were found in symptom burden or history of chest infection between those with 'low MMEF' and those with 'normal MMEF' based on a cut-off value of 80% predicted in the Birmingham cohort. These results were not reflective of those from the study by Stockley et al.[130] where AATD patients with normal FEV_1/FVC ratio but reduced MMEF had worse total health status and a more significant decline in FEV_1 (after at least three years of follow-up) than those with normal MMEF. Although that study included a different group of patients (AATD) to the Early COPD cohort, the difference in results was likely reflective of the lack of power in the Birmingham cohort and the cross-sectional nature of this current analysis.

There was a trend towards older age in the 'low MMEF' group which was similar to that seen in the AATD patients.[130] As lung function data in the current chapter was presented as % predicted, thus accounting for age, the reduced MMEF in some smokers may well relate to the early stages of airway remodelling that precede more significant airflow limitation in COPD.

4.3.7 Data limitations

The study reported in this chapter has several limitations. Notably, only a small proportion of the Birmingham cohort had SAD based on MMEF, and only a proportion had baseline FOT measurements. Thus, the analyses in this chapter will be mainly underpowered. In addition, the COVID-19 pandemic has disrupted access to the lung physiology service and limited data acquisition. For these reasons, only cross-sectional lung function data could be reported and analysed, while longitudinal data has yet to be collected. Follow-up data to detect FEV_1 decline among the participants will be required to determine the importance of SAD identified using both MMEF and FOT for identifying early COPD and the longer-term prognosis of these patients.

4.3.8 Summary

In conclusion, this chapter has provided an analysis of the lung function data as assessed by post-BD spirometry, gas transfer and FOT in the participants from the Birmingham cohort of the national Early COPD cohort. The data reported here have supported the hypothesis that some smokers have evidence of SAD on spirometry or FOT despite having airflow tests in the normal range (FEV_1/FVC >0.7). Furthermore, those with SAD detected by FOT had a higher symptom burden than those without.

Tests related to small airway function may help detect early smoking-related changes that may lead to COPD development, although validation of all tests is required. Tests for SAD (including FOT) have several limitations, as described in section 4.1, and thus it is not feasible for these tests to replace spirometry to diagnose COPD. However, MMEF and FOT may be

helpful adjuncts in assessing smokers in an outpatient or community setting. MMEF and FOT may be central in identifying patients with SAD or early disease at risk of progressing to established disease.

CHAPTER 5 – CHEST COMPUTED

TOMOGRAPHY DENSITOMETRY ANALYSIS

5.1 Introduction

As mentioned in section 1.1.7.1, COPD patients with pulmonary emphysema represent a crucial clinical phenotype. It is consistently shown that COPD patients with severe emphysema have a higher symptom burden,[382 383] exacerbation risk[382 384] and mortality[382 385] than COPD patients with no or mild emphysema. Pathological studies using lung tissue samples from COPD patients have suggested that COPD progresses from small airway disease to emphysematous destruction.[117 118] Koo et al. reported a reduction in the number of small airways in lung tissue samples from COPD patients despite the absence of emphysema. In contrast, the remaining small airways were thickened and/or obstructed.[118] These features involving small airway loss and remodelling in lung tissue not affected by emphysema provide evidence of the progression of small airway disease to emphysematous lesions.

Diagnosing emphysema early in the COPD disease course is vital. CT imaging is widely available and has been used for many years to diagnose emphysema radiologically, providing a non-invasive way of assessing its presence and distribution.[386] Recently, software programmes have allowed quantitative assessment of emphysema severity in CT scans via densitometry analysis.[387] Using these programmes, emphysema can be identified based on the percentage of the lung with low density (where typically -950HU is used as the threshold) or by using the HU threshold below the lowest 15th percentile lung density value is found (Perc15). A detailed explanation of the CT densitometry outputs has been outlined in section 1.2.7.2. Lung CT densitometry has also been shown to have a better correlation with pathological quantification of pulmonary emphysema compared to visual assessment.[388] Thus, lung CT densitometry has been suggested as a method for detecting the pathology of early COPD.

5.1.1 Chapter hypotheses

It was hypothesised that a certain subset of smokers would have abnormalities on CT imaging upon visual inspection that may indicate early COPD disease processes. It was also hypothesised that CT densitometry would help identify smokers with emphysema which is not apparent on visual inspection of CT imaging. These smokers will not necessarily have evidence of airflow obstruction on spirometry but will have worse clinical outcomes with a higher symptom burden and a history of chest infections.

To test these hypotheses, the current chapter had the following aims:

- quantify and describe structural abnormalities found on chest CT imaging among the Birmingham Early COPD cohort
- determine the reliability of the PULMO CMS software in identifying emphysema among the cohort by considering intra-rater and inter-rater reliability
- describe the CT densitometry results among the cohort and compare symptom burden and history of chest infections among smokers with detectable emphysema and those without
- evaluate the agreement between visual assessment of emphysema by a radiologist and qualitative emphysema assessment on CT densitometry
- assess correlations between CT densitometry results and lung function parameters as well as symptom burden

Due to the global COVID-19 pandemic, not all participants in the cohort underwent CT chest scanning due to limited radiological capacity. All methods in this chapter are described in sections 2.5 and 2.7.

5.2 Results

Chest CT scans were performed within six months of recruitment for 33 (47.1%) participants in the Early COPD cohort. Table 5.1 compares demographic features between participants who had undergone chest CT scanning and those who did not. There were no significant differences between the two groups.

5.2.1 Clinical abnormalities in CT scanning

A local thoracic radiologist (DT) reported all chest CT scans performed among the cohort for clinical screening. Eight (24.2%) of the chest CT scans reported abnormalities consistent with early COPD features (air trapping and/or emphysema). Out of the eight scans, three were reported to show emphysema on inspiratory CT scans, while five were reported to show air trapping on expiratory CT scans. All CT scans showing emphysema were reported to be paraseptal and located in the upper lobes. In all scans showing air trapping, it was reported in the lung bases. No participants were found to have co-existing emphysema and air trapping on CT scanning. Figure 5.1 shows typical CT scan images with emphysema and air trapping from the Early COPD cohort.

Two (6.1%) chest CT scans also reported interstitial lung disease. One was consistent with respiratory-bronchiolitis interstitial lung disease (RB-ILD), and the other had features of minor non-specific fibrosis, which was unclassifiable. No participants were found to have co-existing interstitial lung disease and early COPD features. Pulmonary nodules were reported in 13 (39.4%) of reported scans, none of which required further follow-up per Fleischner Society guidelines.[389]

	CT performed (n=33)	CT not performed (n=37)	p-value
Age (years)	34.0 (32.0-40.0)	36.0 (32.5-40.5)	0.61#
Sex, n (% female)	20 (60.6)	23 (62.1)	>0.99+
Smoking history (pack-years)	14.0 (11.0-15.5)	13.0 (11.0-19.0)	0.78#
BMI (kg/m^2)	25.7 (21.6-28.7)	27.0 (22.5-29.9)	0.49*
IMD Decile	3.0 (1.0-5.5)	3.0 (1.0-4.0)	0.36#
Ethnicity, n (%)			
White	25 (75.8)	23 (62.2)	0.39+
Asian/Asian British	6 (18.2)	12 (32.4)	
Black/African/Caribbean	2 (6.1)	2 (5.4)	
Post-BD spirometry			
Post-BD FEV_1 (L)	3.48 (3.19-4.11)	3.48 (2.96-4.11)	0.32#
Post-BD FEV_1 (%predicted)	102.0 (97.0-108.0)	106.0 (95.0-112.0)	0.90*
Post-BD FVC (L)	4.20 (3.72-5.01)	4.19 (3.43-4.98)	0.24#
Post-BD FVC (%predicted)	101.0 (96.5-110.0)	103.0 (93.0-108.5)	0.82*
FEV_1/FVC ratio	0.84 (0.79-0.87)	0.85 (0.80-0.88)	0.76*
MMEF (L)	3.65 (3.27-4.39)	3.88 (3.27-4.46)	0.89*
MMEF (%predicted)	99.0 (86.0-122.0)	109.0 (87.0-127.5)	0.50*
Gas transfer	**n=28**	**n=34**	
TLCO (mmol/min/kPa)	8.25 (6.53-8.91)	7.63 (6.69-8.65)	0.78#
TLCO (%predicted)	78.0 (72.0-88.0)	81.0 (75.-91.0)	0.43*
KCO (mmol/min/kPa/L)	1.40 (1.29-1.55)	1.53 (1.35-1.74)	0.15*
KCO (%predicted)	78.0 (72.0-90.3)	85.0 (75.5-96.0)	0.17*

Table 5.1 – Comparison of baseline demographics and lung function parameters between participants who had chest CT scanning and those who did not

Legend: Continuous data are displayed as median (IQR). Gas transfer results of participants who smoked ≤1 hour before testing were excluded as in section 4.2.3. Statistical differences between the two groups were analysed with the #Mann-Whitney U test, *independent t-test or the +Fisher's exact test. CT: computed tomography; BMI: body mass index; IMD: index of multiple deprivation; Post-BD: post-bronchodilator; FEV_1: forced expiratory volume in the first second; FVC: forced vital capacity; MMEF: maximal mid-expiratory flow; TLCO: transfer capacity for carbon monoxide; KCO: carbon monoxide transfer coefficient

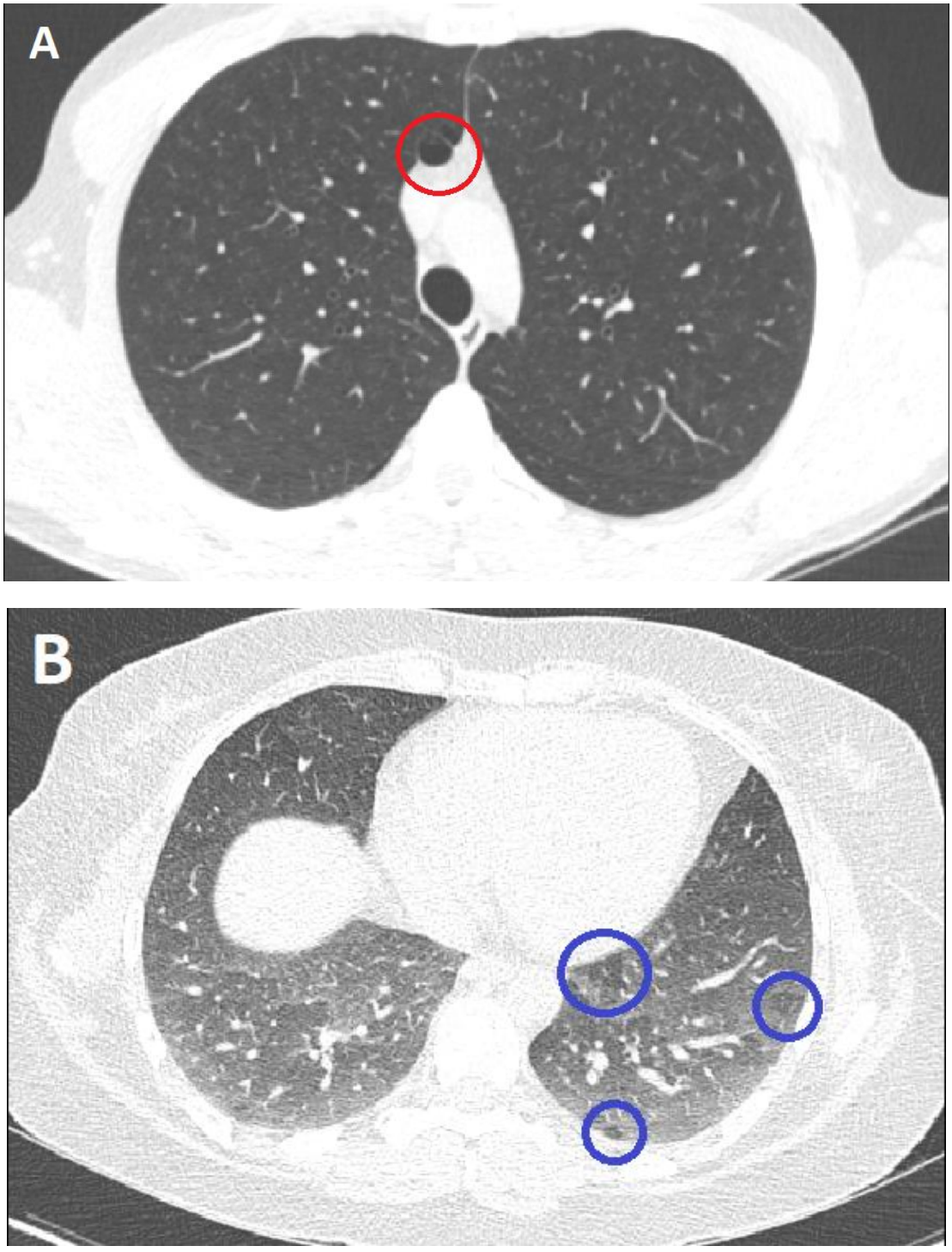


Figure 5.1 – Example of CT chest images showing emphysema (A) and air trapping (B)

Legend: Figure A shows an inspiratory chest CT image of a participant in the Early COPD cohort with paraseptal emphysema (denoted with a red circle). Areas with emphysema appear hypoattenuated compared to surrounding lung parenchyma due to the destruction of alveolar tissue. Figure B shows an expiratory chest CT image in a different participant with air trapping at the lung bases (denoted with blue circles). Air trapping manifests as hypoattenuated areas on expiratory scans due to the retention of excess gas during expiration and can be suggestive of small airways disease.

5.2.2 CT densitometry validation

5.2.2.1 Intraobserver variability

CT densitometry analysis was carried out on eleven CT scans, and the analyses were repeated after three months, as described in section 2.7.3. All ICC results were interpreted using Koo and Li's recommended criteria[124] described in section 2.15. The ICC between two separate readings when the LAA-950% were assessed was 1.0 with a 95% confidence interval (CI) of 1.0-1.0. When evaluated similarly, the ICC for the 15th percentile point (Perc15) was also 1.0 (95% CI 1.0-1.0). Therefore, the expected ICC values of the two CT densitometry parameters showed perfect intra-rater reliability. Figure 5.2 shows the Bland-Altman plots for the two parameters. The plots show no evidence of proportional bias, and apart from one outlier on both plots, all points fall within the 95% limits.

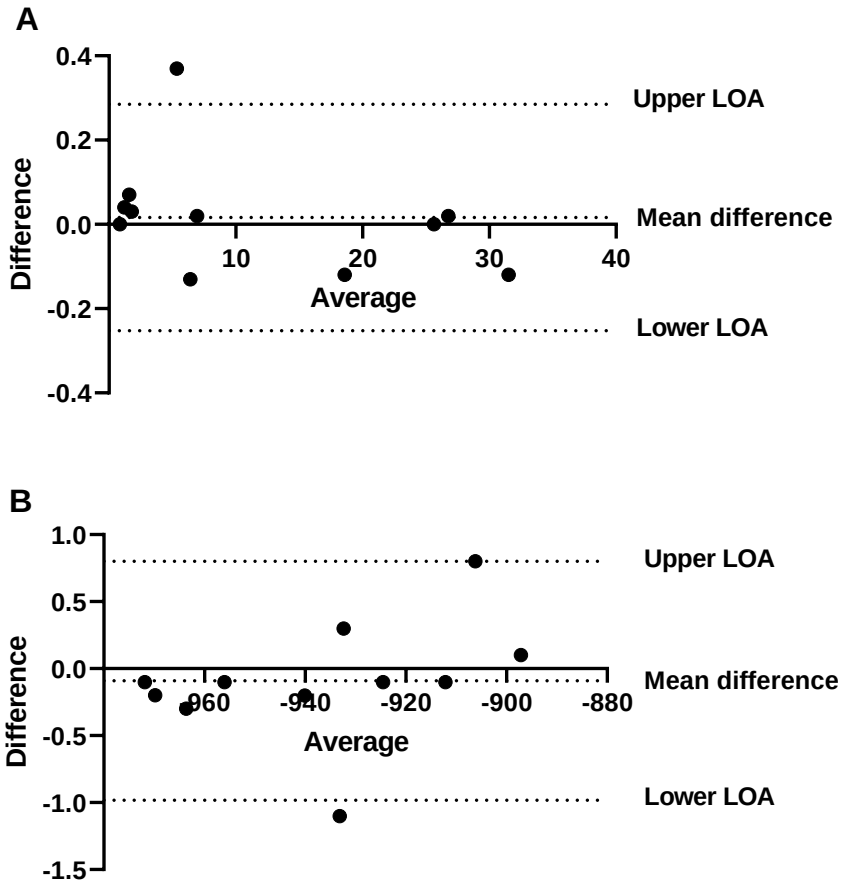


Figure 5.2 – Bland-Altman plots showing intra-rater variability of the CT densitometry parameters

Legend: Bland-Altman plots for the two CT densitometry parameters, LAA-950% (A) and Perc15 (B). The differences between the two separate analyses are plotted against the average of the two analyses. The mean difference, upper and lower LOA, are shown as dotted lines parallel to the x-axis. The upper and lower LOA are calculated as +/-1.96 SD of the mean difference between the two analyses.

5.2.2.2 Inter-observer variability

CT densitometry analysis was performed on eleven CT scans by KPY and then independently reanalysed by a clinical lecturer (DC) who was previously trained in CT densitometry analysis. This analysis was conducted to assess inter-observer variability as described in section 2.7.3. As in section 5.2.2.1, all ICC results in this section were interpreted using Koo and Li's recommended criteria.[124] The ICC for LAA-950% between the two separate analyses by KPY and DC was 1.0 (95% CI 1.0-1.0). The ICC for Perc15, when assessed in the same manner, was also 1.0 (95% CI 1.0-1.0). Therefore, the expected ICC values of the two CT densitometry parameters showed perfect inter-rater reliability. Figure 5.3 shows the Bland-Altman plots for the two parameters. The plots show no evidence of proportional bias, and apart from one outlier on both plots, all points fell within the 95% limits.

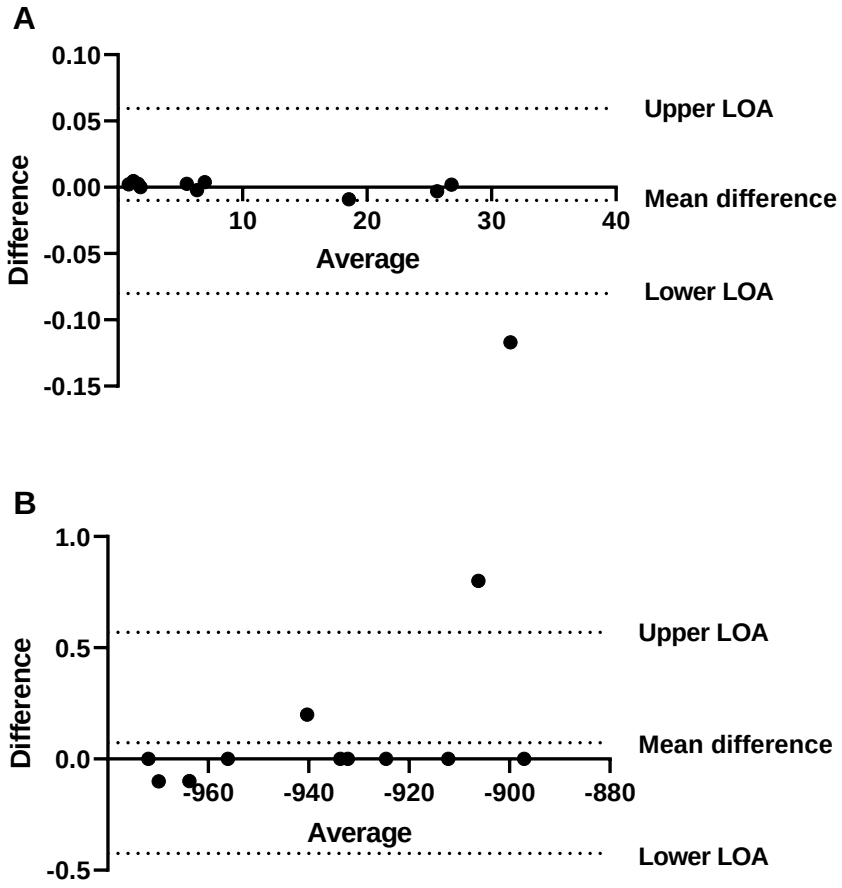


Figure 5.3 – Bland-Altman plots showing inter-rater variability of the CT densitometry parameters

Legend: Bland-Altman plots for the two CT densitometry parameters, namely LAA-950% (A) and Perc15 (B). The analyses differences between two different raters are plotted against the average of the two. The mean difference, upper and lower LOA, are shown as dotted lines parallel to the x-axis. The upper and lower LOA are calculated as +/-1.96 SD of the difference between the two analyses.

5.2.3 CT densitometry in the early COPD cohort

One scan was excluded from CT densitometry analysis due to excessive movement artefact, which made software analysis. All CT densitometry data were listed as median (IQR) unless stated otherwise. The median LAA-950% of the remaining 32 chest CT scans were 1.85% (0.58-3.69) with a median Perc15 of -925.1HU (-931.4 to -909.5). The median craniocaudal locality was -6.9g/L (-15.8 to -2.0). Figure 5.4 show the plots of the different CT densitometry parameters in the cohort.

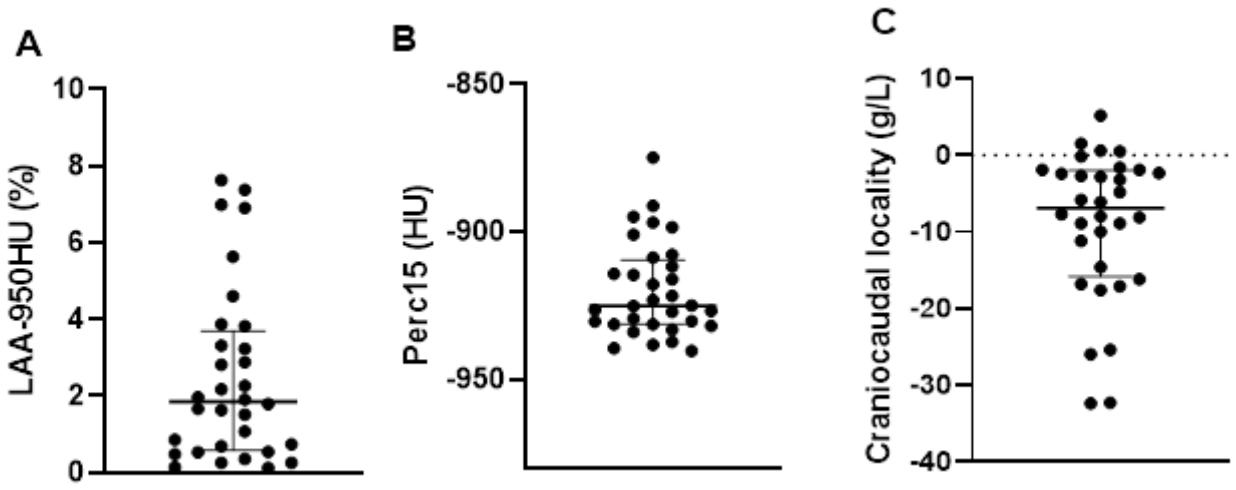


Figure 5.4 – Plot of measured CT densitometry parameters among the Early COPD cohort

Legend: Plots of LAA-950HU% (A), Perc15 (B) and craniocaudal locality (C) among participants who had chest CT scans (n=32). Error bars in the plots represent the median (IQR) of the data. LAA-950HU: low attenuation areas less than a threshold of -950 Hounsfield units; Perc15: 15th percentile point

5.2.4 Comparison between smokers according to emphysema status

According to LAA-950HU% results on chest CT scans, participants were stratified into two groups: those with evidence of emphysema on CT densitometry (Emp+) and those without (Emp-). A threshold of 5% was used for previous studies[382 390] and is discussed later. It was found that five (15.6%) participants had evidence of emphysema on CT densitometry analysis using this threshold.

The Emp- group was found to have a lower FEV_1 (median 4.16 vs 3.35L, p=0.01) and FVC (median 5.23 vs 4.03L, p=0.03) in absolute values, but this was not significant when adjusted for age, sex, height, and race (p=0.35 and p=0.29 for FEV_1 and FVC respectively). The Emp- group also had a higher Perc15 (median -922.5HU vs -938.4HU, p<0.0001) and a higher R_{20} on FOT, suggesting higher large airway resistance (median 90.5% vs 69.5% predicted, p=0.003) than the Emp+ group. Table 5.2 shows the comparison of demographic details, lung function results and CAT scores between the two groups and table 5.3 shows the comparison of FOT and CT densitometry parameters.

In the Emp+ group, two (40.0%) participants reported a chest infection during the 12 months preceding enrolment compared to nine (33.3%) in the Emp- group. This difference in the proportion of participants with previous chest infections was not statistically significant (p=0.77).

	Emp+ (n=5)	Emp- (n=27)	p-value
Age (years)	33.0 (31.0-34.5)	35.0 (32.0-40.0)	0.20[#]
Sex, n (% female)	2 (40.0%)	17 (63.0	0.35[+]
Smoking history (pack-years)	14.0 (10.9-16.5)	14.0 (11.2-15.3)	0.99[#]
BMI (kg/m^2)	29.0 (26.8-29.5)	25.1 (20.8-27.7)	0.26*****
IMD decile	3.0 (1.5-8.5)	4.0 (1.0-5.0)	0.80[#]
Ethnicity, n (%) White Asian/Asian British Black/African/Caribbean	 3 (60.0) 2 (40.0) 0 (0)	 22 (81.5) 3 (11.1) 2 (7.4)	 0.30[+]
Post-BD spirometry Post-BD FEV_1 (L) Post-BD FEV_1 (%predicted) Post-BD FVC (L) Post-BD FVC (%predicted) FEV_1/FVC ratio MMEF (L) MMEF (%predicted)	 4.16 (3.84-4.86) 104.0 (98.5-116.5) 5.23 (4.50-5.71) 110.0 (95.5-118.0) 0.84 (0.82-0.86) 4.53 (3.73-4.80) 100.0 (91.0-125.0)	 3.35 (3.19-3.80) 102.0 (97.0-109.0) 4.03 (3.67-4.70) 101.0 (97.0-108.0) 0.84 (0.79-0.87) 3.43 (3.16-4.25) 99.0 (85.0-121.0)	 **0.01**[#] 0.35***** **0.03**[#] 0.29***** 0.93***** 0.11***** 0.89*****
Gas transfer TLCO (mmol/min/kPa) TLCO (%predicted) KCO (mmol/min/kPa/L) KCO (%predicted)	**n=3** 8.67 (8.39-8.76) 87.0 (78.0-89.0) 1.41 (1.34-1.72) 78.0 (72.0-94.0)	**n=23** 7.28 (6.40-9.15) 75.0 (72.0-88.0) 1.39 (1.28-1.55) 78.0 (69.0-91.0)	 0.33***** 0.39***** 0.70***** 0.68*****
CAT score	12.0 (7.5-19.0)	9.0 (5.0-14.0)	0.47[#]

Table 5.2 – Comparison of baseline demographics, CAT scores, post-BD spirometry and gas transfer parameters between the Emp+ group and the Emp- group

Legend: Continuous data are displayed as median (IQR). Gas transfer results of participants who smoked ≤1 hour before testing were excluded as in section 4.2.3. Statistical differences between the groups were analysed using the [#]Mann-Whitney U test, *****independent t-test or the [+]Fisher's exact test. All significant p-values are in bold. CAT: COPD Assessment Test

	Emp+ (n=5)	Emp- (n=27)	p-value
Resistance parameters	**n=4**	**n=14**	
R_5 (cmH2O/L/s)	2.28 (1.80-3.29)	2.85 (2.45-3.62)	0.23*
R_5 (%predicted)	70.0 (61.3-93.8)	94.0 (85.0-123.0)	0.10*
R_{20} (cmH2O/L/s)	2.15 (1.91-2.58)	2.84 (2.57-3.13)	**0.02***
R_{20} (%predicted)	69.5 (69.0-77.5)	87.0 (81.0-120.0)	**0.003#**
R_{5-20} (cmH2O/L/s)	0.14 (-0.11-0.71)	-0.01 (-0.13-0.49)	0.62*
Reactance parameters			
X_5 (cmH2O/L/s)	-1.10 (-1.13 to -0.95)	-1.16 (-1.36 to -0.78)	0.71*
X_5 (%predicted)	98.5 (88.8-124.8)	98.0 (76.0-122.0)	0.92*
ΔX_5 (cmH2O/L/s)	0.60 (0.32-0.75)	0.45 (0.35-0.57)	0.90*
Ax (cmH2O/L)	3.49 (3.27-4.53)	4.38 (2.48-5.52)	0.64*
CT densitometry parameters			
LAA-950HU (%)	7.00 (6.27-7.51)	1.63 (0.54-2.81)	**<0.0001#**
Perc15 (HU)	-938.4 (-939.9 to -935.7)	-921.8 (-929.5 to -907.8)	**<0.0001#**
Craniocaudal locality (g/L)	-2.4 (-9.5 to -1.8)	-7.7 (-16.8 to -2.3)	0.41*

Table 5.3 – Comparison of FOT and CT densitometry parameters between the Emp+ group and the Emp- group

Legend: Continuous data are displayed as median (IQR). Statistical differences between the groups were analysed using the #Mann-Whitney U test or the *independent t-test. All significant p-values are in bold.

5.2.5 Concordance between the quantitative and visual evaluation of emphysema

Cohen's kappa (κ) was calculated to measure the agreement between quantitative CT percentages of emphysema and visual evidence of emphysema as reported in the clinical report from the radiologist. The κ coefficient was interpreted using Landis and Koch's recommended criteria.[391] A LAA-950HU threshold of 5% was used to differentiate between the presence or absence of emphysema on CT densitometry as in section 5.2.4. Table 5.4 shows the concordance between CT densitometry analysis and visual evaluation of emphysema in chest CT scans by a radiologist for the Early COPD cohort. There was a slight agreement between the two methods, κ = 0.15 (95% CI -0.27 to 0.57).

LAA-950HU (%)	**Visual evidence of emphysema**	
	Yes (n=3)	No (n=29)
≥5% (n=5)	1	4
<5% (n=27)	2	25

Table 5.4 – Concordance of the presence of significant emphysema between CT densitometry analysis and qualitative visual inspection by a radiologist

Legend: LAA-950HU of 5% was set as a threshold to differentiate between the presence or absence of emphysema on CT densitometry analysis. The local thoracic radiologist reported visual absence or presence of emphysema on CT images.

5.2.6 CT densitometry comparison between smoker subtypes

5.2.6.1 Comparison according to the presence of chronic bronchitis

Participants were stratified according to chronic bronchitis (CB) features described in section 2.4.1. CT densitometry parameters of smokers with CB symptoms and asymptomatic smokers (AS) are shown in table 5.5. No significant differences in CT densitometry parameters were found between the two groups.

	CB (n=8)	AS (n=24)	p-value
CT densitometry parameters			
LAA-950HU, %	1.26 (0.32-3.71)	1.85 (0.73-3.69)	0.59
Perc15, HU	-910.3 (-931.1 to -895.8)	-925.8 (-931.8 to -915.0)	0.27
Craniocaudal locality, g/L	-3.0 (-23.3 to -0.23)	-7.9 (-13.8 to -2.0)	0.87

Table 5.5 – Comparison of CT densitometry parameters between smokers with chronic bronchitis (CB) and asymptomatic smokers (AS)

Legend: All data are displayed as median (IQR). Statistical differences between the two groups were analysed using the Mann-Whitney U test.

5.2.6.2 Comparison according to the history of chest infection

Participants were stratified according to the history of chest infection during the preceding 12 months before enrolment. CT densitometry parameters between participants who reported a previous chest infection and those who did not are shown in table 5.3. No significant differences were found in CT density parameters between the two groups.

	Prev LRTI (n=11)	**No prev LRTI (n=21)**	**p-value**
CT densitometry parameters			
LAA-950HU, %	2.26 (0.35-3.87)	1.79 (0.62-3.27)	0.93
Perc15, HU	-926.5 (-933.2 to -901.0)	-923.2 (-931.4 to-911.5)	0.97
Craniocaudal locality, g/L	-3.2 (-17.6 to -1.6)	-7.7 (-15.4 to -2.35)	0.84

Table 5.6 – Comparison of CT densitometry parameters between participants who reported a previous history of chest infection and those who did not

Legend: All data are displayed as median (IQR). Statistical differences between the two groups were analysed using the #Mann-Whitney U test. Prev: previous; LRTI: lower respiratory tract infection

5.2.7 Correlation between CT densitometry parameters

All correlations in this section were assessed using Spearman's correlation coefficient. Significant correlations were found between all three CT densitometry parameters. There was a significant negative correlation between LAA-950HU% and Perc15 (Spearman's rho= -0.95, p<0.0001). Craniocaudal locality was negatively correlated with Perc15 (rho= -0.42, p=0.02) but not with LAA-950HU (p=0.06). Figure 5.5 shows the scatterplots of the correlations described above.

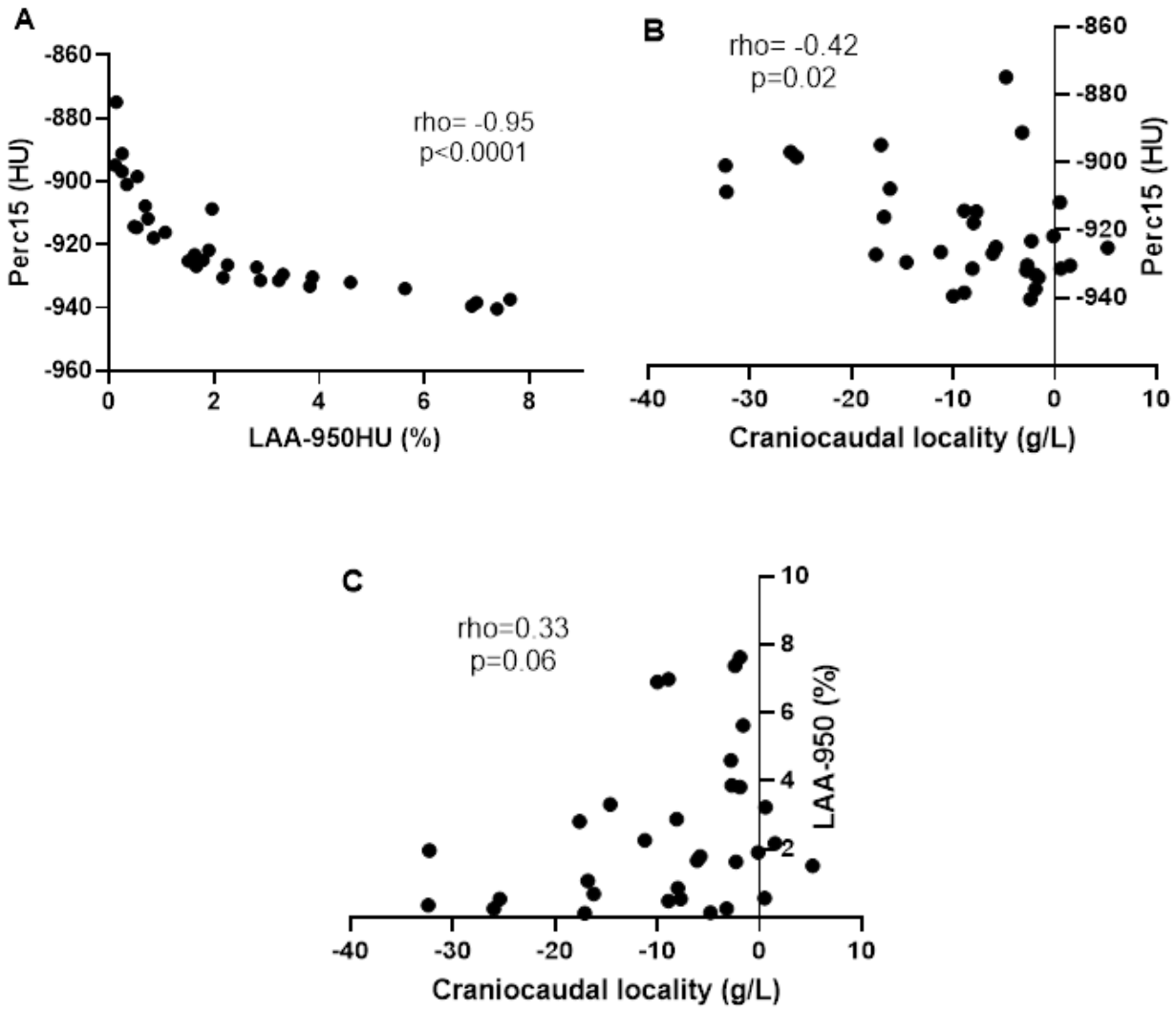


Figure 5.5 – Correlation plots between the CT densitometry parameters

Correlations were assessed using Spearman's correlation coefficient. Thirty-two pairs of data were available for all correlations. Figure A shows the correlation between LAA-950HU% and Perc15, figure B shows the correlation between locality and Perc15, and figure C shows the correlation between locality and LAA-950HU.

5.2.8 Correlation between CT densitometry and other clinical parameters

5.2.8.1 Correlation between CT densitometry and symptom scores

Symptom burden was assessed via quality-of-life questionnaires described in section 2.5, and baseline symptom scores were analysed for all correlations. All correlations in this section were evaluated using Spearman's correlation coefficient. Table 5.7 shows the correlation between CT densitometry parameters and symptom scores at baseline. There were no correlations between the parameters assessed.

5.2.8.2 Correlation between CT densitometry and lung physiology parameters

Lung physiology was assessed using post-bronchodilator spirometry, gas transfer testing and FOT, as described in section 2.6. Baseline results were used for all correlations, which were evaluated using Spearman's correlation coefficient. Thirty-two pairs of data were available for analysing correlation with post-BD spirometry results. Gas transfer results from participants who smoked ≤1 hour before testing were omitted from correlation analysis (as described in section 4.2.3). After omission, 28 pairs of data remained for correlation with gas transfer. When analysing the correlation between CT densitometry and FOT parameters, 19 pairs of data were available. Table 5.8 shows the correlation between baseline CT densitometry and lung function parameters. There were no correlations between the parameters assessed.

	LAA-950HU (%)		Perc15 (HU)	
	Correlation, rho	**p-value**	**Correlation, rho**	**p-value**
mMRC	-0.007 (-0.36 to 0.35)	0.97	0.03 (-0.33 to 0.38)	0.87
LCQ	0.18 (-0.19 to 0.51)	0.32	-0.20 (-0.53 to 0.18)	0.28
CAT	-0.10 (-0.44 to 0.27)	0.60	0.15 (-0.22 to 0.49)	0.40

Table 5.7 – Correlations between CT densitometry parameters (shown in columns) with baseline symptom scores (shown in rows)

Legend: Correlations were assessed using Spearman's correlation coefficient. Spearman's rho was stated together with 95% confidence intervals. Thirty-two pairs of data were available for all correlations. mMRC: modified Medical Research Council dyspnoea scale; LCQ: Leicester Cough Questionnaire

	LAA-950HU (%)		Perc15 (HU)	
	Correlation, rho	**p-value**	**Correlation, rho**	**p-value**
Post-BD spirometry				
Post-BD FEV_1 (%predicted)	-0.17 (-0.50 to 0.20)	0.35	0.15 (-0.21 to 0.49)	0.39
FEV_1/FVC ratio	-0.19 (-0.51 to 0.18)	0.31	0.14 (-0.23 to 0.47)	0.45
MMEF (%predicted)	-0.14 (-0.47 to 0.23)	0.45	0.13 (-0.24 to 0.47)	0.47
KCO (%predicted)	0.20 (-0.20 to 0.54)	0.30	-0.18 (-0.53 to 0.22)	0.36
FOT parameters				
R_5 (%predicted)	-0.29 (-0.66 to 0.21)	0.24	0.16 (-0.33 to 0.58)	0.52
R_{20} (%predicted)	-0.34 (-0.69 to 0.15)	0.16	0.19 (-0.30 to 0.60)	0.44
R_{5-20} (cmH2O/L/s)	-0.007 (-0.47 to 0.46)	0.98	0.15 (-0.34 to 0.58)	0.53
X_5 (%predicted)	-0.18 (-0.59 to 0.32)	0.47	0.15 (-0.34 to 0.58)	0.53
ΔX_5 (cmH2O/L/s)	0.01 (-0.46 to 0.47)	0.97	-0.10 (-0.38 to 0.54)	0.69
Ax (cmH2O/L)	0.08 (-0.40 to 0.53)	0.75	-0.06 (-0.42 to 0.51)	0.81

Table 5.8 – Correlations between CT densitometry parameters (shown in columns) with lung function parameters (shown in rows)

Legend: Correlations were assessed using Spearman's correlation coefficient two-tailed test. Spearman's rho was stated together with 95% confidence intervals. Thirty-two pairs of data were available for correlation with post-BD spirometry measures, 28 for correlation with gas transfer results (KCO %predicted), and 19 for correlation with FOT parameters. FOT: forced oscillometry technique; R_5: resistance at 5Hz; R_{20}: resistance at 20Hz; R_{5-20}: difference between R_5 and R_{20}; X_5: reactance at 5Hz; ΔX_5: difference between inspiratory and expiratory X_5; Ax: reactance area

5.3 Discussion

This chapter has reviewed the CT abnormalities and emphysema severity as assessed by two different techniques – visual assessment by a thoracic radiologist and CT densitometry. Data in this chapter supported the hypothesis that some smokers had evidence of emphysema using both these techniques. Those with emphysema identified using CT densitometry did not have a higher symptom burden or episodes of chest infections than those without emphysema. However, the lack of significant findings might reflect the lack of power associated with the small sample size of the Birmingham Early COPD cohort.

5.3.1 CT abnormalities indicative of early disease in smokers

A significant proportion of smokers in the cohort had abnormalities on CT chest imaging that may indicate early COPD disease processes. This finding is supported by a pilot study of the 102 Early COPD cohort participants in London.[392] In the London cohort, where 42.1% (43/102) of participants demonstrated CT abnormalities (either one or more of visual emphysema, air trapping or bronchial wall thickening), and a majority (88.4%; 38/43) of these were without airflow obstruction on spirometry.[392]

The CT abnormalities found on CT imaging in the Early COPD cohort are likely due to or potentiated by cigarette smoking.[393] These are common incidental findings during lung cancer screening with low-dose chest CT scans. A retrospective review of 320 patients who underwent low-dose CT scanning at the Cleveland Clinic Lung Cancer Screening Programme found that 50.6% of participants had evidence of emphysema, and 39.4% had evidence of

bronchial wall thickening.[394] A prospective study of a large cohort of asymptomatic individuals (n=1929) within NELSON also found a high incidence of emphysema (n=321, 23%) on low-dose CT scanning.[395] Both studies show a higher prevalence of emphysema than in our cohort. However, both studies also involve older patients with a higher smoking history. The participants in the retrospective study described by Morgan et al.[394] had a mean age of 64.4 ± 5.5 years with a mean smoking history of 52.1 ± 19.8 pack-years, while the participants in the NELSON study[395] had a mean age of 59 ± 5.6 years with 65% of them having a smoking history of at least 35 pack-years.

There is also evidence to show that participants without evidence of airflow obstruction on spirometry can have CT abnormalities, which are similar findings to our study. Regan et al. analysed 8980 individuals within the COPDGene study, which included 4388 current or former smokers with normal spirometry results.[10] In the study, 300 scans from the group were inspected, and it was found that 42.3% (n=127) of them were found to have evidence of emphysema or airway disease. Like previous studies, the current or former smokers had a higher mean age (56.7 ± 8.4 years) and smoking history (37.2 ± 20.2 pack-years) than our cohort.

Other CT abnormalities commonly seen among cigarette smokers include pulmonary nodules and interstitial lung disease features, also seen in the Early COPD cohort. Depending on the cut-off size for reporting a pulmonary nodule, these are detected in 20-50% of scanned individuals, with the majority being small and benign, but some will be malignant.[396] RB-ILD is a smoking-related interstitial lung disease that histologically shows excess macrophages filling

the distal airways and alveoli secondary to an immune-mediated response due to smoking. Patients with RB-ILD tend to be asymptomatic and usually regress on smoking cessation.[397]

5.3.2 Reliability of CT densitometry and correlation with visual assessment

For decades, CT has been an established tool for in vivo assessment of pulmonary emphysema. On CT images, pulmonary emphysema appears as lung areas with reduced attenuation, and traditionally visual assessment has been the most common way to detect this in clinical practice. Recently, CT densitometry has been developed to quantify pulmonary emphysema accurately. However, as with FOT, the accuracy, reliability and bias of CT densitometry must be considered before it can be used in either clinical or research settings.

Data presented in section 5.2.2 support the repeatability and reproducibility of CT densitometry as a non-invasive tool for measuring pulmonary emphysema. It demonstrates that a lay clinician or researcher (KPY) could consistently reproduce measurements on CT scans of COPD patients. It was also shown that the same clinician/researcher could produce measurements consistent with those by another trained clinician/researcher (DC). The Bland-Altman plots also do not offer any evidence of systematic bias within repeat measurements by a single rater or measurements taken between two different raters.

Only a slight agreement was found between a radiologist's visual assessment of emphysema and quantitative CT densitometry (using a threshold of >5% LAA-950HU). Similar observations were found in other studies, which showed only slight to fair agreement between visual and quantitative CT analysis.[390 398] Visual assessments and quantitative CT analysis for emphysema

(using a threshold of ≥5%) were performed on 1221 inspiratory CT chest scans from the COPDGene study.[398] It was found that there was a slight to fair agreement between visual assessments by two independent radiologists compared to CT densitometry (κ = 0.16-0.22).[398] A workshop at the American College of Radiology Education Centre to evaluate the concordance between visual assessment and CT densitometry scoring showed similar results.[390] In this workshop, CT scans from non-smokers and smokers with and without COPD were scored by 58 observers using a standardised worksheet, with each scan scored by 9 to 11 observers. There was a fair agreement among observers on the presence of emphysema in CT scans of smokers without COPD (κ = 0.38).

5.3.3 Utility of the different CT densitometry parameters

The relationship between LAA-950HU% and Perc15 was noted to be curvilinear. The differences in the curve gradient in mild emphysema (LAA-950HU% close to zero) and more severe emphysema (LAA-950% further from zero) suggest the difference in sensitivity of these parameters in different emphysema severity. In milder emphysema, the curve is noted to be more vertical, and this would indicate that Perc15 would be more sensitive to changes in lung density than LAA-950HU% at this stage, but the reverse would be true in severe disease.

A similar relationship was seen in the validation study performed by Parr et al.[399] In this study, to validate CT densitometry against FEV_1 decline in AATD patients, 74 patients were grouped according to their COPD disease severity using GOLD criteria[1] and followed up for two years with annual CT imaging and spirometry. The study went on to show that Perc15 is a more consistent measure of lung density change than LAA-950HU% across a broad spectrum of

disease severity and that the relationship between FEV_1 decline and Perc15 was stronger (rho=0.527) than for LAA-950HU% (rho=0.398).[399]

5.3.4 Emphysema presence and distribution on CT densitometry

When assessing CT densitometry in the cohort, an unexpected finding is the data on craniocaudal locality (Figure 5.5C). Conventionally, cigarette smoking is strongly associated with centrilobular emphysema, which usually affects the upper zones of the lungs.[400] However, data presented here suggest that most of the Early COPD cohort had predominantly basal emphysema. However, published data do support this finding. Bakker et al. performed CT densitometry in a cohort of patients with AATD-related COPD (n=50) and a cohort of patients with non-AATD COPD (n=16) to assess the regional progression of emphysema in these patients longitudinally.[290] The study found among the non-AATD COPD patients that, only 18.8% (3/16) had predominantly apical emphysema. However, the craniocaudal locality of the AATD patients was much more strongly negative, indicating a more significant predominance of basal emphysema than the non-AATD COPD patient.[290]

To stratify participants in the Early COPD cohort as smokers with mild emphysema on CT densitometry (Emp-) and those with more significant emphysema (Emp+), a 5% LAA-950HU% threshold was used. Zach et al. reported quantitative CT measures of 92 healthy non-smokers enrolled in the COPDGene study.[401] It was found that the 90th percentile value for LAA-950HU% was approximately 5%.[401] Other studies utilising CT densitometry have subsequently used this threshold.[390 402] A posthoc analysis of two large cohort studies – the SPIROMICS and COPDGene further verified this 5% threshold.[382] In this analysis, when mean exacerbations per

year were plotted against LAA-950HU%, a distinct increase in exacerbation frequency was seen at 5%.[382] This was confirmed when the cohort was split into individuals above and below 5% LAA-950HU. The ≥5% group had higher SGRQ scores in COPDGene (35.1 vs 22.4, p<0.001) and SPIROMICS (39.6 vs 27.1, p<0.001). A higher mean exacerbation frequency was also found in the ≥5% group in both cohorts (COPDGene – 0.69 vs 0.29 exacerbations/year, p=0.03; SPIROMICS – 0.46 vs 0.21 exacerbations/year, p<0.001).

When the 5% threshold was applied in our scanned cohort, it was found that 15.6% had evidence of significant emphysema on CT densitometry. In the Copenhagen Comorbidity in HIV Infection (COCOMO) study, CT densitometry data from 742 HIV-positive individuals were compared to 470 HIV-negative controls.[402] A large majority of individuals in both groups have no evidence of airflow obstruction on spirometry (90.5-92.2%), and it was found that 21.2-24.3% of the cohort had LAA-950HU>5%. This is slightly higher than that found in the Early COPD cohort, but the COCOMO cohort had a higher mean age (54.2-57.4 years) and a higher smoking history (18.0-19.5 pack-years). Apart from a lower R_{20} in the Emp+ group, no differences were found in baseline demographics, lung function results, symptom burden and history of chest infections. The lack of differences in our scanned cohort was most likely due to low participant numbers, especially in the Emp+ group (n=5).

5.3.5 Relationship of CT densitometry with clinical parameters

No statistical differences were found in CT densitometry results when the cohort was stratified according to CB symptoms or a previous chest infection. Although the data in this study is limited by the small number of participants that underwent CT scanning, it has been shown in

some studies that CT densitometry results from smokers with normal spirometry results do not differ significantly from non-smokers.[10 302] Woodruff et al. conducted an observational study involving current and former smokers with normal spirometry results (n=849), current and former smokers with mild-to-moderate COPD (n=963) and non-smoking controls (n=199).[302] In the study, current and former smokers with normal lung function do not have worse emphysema than non-smoking controls, irrespective of symptom burden assessed by CAT.[302] The COPDGene analysis by Regan et al., as described earlier, also showed similar findings.[10]

Although COPD studies show an association between emphysema extent and exacerbation risk,[403 404] this is only consistently seen in patients with severe emphysema. In the study by McAllister et al., 521 COPD participants were studied who were aged ≥60 years with ≥10 pack-year smoking history. It was found that moderate to severe emphysema on visual assessment was associated with acute episodes due to chronic lower respiratory diseases with a RR of 1.89 (95% CI 1.01-3.52).[403] Another study by Han et al. using data from 1002 participants in the COPDGene study also found that increasing emphysema (as defined by LAA-950%) was associated with a significant increase in exacerbation frequency, with a 1.18-fold change with each 5% increase in emphysema.[404] However, this increase in exacerbation frequency was only seen in those with at least 35% emphysema, with no significant changes seen for patients with <10% emphysema and a decrease in exacerbation frequency with 10-35% emphysema.[404] Both studies show that association with exacerbations are more critical with more severe emphysema, unlike the minimal emphysema in our early disease cohort.

There were no correlations between the CT parameters and baseline symptom scores. Other studies have shown a significant relationship between CT parameters and different quality-of-life measures. A systematic review has shown that CT density is consistently associated with SGRQ, especially in multivariate analysis. A meta-analysis was impossible due to the variability in density threshold and the patient groups used.[143] This finding is also similar to that seen in patients with AATD. In a recent initial report of a centralised UK-wide database network involving 187 patients with AATD,[291] it was found that there was a significant relationship between LAA-950HU% and Perc15 with both CAT score and SGRQ. However, the relationships were noted to be weak (r=0.15 to 0.27)[291]

A single-centre study which involved 51 COPD patients also found a significant positive correlation between LAA-950HU% and mMRC dyspnoea scale (r=0.47).[405] Another study involving 115 COPD patients did not find a significant association between the quantified extent of emphysema and clinical parameters (total SGRQ and mMRC).[406] The main difference between that study[406] and previously described studies[143 405] is that the former only involved patients with mild and moderate COPD, whereas the latter also included patients with more severe disease. A possible explanation for these study results is that the association between CT densitometry parameters and symptoms is much weaker or does not exist in patients with early or mild disease. However, it is also likely that the lack of significant correlations between CT densitometry results and symptom scores in current data reflects the low power related to the Birmingham Early COPD cohort.

No significant correlations were also found between CT densitometry parameters and lung function results as assessed by spirometry, gas transfer and FOT. This is likely due to our

study's low number of paired data. Multiple studies have previously established the relationship between CT parameters and spirometry/gas transfer. A systematic review has shown many studies demonstrating a significant correlation between CT densitometry and spirometry/gas transfer parameters.[143] In the subsequent meta-analyses that considered studies using the same CT acquisition parameters, the correlation between LAA-950HU% with FEV_1, FEV_1/FVC and TLCO %predicted were -0.66, 0.53 and 0.69, respectively.[143]

The relationship between CT densitometry results and FOT parameters is less clear. Crim et al. performed CT densitometry and lung function in 233 healthy non-smokers, 322 healthy former smokers and 2054 patients with COPD as part of the ECLIPSE trial.[138] The trial shows that the relationship between LAA-950HU% and R_5, R_{5-20} and Ax was poor (Pearson's r≤0.16)[138] Similar findings were also found in a retrospective analysis of 66 patients with stable COPD within the Korean Obstructive Lung Disease (KOLD) cohort.[407] Apart from R_{20} (r=-0.28, p=0.024), there were no significant correlations found between LAA-950HU% and R_5, X_5, R_{5-20} and Ax in the KOLD cohort.[407]

5.3.6 Data limitations

The data reported in this chapter has several limitations. Firstly, only a small number of participants (n=33) underwent CT scanning, and only a few patients (n=5) were found to have significant emphysema on CT densitometry. The analyses performed are thus underpowered, making it difficult to identify any definitive trends. Secondly, only cross-sectional lung function data were used for comparison due to reduced access to spirometry and gas transfer testing caused by the COVID-19 pandemic. Therefore, comparing CT densitometry data and FEV_1

decline longitudinally was not possible. It would have been essential to assess whether those with evidence of emphysema as defined by CT densitometry would have a more significant FEV_1 decline on follow-up as described by others.[146 172 408]

CT densitometry may help detect smokers with normal spirometry but with emphysema. This non-invasive technique may help identify patients at increased risk of disease progression to COPD, but further longitudinal studies are needed to validate its predictive ability. Several limitations must be overcome before CT densitometry can be adopted into routine care. Although an LAA-950HU>5% threshold was used to define significant emphysema in this chapter, the sensitivity and specificity for exact thresholds of CT abnormalities have yet to be determined. Further efforts are needed to obtain normative values relating to lung density, similar to reference values seen in pulmonary function tests.[409] Furthermore, variations in CT acquisition protocols and quantitative analysis contribute to heterogeneity in study findings.[143] A consensus will need to be reached to standardise CT image acquisition and analysis for future studies.

5.3.7 Summary

In conclusion, this chapter has provided an analysis of the CT chest data (as assessed visually and quantitatively) among the Birmingham participants of the Early COPD cohort. Data in this chapter has shown evidence to support the hypothesis that some smokers have evidence of CT abnormalities when assessed visually (section 5.2.1) and have significant emphysema detected using CT densitometry (section 5.2.4). The hypothesis that smokers with significant

emphysema have increased symptom burden, history of chest infections or lung function abnormalities could not be confirmed.

CHAPTER 6 – NEUTROPHIL DYSFUNCTION AND PHENOTYPE IN EARLY COPD

6.1 Introduction

Neutrophils and proteolytic enzymes are central to the development of chronic obstructive pulmonary disease (COPD) and its progression.[410] It has been shown that airway neutrophil numbers and their secretory products relate to lung function decline as assessed by spirometry and gas transfer, as well as the progression of emphysema measured by CT densitometry longitudinally.[411] [412] However, despite airway neutrophilia, COPD patients frequently demonstrate airway bacterial colonisation (defined by the detection of pathogenic bacterial isolates using culture-based methods in sputum samples), which may affect the frequency of exacerbations.[413] This raises the possibility of impaired neutrophil function, which not only results in reduced antimicrobial function locally but may also precipitate bystander lung tissue damage simultaneously.

As mentioned in section 1.4.4, accurate neutrophil migration is imperative for an effective innate immune response. However, previous work has shown that peripheral neutrophils from COPD patients migrate with reduced directional accuracy towards chemoattractants compared with age-matched healthy control subjects.[237] As neutrophils migrate, they cause obligate tissue damage by releasing serine proteases (such as NE and PR3) contained within azurophilic granules into the extracellular space. Thus, reduced migratory accuracy of neutrophils in COPD may have implications for disease pathology due to the increased area of obligate tissue damage caused by proteinase release during poorly directed migration.[209]

It has also been increasingly shown that in addition to serine proteases, MMPs can degrade various extracellular components and, for this reason, have also been implicated in both pulmonary emphysema development and small airway remodelling[235] [414] as discussed in

section 1.5.2. In particular, MMP-8 and MMP-9 are both contained within the specific and gelatinase granules of neutrophils and are released into the extracellular space upon neutrophil activation[194] and may also have the potential to cause obligate tissue damage during inaccurate neutrophil migration.

There has been emerging interest in the concept of differing neutrophil phenotypes, but there are few studies on COPD and none in smokers in the early COPD phase. Proteomic profiling of peripheral blood neutrophils from COPD patients has shown two distinct clusters despite no differences in symptoms or lung function between the two COPD patient groups. However, neutrophils from one of the clusters demonstrated higher ROS production than neutrophils from the other.[415] These results suggest that COPD patients may be characterised by subtle differences in inflammatory responses that classical markers cannot identify. Identifying neutrophil phenotypes has broader implications in a disease setting (including COPD) to guide future therapies.[416] For example, can a neutrophil phenotype be more pathologically detrimental, and if so, can it be modified to prevent or limit disease progression?

6.1.1 Chapter hypotheses

It was hypothesised that a certain subset of smokers might have evidence of inaccurate neutrophil migration, which could contribute to the early disease process in COPD. These individuals would have evidence of increased NE and PR3 activity and increased levels of MMP-8 and MMP-9 detectable in plasma. It was also hypothesised that fundamental differences in neutrophil phenotypes exist between this subset of smokers and other smokers within the cohort, especially regarding activation status and senescence.

To test these hypotheses, this chapter had the following aims:

- To assess neutrophil migratory dynamics in a group of participants within the Early COPD cohort as stratified by symptoms (chronic bronchitis)
- To assess neutrophil activity indirectly *in vivo* by quantifying footprints of NE and PR3 activity as well as MMP levels in plasma
- To describe differences in neutrophil phenotypes among participants in the cohort by measuring neutrophil surface expression of receptors and ligands

All methods in this chapter were described in sections 2.8 to 2.14. Studies of this nature have not been previously conducted, and the number of patients required to power such investigations appropriately was unknown. Investigations were considered pilot studies that could provide data to perform power calculations for future studies.

6.2 Results

6.2.1 Validation of chemoattractant choice

As part of assay validation, RPMI-1640 was used as the vehicle control as this was the diluent for the study. To validate interleukin-8 (CXCL8) or fMLP as chemoattractants for isolated neutrophils in the assay, neutrophil migration parameters were compared using vehicle control and 100nM CXCL8 or 10nM fMLP in cells from six healthy individuals. These individuals were 26-32 years old and were lifelong non-smokers with no known history of respiratory illnesses.

There was a significant increase in neutrophil speed (median 2.99 (IQR 2.80-3.70) vs 4.76 (IQR 4.34-5.10) μm/min, p=0.03), velocity (median 0.17 (IQR -0.06-0.54) vs 1.77 (IQR 1.22-2.22) μm/min, p=0.03) and chemotactic index (median 0.04 (IQR -0.02-0.10) vs 0.32 (IQR 0.17-0.37), p=0.03) when CXCL8 was used as the chemoattractant compared to vehicle control. A similar significant increase was also seen in neutrophil speed (median 5.29 (IQR 4.85-5.60) μm/min, p=0.03), velocity (median 1.60 (IQR 1.27-1.80) μm/min, p=0.03) and chemotactic index (median 0.28 (IQR 0.24-0.33), p=0.03) when fMLP was used as the chemoattractant compared to vehicle control. Figure 6.1 shows an example of neutrophil migratory pathways with vehicle control and CXCL8 as the chemoattractant, while figure 6.2 shows the neutrophil migration parameters between vehicle control and CXCL8/fMLP.

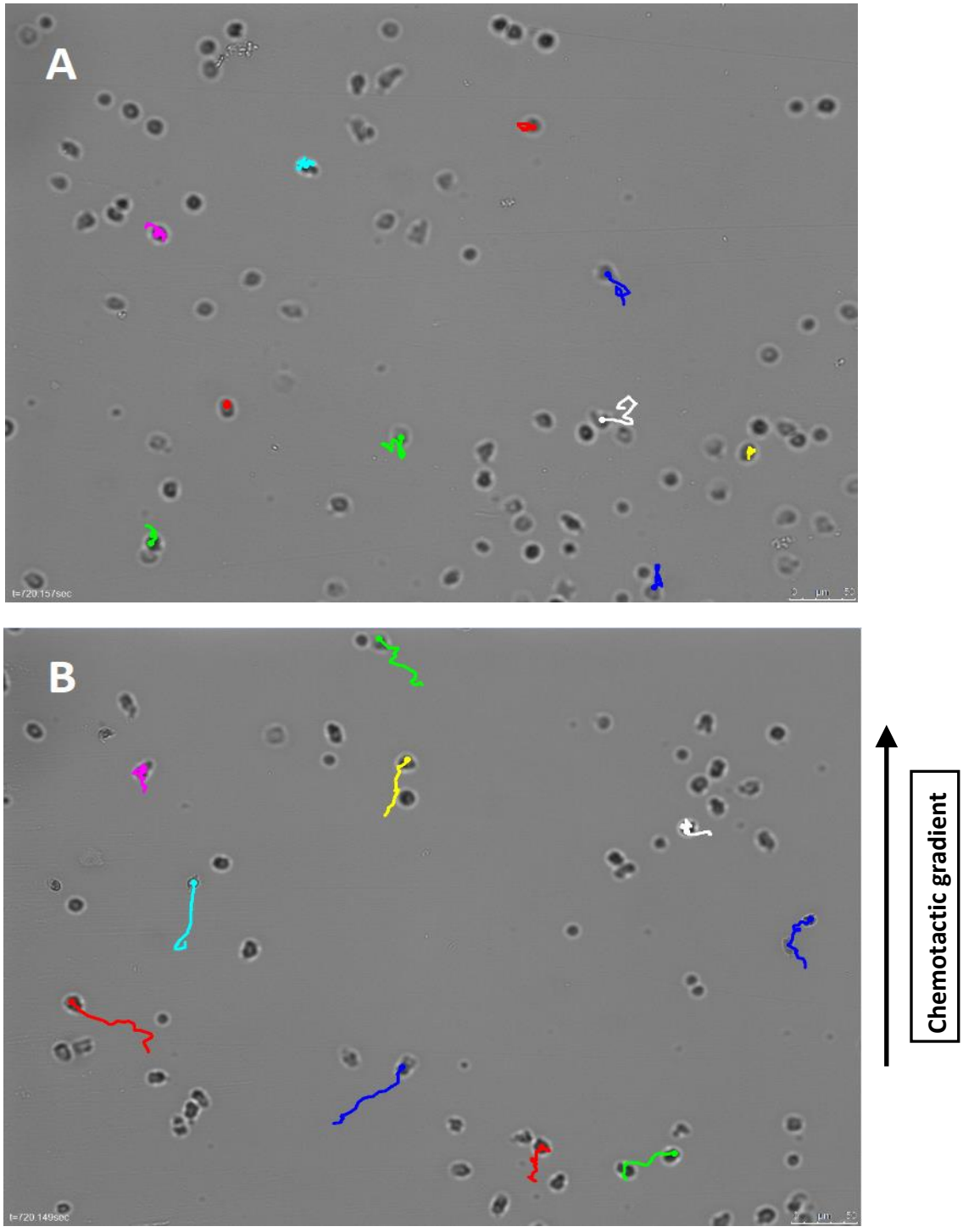


Figure 6.1 – Representative migratory pathways of isolated neutrophils from a healthy non-smoker

Legend: Migratory pathways of neutrophils using RPMI-1640 as vehicle control (A) and 100nM CXCL8 (B) were shown. The chemotactic gradient is illustrated by an arrow in figure B from low (bottom) to high (top). Peripheral neutrophils shown were isolated from the same individual. The coloured tracks indicate the path of migration for each cell analysed. RPMI = Roswell Park Memorial Institute; CXCL8: interleukin-8

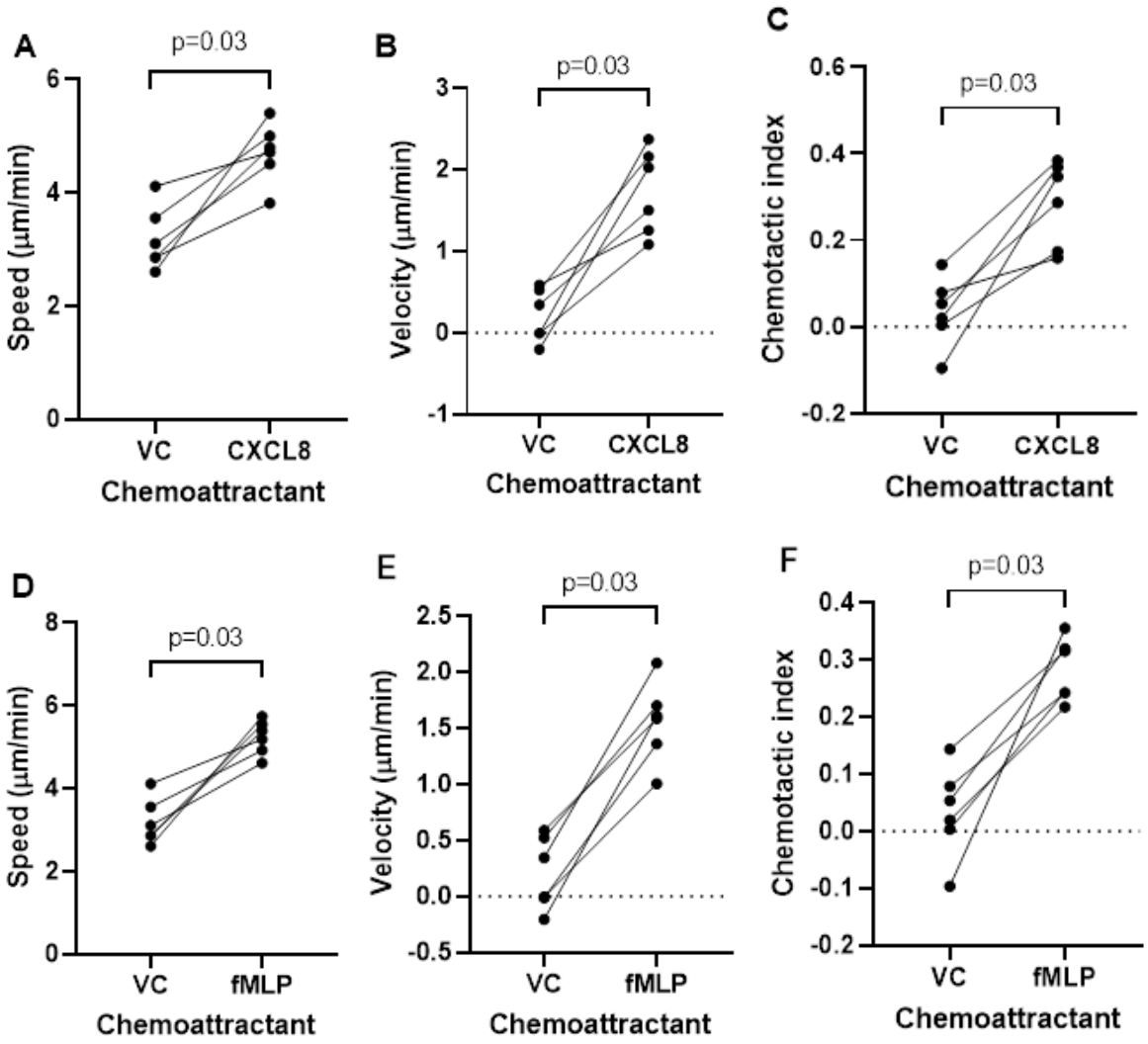


Figure 6.2 – Comparison of neutrophil migration parameters using vehicle control compared to CXCL8 or fMLP as the chemoattractant

Legend: Migration parameters of isolated neutrophils were assessed consecutively using vehicle control (VC) and either 100nM CXCL8 (figure A, B and C) or 10nM fMLP (figure D, E and F) as chemoattractant. Six pairs of data were available for comparison. All comparisons of neutrophil migration parameters were made using the Wilcoxon signed-rank test.

6.2.2 Neutrophil migration

Peripheral neutrophils were isolated from blood samples obtained from 22 participants of the Early COPD cohort, with 11 reporting symptoms of chronic bronchitis (CB) and 11 who were asymptomatic smokers (AS). As a control group, peripheral neutrophils were also isolated from age-matched healthy non-smoker controls (HNS) and age-matched subjects with established COPD (the latter data provided by ES, who assessed neutrophil migration using the same methods). For all studies, neutrophil migration was assessed using the assay described in section 2.10.1 and analysed as described in section 2.10.2. All neutrophil migration parameters are listed as mean ± SD unless stated otherwise. Table 6.1 compares demographic details and basic lung function parameters between the groups. Apart from the expected worse values of post-bronchodilator (BD) spirometry parameters in the COPD group, there were no significant differences between the four groups.

	CB (n=11)	AS (n=11)	COPD (n=10)	HNS (n=10)	p-value
Age (years)	39.0 (32.0-40.0)	37.0 (32.0-42.0)	42.5 (40.5-43.3)	35.5 (33.5-37.5)	0.08[#]
Sex, n (% female)	8 (72.7)	5 (45.5)	4 (40.0)	6 (60.0)	0.44[+]
Smoking history (pack-years)	14.0 (10.8-24.0)	14.0 (11.0-18.0)	14.0 (12.0-17.0)	0 (0-0)	0.91[#]
Post-BD spirometry					
Post-BD FEV_1 (L)	3.37 ± 0.69	3.79 ± 0.66	2.92 ± 0.64	N/A	**0.02***
Post-BD FEV_1 (%predicted)	103.4 ± 8.4	102.0 ± 12.6	79.9 ± 12.1	N/A	**<0.0001***
FEV_1/FVC ratio	0.82 ± 0.06	0.81 ± 0.06	0.65 ± 0.07	N/A	**<0.0001***

Table 6.1 – Comparison of baseline demographics and basic lung function parameters between the CB group, AS group and HNS group.

Legend: Data in the post-BD spirometry section are displayed as mean ± SD, while age and smoking history are expressed as median (IQR). Statistical differences between the groups were analysed using the *one-way ANOVA test with Tukey's comparison test between groups, the [#]Kruskal-Wallis test with Dunn's comparison test between groups or the [+]Fisher's exact test. The HNS group were not included in comparative analyses for the smoking history and post-BD spirometry parameters. All significant p-values are in bold. CB: chronic bronchitis; AS: asymptomatic smokers; HNS: healthy non-smokers; Post-BD: post-bronchodilator; FEV_1: forced expiratory volume in the first second; FVC: forced vital capacity

6.2.2.1 Comparison of neutrophil migration between Early COPD cohort participants and healthy non-smokers

Neutrophil migration for the Early COPD participants was initially compared to HNS. When 100nM CXCL8 was used as the chemoattractant, no differences were found in the neutrophil migration speed between the CB, AS and HNS groups (p=0.25). However, peripheral neutrophils from the CB group were found to migrate with a decreased velocity compared with those from AS (0.78 ± 0.36 vs 1.72 ± 0.59μm/min, p=0.0002) and HNS groups (1.68 ± 0.43μm/min, p=0.0004). There was no difference in neutrophil migration velocity between the AS and HNS groups (p=0.98). Neutrophils from CB smokers were also found to have a significantly lower chemotactic index compared to neutrophils from the AS group (0.162 ± 0.050 vs 0.342 ± 0.066, p<0.0001) and the HNS group (0.276 ± 0.082, p=0.001). There were no significant differences in chemotactic index between neutrophils from the AS and HNS groups (p=0.08). Figure 6.3 compares neutrophil migration parameters between the groups using 100nM CXCL8.

When fMLP was used as the chemoattractant, the two groups found no differences between the neutrophil migration speed (p=0.20) and chemotactic index (p=0.08). There was a stepwise reduction in neutrophil migration velocity from the HNS group (1.65 ± 0.29μm/min) to the AS group (1.45 ± 0.65μm/min) and CB group (1.02 ± 0.77μm/min), but this did not reach statistical significance (p=0.07). Figure 6.4 compares neutrophil migration parameters between the groups using 10nM fMLP.

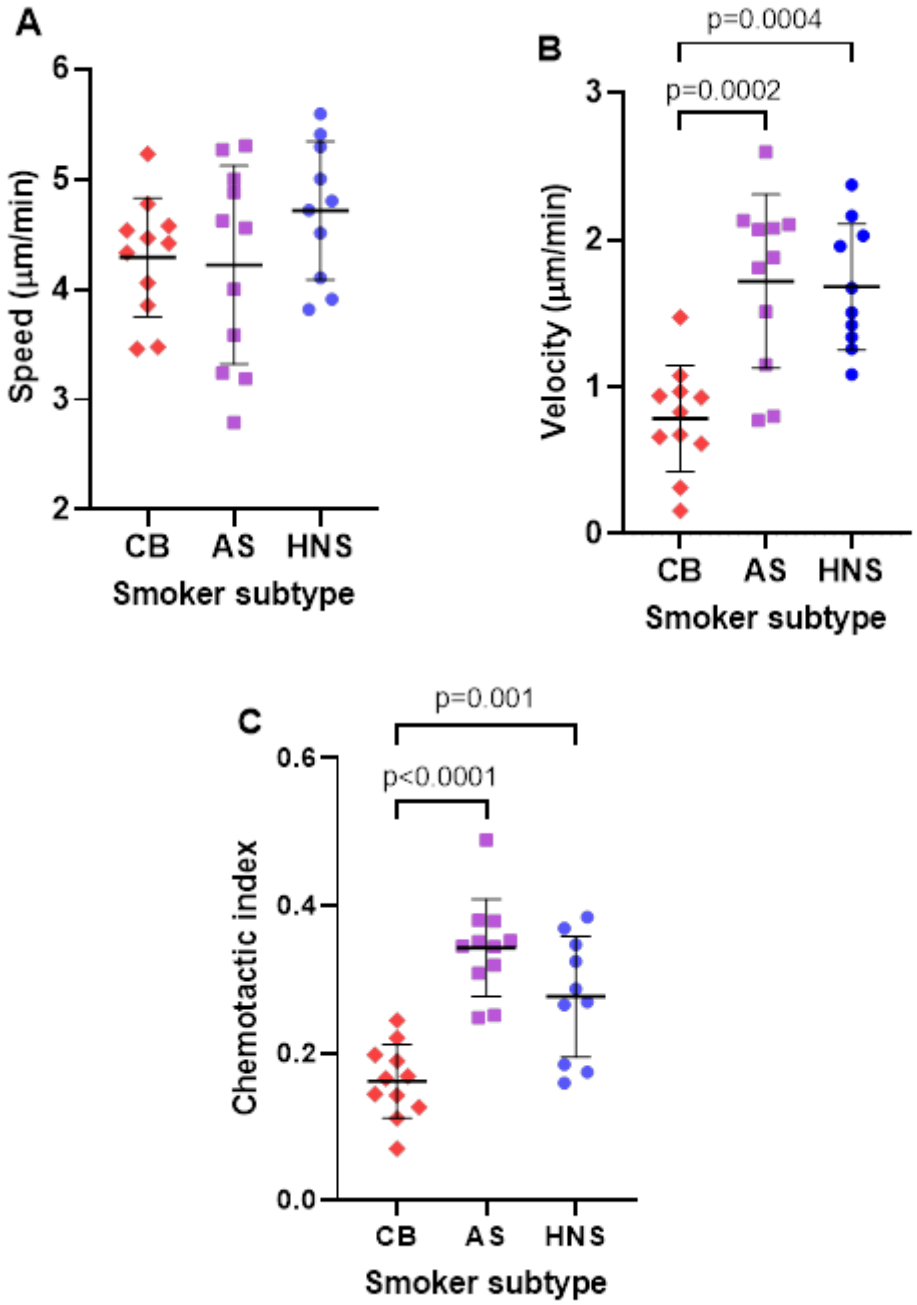


Figure 6.3 – Plots of neutrophil migration parameters using CXCL8 as the chemoattractant

Legend: Plots of neutrophil speed (A), neutrophil velocity (B) and chemotactic index (C) using 100nM CXCL8 as chemoattractant. Error bars in the plots represent the mean ± SD of the data. Comparisons were made using the one-way ANOVA test with Tukey's comparison test between groups. *Statistically significant difference in comparison to AS and HNS groups

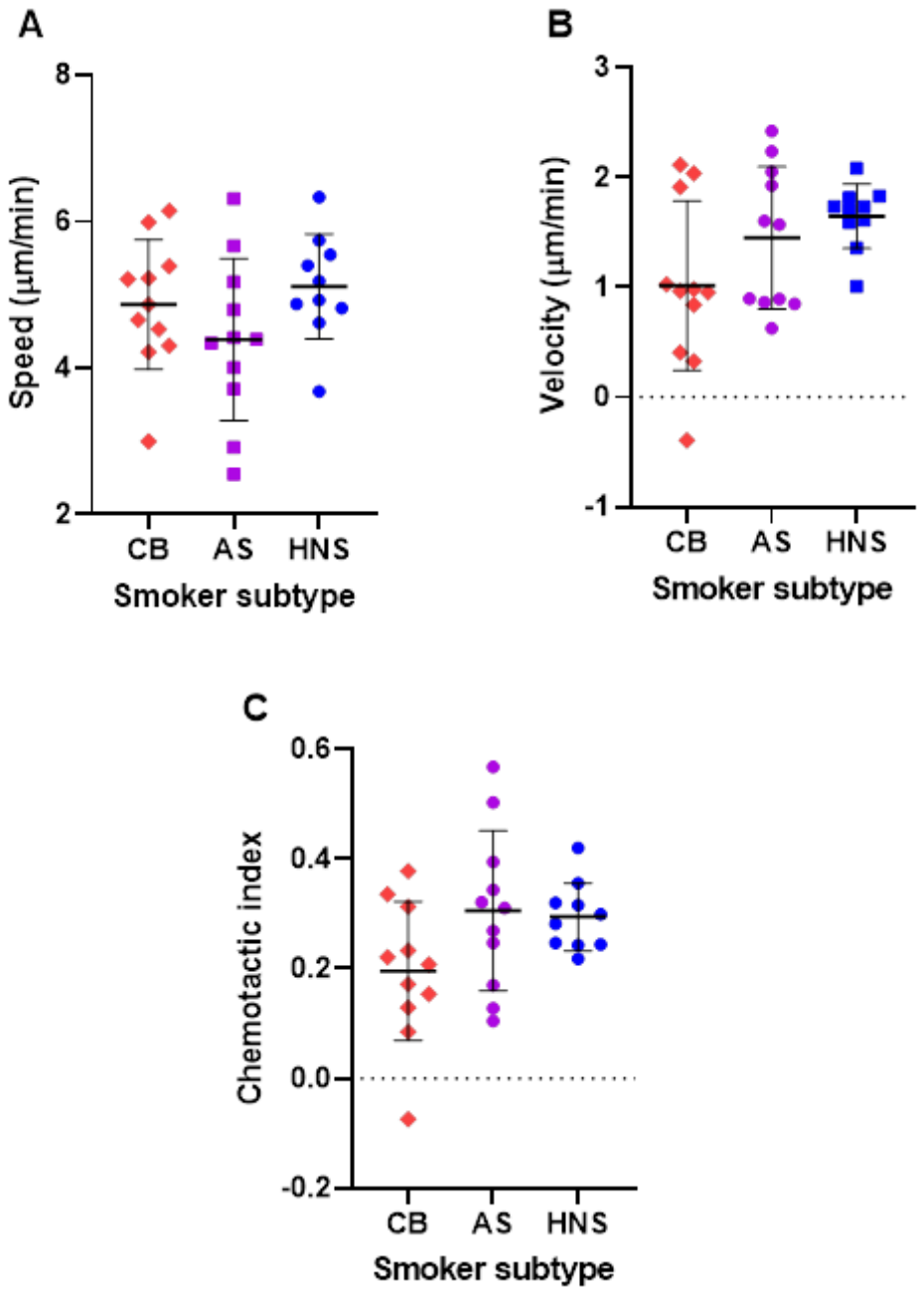


Figure 6.4 – Plots of neutrophil migration parameters using fMLP as the chemoattractant

Legend: Plots of neutrophil speed (A), neutrophil velocity (B) and chemotactic index (C) using 10nM fMLP as chemoattractant. Error bars in the plots represent the mean ± SD of the data. Comparisons were made using the one-way ANOVA test with Tukey's comparison test between groups.

6.2.2.2 Comparison of neutrophil migration between Early COPD cohort participants and patients with COPD

Neutrophils from Early COPD participants were then compared to previous data from patients with established COPD. When 100nM CXCL8 was used as the chemoattractant, peripheral neutrophils from the CB (4.29 ± 0.54μm/min) and AS groups (4.22 ± 0.90 μm/min) both migrated with a decreased speed compared to those from the COPD group (5.55 ± 0.84μm/min, p=0.002 and p=0.001 compared to CB and AS groups respectively). However, neutrophils from the AS group were found to migrate with an increased velocity and had a higher chemotactic index compared to those from the COPD group (velocity: 1.72 ± 0.59 vs 0.56 ± 0.44μm/min, p<0.0001; chemotactic index: 0.34 ± 0.07 vs 0.18 ± 0.11, p=0.0002). There were no differences in neutrophil migration velocity and chemotactic index between the CB and COPD groups (p=0.54 and p=0.79, respectively). Figure 6.5 compares neutrophil migration parameters between the groups using 100nM CXCL8.

When 10nM fMLP was used as the chemoattractant, AS neutrophils had a lower migration speed (4.39 ± 1.11 vs 5.86 ± 0.59μm/min, p=0.005) and higher chemotactic index (0.31 ± 0.15 vs 0.13 ± 0.13, p=0.02) compared to those from the COPD group. Peripheral neutrophils from the CB (1.02 ± 0.77μm/min) and AS groups (1.45 ± 0.65μm/min) both migrated with an increased velocity compared to COPD neutrophils (0.09 ± 0.43μm/min, p=0.007 and p=0.0001 compared to CB and AS groups respectively). There were no differences in neutrophil migration speed and chemotactic index between the CB and COPD groups (p=0.08 and p=0.53, respectively). Figure 6.6 compares neutrophil migration parameters between the groups using 10nM fMLP.

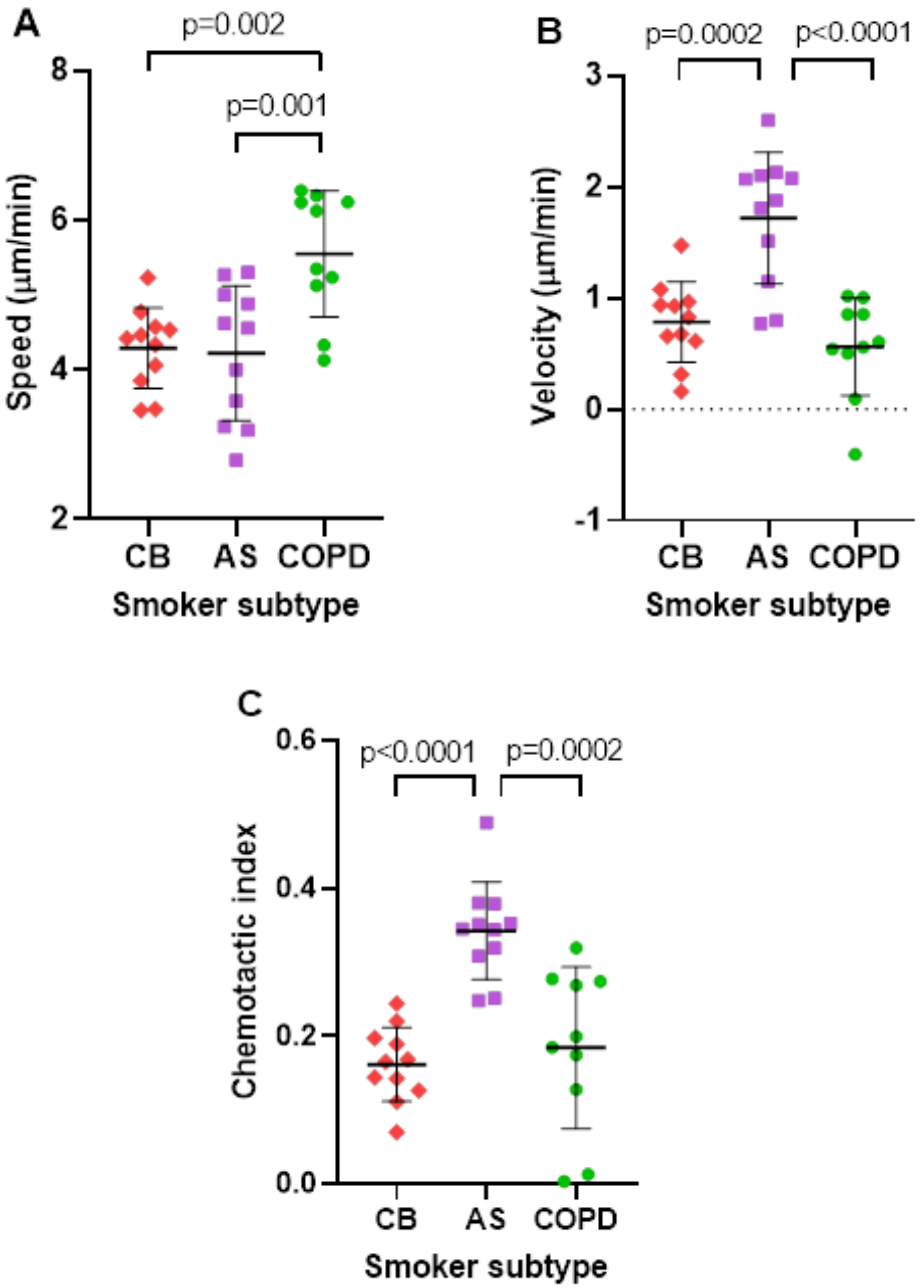


Figure 6. 5 – Plots of neutrophil migration parameters using CXCL8 as the chemoattractant

Legend: Plots of neutrophil speed (A), neutrophil velocity (B) and chemotactic index (C) using CXCL8 as chemoattractant. Error bars in the plots represent the mean ± SD of the data. Comparisons were made using the one-way ANOVA test with Tukey's comparison test between groups. COPD: chronic obstructive pulmonary disease

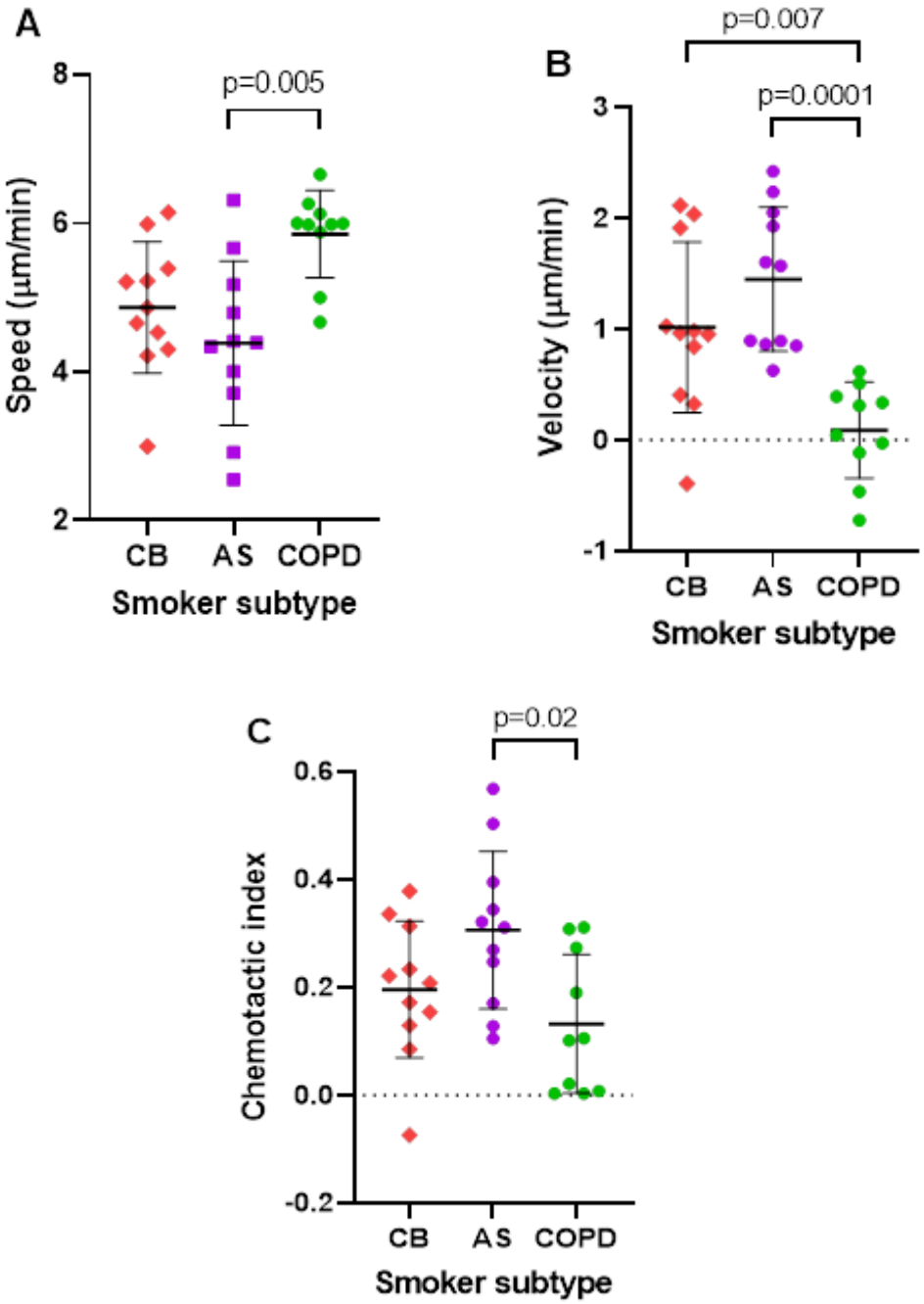


Figure 6.6 – Plots of neutrophil migration parameters using fMLP as the chemoattractant

Legend: Plots of neutrophil speed (A), neutrophil velocity (B) and chemotactic index (C) using fMLP as chemoattractant. Error bars in the plots represent the mean ± SD of the data. Comparisons were made using the one-way ANOVA test with Tukey's comparison test between groups.

6.2.3 Neutrophil degranulation – NE and PR3

6.2.3.1 Participant demographics

NE and PR3 footprint activity were determined in plasma samples obtained from Early COPD cohort participants and age-matched HNS individuals. They were quantified using ELISA, as detailed in section 2.13. All continuous data in this section are listed as median (IQR) unless stated otherwise. Table 6.2 compares demographic details and basic lung function parameters for those who had NE activity quantified, and Table 6.3 shows the same comparisons for those who had PR3 activity quantified. There were no significant differences in demographic details and lung function parameters between the CB and AS groups.

	CB (n=18)	AS (n=18)	HNS (n=19)	p-value
Age (years)	36.5 (32.0-40.3)	34.0 (32.0-41.0)	35.0 (33.0-37.0)	0.59*
Sex, n (% female)	12 (66.7)	11 (61.1)	10 (52.6)	>0.99^{+}
Smoking history (pack-years)	14.0 (11.0-20.3)	12.6 (11.2-14.0)	0 (0-0)	0.18$^{\#}$
Post-BD spirometry				
Post-BD FEV_1 (L)	3.35 ± 0.60	3.58 ± 0.54	N/A	0.23$^{\wedge}$
Post-BD FEV_1 (%predicted)	101.3 ± 11.0	102.4 ± 8.07	N/A	0.75$^{\wedge}$
FEV_1/FVC ratio	0.84 ± 0.06	0.84 ± 0.06	N/A	0.89$^{\wedge}$

Table 6.2 – Comparison of baseline demographics and basic lung function parameters for participants who had NE footprint activity quantified in plasma samples

Legend: Continuous data are displayed as median (IQR) apart from post-BD spirometry parameters, which are expressed as mean ± SD. Statistical differences between the groups were analysed using the *Kruskal-Wallis test with Dunn's comparison test between groups, $^{\#}$Mann-Whitney U test, $^{\wedge}$independent t-test or the $^{+}$Fisher's exact test. Only the CB and AS groups were compared for the smoking history and post-BD spirometry parameters.

	CB (n=19)	AS (n=18)	HNS (n=18)	p-value
Age (years)	36.0 (33.0-40.0)	33.5 (32.0-41.0)	35.0 (33.8-37.3)	0.38*
Sex, n (% female)	11 (57.9)	11 (61.1)	11 (61.1)	>0.99^{+}
Smoking history (pack-years)	14.0 (11.0-24.0)	12.6 (11.0-14.0)	0 (0-0)	0.16$^{\#}$
Post-BD spirometry				
Post-BD FEV_1 (L)	3.49 ± 0.68	3.53 ± 0.61	N/A	0.83$^{\wedge}$
Post-BD FEV_1, %predicted	99.7 ± 9.9	100.9 ± 9.4	N/A	0.71$^{\wedge}$
FEV_1/FVC ratio	0.83 ± 0.05	0.84 ± 0.06	N/A	0.76$^{\wedge}$

Table 6.3 – Comparison of baseline demographics and basic lung function parameters for participants who had PR3 footprint activity quantified in plasma samples

Legend: Continuous data are displayed as median (IQR) apart from post-BD spirometry parameters, which are shown as mean ± SD. Statistical differences between the groups were analysed using the *Kruskal-Wallis test with Dunn's comparison test between groups, $^{\#}$Mann-Whitney U test, $^{\wedge}$independent t-test or the $^{+}$Fisher's exact test. Only the CB and AS groups were compared for the smoking history and post-BD spirometry parameters.

6.2.3.2 NE footprint activity between smoker subtypes

As described in section 2.13, NE footprint activity was determined in plasma samples by quantifying the NE-specific fibrinogen cleavage product present, Aα-Val^{360}. There were no significant differences in Aα-Val^{360} plasma levels among the CB, AS and HNS groups (p=0.44). Figure 6.7 shows the plot comparing plasma Aα-Val^{360} levels among the different groups.

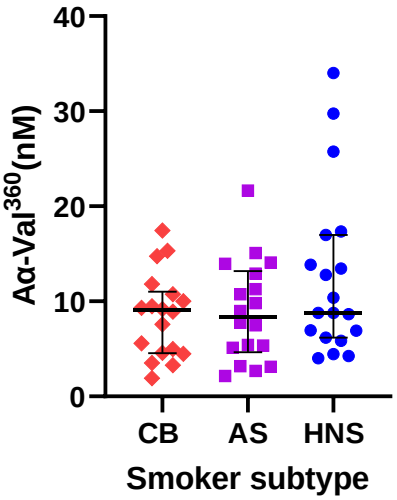


Figure 6.7 – Plots of NE activity marker

Legend: Plots of Aα-Val^{360} levels in plasma samples. Aα-Val^{360} levels were quantified in plasma samples using ELISA. Error bars in the plots represent the median (IQR) of the data. n=18 for the CB group, n=18 for AS group and n=19 for the HNS group. Comparisons were made using the Kruskal-Wallis test with Dunn's comparison test between groups.

6.2.3.2 PR3 footprint activity between smoker subtype

PR3 footprint activity was determined in plasma samples by quantifying the amount of PR3-specific fibrinogen cleavage product, Aα-Val^{541}, as described in section 2.13. There were no differences in plasma Aα-Val^{541} levels between the three groups (p=0.08). Figure 6.8 shows the plot comparing plasma Aα-Val^{541} levels for the different groups.

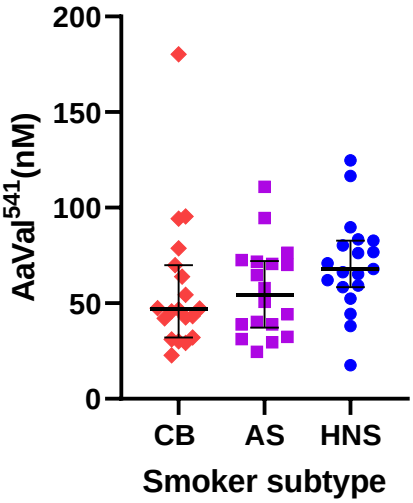


Figure 6.8 – Plots of PR3 footprint activity marker

Legend: Plots of Aα-Val^{541} levels in plasma samples. Aα-Val^{541} levels were quantified in plasma samples using indirect ELISA. Error bars in the plots represent the median (IQR) of the data. n=19 for the CB group, n=18 for AS group and n=19 for the HNS group. Comparisons were made using the Kruskal-Wallis test with Dunn's comparison test between groups.

6.2.3.3 Key correlation with clinical parameters

Correlation analyses were performed to assess the relationship between NE/PR3 footprint activity and key clinical parameters in the Early COPD cohort. Key clinical parameters assessed include pulmonary function tests (post-BD spirometry and gas transfer testing), CAT score and CT densitometry output (as described in Chapter 5). Baseline pulmonary function test results and CAT scores were used for all correlations and assessed using Spearman's two-tailed correlation coefficient.

There was no significant relationship between plasma Aα-Val^{360} levels and any of the key clinical parameters assessed or between plasma Aα-Val^{541} levels and any of the key clinical parameters assessed. Table 6.4 shows the correlations between NE footprint activity and key clinical parameters, while table 6.5 shows the same results with PR3 footprint activity.

	Correlation, rho	95% CI	p-value
FEV_1 (%predicted)	-0.29	-0.56 to 0.05	0.08
FEV_1/FVC ratio	0.28	-0.06 to 0.56	0.09
MMEF (%predicted)	0.21	-0.13 to 0.51	0.22
KCO (%predicted)	0.08	-0.29 to 0.42	0.67
CAT score	0.08	-0.26 to 0.40	0.64
LAA-950HU (%)	0.09	-0.37 to 0.51	0.70
Perc15 (HU)	-0.01	-0.45 to 0.43	0.96

Table 6.4 – Correlations between NE footprint activity with key clinical parameters

Legend: Correlations were assessed using Spearman's correlation coefficient two-tailed test. Spearman's rho was stated together with 95% CI. n=36 for FEV_1, FEV1/FVC ratio, MMEF and CAT score correlations, n=31 for KCO correlation and n=21 for LAA-950HU% and Perc15 correlations. KCO: carbon monoxide transfer coefficient; CAT: COPD Assessment Test; LAA-950HU: low attenuation areas less than a threshold of -950 Hounsfield units; Perc15: 15^{th} percentile point; HU: Hounsfield unit

	Correlation, rho	95% CI	p-value
FEV_1 (%predicted)	-0.05	-0.37 to 0.29	0.76
FEV_1/FVC ratio	-0.03	-0.36 to 0.30	0.87
MMEF (%predicted)	-0.04	-0.37 to 0.29	0.80
KCO (%predicted)	-0.24	-0.56 to 0.13	0.19
CAT score	-0.0009	-0.33 to 0.33	>0.99
LAA-950HU (%)	-0.24	-0.63 to 0.26	0.33
Perc15 (HU)	0.33	-0.16 to 0.69	0.16

Table 6.5 – Correlations between PR3 footprint activity with key clinical parameters

Legend: Correlations were assessed using Spearman's correlation coefficient two-tailed test. Spearman's rho was stated together with 95% CI. n=37 for FEV_1, FEV_1/FVC ratio, MMEF and CAT score correlations, n=30 for KCO correlation and n=19 for LAA-950HU% and Perc15 correlations.

6.2.4 Neutrophil degranulation – MMPs

6.2.4.1 Participant demographics

MMP-8 and MMP-9 concentrations were determined in plasma samples obtained from Early COPD cohort participants and age-matched HNS individuals. Analytes were quantified using Quantikine ELISA kits (Bio-techne, Minnesota, USA), as detailed in section 2.14. All continuous data in this section are listed as median (IQR) unless stated otherwise. Table 6.6 compares demographic details and basic lung function parameters for those who had plasma MMP-8 and MMP-9 quantified. There were no significant differences found between the CB and the AS group.

	CB (n=10)	**AS (n=12)**	**HNS (n=12)**	**p-value**
Age (years)	38.0 (32.0-40.3)	34.0 (32.0-40.3)	34.0 (33.3-39.5)	0.67*
Sex, n (% female)	7 (70.0)	9 (75.0)	8 (66.7)	>0.99^{+}
Smoking history (pack-years)	14.9 (10.7-25.0)	13.4 (11.1-15.0)	0 (0-0)	0.41$^{\#}$
Post-BD spirometry				
Post-BD FEV_1 (L)	3.52 ± 0.60	3.50 ± 0.57	N/A	0.91$^{\wedge}$
Post-BD FEV_1 (%predicted)	106.0 ± 8.1	100.4 ± 8.4	N/A	0.13$^{\wedge}$
FEV_1/FVC ratio	0.84 ± 0.06	0.82 ± 0.06	N/A	0.48$^{\wedge}$

Table 6.6 – Comparison of baseline demographics and basic lung function parameters for participants who had MMPs quantified in plasma samples

Legend: Continuous data are displayed as median (IQR) apart from post-BD spirometry parameters, which are expressed as mean ± SD. Statistical differences between the groups were analysed using the *Kruskal-Wallis test with Dunn's comparison test between groups, $^{\#}$Mann-Whitney U test, $^{\wedge}$independent t-test or the $^{+}$Fisher's exact test. Only the CB and AS groups were compared in the smoking history and post-BD spirometry parameters.

6.2.4.2 Plasma MMP concentrations between smoker subtype

No difference was seen in plasma MMP-8 concentration among the CB, AS and HNS groups (p=0.94). There was also no difference in plasma MMP-9 concentration among the three groups (p=0.78). Figure 6.9 shows the plot comparing plasma MMP-8 levels, and figure 6.10 shows the plot comparing plasma MMP-9 levels among the different groups.

6.2.4.3 Key correlation with clinical parameters

Correlation analyses were performed to assess the relationship between plasma MMP-8 and MMP-9 levels with key clinical parameters in the Early COPD cohort. Key clinical parameters assessed include pulmonary function tests (post-BD spirometry and gas transfer testing), CAT score and CT densitometry output (as described in Chapter 5). Baseline pulmonary function test results and CAT scores were used for all correlations and assessed using Spearman's two-tailed correlation coefficient.

No relationships were found between plasma MMP-8 and any key clinical parameters. There were also no relationships between plasma MMP-9 levels and any key clinical parameters assessed. Table 6.7 shows the correlations between plasma MMP-8 concentration and key clinical parameters, while table 6.8 shows the same results with plasma MMP-9 concentration.

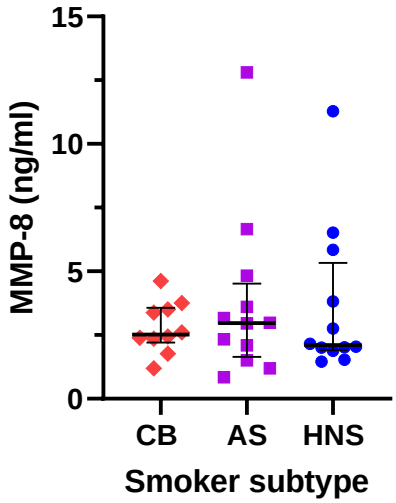


Figure 6.9 – Plots of plasma MMP-8 concentration

Legend: Plots of MMP-8 levels in plasma samples. MMP-8 levels were quantified in plasma samples using ELISA. Error bars in the plots represent the median (IQR) of the data. Comparisons were made using the one-way ANOVA test with Tukey's comparison test between groups. MMP: matrix metalloproteinase

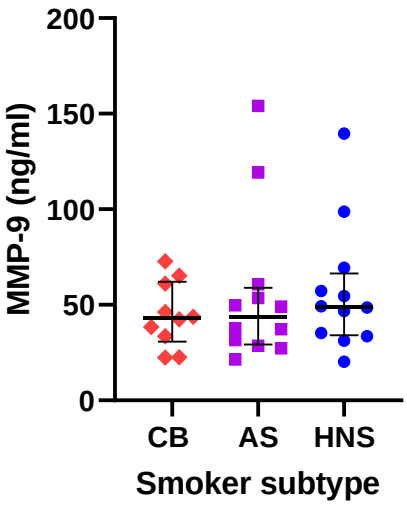


Figure 6.10 – Plots of plasma MMP-9 concentration

Legend: Plots of MMP-9 levels in plasma samples. MMP-9 levels were quantified in plasma samples using ELISA. Error bars in the plots represent the median (IQR) of the data. Comparisons were made using the one-way ANOVA test with Tukey's comparison test between groups.

	Correlation, rho	95% CI	p-value
FEV_1 (%predicted)	0.13	-0.32 to 0.53	0.56
FEV_1/FVC ratio	0.04	-0.40 to 0.46	0.87
MMEF (%predicted)	-0.14	-0.54 to 0.31	0.53
KCO (%predicted)	-0.007	-0.50 to 0.49	0.98
CAT score	-0.19	-0.58 to 0.26	0.40
LAA-950HU (%)	-0.29	-0.72 to 0.30	0.32
Perc15 (HU)	0.40	-0.18 to 0.78	0.16

Table 6.7 – Correlations between plasma MMP-8 levels with key clinical parameters

Legend: Correlations were assessed using Spearman's correlation coefficient two-tailed test. Spearman's rho was stated together with 95% CI. n=22 for FEV_1, FEV_1/FVC ratio, MMEF and CAT score correlations, n=17 for KCO correlation and n=14 for LAA-950HU% and Perc15 correlations.

	Correlation, rho	95% CI	p-value
FEV_1 (%predicted)	0.21	-0.24 to 0.59	0.34
FEV_1/FVC ratio	-0.17	-0.56 to 0.29	0.46
MMEF (%predicted)	-0.26	0.63 to 0.19	0.23
KCO (%predicted)	0.10	-0.42 to 0.56	0.71
CAT score	-0.19	-0.57 to 0.27	0.40
LAA-950HU (%)	-0.37	-0.76 to 0.21	0.19
Perc15 (HU)	0.45	-0.12 to 0.80	0.10

Table 6.8 – Correlations between plasma MMP-8 levels with key clinical parameters

Legend: Correlations were assessed using Spearman's correlation coefficient two-tailed test. Spearman's rho was stated together with 95% CI. n=22 for FEV_1, FEV_1/FVC ratio, MMEF and CAT score correlations, n=17 for KCO correlation and n=14 for LAA-950HU% and Perc15 correlations.

6.2.5 Neutrophil phenotyping by surface marker expression

The expression of cell surface markers from isolated neutrophils was assessed using flow cytometry over two panels, as described in section 2.11. Cell surface marker expression was conveyed as MFI and listed as median (IQR) unless stated otherwise. These markers were split into groups for analysis to assess neutrophil status, namely activation state (CD11b, CD66b and CD62L); senescence (CXCR2 and CXCR4); inflammatory status (HLA-DR, PD-L1 and CD11c); reverse transmigration (CD54) and neutrophil maturity (CD10 and CD16). The reasoning for selecting and grouping these markers was explained in section 1.6.

6.2.5.1 Sample quality control

All isolated neutrophil samples were measured for viability, and phenotype analysis was performed only on live cells. Neutrophil viability was assessed using annexin V and 7AAD, and the gating strategy for live cells has been explained in section 2.11.2. Samples containing <25% viable neutrophils were excluded from further analysis due to low cell numbers for analysis and variations in the fluorescence intensity readings. The rationale for using 25% as the cut-off was explained by a previous PhD student in the group.[295] Using this viability criterion, seven samples (five from the Early COPD cohort and two HNS) were excluded from further analysis. The remaining samples were deemed to have accurate fluorescence readings from a large enough sample of live neutrophils for phenotyping.

As shown in table 2.4, two of the surface markers assessed were present on both antibody panels (CD11b and CD10), allowing inter-plate variations to be evaluated. There was a significantly strong relationship between MFIs for both markers over the two panels. The

Pearson's correlation coefficient, *r* for CD11b between the two panels, was 0.85 (95% confidence interval (CI) 0.75-0.92, p<0.0001), and *r* for CD10 was 0.94 (95% CI 0.90-0.97, p<0.0001). These indicate that there was minimal MFI variation between the two panels. Figure 6.11 shows the scatterplots for both markers over the two panels.

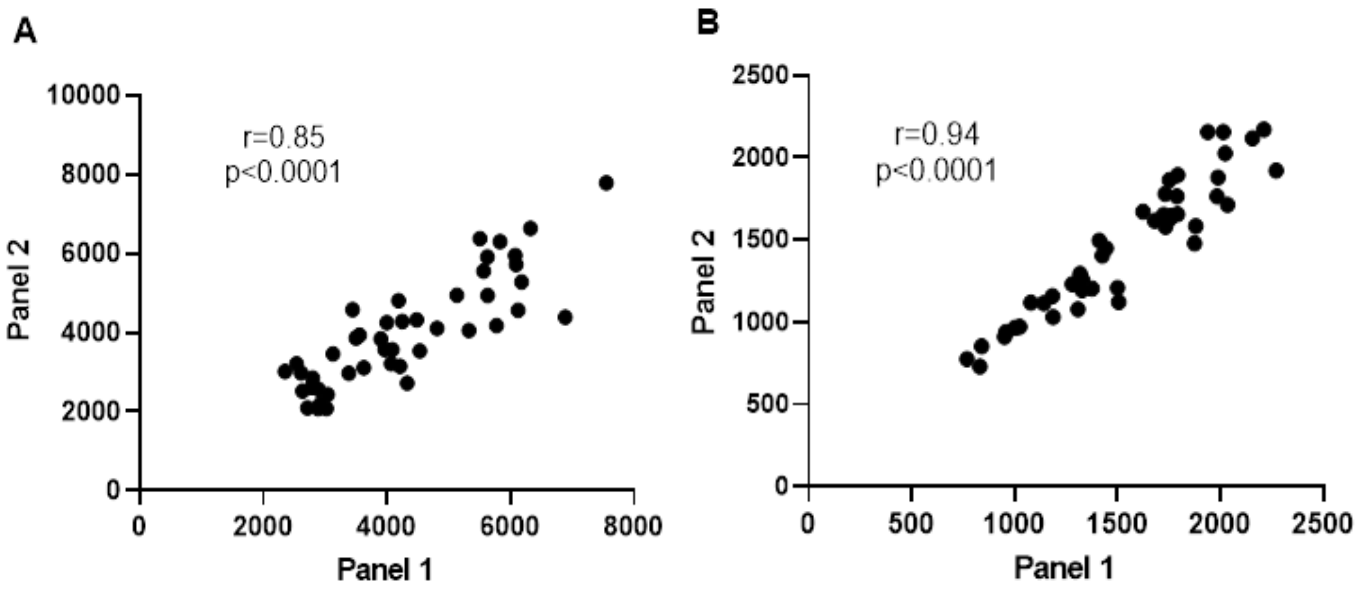


Figure 6.11 – Correlation plots of CD11b (A) and CD10 (B) between the two flow cytometry panels

Legend: CD11b and CD10 expression were assessed on isolated neutrophils on two antibody panels using MFI. Correlations on CD11b and CD10 MFI readings in both panels were assessed using Pearson's correlation coefficient two-tailed test. Forty-four pairs of data were available for both correlations. Figure A: r=0.85, p<0.0001; Figure B: r=0.94, p<0.0001

6.2.5.2 Participant demographics

Peripheral neutrophils were isolated, and cell surface marker expression was measured in 34 participants from the Early COPD cohort, including 17 each from the CB and the AS groups and 17 HNS individuals. As mentioned in section 6.2.4.1, seven samples were excluded from the final analysis. Table 6.9 compares demographic details and basic lung function parameters between participant groups included in the final analysis. There were no significant differences found between the three groups.

	CB (n=14)	**AS (n=15)**	**HNS (n=15)**	**p-value**
Age (years)	39.0 (35.8-44.0)	33.0 (31.0-41.0)	34.0 (33.0-37.0)	0.06*
Sex, n (% female)	8 (57.1)	10 (66.6)	9 (60.0)	0.93^{+}
Smoking history (pack-years)	14.0 (11.8-20.0)	11.5 (11.0-18.0)	0 (0-0)	0.41$^{\#}$
Post-BD spirometry				
Post-BD FEV_1 (L)	3.41 ± 0.74	3.44 ± 0.60	N/A	0.59$^{\wedge}$
Post-BD FEV_1 (%predicted)	98.9 ± 12.6	105.1 ± 11.1	N/A	0.17$^{\wedge}$
FEV_1/FVC ratio	0.80 ± 0.10	0.83 ± 0.06	N/A	0.45$^{\wedge}$

Table 6.9 – Comparison of baseline demographics and basic lung function parameters between the CB, AS, and HNS groups.

Legend: Continuous data are displayed as median (IQR) apart from post-BD spirometry parameters, which are expressed as mean ± SD. Statistical differences between the groups were analysed using the *Kruskal-Wallis test with Dunn's comparison test between groups, $^{\#}$Mann-Whitney U test, $^{\wedge}$independent t-test or the $^{+}$Fisher's exact test. Only the CB and AS groups were compared in the smoking history and post-BD spirometry parameters.

6.2.5.3 Neutrophil activation status between smoker subtypes

There were no differences in the expression of CD62L, CD66b or CD11b in peripheral neutrophils isolated from participants in the CB, AS, or HNS groups (p=0.97). Figure 6.12 shows the plots comparing the cell surface marker expression among the different groups. A positive correlation between CD11b and CD66b was observed (*r*=0.43, p=0.004), supporting CD11b and CD66b double-positive events to identify neutrophil activation. Figure 6.13 shows the scatterplot for the correlation between CD11b and CD66b.

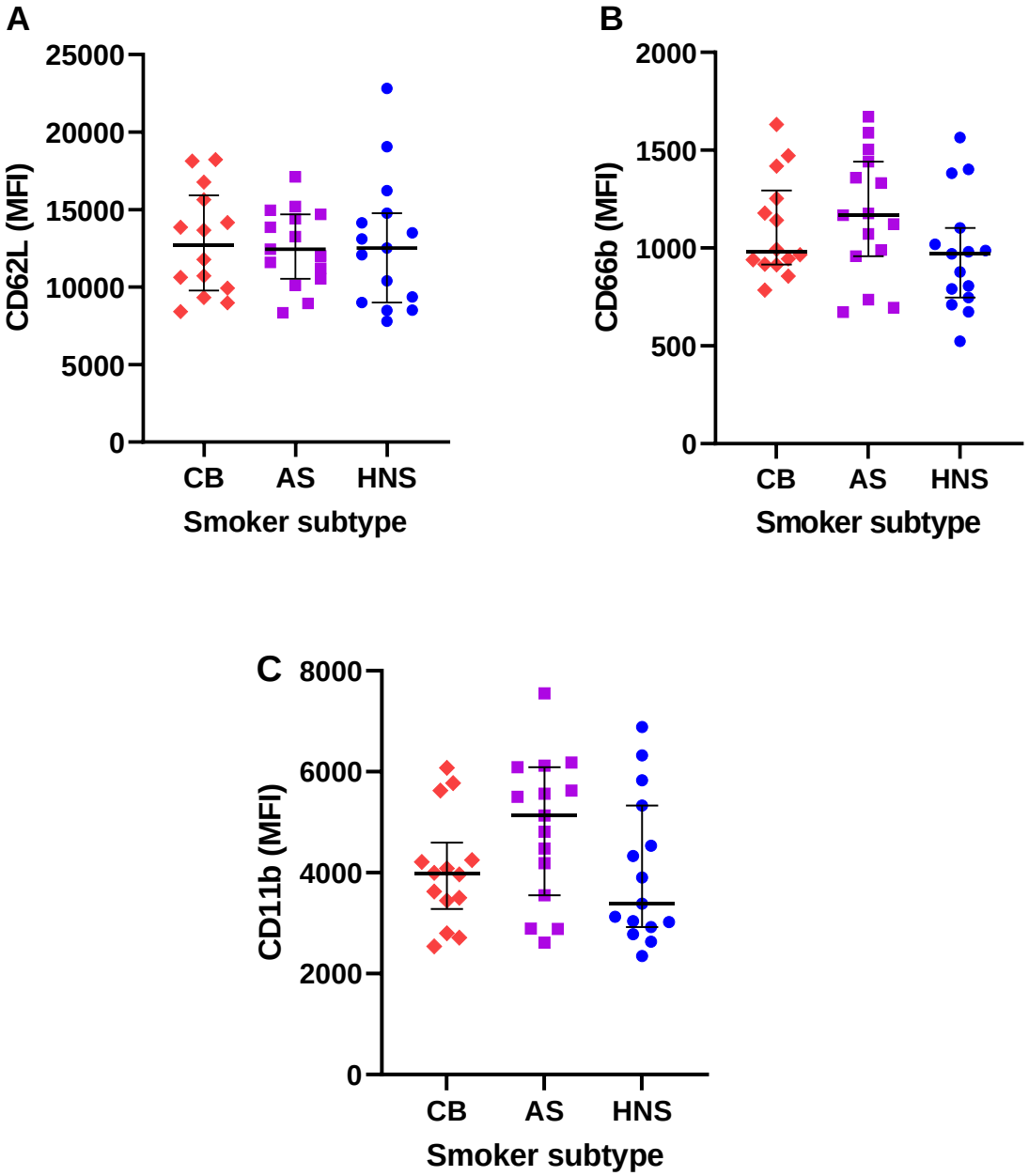


Figure 6.12 – Plots of neutrophil surface activation marker expression

Legend: Plots of CD62L (A), CD66b (B) and CD11b (C) expression on isolated peripheral neutrophils from whole blood. Neutrophils were stained with antibodies, and the MFI was measured via flow cytometry. Error bars in the plots represent the median (IQR) of the data. Comparisons were made using the one-way ANOVA test with Tukey's comparison test between groups.

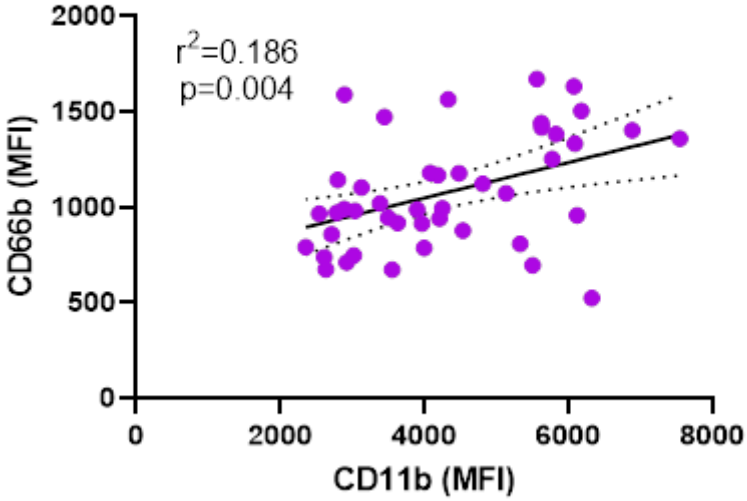


Figure 6.13 – Correlation of CD66b expression with CD11b expression on peripheral neutrophils

Legend: Correlations were assessed using Pearson's correlation coefficient two-tailed test. Forty-four pairs of data were available for the analysis. Linear regression (solid line) is shown together with 95% CI (dotted line). The goodness of fit (r^2) is indicated for the linear regression.

6.2.5.4 Neutrophil senescence between smoker subtypes

No differences were observed in CXCR2 and CXCR4 expression in peripheral neutrophils isolated from participants in the CB, AS or HNS groups (p=0.31 and p=0.07, respectively). Figure 6.14 shows the plots comparing CXCR2 and CXCR4 expressions for the different groups. A correlation analysis was performed between the two markers to assess the relationship between the senescence markers. However, no correlation was found between the two markers (*r*=0.16, p=0.31).

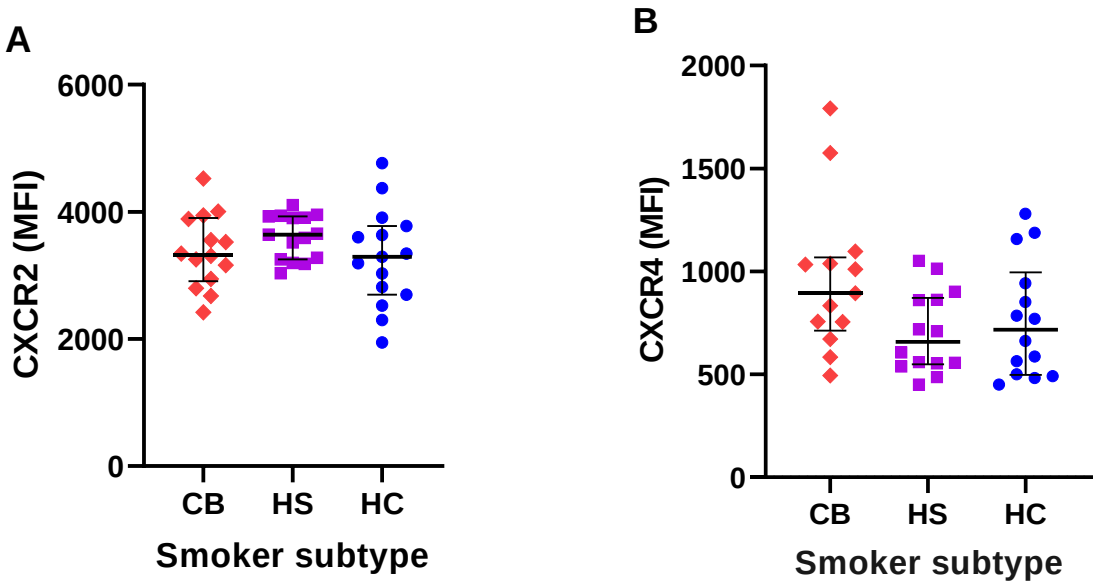


Figure 6.14 – Plots of neutrophil surface senescence marker expression

Legend: Plots of CXCR2 (A) and CXCR4 (B) expression on isolated peripheral neutrophils from whole blood. Neutrophils were stained with antibodies, and the MFI was measured via flow cytometry. Error bars in the plots represent the median (IQR) of the data. Comparisons were made using the one-way ANOVA test with Tukey's comparison test between groups.

6.2.5.5 Neutrophil inflammatory status between smoker subtypes

The expression of CD11c, HLA-DR and PD-L1 were measured on peripheral neutrophils to assess their inflammatory status. No differences were observed in the expression of the three markers between isolated neutrophils from the different groups (p=0.10, 0.45 and 0.58 for CD11c, HLA-DR and PD-L1, respectively). Figure 6.15 shows the plots comparing the expression of these cell surface markers among the different groups.

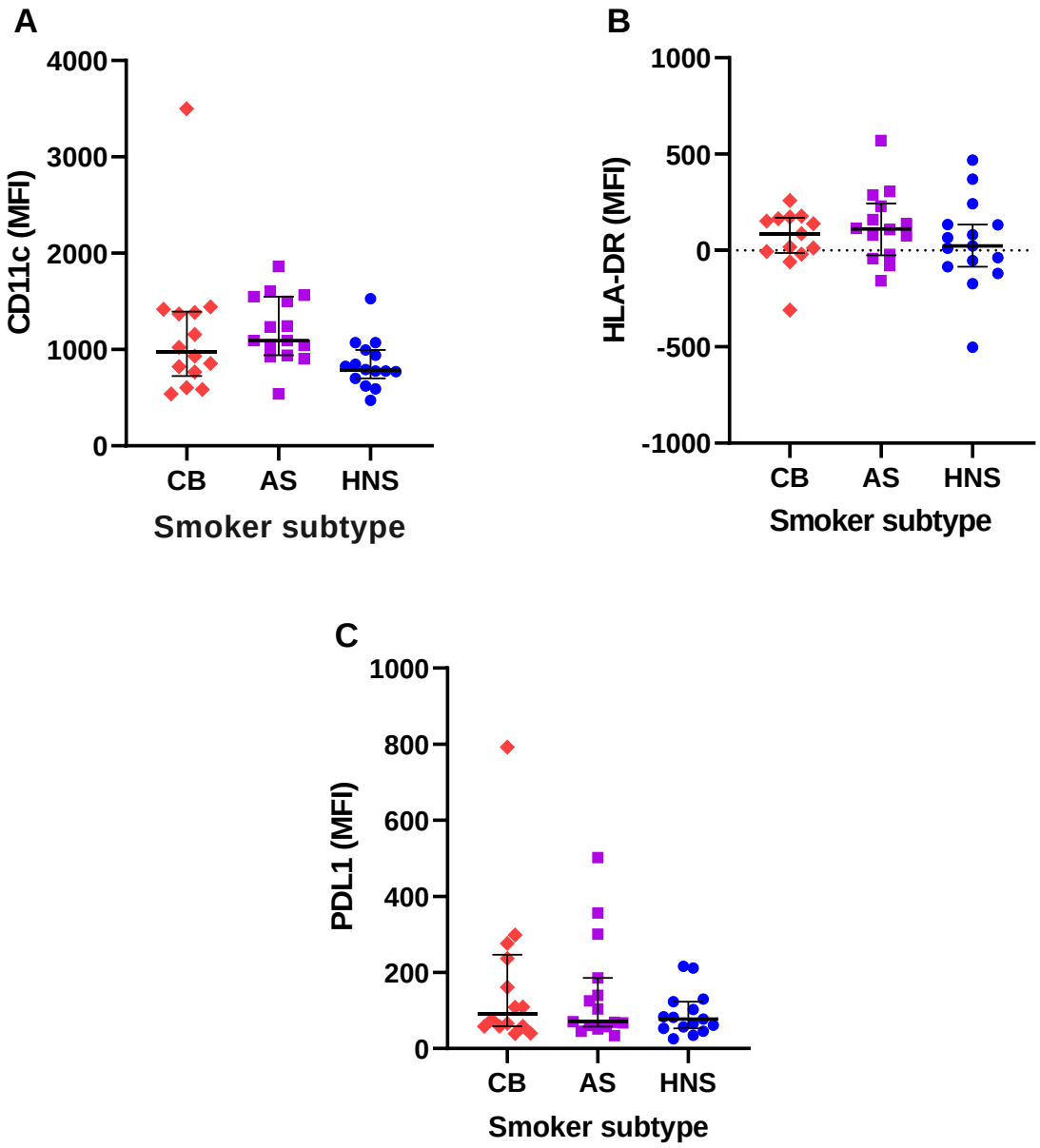


Figure 6.15 – Plots of neutrophil surface inflammation marker expression

Legend: Plots of CD11c (A), HLA-DR (B) and PD-L1 (C) expression on isolated peripheral neutrophils from whole blood. Neutrophils were stained with antibodies, and the MFI was measured via flow cytometry. Error bars in the plots represent the median (IQR) of the data. Comparisons for CD11c and HLA-DR used the one-way ANOVA test with Tukey's comparison test between groups, while comparisons for PD-L1 used the Kruskal-Wallis test with Dunn's comparison test.

6.2.5.6 Reverse transmigrated neutrophils between smoker subtypes

The expression of CD54 was measured on peripheral neutrophils to assess neutrophil reverse transmigration. No differences were found in the expression of CD54 between neutrophils from the different groups (p=0.08). Figure 6.16 shows the plot comparing the neutrophil expression of CD54 for the different groups.

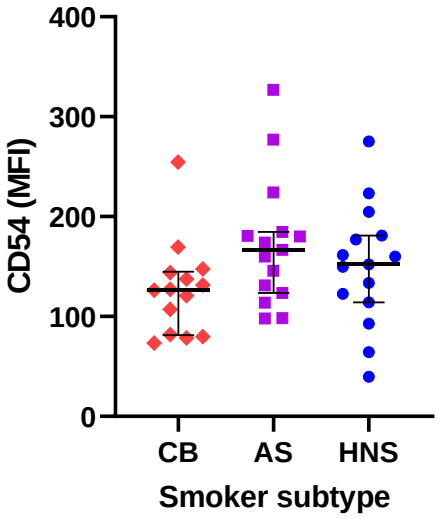


Figure 6.16 – Plot of neutrophil reverse transmigration marker expression

Legend: The plot of CD54 expression on isolated peripheral neutrophils from whole blood. Neutrophils were stained with antibodies, and the MFI was measured via flow cytometry. Error bars in the plots represent the median (IQR) of the data. Comparisons were made using the Kruskal-Wallis test with Dunn's comparison test between groups.

6.2.5.7 Neutrophil maturity between smoker subtypes

The expression of CD10 and CD16 was measured on peripheral neutrophils to assess their maturity. No differences were observed in CD10 and CD16 expression on isolated neutrophils from the different groups (p=0.42 and 0.77 for CD10 and CD16, respectively). Figure 6.17 shows the plots comparing the neutrophil expression of the two markers for the different groups.

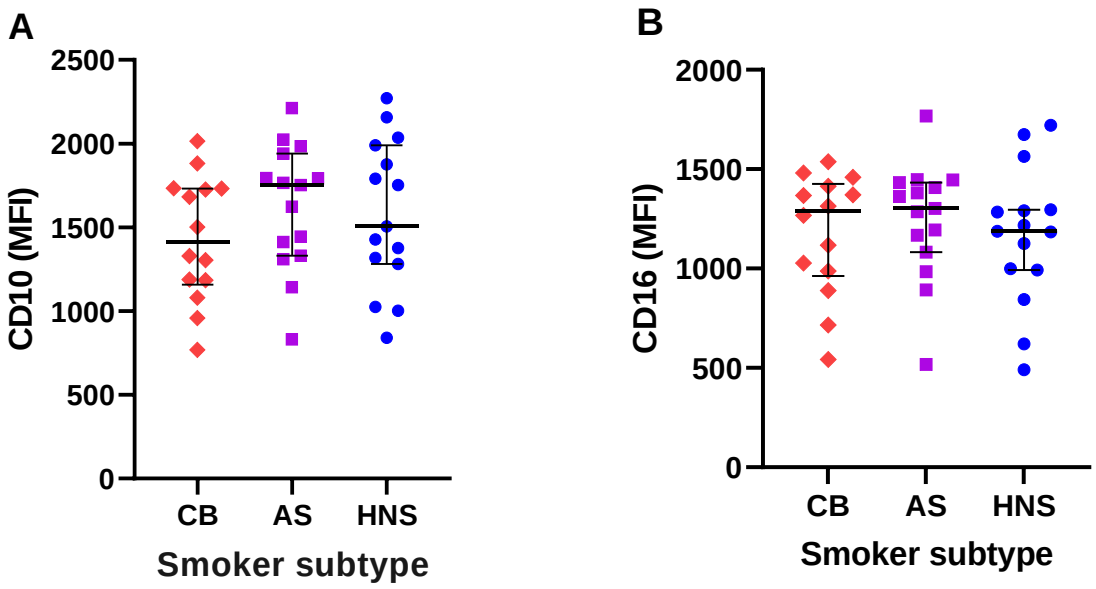


Figure 6.17 – Plots of neutrophil maturity marker expression

Legend: Plots of CD10 (A) and CD16 (B) expression on isolated peripheral neutrophils from whole blood. Neutrophils were stained with antibodies, and the MFI was measured via flow cytometry. Error bars in the plots represent the median (IQR) of the data. Comparisons were made using the one-way ANOVA test with Tukey's comparison test between groups.

6.2.6 Neutrophil phenotyping using t-SNE and Rphenograph clustering

t-SNE visualisation with a clustering algorithm called Rphenograph was utilised to perform a multi-dimensional analysis of gated live neutrophils in the same groups previously designated: CB, AS and HNS. The methods and reasoning of this analysis are described in section 2.11.2. The resulting t-SNE plot shows the cells' distribution based on the clusters' similarities, where the closer they are, the greater the similarity of the clusters. The percentage of cells from each sample within each cluster and the expression of each marker for each cluster can also be quantified.

6.2.6.1 Rphenograph analysis of the neutrophil phenotypes between smoker subtypes

Primary antibodies used in panels 1 and 2 are shown in Table 2.4. Analysis of neutrophils from the CB, AS, and HNS groups revealed 20 clusters stained with panel 1 antibodies. There were no apparent differences between these clusters, with small regions with a higher expression for CD54, CXCR4, CD10, CD62L and CD11b. Areas with higher CD11b expression overlapped regions of higher CD10 expression and lower CD62L expression. This indicates a positive correlation between CD11b expression and CD10 expression but a negative one with CD62L. However, a uniform expression profile was noted between all neutrophils. There was a homogenous distribution of CD16 and CXCR2 found in these plots. Figure 6.18 shows the t-SNE plots with the resulting clusters. Figure 6.19 illustrates the t-SNE plots with the combined relative surface marker expression for antibody panel 1 after Rphenograph clustering analysis.

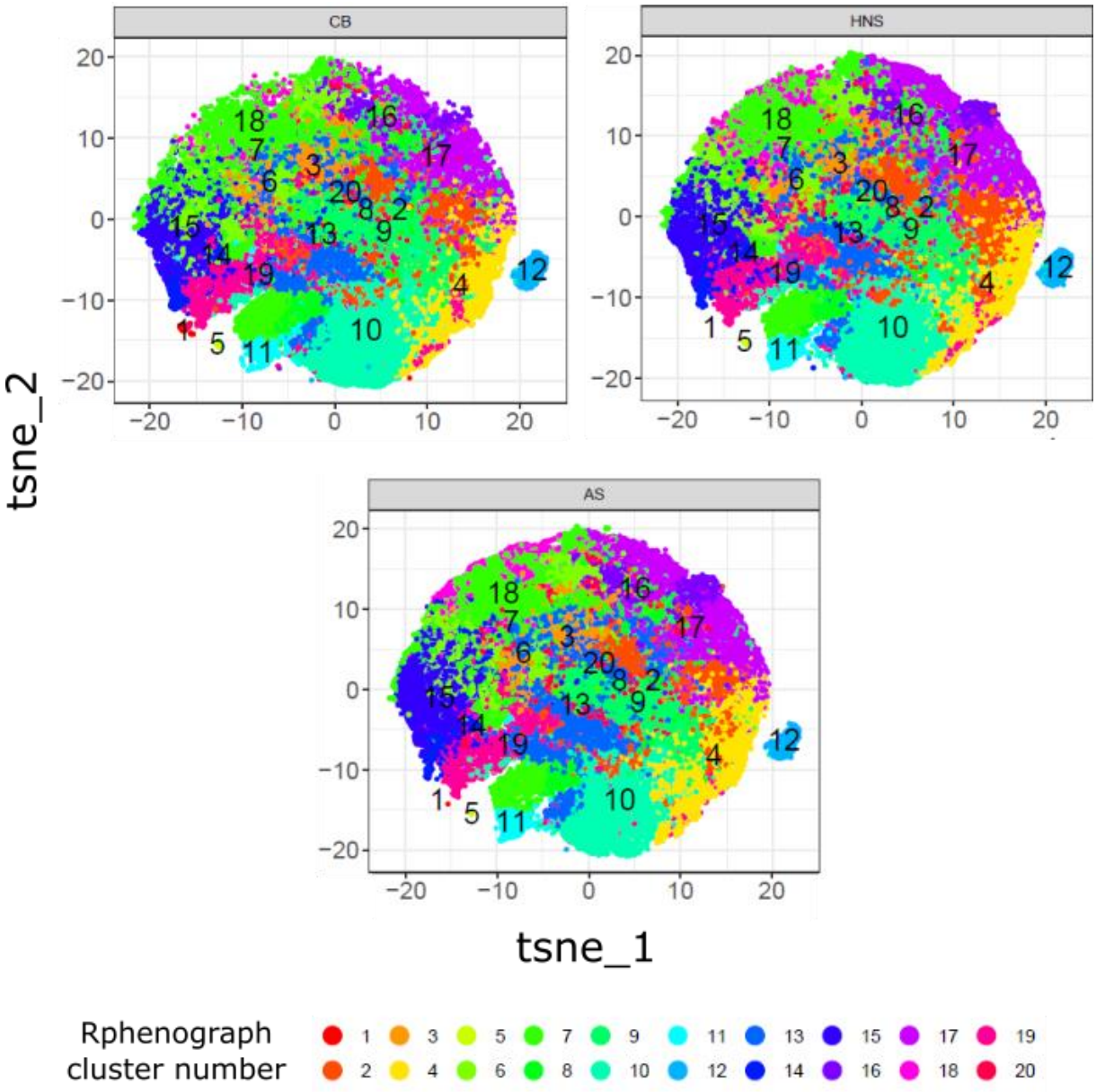


Figure 6.18 – t-SNE plot showing resulting clusters after Rphenograph clustering analysis based on surface marker expression from antibody panel 1.

Legend: Isolated neutrophils were stained with panel 1 antibodies and gated for live cells. Live neutrophils from each sample were then clustered using the Rphenograph algorithm based on surface marker expression. Clusters are presented for each participant group (CB, AS and HNS group) and coloured by cluster numbers.

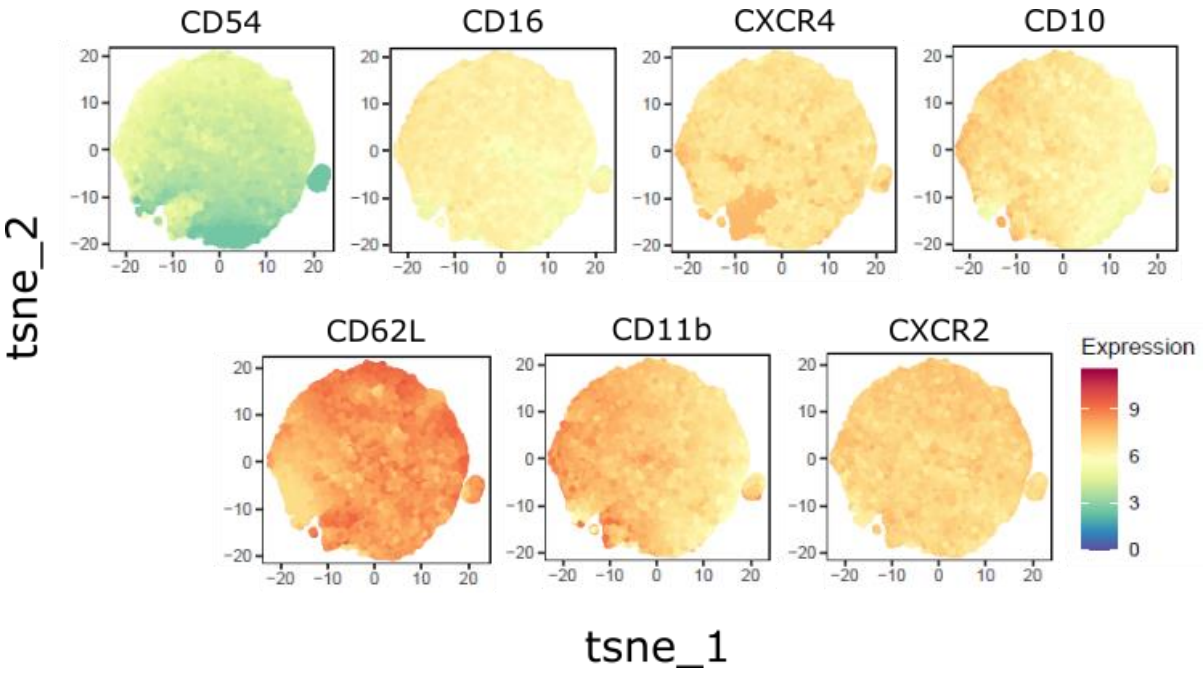


Figure 6. 19 – t-SNE plot showing combined relative surface marker expression from antibody panel 1 after Rphenograph clustering analysis.

Legend: Isolated neutrophils were stained with panel 1 antibodies and gated for live cells. Live neutrophils from each sample were then clustered using the Rphenograph algorithm based on surface marker expression. Plots here are presented as a combined relative surface expression, where red indicates high expression. These plots represent the relative expression of each surface marker within clusters and are not stratified according to the participant group.

Analysis of neutrophils from the different groups revealed 23 clusters when stained with panel 2 antibodies. As with panel 1, there were minimal differences between the clusters, with only clear regions of low PD-L1 expression separating from neighbouring clusters. Regions with higher CD11b expression regions were noted to match regions of higher CD10, CD11c and CD66b expressions. These findings support a relationship between CD11b and CD66b expression (Figure 6.13) and indicate that CD11c and CD10 expression may be associated with individual neutrophils. Figure 6.20 shows the t-SNE plots with the resulting clusters. Figure 6.21 illustrates the t-SNE plots with the combined relative surface marker expression for antibody panel 2 after Rphenograph clustering analysis.

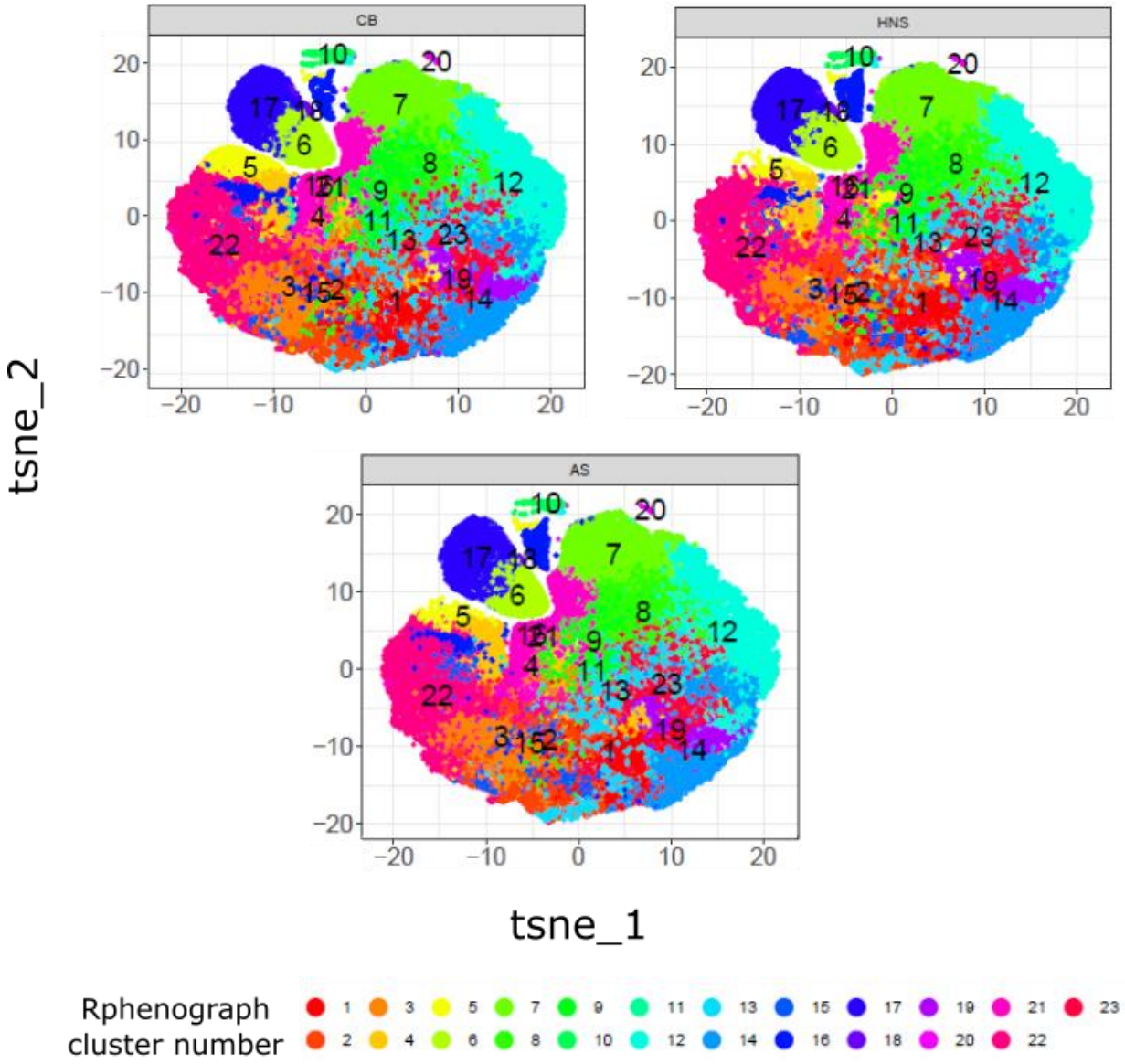


Figure 6.20 – t-SNE plot showing resulting clusters after Rphenograph clustering analysis based on surface marker expression from antibody panel 2.

Legend: Isolated neutrophils were stained with panel 2 antibodies and gated for live cells. Live neutrophils from each sample were then clustered using the Rphenograph algorithm based on surface marker expression. Clusters are presented for each participant group (CB, AS and HNS group) and coloured by cluster numbers.

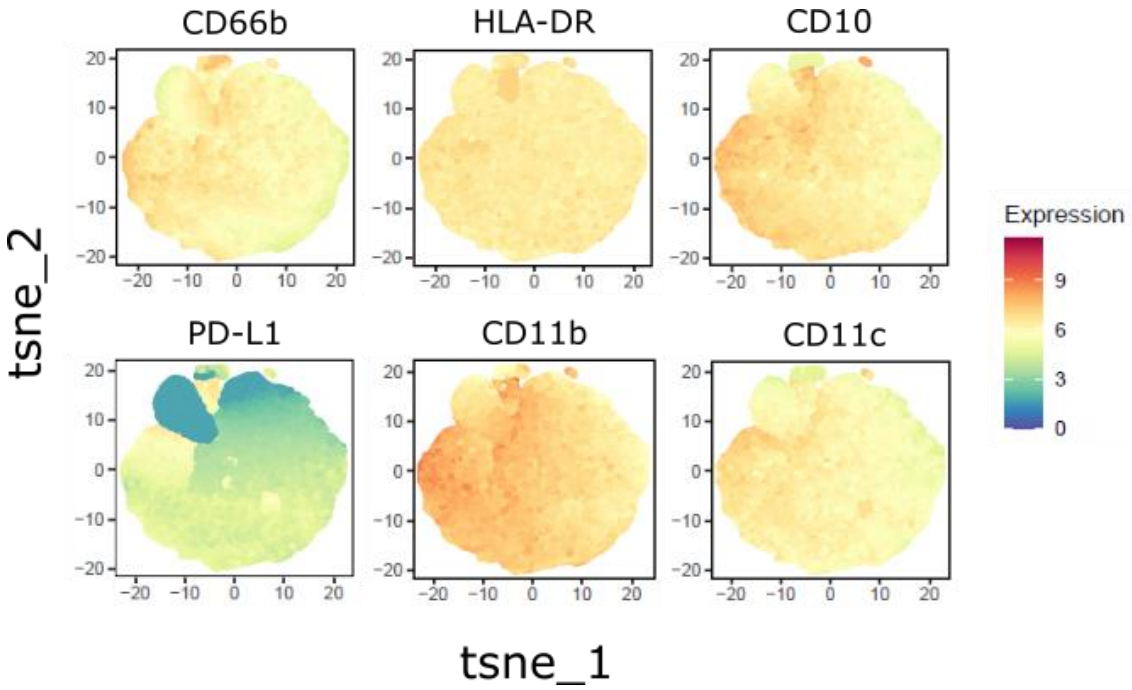


Figure 6.21 – t-SNE plot showing combined relative surface marker expression from antibody panel 2 after Rphenograph clustering analysis.

Legend: Isolated neutrophils were stained with panel 2 antibodies and gated for live cells. Live neutrophils from each sample were then clustered using the Rphenograph algorithm based on surface marker expression. Plots here are presented as a combined relative surface expression, where red indicates high expression. These plots represent the relative expression of each surface marker within clusters and are not stratified according to the participant group.

6.2.6.2 Cluster neutrophil percentage between smoker subtypes

There was similar quantification of the percentage of neutrophils within each cluster between smoker subtypes groups. Five clusters from panel 2 showed statistically significant differences between groups (see Figure 6.22). Of note, cluster 5 from panel 2 showed that the CB group had higher proportions of neutrophils in this cluster compared to the AS and HNS groups. In

contrast, cluster 22 from panel 2 showed that the AS group had a higher proportion of neutrophils in this cluster compared to the CB and HNS group (see Figure 6.23).

When comparing the expression profile within panel 2, cluster 5 showed a higher expression of CD11c than the other clusters. Cluster 22 showed a higher expression of CD10 than the other clusters (see Figure 6.24). These may suggest the presence of more immunosuppressive neutrophils[266 267] in the circulation of smokers with CB and more mature neutrophils[263 264] in AS.

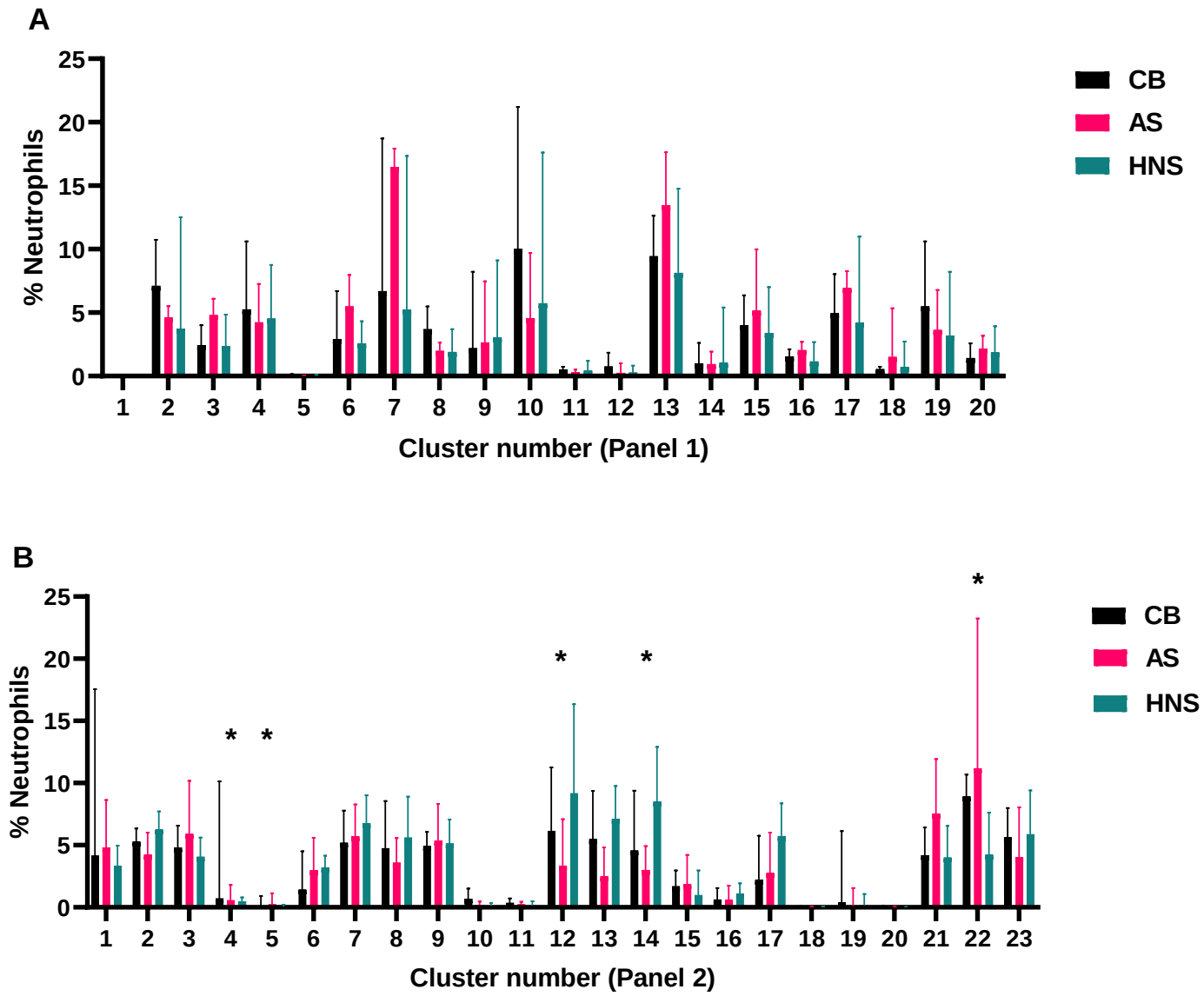


Figure 6.22 – Cluster abundance of neutrophils following Rphenograph cluster analysis

Legend: The proportion of live neutrophils identified within each cluster (shown in Figure 6.18 and Figure 6.20) are shown for panels 1 (A) and 2 (B). The asterisks indicate clusters with significant differences between the groups and are summarised in figure 6.23 below. Error bars represent the median (IQR) of the data. Comparisons were made by the two-way ANOVA test with Tukey's comparison test between groups.

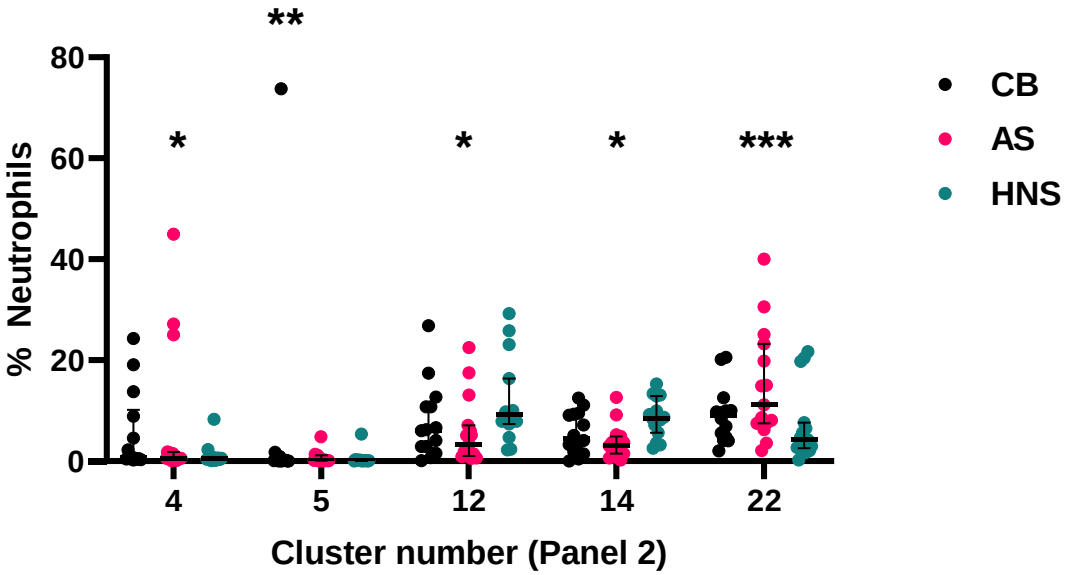


Figure 6.23 – Clusters with statistically significant changes in cluster percentages between groups in panel 2

Legend: A more detailed illustration of the clusters with statistically significant changes in cluster percentages in panel 2. (Figure 6.22). Error bars represent the median (IQR) of the data. Comparisons were made using the two-way ANOVA test with Tukey's comparison test between groups. *Statistically different in the AS group compared to the HNS group (p=0.005, p=0.008 and p=0.03 for cluster 4, cluster 12, and cluster 14, respectively); **Statistically different in the CB group compared to the AS and HNS group (p=0.04 and p=0.02 for AS and HNS respectively); ***Statistically different in the AS group compared to the CB and HNS group (p=0.009 and p=0.0001 for CB and HNS respectively)

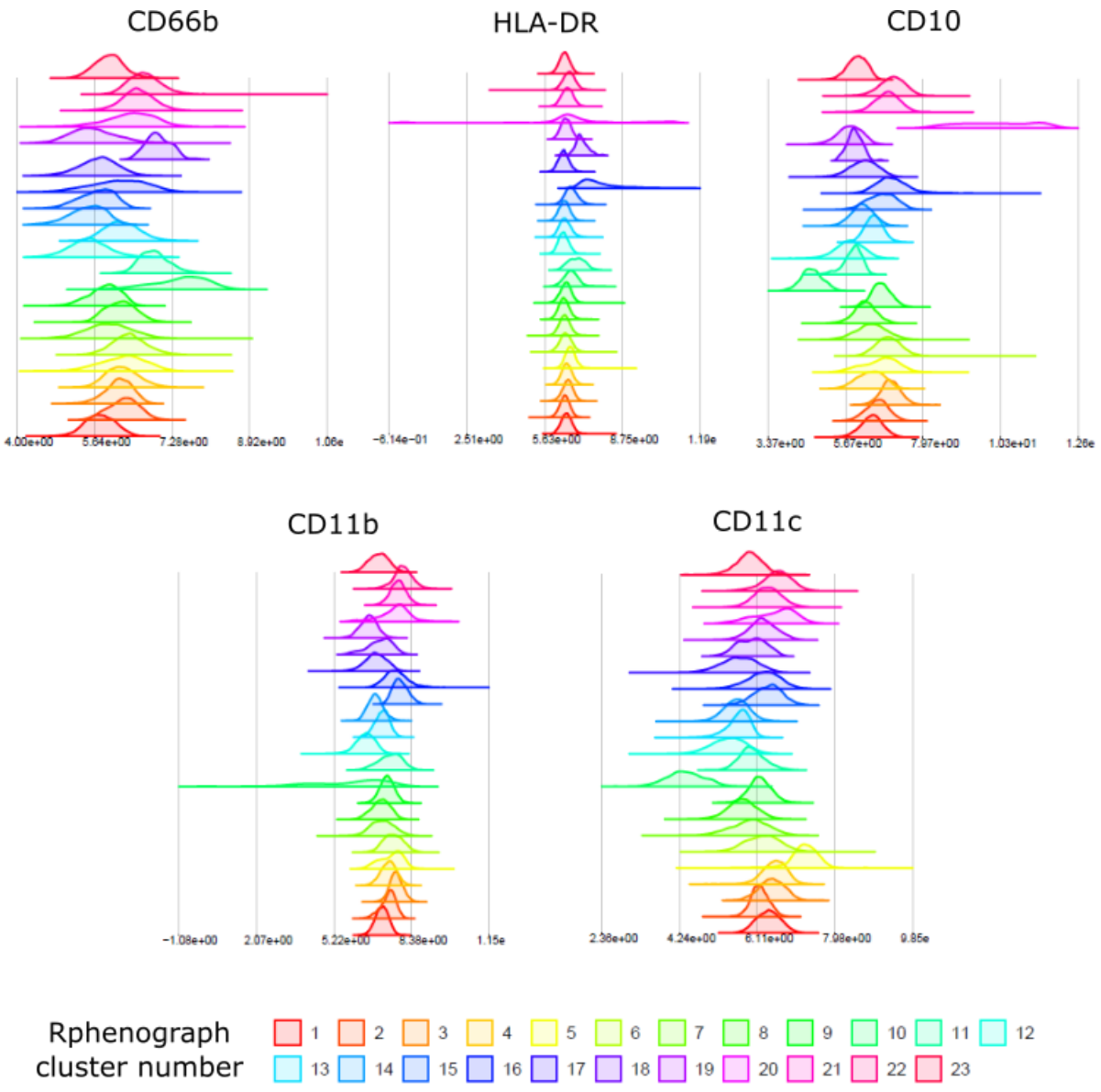


Figure 6.24 – Surface marker expression for neutrophils within each cluster identified for panel 2

Legend: Isolated neutrophils were stained with antibodies, and live neutrophils from each sample were clustered using the Rphenograph algorithm based on surface marker expression. The expression of each marker among the identified clusters using panel 2 antibodies are shown with cluster 1 at the bottom and cluster 23 at the top. The further the curve is to the right, the higher the surface marker expression within the cluster. Cluster expression of PD-L1 was not shown due to very low expression in most clusters.

6.2.7 Power calculation for future studies

Data reported in sections 6.2.3 to 6.2.5 represent pilot data from exploratory assessments. These data can be used to perform power calculations to identify the number of individuals needed to observe significant neutrophil degranulation and phenotypic differences between the smoking subtypes. The power calculations in this section would provide the necessary sample numbers to provide an 80% power to detect a difference at the 5% significance level.

6.2.7.1 Neutrophil degranulation studies

Using data from section 6.2.3, 1598 smokers per arm are needed in the CB and AS groups to detect a statistically significant difference in plasma Aα-Val^{360} and 18399 smokers per arm for Aα-Val^{541} plasma levels. Eighteen individuals per arm are needed for plasma Aα-Val^{360} and 60 individuals per arm for plasma Aα-Val^{541} based on the mean data to detect a difference between the CB and HNS groups.

Ninety-eight smokers per arm are needed in the CB and AS groups. In contrast, 109 individuals per arm are required to detect a difference between the CB and HNS groups using data from section 6.2.4 to detect a difference in plasma MMP-8 levels. For plasma MMP-9 levels, 106 smokers per arm are needed between the CB and AS groups, while 80 individuals per arm are required between the CB and HNS groups.

6.2.7.2 Neutrophil phenotyping studies

Using data from section 6.2.5, a minimum of 2089 smokers per arm is needed to detect a statistically significant difference in mean cell surface marker expression between the CB and AS groups. A minimum of 67408 individuals (a total of 134816) are needed to detect a difference between the CB and HNS group. Table 6.10 shows the sample size required to detect a mean difference in neutrophil expression of each surface marker between smoker subtypes.

	Sample number required (n)	
	CB vs AS	**CB vs HNS**
Activation status		
CD62L	2089	24984
CD66b	246	156
CD11b	48	67408
Senescence status		
CXCR2	105	504
CXCR4	20	33
Inflammatory status		
CD11c	1239	20
HLA-DR	126	959
PD-L1	466	49
Reverse transmigration		
CD54	29	109
Maturity status		
CD10	63	111
CD16	339	10235

Table 6.10 – Samples number needed to detect a statistically significant difference in neutrophil surface marker expression between smoker subtypes

Legend: Power calculations were performed using pilot data described in section 6.2.5. The calculated sample number required represents the number of individuals needed per group. It would provide 80% power to detect a difference in neutrophil surface marker expression between groups at the 5% significance levels.

6.3 Discussion

The biological investigations performed in this chapter represent the first assessment of smokers who would be considered to have early COPD disease or GOLD 0 COPD. This chapter reviewed the neutrophil migration dynamics and indirect evidence of neutrophil degranulation via the use of previously validated indirect ELISA techniques[223 231] (for NE and PR3 footprint activity) and commercially available ELISA kits (for MMP-8 and MMP-9) using isolated peripheral blood neutrophils. The concept of multiple neutrophil phenotypes (as assessed using a combination of markers linked to neutrophil function) was also evaluated. Data in this chapter supported the initial hypothesis that neutrophil migration is impaired in a subset of smokers (particularly those with CB symptoms) who did not have airflow obstruction on spirometry. However, there was insufficient evidence to support the hypothesis that peripheral neutrophils from these smokers have increased degranulation or expressed different cell surface markers compared to other smokers.

6.3.1 Neutrophil migration in non-COPD smokers with chronic bronchitis

Power calculations performed previously in our group have suggested that including ten subjects per group can provide 80% power to detect a difference in neutrophil migration parameters.[417] The present data was adequately powered based on previous calculations and has shown that peripheral neutrophils from smokers with CB had reduced neutrophil migration accuracy (velocity) compared to AS and HNS when CXCL8 was used as the chemoattractant. These neutrophil migration characteristics were also observed in COPD reported previously,[237], although an increased neutrophil migration speed was not observed.

In an *in vitro* study assessing neutrophil migration dynamics, neutrophils isolated from COPD patients (n=20) were found to be dysfunctional in their ability to migrate accurately towards the various host and bacterial-derived chemoattractants.[237] These neutrophils had an increased migration speed but reduced velocity compared to neutrophils from healthy smokers (n=16), healthy never-smokers (n=15) and patients with alpha-1 antitrypsin deficiency (AATD; n=20).[237] While this previous study did not show any differences in neutrophil migration parameters between healthy smokers and healthy never-smokers, the healthy smokers in the study were not stratified according to symptom presence.[237] Data in this chapter have shown that a subset of smokers, particularly those with CB symptoms, have impaired neutrophil migration similar to COPD patients, which may be an early marker of disease susceptibility.[120 121]

The current data observed that the reduced chemotactic accuracy of neutrophils from smokers with CB symptoms seen towards CXCL8 was not replicated using fMLP. The reason is unclear, but there are several plausible explanations. Heit et al. previously demonstrated *in vitro* in an under-agarose migration assay that neutrophil migration towards fMLP depends on the p38 mitogen-activated protein kinases (p38 MAPK) pathway.[418] On the other hand, neutrophil migration towards CXCL8 depends on the PI3K pathway. Although PI3K accelerates the migration response to fMLP, it plays a minor role in this process.[418] It may be that the PI3K pathway is affected earlier in the COPD disease process, whereas the p38 MAPK pathway becomes a later mechanism in established disease. Although there is no evidence to support this, it has already been shown that defective migratory dynamics in neutrophils from COPD patients can be normalised by specific PI3K inhibitors suggesting that the PI3K pathway is central.[237]

Another possible explanation for the disparate neutrophil chemotactic response to fMLP is that neutrophils follow a hierarchical preference of chemoattractants under physiological conditions.[197] Kim et al. used a microfluidic platform to study the dynamics of neutrophil chemotaxis under competing chemoattractant gradients. The results suggest that neutrophils preferentially migrate towards bacterial-derived chemoattractants (such as fMLP) rather than host-derived chemoattractants such as CXCL8.[197] It is possible that in early disease, stimuli from fMLP as a chemoattractant is sufficient to avoid any chemotactic response seen here.

Previous studies of neutrophil migration in COPD have used Boyden chamber assays, in which cells migrate from an upper well through a porous membrane into a lower well containing the chemoattractant. The number of cells reaching the underside of the membrane (closest to the chemoattractant) is then counted.[236 419 420] However, using an Insall chamber allows more insight into neutrophil migration properties towards a chemoattractant in a two-dimensional format.[421] Computer tracking of individual cell paths in the Insall chamber allows a more comprehensive analysis of migratory dynamics, including overall speed of movement but also speed of movement in the direction of the chemoattractant (allowing assessment of migration accuracy). This assay was chosen for the current studies as it provides more information about migration mechanics. This assay has allowed the differences in speed and migration between healthy controls and Early COPD cohort smokers to be determined in a manner that would not be possible with traditional Boyden chamber assays.

6.3.2 Implications of inaccurate neutrophil migration in early disease

As mentioned in section 1.2.1, smoking cessation remains the best way to prevent COPD development and progression. However, it has been shown that airway inflammation persists in COPD smokers who successfully quit smoking.[422] Willemse et al. investigated serial sputum samples and bronchial biopsies in 28 COPD and 25 non-COPD smokers over 12 months. COPD smokers who successfully quit smoking had persistently high sputum neutrophils, lymphocytes and CXCL8, and airway inflammation on bronchial biopsy. Some of these changes were also observed in non-COPD quitters.[422] These findings show that whilst smoking cessation is essential, it may not be enough to prevent the damage from persistent chronic inflammation that may eventually lead to COPD in a proportion of smokers.

Several components of the disease can become self-perpetuating as the disease progresses. As inflammatory mediators increase, a cycle of ongoing inflammation and pulmonary damage sets in. If an altered neutrophil function is causative to COPD pathology, then identifying this early and correcting any amenable defect might halt this self-perpetuating cycle of damage. Data shown here have shown an altered neutrophil migration in CB smokers. A neutrophil-focused targeted approach in this subset of smokers may be effective in preventing subsequent lung damage, but further work is needed to establish this. It is unclear whether altered neutrophil migration in CB smokers represents a reversible phenotype. If this defect is still observed after smoking cessation, it would further support the hypothesis that neutrophils are involved in the initiation and persistence of the chronic inflammatory process. Data described here consists of the assessment of neutrophil migration *in vitro,* but this should also be assessed in physiological lung models. This may be achievable by the use of precision

lung cut slices where neutrophil migration through the tissue can be observed and can be linked to subsequent tissue damage detected microscopically.[423]

6.3.3 Neutrophil degranulation in early disease

Inaccurate neutrophil migration through the lung tissue can increase the area of tissue exposed to proteolytic enzymes and result in a larger area of tissue damage due to the process of 'quantum proteolysis' (described in section 1.4.6). Our group had previously developed and validated assays that indirectly assess NE and PR3 activity by quantifying NE-specific[223] and PR3-specific[231] breakdown products of fibrinogen (Aα-Val^{360} and Aα-Val^{541} respectively) as a footprint of the relevant activity detectable in plasma samples. PR3 and NE can induce excess mucus production from airway submucosal glands and impair mucus clearance, both of which are features of CB.[204 424]

It was initially hypothesised that smokers with CB symptoms would have evidence of increased NE or PR3 activity. However, the present data do not support this hypothesis, as no difference in Aα-Val^{360} or Aα-Val^{541} was noted between the CB, AS, and HNS groups. Concerning plasma Aα-Val^{360}, this was previously measured in 81 patients with CB and exertional dyspnoea (of which 58 had an FEV_1/FVC < lower limit of normal (LLN) on spirometry) and 39 healthy controls. Patients with CB and exertional dyspnoea had higher Aα-Val^{360} levels (median 20.8nM, IQR 14.0-25.4) than the control group (median 3.5nM, IQR 2.4-5.1).[224] However, the symptomatic patient cohort in the study by Carter et al. was made up of both COPD and non-COPD patients, making direct comparisons of results difficult.[224]

Large datasets had not found a difference between Aα-Val^{541} levels in non-AATD COPD and healthy controls. Newby et al. quantified plasma Aα-Val^{541} levels in a cohort of AATD patients (n=94 for PiSZ genotype and n=239 for PiZZ genotype), non-deficient COPD patients (n=78) and healthy smokers as controls (n=53).[231] Although Aα-Val^{541} levels were markedly higher in PiZZ (median 270.0nM, IQR 158.9-440.6) and PiSZ AATD patients (median 58.8nM, IQR 39.4-87.1) compared to healthy smokers (median 27.6nM, IQR 15.0-40.0), there were no significant differences in Aα-Val^{541} levels between non-deficient COPD patients (median 20.0, IQR 13.3-32.1) and healthy smokers.[231]

No differences in MMP-8 and MMP-9 levels were observed in plasma samples between the CB, AS and HNS groups in the present data. There are currently no studies assessing MMP-8 and MMP-9 levels in non-COPD smokers, but both MMPs are increased in plasma[425] and bronchoalveolar lavage (BAL) fluid[235] of COPD patients compared to healthy controls. Sng et al. compared plasma MMP-8 and MMP-9 in stable COPD patients and healthy controls (n=23) and found that plasma levels of both MMPs were higher in COPD patients compared to healthy controls. In another study, Ostridge assessed levels of multiple MMPs in BAL fluid from 24 mild and moderate COPD patients (as defined by GOLD[1]) and eight healthy controls to determine their relationship with CT parameters and lung function.[235] Higher MMP-8 and MMP-9 were found in the BAL fluid of COPD patients compared to healthy controls. The levels of both MMPs had the strongest association with FEV1 %predicted (MMP-8: rho= -0.60, $p<0.01$; MMP-9: rho= -0.59, $p<0.01$) MMEF %predicted (MMP-8: rho= -0.61, $p<0.01$; MMP-9: rho= -0.58, $p<0.01$) and marker of small airways disease on CT (MMP-8: rho=0.60, $p<0.05$; MMP-9: rho=0.56, $p<0.05$) in COPD patients.[235]

6.3.4 Lack of neutrophil degranulation signal

The lack of difference in neutrophil degranulation observed in the current study is likely because the study is underpowered to detect a meaningful difference between the smoker subtypes. However, there may be several other possible explanations for this finding. Dysfunctional neutrophil migration in young CB smokers is likely insufficient to cause a rise in systemic protease activity detectable in the peripheral circulation. Instead, using lung media such as sputum or BAL to replace plasma may have better sensitivity in detecting increased protease activity. Another explanation may be that in these young CB smokers, the anti-proteinase activity in the lung is sufficient to nullify the increased protease activity from dysfunctional neutrophil migration.

As mentioned in section 1.3.3, the relationship between lung disease and biomarkers is complex.[160] Correlations of either NE and PR3 activity, as well as MMPs with clinical parameters in other published COPD studies, raise the issue of 'cause and effect'. As most of these studies were done in individuals with COPD, elevated markers may reflect physiological responses to established disease and help explain the lack of significant findings in the present study, where the disease process is mild and not established. As the data described here is only cross-sectional, assessing the relationship between neutrophil degranulation and markers of disease activity (such as FEV_1 decline) has not been possible.

6.3.5 Neutrophil phenotypes in early disease

There is limited literature surrounding neutrophil phenotypes and how this may be linked to the COPD disease process. Here, an integrated panel was used to assess a variety of neutrophil

phenotypes in smokers possibly at risk of developing COPD. It was hypothesised that neutrophils from CB smokers who may be at increased risk of progression to COPD would have an activated phenotype (increased CD11b and CD66b but reduced CD62L expression) and a senescent phenotype (decreased CXCR2 and increased CXCR4 expression).

Neutrophil phenotypes were similar in the CB, AS and HNS groups. There was no change in CD11b, CD66b and CD62L expression observed between the three groups, suggesting no measurable systemic activation of neutrophils with smoking or in the presence of respiratory symptoms. Lokwani et al. quantified the neutrophil surface expression of CD62L, CD11b, CD11c and CD54 in peripheral neutrophils in a cohort of COPD patients (n=17), asthma patients (n=20) and healthy non-smokers (n=19).[252] In this study, peripheral CD11b expression was not altered in COPD, but CD62L expression was reduced. Another study by Blidberg et al. also assessed CD11b, CD62L and CD162 expression on peripheral blood neutrophils from COPD smokers (n=18), non-COPD smokers (n=21) and healthy non-smokers (n=22).[426] In contrast to Lokwani et al.'s study results,[252] CD11b expression was increased in COPD blood neutrophils compared to non-COPD smokers and healthy non-smokers. Still, no difference was seen in CD62L expression among the different groups.[426] Of note, no difference was noted in CD11b expression between smokers without COPD and healthy non-smokers, similar to the present data.[426]

The variation in the number of activated neutrophils detected between the different studies may be explained by differences in patient cohorts and antibody staining and neutrophil isolation methods. In the study by Lokwani et al.[252] and Blidberg et al.[426], whole blood was used for antibody staining, followed by red cell lysis. In the current study, neutrophils were

isolated using Percoll gradient separation. Antibody staining performed in whole blood in the presence of other cell types may alter priming signals received by these neutrophils. The red blood cell lysis step can also change neutrophil behaviour and the ability to be activated.[427] Given the range of neutrophil isolation procedures used and the impact these can have on cellular activation, there remains difficulty in comparing outcomes between different studies.

It is thought that senescent neutrophils display increased CXCR4, which allows 'homing' back to the bone marrow as a mechanism of clearance from the circulation.[183 428] However, little evidence exists for human neutrophil senescence *in vivo*, with neutrophil 'homing' only shown in murine models[183]. Data on similar human processes are limited and rely on *in vitro* ageing methodology.[429] The findings from murine studies may not directly relate to a similar phenotype in humans. Nevertheless, paired assessment of CXCR4 and CXCR2 may identify a senescent phenotype. The lack of difference in CXCR4 and CXCR2 expression between smoker subtypes in present data suggest that peripheral neutrophil senescence is not a feature of smoking alone or in the presence of respiratory symptoms. Thus, it is unlikely that the early disease process in COPD is linked to an increased burden of circulating senescent neutrophils.

The lack of difference in CXCR2 expression among the three groups was supported by study findings from Traves et al., who assessed CXCR2 expression in neutrophils, monocytes and lymphocytes in COPD patients (n=37), non-COPD smokers (n=33) and healthy non-smokers (n=30).[430] The MFI value for CXCR2 in neutrophils was noted to be lower in COPD patients (MFI 6.8 ± 1.0) than smokers without COPD (MFI 8.5 ± 1.0) and healthy non-smokers (MFI 8.4 ± 1.2) although this did not reach statistical significance.[430] However, the CXCR2 MFI value in smokers without COPD and healthy non-smokers were almost identical, which supports the

lack of difference in the current study.[430] This finding suggests that the neutrophil migratory defect seen in response to CXCL8 in CB smokers in section 6.2.2.1 was not due to a decrease in the expression of chemoattractant receptors on the neutrophil cell surface. Receptor expression, internalisation, recycling, and degradation are complex processes, and the neutrophil migratory defect seen may instead be due to differences in receptor function or downstream signalling events.

Immature neutrophils are rarely seen in the circulation of healthy individuals as maturation occurs almost exclusively within the bone marrow, only entering the circulation as CD10-expressing mature neutrophils.[431] However, previous studies have shown that acute inflammation can lead to the premature release of neutrophils into circulation. This occurrence has been termed emergency granulopoiesis and has been observed in patients with sepsis[432] and after cardiac surgery,[433] associated with reduced CD10 expression in peripheral neutrophils. Studies researching CD10 expression in smokers and COPD are lacking. Still, data presented here had shown no changes in the maturity of circulating neutrophils regardless of smoking status or respiratory symptoms. It is unlikely that an immature neutrophil phenotype has a role in the early COPD disease process.

Neutrophils have been reported to display pro-inflammatory or anti-inflammatory properties through either expression of HLA-DR[434] or inhibiting T-cell responses via PD-L1.[435] CD11c has been linked with an immunosuppressive neutrophil phenotype,[266 267] and CD54 has been linked with neutrophil reverse transmigration.[258] Limited HLA-DR expression was detected on neutrophils from any participant group in the current study, with no changes in PD-L1, CD11c expression or CD54 expression in smokers with or without respiratory symptoms and healthy

non-smokers. These results suggest neutrophils do not display pro-inflammatory, anti-inflammatory or reverse transmigration properties in patient groups studied here. These phenotypes have not been detailed in smokers or those with established COPD. The data here suggest that they exist in low numbers in the circulation and are unlikely to contribute to the early disease process in COPD. The lack of CD54 expression also makes it doubtful that pro-inflammatory conditions in the lung due to smoking could be detected systemically as alterations in the neutrophil phenotype.

6.3.6 Use of dimension-reduction algorithms in neutrophil phenotyping

Discussion of flow cytometry data so far has focused on average differences between smoker subtypes, but this does not address the heterogeneity of the neutrophil population or patient susceptibility. The role of different neutrophil phenotypes in disease pathogenesis is largely unknown as it is challenging to determine if neutrophil phenotype changes occur before disease pathogenesis or as a result of the disease itself.[436]

Using dimension-reduction algorithms, such as t-SNE, has helped identify complex surface marker expression changes. The use of t-SNE in this chapter has highlighted that neutrophils are broadly similar within a given population of non-COPD smokers. Between healthy non-smokers and smokers with CB symptoms, similar phenotypes were observed, suggesting that, at best, a subtle shift in surface expression within these populations. The ability of t-SNE to analyse non-discrete datasets has been previously investigated in healthy individuals, showing that clear separation is not seen when PBMCs are analysed.[437] The use of multi-dimension

analysis in neutrophils is not identifying discrete subsets but suggesting where subtle differences may exist within neutrophil populations.

From current data, there is an overlap of neutrophils expressing relatively higher levels of CD11b, CD66b and CD11c. This suggests that CD11c has a relationship with neutrophil activation, typically denoted using CD11b and CD66b, despite none of the marker expressions being significantly different when investigated alone. Indeed, neutrophils from CB smokers had a higher proportion of neutrophils in a cluster expressing higher levels of CD11c, suggesting an immunosuppressive phenotype. Neutrophils from AS smokers had a higher proportion of neutrophils expressing higher levels of CD10, suggesting a mature neutrophil phenotype. While it is unexpected that CB smokers would have a higher population of immunosuppressive neutrophil phenotype than AS and HNS, this may be a physiological response to increased lung inflammation. These analyses highlight that neutrophil phenotypes are more complex than those identified by the expression of a single marker and also support that the expression of neutrophil surface markers is a gradient with no apparent clustering.

6.3.7 Limitations and summary

One limitation of this study is that the analyses performed, especially in this chapter, focused on cross-sectional data obtained at baseline. Longitudinal data on lung function and complete CT densitometry data on all participants could not be obtained due to disruptions caused by the COVID-19 pandemic. Therefore, the relationship between neutrophil function and disease progression could not be assessed. This chapter's data on neutrophil function was descriptive and did not provide any mechanistic insight. Although peripheral neutrophils from smokers

with CB were demonstrated to have impaired migratory dynamics, it was impossible to examine the exact nature of this functional defect.

As detailed in section 1.4, neutrophils have other known functions, such as phagocytosis and the formation of neutrophil extracellular traps. Investigations on these neutrophil functions in the Early COPD cohort could not be performed due to laboratory restrictions imposed during the COVID-19 pandemic. Another major limitation of studies described in this chapter is that they are underpowered to detect a significant difference in neutrophil function and features. However, power calculations described in section 6.2.7 have shown that a high number of participants would be needed to detect differences in neutrophil serine protease activity and neutrophil phenotypes between the smoker subtypes. This makes such studies non-feasible and indicates that any changes are likely to be of minimal relevance to the pathophysiology of COPD. However, fewer participants would be required to adequately power studies to detect differences in plasma MMP levels between the smoker subtypes and suggest such enzymes may be more central to the initial disease process.

In summary, the present study found dysfunctional migratory dynamics in neutrophils from symptomatic smokers, similar to those in COPD patients.[237] There is no evidence of increased neutrophil degranulation in symptomatic smokers. High levels of neutrophil degranulation may reflect disease severity rather than disease activity. Comprehensive neutrophil phenotyping revealed subtle phenotype differences in individuals based on smoking status and the presence of respiratory symptoms. These changes may provide mechanisms of neutrophil dysfunction and therapeutic avenues, but further studies are required to characterise the functional and clinical impact of these phenotypes

CHAPTER 7 – GENERAL DISCUSSION

7.1 Looking for early disease in COPD

COPD remains a major non-communicable disease that causes significant morbidity and mortality worldwide.[438 439] Diagnosing COPD as early as possible in the disease process is essential, providing opportunities to modify risk factors and preserve lung function. However, only 25% of smokers develop COPD, and the pathology is likely to progress over many years, making detection difficult. The current spirometric definition of COPD of an FEV_1/FVC ratio <0.7 may not be sensitive enough to detect abnormalities such as SAD earlier in the disease process.

Smokers with early COPD are at increased risk for disease progression and, in some cases, experience significant morbidity. These individuals likely have underlying pathophysiological abnormalities that may require targeted treatment. The ability to detect smokers at risk of progression from early to clinically significant disease is limited in clinical practice. Furthermore, the evidence base surrounding treatment options in such individuals remains unclear as they are usually excluded from clinical trials. Studies have suggested that assessing clinical metrics such as symptoms,[120 121] lung function,[128 440] and emphysema quantification using CT densitometry in smokers[146 147] could help identify individuals in whom the disease is likely to progress to overt airflow obstruction. However, we currently have little data on the sensitivity or specificity of any individual metric or combination of metrics to accurately identify such individuals.

7.2 Role of neutrophils

Neutrophils play a role in COPD pathogenesis and are the most abundant leukocytes found in lung secretions of COPD patients.[441] Both neutrophil numbers and products are linked with multiple aspects of disease in animal and cell models.[221 442] There is a need for disease-modifying therapies that can be utilised early in the disease course before irreversible lung damage. Therapeutics targeting neutrophils may allow for fine-tuning of the immune response to reduce collateral lung tissue damage whilst maintaining immune protection from pathogens. Although there have been numerous studies involving neutrophils in patients with established disease, the role of neutrophils in early disease is unknown. Uncovering evidence of neutrophil dysfunction in early disease and identifying underlying mechanisms leading to this may help identify where repurposed or novel therapeutics could be deployed.

7.3 Summary of findings

This thesis tested the hypothesis that smokers at risk of developing COPD would have a significant symptom burden. There was evidence of SAD on lung function testing and/or emphysema detected using CT densitometry. Peripheral neutrophils from these smokers would also have impaired migration and excessive degranulation with changes in phenotype, specifically pro-inflammatory and senescence. It sought to investigate the clinical symptoms, lung physiology (using both conventional spirometry and physiological markers of SAD (MMEF and FOT parameters)) and CT changes of smoking adults in the Birmingham Early COPD cohort. This cohort consisted of young adult smokers with significant smoking history who may be at

risk of developing COPD. Further, this thesis aimed to characterise neutrophil function (migration and degranulation) and neutrophil phenotype in this cohort.

Due to disruption caused by the COVID-19 pandemic, there was reduced access to local lung function testing, preventing longitudinal lung physiology assessment. As there has been compelling evidence for a relationship between CB symptoms and subsequent development of COPD,[120 121] smokers with CB symptoms were used in this thesis as a surrogate for smokers at risk of developing COPD, similar to the GOLD 0 concept.[443]

7.3.1 Assessment of clinical parameters

7.3.1.1 Impact of symptoms among non-COPD smokers

Data in chapter 3 showed that a significant proportion (30.0%) of smokers within the current cohort had CB symptoms and that these patients (with CB) had a worse quality of life than those seen in asymptomatic smokers. This finding is similar to those reported in other large studies with cohorts of smokers or ex-smokers with normal spirometry, such as the COPDGene study[304] and the Lovelace Smoker's Cohort.[303] The fact that smoking is associated with respiratory symptoms and poor health-related quality of life in some individuals who do not meet the criteria for COPD could have important implications for global health economics.

No studies have assessed the health-related costs of non-COPD smokers with chronic bronchitis. However, a retrospective case-control analysis by Blanchette et al. in the USA showed that patients with CB (both COPD and non-COPD) had higher mean total health-associated costs than matched controls (n=11674 both in the CB group and matched controls)

both before CB diagnosis and up to two years postdiagnosis.[444] This equated to an approximate increased cost of $2000-2800 for medical services and $500-600 for pharmacy costs per patient with CB over six months compared to the control cohort.[444] Furthermore, the COPDGene study[304] and Lovelace Smoker's Cohort[303] found smokers or ex-smokers with CB had a higher rate of exacerbations requiring hospital admission and/or antibiotic or steroid use compared to those without CB.[303 304] Taking these findings, smokers with CB symptoms likely incur a considerable cost to the global health economy even if they do not reach the spirometric criteria for COPD diagnosis.

There are also no treatment guidelines or clinical trial evidence to guide the treatment of non-COPD patients with CB symptoms. The Redefining Therapy in Early COPD (RETHINC) study assesses the impact of dual bronchodilator therapy in this patient population[445] but has yet to report any outcomes. This study should help address some aspects of the pharmacologic treatment of symptomatic smokers without obstruction and could provide further information about early disease.

7.3.1.2 Physiological and imaging characteristics among at-risk smokers

Data presented in chapters 4 and 5 did not support the hypothesis that smokers with CB symptoms have increased SAD or emphysema as quantified by CT densitometry compared to AS. This likely reflects both the intra and inter-patient variability in the results of tests of small airway function and that symptomatic non-COPD smokers represent a markedly heterogeneous group with various physiological and radiological abnormalities. The study was underpowered to detect differences using physiological tests of small airways with significant

variance across heterogeneous groups. Data from the current study identified that a proportion of the Birmingham Early COPD smokers, both with CB symptoms or otherwise, had physiological and radiological abnormalities seen in other published studies.[146 147 440 446 447]

Studies have shown that a proportion of non-COPD smokers or ex-smokers have low TLCO[440] or evidence of emphysema, based on both Perc15[146] and LAA-950HU%[147] quantified by CT densitometry. These smokers were at increased risk of developing spirometric-defined COPD at follow-up compared to non-COPD or ex-smokers without these abnormalities. Other studies have also demonstrated that some smokers have evidence of SAD, detected using MMEF and FOT. Data on longitudinal progression to established COPD in such individuals is lacking but a data from a study among AATD patients[130] supports the hypothesis that SAD is a risk factor for progression to COPD. In the study by Stockley et al.,[130] AATD patients with FEV_1/FVC >0.7 but MMEF <80% predicted on spirometry (n=40) had a greater median FEV_1 decline than those with FEV_1/FVC >0.7 and MMEF >80% predicted (n=43) after 3 years follow-up (-1.09% (IQR -1.91 to -0.04% predicted/year) vs -0.04 (IQR -0.67 to -0.03% predicted/year), p=0.007). Smokers with SAD could also have a similar picture with excess FEV_1 decline leading to COPD, but this will need to be confirmed with further prospective longitudinal studies.

Smokers within the Birmingham Early COPD cohort with similar abnormalities may also be at an increased risk for future COPD, although this could not be proven with current data. The use of CB smokers as a surrogate in current analyses may give an incomplete picture of the role of detecting such abnormalities among at-risk smokers. Although CB symptoms are strongly associated with progression to COPD, not all patients with CB eventually progress to established COPD.[119] These patients represent only a subset at risk for ultimate disease

progression. Thus, a combined assessment may better identify at-risk smokers, which will be discussed in detail below.

7.3.1.3 Combined assessment and implications for screening

Results in sections 3 through 5 have shown that the Early COPD cohort is a heterogenous group. Some abnormalities are detected only in some smokers, and there is no apparent overlap between the groups. For example, not all smokers with CB have evidence of significant emphysema on CT or SAD on physiological testing. Conversely, not all smokers with SAD have CB or emphysema on CT. In recognition of this, the next step forward in early COPD assessment should incorporate symptoms, lung function and CT imaging to stage smokers for risk for COPD development. Several groups have already proposed this approach.[448 449] In particular, the COPDGene research group used data from 8784 current and ex-smokers to propose a system which utilised a combination of symptoms, spirometric abnormalities and CT abnormalities to classify patients as possible, probable and definite COPD.[449] Incrementally worse outcomes were demonstrated, as reflected by FEV_1 decline and mortality with increasing disease manifestations.

In that study, using patients without disease features as a reference, those having possible COPD (n=2095) had an OR of 1.26 (95% CI 1.03-1.53) for change in FEV_1 >350mls at five years and an HR for all-cause mortality of 1.28 (0.99-1.66). Those classified as having definite COPD (n=2875) had an OR of 2.88 (95% CI 2.23-3.71) and an HR of 5.21 (4.17-6.52) for all-cause mortality.[449] The clinical utility of categorising individuals in this manner is currently unclear. Still, it may improve access of such individuals to prevention strategies such as intensive

smoking cessation programmes and interventions such as pulmonary rehabilitation. Further prospective data will be needed to understand disease evolution from these categories and response to treatment before it can be implemented more widely.

Currently, the USA Preventive Services Task Force[450] and the UK National Screening Committee[451] do not advocate a systematic population screening programme for COPD. This is due to uncertainties about the diagnostic accuracy of risk assessment questionnaires and pulmonary function tests in a primary care setting. There has not been any health economic evaluation examining the cost of implementing screening programmes for COPD. Even if a combined COPD assessment were proven effective at identifying smokers at risk of COPD development, the cost and impact on health services would need to be considered before this can be adapted for screening purposes.

The timing of when smokers will need to be screened and the screening interval also need to be considered. It is unknown whether it is better to screen smokers once they have hit an age threshold or once they have accumulated a minimum smoking history. Furthermore, if they are deemed low risk, another question will be whether they will need to be rescreened in the future and, if so, how often should this occur? If a rescreen is required after a certain period, this will further increase the cost of screening, which is growing exponentially in ageing populations and will need to be considered.

7.3.1.4 BMI and SES – are they relevant?

Current data has suggested that BMI and SES may be essential factors to consider in assessing smokers at risk of COPD. Data presented in chapters 3 and 4 showed that in the current cohort,

smokers with CB had a higher BMI and lived in more deprived neighbourhoods than asymptomatic smokers. Furthermore, those with evidence of SAD on FOT also had a higher BMI than those who did not. Published studies have demonstrated a link between SES and respiratory symptoms.[328] Other studies have also supported the link between obesity and the presence of respiratory symptoms[326 327] and SAD.[380 381] The relationship between SES and COPD incidence and clinical outcomes has been established. A lower SES has been associated with increased COPD incidence,[99] greater FEV_1 decline[452] and exacerbation risk[98] as well as lower exercise capacity.[98]

Several factors may contribute to worse clinical outcomes among smokers within deprived communities. Firstly, smoking is more prevalent among low SES communities,[96] and they are also less likely to quit.[97] Secondly, studies have shown that areas where low SES communities dwell experience higher air pollutants.[453] Thirdly, inequalities of access to healthcare exist, with patients from deprived areas suffering from more prolonged waiting times for elective procedures.[454] It is thus plausible that smoking cessation services may not be readily available among low SES communities. A higher burden of tobacco and environmental-related harms and poorer access to smoking cessation options may help explain why low SES communities are disproportionately affected by smoking. Social deprivation must be addressed in any public health effort to decrease the burden of tobacco-related harms and COPD. In the UK, this has been recognised and included in the NHS Long Term Plan to mitigate the impact of social deprivation on respiratory diseases.[455]

The relationship between BMI and COPD outcomes in severe disease is also well established. An analysis of data from 2132 COPD patients as part of the Copenhagen City Heart Study has shown that mortality was highest in low BMI subjects and decreased with increasing BMI, with

the strongest association seen in those with severe disease.[456] This contrasts with epidemiological data from the general population, where obesity is associated with decreased life expectancy, irrespective of smoking status.[457] These contrasting findings between COPD patients and the general population are referred to as the 'obesity paradox' and are well recognised. A systematic review which included data from 33021 COPD patients from five randomised controlled trials, also showed that compared to normal BMI, low BMI was a risk factor for accelerated FEV_1 decline, whilst high BMI had a protective effect.[458]

However, the relationship between obesity and outcomes in early disease is less well understood. In the Tucson prospective cohort study, an increased prevalence of pre-obesity (BMI ≥28 kg/m^2) was reported in patients with CB symptoms (25% vs 16%, p<0.001). At the same time, emphysema was associated with a low BMI (BMI <18.5) when compared to controls (OR 2.97, 95% CI 1.33-6.68).[326] Another study which included data analyses from 3673 participants from the ECRHS study, has shown that weight gain over 20 years was associated with accelerated FEV_1 decline, while individuals who lost weight exhibited an attenuation of FEV_1 decline.[459]

Common explanations may help clarify the relationship between BMI and COPD outcomes in early and late diseases. In early disease, obesity causes airflow limitation and reduction in respiratory system compliance. The excess weight compressing the thoracic cage causes obese individuals to take shallower breaths than non-obese individuals, which may result in dyspnoea and symptoms of CB.[460] Gastroesophageal reflux disease (GORD) and obesity are also related in both prevalence and causality associations, with GORD symptoms increasing in severity with increasing BMI.[461] Presence of GORD symptoms has been associated with chronic bronchitis symptoms[462] and FEV_1 decline[463] among COPD patients, possibly from

pulmonary micro-aspiration of refluxed gastric material. This may explain why CB symptoms are more prevalent among obese individuals who smoke and may be at higher risk of established COPD. Conversely, once COPD becomes established and the disease progresses, emphysema may become a prominent feature, and patients require increased energy to breathe due to reduced ventilatory muscle efficiency.[464] COPD is also recognised as a systemic disease, with systemic inflammation among COPD patients.[411 465] These features cause catabolic changes in skeletal muscle and resultant weight loss in established COPD[466].

Although current results suggest a relationship between BMI and early COPD, BMI also has a complex relationship with other COPD risk factors such as SES[467] and smoking habits.[468] It is thus difficult to draw definitive conclusions on the role of assessing BMI in early COPD, and larger prospective studies will be needed to address this knowledge gap.

7.3.2 Neutrophils in early COPD disease

7.3.2.1 Investigating neutrophils

Careful handling of neutrophils is essential to ensure reliable and accurate conclusions. In studies described in Chapter 6, neutrophils were isolated from heparinised whole blood by dextran sedimentation of red cells followed by the use of a discontinuous Percoll gradient, which allowed for the isolation of live neutrophils for assessment of neutrophil function and comparison of cell surface markers. Recently published data have demonstrated that isolation in this manner resulted in a higher neutrophil purity compared with similar gradient-based Ficoll isolation following dextran sedimentation. A priming response can be stimulated *in vitro*

with LPS and tumour necrosis factor-alpha (TNF-α), suggesting that this isolation method does not cause substantial alteration of the neutrophil priming response.[469]

7.3.2.2 Neutrophil function in early disease

Data presented in chapter 6 has shown reduced migration velocity of peripheral blood neutrophils in smokers with CB symptoms, which was similar to the dysfunctional neutrophil migration among peripheral neutrophils in COPD patients.[237] Reduced neutrophil migration accuracy in these smokers may have implications for disease pathology owing to the potential for increased areas of obligate tissue damage resulting from protease release during inaccurate migration. However, the current data did not support increased neutrophil degranulation in smokers with CB symptoms. Therefore the hypothesis regarding degranulation could not be confirmed. Possible reasons for the lack of neutrophil degranulation signals in CB smokers have been explored in section 6.3.4.

The precise signalling mechanisms responsible for impairment in neutrophil migration in symptomatic smokers have not been explored. Other studies which demonstrated similar neutrophil migratory defects in COPD patients[237], older adults[417] and frailty[293] have suggested a crucial role of PI3K in this regard. PI3K-inhibition strategies, specifically PI3Kγ or PI3Kδ, have improved neutrophil migration velocity from these cohorts to levels comparable to healthy controls. These findings suggest that impaired neutrophil migration in these states may result from increased or constitutive PI3K activity.

Precisely how alterations to PI3K signalling are mediated and how the downstream effects arise remain unclear. However, as shown in Figure 1.7, PTEN represents a major regulator of

PI3K activity by reversing PI3K action and converting PIP3 into PIP2, and thus PTEN expression and activity should be investigated. Western blotting could determine if protein expression is altered in early COPD, while phosphatase activity assay could assess enzyme activity. Such experiments would offer insight into whether increased PI3K activity is due to impaired regulation of PI3K activity rather than PI3K itself.

7.3.2.3 Neutrophil heterogeneity and phenotypes

Neutrophil heterogeneity has become a topic of debate over the last decade.[436 470] Although not widely replicated, altered neutrophil phenotypes have been identified in the circulation of COPD patients.[431] These changes may provide insight into disease pathogenesis and avenues for therapeutic intervention. Data in section 6.2.6 have shown that whilst differences in individual markers between smokers with CB, AS, and HNS were limited, multi-dimensional cluster analysis revealed a subtle gradient of each cell surface marker rather than distinct subpopulations. These changes suggest neutrophil phenotypes are fluid and less discrete than absolute changes in a single marker. This has two important implications – firstly, it is unlikely a distinct pathogenic neutrophil subpopulation exists in at-risk smokers. Secondly, therapeutics aiming to cause slight changes in neutrophil function that allow them to perform their protective role may provide more clinical benefit than those that cause significant shifts in neutrophil behaviour that inadvertently affect other normal functions.

There has been evidence of systemic inflammation in COPD patients, with raised inflammatory cytokines, including TNF-α[471] and IL-6[465] in these patients. Studies have also suggested neutrophil priming occurs in peripheral neutrophils from COPD patients, identified by a

reduction in CD62L[251 252] or an increase in CD11b expression.[251 472] Evidence of systemic neutrophil activation has yet to be explored in non-COPD smokers with CB symptoms. Data presented in this thesis shows that systemic neutrophil activation, measured using CD11b, CD66b and CD62L, was not significantly altered in cigarette smoking or respiratory symptoms. There are several potential reasons why a signal of activation was not seen in smoking non-COPD participants with CB. One possible hypothesis to explain the lack of systemic activation in CB smokers is that although there are increased concentrations of cytokines in the lungs of smokers that can induce neutrophil activation (e.g., TNF and CXCL8),[473] this may not hold in the general circulation. Another reason may be that activated neutrophils in these smokers leave the circulation and enter the lung following chemotactic gradients.

Previous work has suggested that COPD is a disease of accelerated ageing, such as increased cellular senescence and loss of anti-ageing processes.[474] Neutrophil senescence leads to distinct functional alterations that impair response to infection and increase the potential for host damage[475] and have yet to be explored in COPD. However, the lack of difference in CXCR4 and CXCR2 expression on peripheral neutrophils suggests that neutrophil senescence is not a feature of early disease. It is important to note that these data only highlight neutrophil changes and do not conflict with the evidence of senescence in other cell types (such as endothelial cells and fibroblasts) previously described.[474]

Multidimensional cluster analyses have shown that smokers with CB have a higher proportion of circulating neutrophils in a cluster with a higher expression of CD11c. Expression of HLA-DR, CD11c and PD-L1 on neutrophils have been linked with inflammatory status, where HLA-DR indicates a pro-inflammatory phenotype while PD-L1 and CD11c indicate an anti-inflammatory phenotype. In particular, Pillay et al. have shown that suppression of T-cell

function was accomplished by a subset of neutrophils with higher expression of CD11c that was systematically induced in response to acute inflammation, in this case via intravenous injection of LPS.[266] The increase in CD11c seen in a subset of CB neutrophils may represent a physiological balancing mechanism that reduces the systemic inflammatory response and might also help explain the lack of systemic activation in neutrophils from these individuals. Evidence supports this in patients with type 2 diabetes, where peripheral neutrophils showed increased CD11c surface expression and blunted upregulation of CD11b expression in response to fMLP compared to non-diabetic subjects.[267]

7.4 Study limitations

The study limitations have been discussed in the respective chapters, but the main limitations are discussed below. Work described in this thesis had not previously been performed in a population of smokers who may be at risk of developing COPD. Thus, calculating sample sizes that were adequately powered to detect differences was not possible. Recruitment into the study and laboratory cellular work was halted prematurely due to the COVID-19 pandemic. Furthermore, due to clinical service priority, the pandemic disrupted lung function testing and chest CT scanning capability. The data reported here has to be considered pilot data, and as a consequence, some of the analyses in this thesis were underpowered. This makes drawing definitive conclusions from current data difficult.

Apart from symptom questionnaire scores described in chapter 3, the data presented in this study was cross-sectional. The inability to measure lung function longitudinally made it impossible to identify smokers with rapid FEV_1 decline who were thought to be most at risk of

developing COPD. Thus, smokers with CB were used as a surrogate for these at-risk smokers, but this approach comes with several caveats, as described in section 7.3.1.2.

The COVID-19 pandemic allowed the opportunity to study the effects of a national lockdown and social distancing measures on symptoms and rates of chest infections in the Birmingham Early COPD cohort, as detailed in section 3.2.6. Data in this section described improvement in physical symptoms and worsening psychological symptoms with the implementation of the national lockdown. However, the introduction of such measures also made longitudinal analyses of symptoms and incidence of chest infections among the cohort less generalisable as their behaviour has been fundamentally altered during this period.

Neutrophil function and phenotyping studies were performed using isolated cells. The process of isolating neutrophils for *in vitro* studies, including the methodology employed in this thesis, can alter neutrophil functional responses and phenotype. The sedimentation of red cells using dextran was previously shown to increase neutrophil activation, potentially due to monocyte interactions with neutrophils.[476] A Percoll gradient-based isolation may also reduce the ability of neutrophils to respond to subsequent stimulation.[477] These changes may influence current data in this thesis, although a consistent neutrophil isolation method should not alter comparisons between groups.

Using neutrophils isolated from peripheral blood represents the simplest and non-invasive way to study neutrophil function and phenotype. However, it is known that circulating neutrophils are fundamentally different compared to neutrophils within the lungs,[259] of which are thought to exert their pathogenic effects leading to COPD. It is currently unknown whether neutrophils from lung secretions of CB smokers are fundamentally different compared to

those from AS or HNS. Whilst studies of peripheral blood neutrophils describe differences in neutrophil migration, it is unknown whether this phenotype is maintained within the lung following transmigration.

7.5 Future directions

Multiple aspects of clinical assessments among smokers who may have early COPD disease process have been investigated in this thesis. Neutrophil function and phenotypes were also investigated, building upon data from other Birmingham Respiratory research group members. The presented data offers several novel insights and raises questions requiring further investigation.

1. Assessing lung function over multiple time points would allow for longitudinal assessment of lung function trajectory, such as FEV_1 decline. Those with excess FEV_1 decline are at high risk of developing COPD. It is essential to characterise the symptom burden, lung function and chest CT parameters in such smokers before airflow obstruction is diagnosed spirometrically. Such data will contribute to creating models that allow risk stratification of smokers who may develop COPD.
2. As mentioned in section 7.3.1.4, there is a suggestion that BMI may be a factor to consider in assessing at-risk smokers. The impact of BMI on COPD risk has not been fully explored in epidemiological studies. Recruitment of more participants would allow enough data to perform analyses such as multivariable regression analyses that control for confounding factors. This allows a closer assessment of the relationship between BMI and important clinical outcomes such as symptom burden and FEV_1 decline. If this relationship were

found to be substantial, collecting blood and sputum samples would allow cellular and inflammatory studies to identify the underlying mechanisms.

3. The small airways are affected early in the COPD disease course. However, there is currently no gold standard to detect SAD using lung function testing. MMEF and, increasingly, FOT have been used in clinical practice to assess small airways in patients with respiratory disease. Still, other physiological assessment methods exist, each having potential advantages and disadvantages.[478] These tests will need to be assessed on which is most reliable at identifying at-risk smokers, acceptable to patients, practical to deliver and have utility within clinical trials to help improve clinical outcomes.
4. CT densitometry allows the detection and quantification of emphysema that may not be apparent on visual inspection. However, it would be of interest to assess for functional small airway disease on CT scans obtained from the Early COPD cohort using techniques such as parametric response mapping. Previous studies have shown that functional small airway disease has been associated with FEV_1 decline in patients with mild-to-moderate COPD.[104] Thus, it would be helpful to see whether such results can be replicated in those without spirometric airflow obstruction. However, limitations to these techniques will have to be taken into consideration. Exposure to radiation limits repeated assessment for monitoring, and CT quantification variability described in section 1.2.7.3 makes longitudinal comparison difficult.
5. There is widespread agreement that neutrophils are causally associated with the damage present in the lungs of COPD patients. It is imperative to understand the function and phenotype of neutrophils in the lung of at-risk smokers, and the collection of both blood and lung secretions would enable more detailed phenotyping in such smokers. It is

possible that although there were no differences found in neutrophil phenotypes between the smoker subtypes in the current data, there may be differences seen when neutrophils from lung secretions are compared. It would also be essential to see whether the altered neutrophil migratory defect in CB smokers can be replicated using neutrophils from lung secretions to determine whether transmigration into lung tissue alters this defect.

6. Although a neutrophil migratory defect had been demonstrated in peripheral neutrophils from smokers with CB, the mechanism underpinning this is unknown. Previous work from other members of the research group has suggested that the PI3K pathway has a role to play in this regard in other diseases.[237 293 417] It would be helpful to see whether the neutrophil migratory defect can be corrected using PI3K inhibitors to determine whether the PI3K pathway is implicated in the neutrophil migratory defect seen in CB smokers.
7. As described in section 1.4, neutrophils have other functions apart from those studied here, including phagocytosis of pathogens and the formation of NETs. A study of these using functional assays using neutrophils from smokers with CB or those with excess FEV_1 decline would be of interest to determine whether there are any other defects in neutrophil function that could potentially contribute to lung damage in early disease.

7.6 Conclusion

There is increasing recognition that understanding the early stages of disease in COPD will help identify therapeutic targets to prevent disease progression and mitigate risk factors. Understanding early COPD may determine optimal treatment options for individuals with early disease and help discover robust biomarkers closely linked to the underlying disease

mechanisms to accelerate the discovery of novel therapeutic agents. However, the ability to detect patients at risk of progression from early to established disease is limited in clinical practice. In this thesis, the Early COPD cohort represents a well-characterised cohort of young smokers who may have an early COPD disease process. This novel cohort was assessed using a combination of symptom questionnaires, lung function and CT imaging. Neutrophil function and surface expression of key markers were also evaluated among cohort participants and represent the first biological assessment among smokers who may have early COPD.

A proportion of the cohort has a significant physical and psychological symptom burden. Some cohort participants had SAD on lung function testing or emphysema detected using CT densitometry. Furthermore, neutrophils from smokers with CB were found to have impaired migratory function but not from AS. However, there was no evidence of excess neutrophil degranulation or altered phenotype based on cell surface marker expression among neutrophils from smokers with CB compared to neutrophils from AS. These data suggest that smokers with possible early COPD are heterogeneous regarding their symptoms, lung function and radiological features. These smokers have evidence of neutrophil dysfunction, which may result in increased collateral tissue damage, but distinct alterations do not explain this in neutrophil phenotypes.

CHAPTER 8 – REFERENCES

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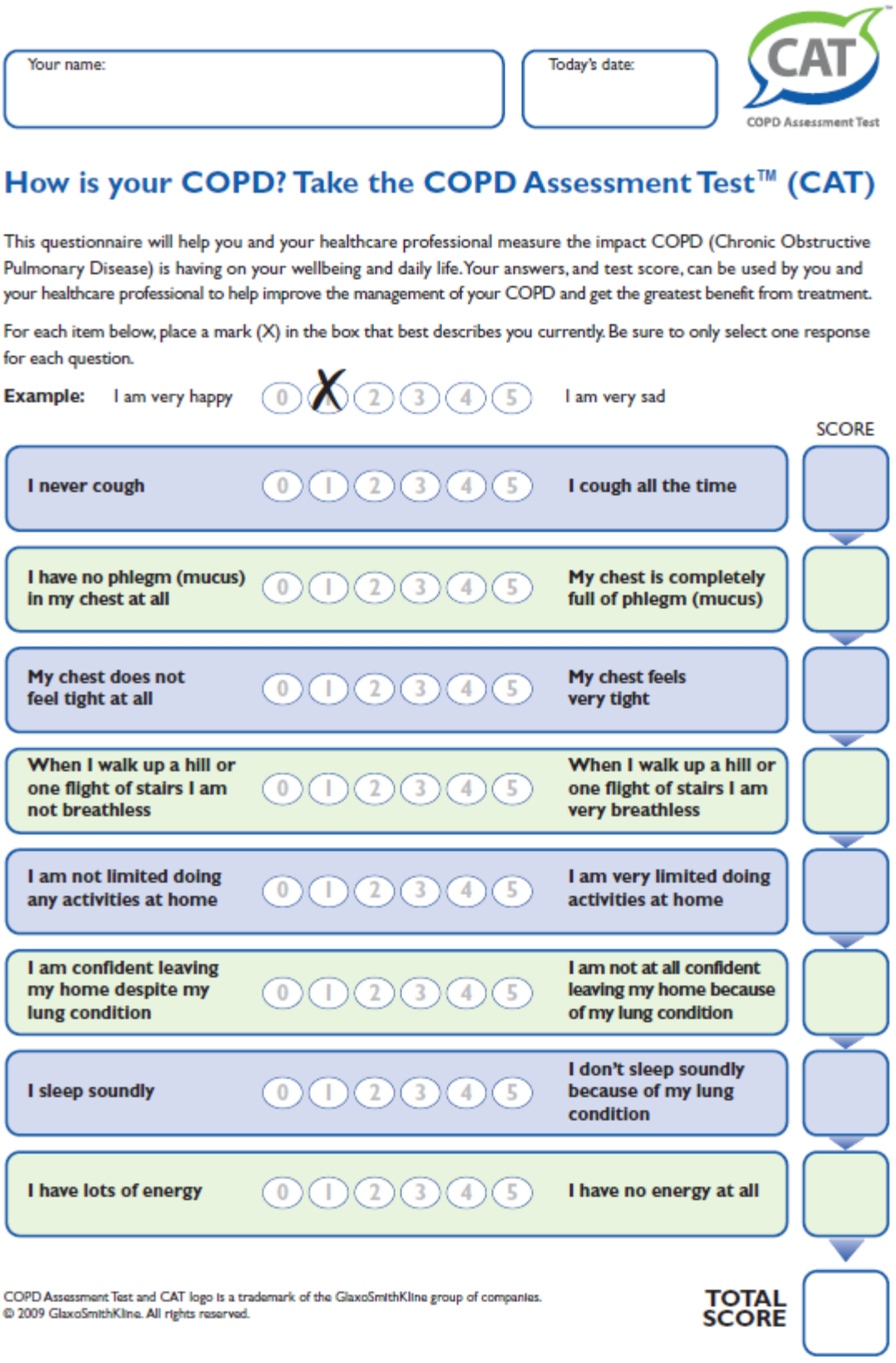
CHAPTER 9 – APPENDICES

9.1 Appendix 1 – mMRC dyspnoea scale

Modified MRC Dyspnoea Scale

0	I only get breathless with strenuous exercise.	
1	I get short of breath when hurrying on the level or walking up a slight hill.	
2	I walk slower than people of the same age on the level because of breathlessness or have to stop for breath when walking at my own-pace on the level.	
3	I stop for breath after walking about 100 yards or after a few minutes on the level.	
4	I am too breathless to leave the house.	

9.2 Appendix 2 – COPD Assessment Test



Your name:

Today's date:

CAT
COPD Assessment Test

How is your COPD? Take the COPD Assessment Test™ (CAT)

This questionnaire will help you and your healthcare professional measure the impact COPD (Chronic Obstructive Pulmonary Disease) is having on your wellbeing and daily life. Your answers, and test score, can be used by you and your healthcare professional to help improve the management of your COPD and get the greatest benefit from treatment.

For each item below, place a mark (X) in the box that best describes you currently. Be sure to only select one response for each question.

Example: I am very happy 0 X 2 3 4 5 I am very sad

			SCORE
I never cough	0 1 2 3 4 5	I cough all the time	
I have no phlegm (mucus) in my chest at all	0 1 2 3 4 5	My chest is completely full of phlegm (mucus)	
My chest does not feel tight at all	0 1 2 3 4 5	My chest feels very tight	
When I walk up a hill or one flight of stairs I am not breathless	0 1 2 3 4 5	When I walk up a hill or one flight of stairs I am very breathless	
I am not limited doing any activities at home	0 1 2 3 4 5	I am very limited doing activities at home	
I am confident leaving my home despite my lung condition	0 1 2 3 4 5	I am not at all confident leaving my home because of my lung condition	
I sleep soundly	0 1 2 3 4 5	I don't sleep soundly because of my lung condition	
I have lots of energy	0 1 2 3 4 5	I have no energy at all	

TOTAL SCORE

9.3 Appendix 3 – Leicester Cough Questionnaire

APPENDIX 1: Leicester Cough Questionnaire. © 2001

This questionnaire is designed to assess the impact of cough on various aspects of your life. Read each question carefully and answer by CIRCLING the response that best applies to you. Please answer ALL questions, as honestly as you can.

1. In the last 2 weeks, have you had chest or stomach pains as a result of your cough?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

2. In the last 2 weeks, have you been bothered by sputum (phlegm) production when you cough?

1	2	3	4	5	6	7
Every time	Most times	Several times	Some times	Occasionally	Rarely	Never

3. In the last 2 weeks, have you been tired because of your cough?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

4. In the last 2 weeks, have you felt in control of your cough?

1	2	3	4	5	6	7
None of the time	Hardly any of the time	A little of the time	Some of the time	A good bit of the time	Most of the time	All of the time

5. How often during the last 2 weeks have you felt embarrassed by your coughing?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

6. In the last 2 weeks, my cough has made me feel anxious

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

7. In the last 2 weeks, my cough has interfered with my job, or other daily tasks

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

8. In the last 2 weeks, I felt that my cough interfered with the overall enjoyment of my life

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

9. In the last 2 weeks, exposure to paints or fumes has made me cough

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

10. In the last 2 weeks, has your cough disturbed your sleep?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

11. In the last 2 weeks, how many times a day have you had coughing bouts?

1	2	3	4	5	6	7
All of the time (continuously)	Most times during the day	Several times during the day	Some times during the day	Occasionally through the day	Rarely	None

12. In the last 2 weeks, my cough has made me feel frustrated

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

13. In the last 2 weeks, my cough has made me feel fed up

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

14. In the last 2 weeks, have you suffered from a hoarse voice as a result of your cough?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

15. In the last 2 weeks, have you had a lot of energy?

1	2	3	4	5	6	7
None of the time	Hardly any of the time	A little of the time	Some of the time	A good bit of the time	Most of the time	All of the time

16. In the last 2 weeks, have you worried that your cough may indicate serious illness?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

17. In the last 2 weeks, have you been concerned that other people think something is wrong with you, because of your cough?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

18. In the last 2 weeks, my cough has interrupted conversation or telephone calls

1	2	3	4	5	6	7
Every time	Most times	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

19. In the last 2 weeks, I feel that my cough has annoyed my partner, family or friends

1	2	3	4	5	6	7
Every time I cough	Most times when I cough	Several times when I cough	Some times when I cough	Occasionally when I cough	Rarely	Never

Thank you for completing this questionnaire.

9.4 Appendix 4 – Hospital Anxiety and Depression Scale

Hospital Anxiety and Depression Scale (HADS)

Tick the box beside the reply that is closest to how you have been feeling in the past week.
Don't take too long over you replies: your immediate is best.

D	A		D	A	
		I feel tense or 'wound up':			**I feel as if I am slowed down:**
	3	Most of the time	3		Nearly all the time
	2	A lot of the time	2		Very often
	1	From time to time, occasionally	1		Sometimes
	0	Not at all	0		Not at all
		I still enjoy the things I used to enjoy:			**I get a sort of frightened feeling like 'butterflies' in the stomach:**
0		Definitely as much		0	Not at all
1		Not quite so much		1	Occasionally
2		Only a little		2	Quite Often
3		Hardly at all		3	Very Often
		I get a sort of frightened feeling as if something awful is about to happen:			**I have lost interest in my appearance:**
	3	Very definitely and quite badly	3		Definitely
	2	Yes, but not too badly	2		I don't take as much care as I should
	1	A little, but it doesn't worry me	1		I may not take quite as much care
	0	Not at all	0		I take just as much care as ever
		I can laugh and see the funny side of things:			**I feel restless as I have to be on the move:**
0		As much as I always could		3	Very much indeed
1		Not quite so much now		2	Quite a lot
2		Definitely not so much now		1	Not very much
3		Not at all		0	Not at all
		Worrying thoughts go through my mind:			**I look forward with enjoyment to things:**
	3	A great deal of the time	0		As much as I ever did
	2	A lot of the time	1		Rather less than I used to
	1	From time to time, but not too often	2		Definitely less than I used to
	0	Only occasionally	3		Hardly at all
		I feel cheerful:			**I get sudden feelings of panic:**
3		Not at all		3	Very often indeed
2		Not often		2	Quite often
1		Sometimes		1	Not very often
0		Most of the time		0	Not at all
		I can sit at ease and feel relaxed:			**I can enjoy a good book or radio or TV program:**
	0	Definitely	0		Often
	1	Usually	1		Sometimes
	2	Not Often	2		Not often
	3	Not at all	3		Very seldom

Please check you have answered all the questions

Scoring:

Total score: Depression (D) _____ Anxiety (A) _____

0-7 = Normal

8-10 = Borderline abnormal (borderline case)

11-21 = Abnormal (case)

9.5 Appendix 5 – Work during the COVID-19 pandemic

During periods when data collection was impossible during the COVID-19 pandemic, I have focused efforts on publishing a narrative review article in a peer-reviewed journal and contributed to other studies, which are listed below:-

Review article: Yip KP, Stockley RA, Sapey E. Catching "Early" COPD – The Diagnostic Conundrum. *Int J Chron Obstruct Pulmon Dis*. 2021;16:957-968. PMID: 33880020

Framework article: Walker EM, Jasper AE, Davis L, Yip KP, Faniyi AA, Hughes MJ, Crisford HA, Spittle DA, Sapey E, Belchamber KBR, Scott A. Mitigating Health Risks to Reopen a Clinical Research Laboratory During the COVID-19 Pandemic: A Framework. JMIR Res Protoc. 2020 Dec 4;9(12):e22570. PMID: 33146625

Systematic review: Chin IS, Galavotti S, Yip KP, Curley H, Arnold R, Sharma-Oates A, Chedwidden L, Lee SI, Lee LYW, Pinato DJ, Dettorre GM, Palles C. Influence of clinical characteristics and anti-cancer therapy on outcomes from SARS-CoV-2 infection: a systematic review and meta-analysis of 5,678 cancer patients. medRxiv. doi:10.1101/2020.12.15.20248195.

Research article: Yip KP, Gompertz S, Snelson C, et al. Increase in recruitment upon integration of trial into a clinical care pathway: an observational study. BMJ Open Respiratory Research 2021;8:e000967. PMID: 34230034

Research article: Belchamber KBR, Thein OS, Hazeldine J, Grudzinska FS, Hughes MJ, Jasper AE, Yip KP, Sapey E, Parekh D, Thickett DR, Scott A. Altered neutrophil phenotype and function in non-ICU hospitalised COVID-19 patients correlated with disease severity. medRxiv. doi:10.1101/2021.06.08.21258535