

# Eosinophilic airways inflammation in Chronic Obstructive Pulmonary Disease

Thesis submitted for the degree of

Doctor of Philosophy

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## Abstract

**Background:** Eosinophilic airway inflammation (>3% sputum eosinophils) is a feature of subgroup of subjects with chronic obstructive pulmonary disease (COPD). My objectives were to investigate the clinical characteristics of eosinophilic COPD, its stability over time and extent in bronchial tissue, whether it is related to parasite exposure or atopy and whether its persistence is due to abnormal clearance by macrophages.

**Methods:** Subjects were studied that had participated in previous observational studies. The repeatability of sputum eosinophils was measured between 3 monthly visits for 1 year. The extent of eosinophilic inflammation in bronchial tissue was assessed using immunohistology on bronchial tissue from COPD and control subjects. Positive serology for parasites was tested in serum samples for 4 helminth species. Atopy was assessed in the subjects using serum total Ig-E and skin prick test. Eosinophil efferocytosis by macrophages was investigated *in vivo* using cytoplasmic area of red hue of macrophages and *in vitro* using apoptotic eosinophils fed to monocyte-derived macrophages from COPD and healthy controls. The dynamics of eosinophil clearance during exacerbations was explored using the red hue technique.

**Results:** Eosinophilic and non-eosinophilic COPD have similar lung function and exacerbation frequency. In eosinophilic COPD the health status is better and bacterial colonisation is lower. This phenotype is stable over time. Airway tissue eosinophils are increased in COPD subjects with high blood eosinophils and are positively correlated with features of remodelling. Eosinophilic COPD is not associated with helminth exposure, but is related to elevated total Ig-E. Macrophage efferocytosis of eosinophils is impaired in COPD and is associated with the severity and frequency of COPD exacerbations. Efferocytosis of eosinophils by macrophages is increased following oral corticosteroid therapy at exacerbation.

**Conclusion:** Eosinophilic COPD is a distinct and stable phenotype that persists in the blood, bronchus and sputum. Its persistence is partly related to atopy and impaired clearance by macrophages with the latter associated with COPD exacerbation severity and frequency.

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## Statement of Originality

This thesis entitled 'Eosinophilic airways inflammation in Chronic Obstructive Pulmonary Disease' is submitted for the degree of Doctor of Philosophy. This thesis is based on work conducted by the author in the Department of Infection, Immunity and Inflammation at the University of Leicester during the period between October 2010 and September 2014. All the work recorded in this thesis is original unless otherwise acknowledged in the text or referenced.

This work has not been submitted for another degree in this or any other University.

Signed ..... *Osama* ..... Date ..... 06/10/2014 .....

## **Statement of Work Performed**

The hypothesis and design of the studies were designed by myself in conjunction with Professor Christopher Brightling.

Data collection, recruiting volunteers, blood collection, processing and purification in the laboratory for chapter 5 and 7 were performed by myself. Data for chapter 3, 4, 6 and 8 were generated from previous studies as will be mentioned in each individual chapter. I designed and conducted all the macrophage colour assessment, cell culture experiments including phagocytosis assay, cytospin preparation, immunohistochemical staining and quantification of cells. I also carried out the microscopy including assessment and quantification of immunohistochemical stained inflammatory cells. I performed all the statistical data analysis.

Sputum induction and spirometry was performed by the designated nurses. Sputum processing was carried out in the sputum laboratory by the designated team. Blood differential count and CRP were performed in the pathology laboratory at University Hospitals of Leicester.

GMA embedded bronchial tissue biopsy collection, cutting and immunohistochemical staining were completed by Vijay Mistry. Haematoxylin and Eosin staining of bronchial slides was carried out by the histology department at University Hospitals of Leicester. Serum samples for helminths were analysed in the Parasitology Reference Laboratory at the Hospital for Tropical Diseases, London

Finally I prepared all the manuscripts and abstracts that were presented at internal and national conferences. The work arising from this thesis was published in peer-reviewed journals.

## **Publications and Abstracts arising from this thesis**

**Eltboli O**, Brightling CE. Eosinophils as diagnostic tools in chronic lung disease. *Expert Rev Respir Med.* 2013; 7(1):33-42.

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**Eltboli O**, Bafadhel M, Hollins F, Wright A, Hargadon B, Kulkarni N, Brightling C. COPD exacerbation severity and frequency is associated with impaired macrophage efferocytosis of eosinophils. *BMC Pulm Med.* 2014; 9;14:112.

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## **Abstracts**

**Eltboli O**, Bafadhel M, Kulkarni N, Brightling C. COPD exacerbation severity is associated with impaired macrophage phagocytosis of eosinophils. *ERJ* September 1, 2013 vol. 42 no. Suppl 57 P879.

## **List of Abbreviations**

ACCP American College of Chest Physicians

ACOS Asthma-COPD overlap syndrome

ADCC Antibody dependent cell-mediated cytotoxicity

ANOVA Analysis of variance

ASM Airway smooth muscle

ATS American Thoracic society

AUC Area under the curve

BAL Bronchoalveolar lavage

BALT Bronchus associated lymphoid tissue

BDP Beclometasone Dipropionate

BMI Body mass index

BSA Bovine serum albumin

CC Chemokine

CCR Chemokine receptor

CD Cluster of differentiation

CFU Colony forming units

CI Confidence interval

CO<sub>2</sub> Carbon dioxide

COPD Chronic obstructive pulmonary disease

CRP C-reactive protein

CRQ Chronic respiratory disease questionnaire

CT Computed tomography

CXCL Chemokine (C-X-C motif) ligand



CXR Chest X-ray

DAB 3,3'-diaminobenzidine tetrahydrochloride

DAPI 4',6-diamidino-2-phenylindole

DMEM Dulbecco modified Eagle medium

D-PBS Dulbecco's phosphate buffered saline

DREAM Dose Ranging Efficacy And safety with Mepolizumab

DTT Dithiothreitol

ECLIPSE: Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points

ECP Eosinophil cationic protein

EDN Eosinophil derived neurotoxin

EDTA Ethylenediaminetetraacetic acid

ELISA Enzyme linked immunosorbent assay

EPO Eosinophil peroxidase

ERS European Respiratory Society

FBS Foetal bovine serum

FeNO Fraction of exhaled nitric oxide

FEV<sub>1</sub> Forced expiratory volume in 1 second

FITC Fluorescein isothiocyanate

FVC Forced vital capacity

G-CSF Granulocyte colony stimulating factor

GMA Glycomethacrylate

GMCSF Granulocyte macrophage colony stimulating factor

GOLD Global initiative for chronic obstructive lung disease

GRO Growth-related oncogene

HBSS Hank's Balanced Salt Solution

HDAC2 Histone deacetylase 2

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

H<sub>2</sub>O<sub>2</sub> hydrogen peroxide

IFN- $\gamma$  Interferon gamma

Ig Immunoglobulin

IL Interleukin

IL-5R Interleukin-5 receptor

iNOS inducible Nitric Oxide synthase

IP Interferon- $\gamma$  inducible protein

IQR Interquartile range

kPa Kilopascal

LT leukotriene

MBP Major basic protein

MCP Macrophage chemotactic protein

M-CSF Macrophage colony-stimulating factor

MDM monocyte–derived macrophages

mg Milligram

MIG Monokine induced by interferon-gamma

mL Milliliter

mM millimole

MMP Matrix metalloproteinase

MRC Medical Research Council

NEAA Non-essential amino acids

NHS National Health Service

NICE National Institute for Health and Care Excellence

NIH National Institutes of Health

NIHR National institute for health research

PB Peripheral blood

PBMC Peripheral blood mononuclear cells

PCR Polymerase chain reaction

PCT Procalcitonin

PSA principal component analysis

QoL Quality of life

qPCR quantitative polymerase chain reaction

RANTES (regulated upon activation normal T cell-expressed and secreted)

RBM reticular basement membrane

ROI Region of interest

rpm Round per minute

RT-PCR Reverse transcriptase polymerase chain reaction

SD Standard deviation

SEM Standard error of mean

SGRQ St George's Respiratory Questionnaire

SP Sodium Pyruvate

SP-D Surfactant protein D

SPSS Software package for statistical analysis

TCC total cell count

T<sub>h</sub> T helper cells

TNF Tumour necrosis factor

VAS Visual analogue scale

VCAM-1 Vascular cell adhesion molecule-1

VLA Very late antigen

WHO World Health Organization

μg Microgram

μL Microlitre

μm Microns/micrometer

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# **Chapter 1   Introduction**

## **1.1 Background**

The airway inflammatory and remodelling profiles of chronic obstructive pulmonary disease (COPD) are heterogeneous. Understanding this heterogeneity is likely to shed light upon the underlying pathogenesis of disease and provide potential biomarkers to target using current and future therapy. Exacerbations are sudden and sustained deterioration of the disease symptoms beyond day to day variability. COPD exacerbations have a significant burden not only on the community, but also at the personal level in terms of the overall disease severity, deterioration of lung function, susceptibility of developing frequent exacerbations in the future and hospitalizations.

## **1.2 COPD definition, clinical features and diagnosis**

Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines COPD as a common preventable disease that is characterized by progressive and fixed airflow obstruction with limited reversibility, which is the hallmark of this disease, and an amplified and persistent inflammatory response to noxious stimuli, punctuated by development of episodes of acute attacks known as exacerbations and associated with extra pulmonary comorbidities that may contribute to the severity of the disease (Rabe, Hurd *et al.* 2007). The diagnosis of COPD is usually made on clinical grounds typically in middle aged smoker subjects, or in those exposed to other noxious agents who present with breathlessness, wheeze, chronic cough or increased production of sputum with reduced breath sounds and rhonchi on chest examination. However, spirometric finding of airway obstruction with post-bronchodilator  $FEV_1/FVC$  ratio less than 0.70 is needed as well to make the diagnosis. The Global Initiative for Chronic Obstructive Lung Disease has categorised COPD into four forms in terms of disease severity based on the degree of airway flow obstruction in patients with  $FEV_1/FVC$

ratio  $<0.70$ : mild; moderate; severe and very severe COPD or GOLD1; GOLD2; GOLD3 and GOLD4 if the post-bronchodilator  $FEV_1 \geq 80\%$ ; 50%-79%; 30%-49%;  $<30\%$  predicted  $FEV_1$  (or  $<50\%$  predicted plus the evidence of chronic respiratory failure (defined as  $PaO_2 < 8.0$  kPa measured at room air and/or presence of cor pulmonale)) respectively (GOLD 2014).

Importantly, the current classification of COPD severity using  $FEV_1$  alone is not very precise in describing or defining the disease as it may over- or underestimate the diagnosis especially at extremes of age and also does not consider the airways inflammation (Bhatt, Wood 2008; Swanney, Ruppel *et al.* 2008, Hnizdo, Glindmeyer *et al.* 2006), in addition to heterogeneity of the disease. Thus the “combined assessment of COPD” might be more accurate than spirometric grading systems as it does take into account not only  $FEV_1$ , but also symptoms and exacerbation rate (GOLD 2014).

### **1.3 Epidemiology and burden of COPD**

COPD is currently the fourth major cause of mortality worldwide and is predicted to become the third commonest cause by 2030 (WHO 2004). Approximately 64-65 million individuals worldwide are diagnosed nowadays with COPD, with over 3 million patients died of COPD in 2005 (WHO 2014a). Developing countries represent about 90% of the death cases that take place globally. The COPD prevalence is more than 5% for people with 65 years of age or older. At present, approximately the male: female affected ratio is 2:1, but with the increase of cigarette smoking among women, especially in western world, both genders will be equally affected by the disease (Raheison, Girodet 2009). It is estimated that almost 3 million people in the UK have COPD (Stang, Lydick *et al.* 2000), with a loss of greater than 27,000 lives annually (Hubbard 2006). Furthermore, it costs more than £800 million to the

National Health Service (NHS) as a direct cost, in addition to about £2.7 billion annually due to loss of productivity (NICE report 2011). Unlike some other chronic diseases like cardiovascular disorders, few therapeutic discoveries have been revealed that make a large difference in terms of reducing the morbidity and mortality associated with the disease.

#### **1.4 Aetiology and risk factors**

COPD is predominately caused by cigarette smoking, including second-hand or passive exposure, especially in western countries in susceptible individuals, but other factors such as occupational exposure to dust, gases or fumes and indoor air pollution including the use of biomass fuels for cooking and heating may also be important particularly in the third world (Mehta, Miedinger *et al.* 2012, WHO 2014b). However, large numbers of people who smoke never develop the disease. Small proportions of subjects have congenital deficiency of  $\alpha_1$ -antitrypsin enzyme and develop COPD even without smoking as a result of imbalance between proteinases and antiproteinases (Turino, Seniorrm *et al.* 1969).

#### **1.5 Pathophysiology**

COPD is characterized by small and large airways disease. Airway inflammation, fibrosis, luminal plugs, airway resistance and parenchymal destruction are the main causes of airway limitations (GOLD 2010). The extravagant inflammatory response is the main culprit behind these structural abnormalities. Emphysema is the end result of COPD morphological damage. The pathological definition of emphysema is “an abnormal permanent enlargement of air spaces distal to the terminal bronchiole and destruction of their alveolar walls without obvious fibrosis”, which causes an air trapping and increase in lung compliance (Hogg, Senior 2002). Chronic bronchitis is the condition that results from hyperplasia of mucus-

producing glands, metaplasia of goblet cells and chronic inflammation adjacent to bronchi resulting in a disproportionate mucus production and obstruction of airways. Historically the terms “pink buffer” and “blue bloater” were used to describe the pathophysiological abnormalities that take place in emphysema and chronic bronchitis phenotype respectively, where there is a hyperventilation and normal oxygenation in the emphysema, unlike in bronchitis where the hypoxia and hypercapnoea predominates.

## **1.6 Immunopathogenesis and airway inflammation in COPD**

Airway inflammation plays a pivotal role in the pathogenesis of COPD and other airway diseases. The new definition of COPD does include the amplified and abnormal inflammatory response to noxious stimuli, (GOLD 2014). The immunopathogenesis of the disease is complex, poorly understood and involves many inflammatory cells and mediators.

Airway inflammation can be feasibly measured in peripheral blood and in the airway invasively by bronchoscopic sampling or non-invasively by sputum induction. The use of sputum cytology to explore airways inflammation has been in existence since the first descriptions by Ernst Leyden and Jean Charcot of Charcot-Leyden crystals and was used clinically in the 1950s by Dr Morrow-Brown and then optimised in the 1990s by Freddy Hargreaves and colleagues (Pavord, Pizzichini *et al.* 1997). Despite the innovation of many other techniques to examine airway inflammation, sputum differential cell count remains the method of choice to investigate the bronchial tree. It is a safe, non-invasive and well-tolerated procedure in all stages of disease severity and most importantly is useful in differentiating various diseases phenotypes and their relationship with response to treatment (Brightling

2006). It requires minimal training and equipment that is available in routine pathology laboratories.

Neutrophils seem to be the predominant cells in most COPD patients, but significant proportion of subjects have eosinophilic airway inflammation or mixture of different cells.

### **1.6.1 Neutrophils**

Typically the airways inflammation in COPD is neutrophilic. Neutrophils are the principal inflammatory cells in sputum, broncho-alveolar lavage (BAL) fluid and to a lesser extent in bronchial tissues in COPD (Barnes, Shapiro *et al.* 2003). Many serine proteases such as neutrophil elastase, cathepsin G and proteinase-3, in addition to matrix metalloproteins (MMPs) are secreted by neutrophils. Numerous effects such as destruction of alveoli and oversecretion of mucus are attributed to these catalytic enzymes which supports the protease/antiprotease imbalance theory for development of pulmonary emphysema (Tetley 1993). This involves activation of MMP-2, MMP-9, MMP-12, cathepsin K, L and S and neutrophil elastase proteolytic enzymes as a result of oxidative stress and inflammatory process in COPD (Angelis, Porpodis *et al.* 2014), but it appears that other factors contribute to the pathogenesis of emphysema in COPD, since the mechanism in some other diseases such as cystic fibrosis and bronchiectasis does not implicate a damage of elastic tissue, despite the increased numbers of neutrophils in such diseases (Barnes, Shapiro *et al.* 2003).

The recruitment of neutrophils into the airways and lungs is mainly influenced by (IL)-8 and leukotriene B<sub>4</sub> (LTB<sub>4</sub>), which are secreted by activated macrophages. Whereas granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor



(G-CSF) are responsible for the survival of neutrophils in the respiratory system (Barnes, Shapiro *et al.* 2003). Neutrophils elastase, cathepsin-G and proteinase-3, which are released by neutrophils, promote submucosal mucous glands and goblet cells to secrete proteinases (Swanney, Ruppel *et al.* 2008). The increased numbers of neutrophils in sputum of COPD subjects is correlated with a reduction in FEV<sub>1</sub> (Stanescu, Sanna *et al.* 1996) and severity of the disease (Barnes, Shapiro *et al.* 2003).

### **1.6.2 Macrophages**

It is well established that tissue macrophages are derived from circulating monocytes (Tacke, Randolph 2006, Yang, Zhang *et al.* 2014) under the influence of certain growth factors such as Granulocyte macrophage colony stimulating factor GM-CSF and macrophage-colony stimulating factor (M-CSF) in the case of alveolar macrophages (Guilliams, De Kleer *et al.* 2013, Lenzo, Turner *et al.* 2012), which takes place in foetal life according to recent studies (Guilliams, De Kleer *et al.* 2013, Ginhoux, Jung 2014). The role of macrophages in COPD is very crucial. It is reported that the number of macrophages is significantly raised in sputum, BAL and tissue in COPD subjects, especially at sites with damaged alveoli in contrast to control smokers (O'Donnell, Breen *et al.* 2006). It has been also revealed that severity of COPD is associated with macrophages numbers, in addition to neutrophils in the airways (Di Stefano, Capelli *et al.* 1998).

Under the influence of cigarette smoke, alveolar macrophages are activated and release many inflammatory mediators and chemotactic factors that regulate other inflammatory cells including, but not limited to the following mediators: interleukin (IL)-8; interleukin IL-6; tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ); growth-related oncogene- $\alpha$  (GRO- $\alpha$ ) and LTB<sub>4</sub>, which

stimulates recruitment of neutrophils; macrophage chemotactic protein-1 (MCP-1); chemoattract monocytes and interferon- $\gamma$  inducible protein-10 (IP-10), which induces CD8<sup>+</sup> lymphocytes recruitment. Furthermore, many other proteolytic enzymes particularly MMP-9, MMP-12 are released by macrophages and associated with elastic tissue damage, as well as reactive oxygen species and nitric oxide that are related to steroids resistance (Agusti 2013, Barnes, Shapiro *et al.* 2003).

Macrophage is known to be one of the major cells responsible for phagocytosis of apoptotic eosinophils (efferocytosis) and other inflammatory cells (Stern, Meagher *et al.* 1992, Stern, Savill *et al.* 1996, Matsumoto, Terakawa *et al.* 2007, Mahajan, Madan *et al.* 2008), although airway epithelial cells may also be involved in engulfing eosinophils (Walsh, Sexton *et al.* 1999, Sexton, Al-Rabia *et al.* 2004, Sexton, Blaylock *et al.* 2001), but not neutrophils (Sexton, Al-Rabia *et al.* 2004). However, other cells such as fibroblasts and mesangial cells may also have some phagocytic ability (Ward, Dransfield *et al.* 1999).

### **1.6.3 T-Lymphocytes**

It has been recognised that T cells are the most abundant cells in the airways of COPD patients (Finkelstein, Fraser *et al.* 1995). Their numbers are found to be higher in lungs of patients with emphysema than normal smokers (Majo, Ghezzi *et al.* 2001). They are also shown to be agglomerated in sub-epithelial lymphoid masses known as bronchus associated lymphoid tissue (BALT) in COPD small airways. The degree of airflow obstruction is negatively correlated with the number of BALT follicles (Hogg, Chu *et al.* 2004) and CD8<sup>+</sup> lymphocytes (Saetta, Di Stefano *et al.* 1998). The T-cells inducible damage to the lungs can be either a result of direct catalytic action of CD8<sup>+</sup> lymphocytes through release of cytotoxic

mediators and Fas ligand or by the release of inflammatory mediators  $T_h1$  predominant cytokines such as IP-10 and monokine induced by interferon-gamma (MIG) that stimulate the production of MMPs by macrophages and other cells (Gadgil, Duncan 2008). In addition, the lungs of subjects with emphysema are also shown to have large numbers of  $CD4^+$  cells, although their number and role are less significant than  $CD8^+$  cells.  $CD4^+$  lymphocytes act synergistically with  $CD8^+$  T-cells and other immune cells. They also prolong the survival of  $CD8^+$  lymphocytes (Gadgil, Duncan 2008) and promote the release of Ig-G autoantibodies by B-cells (Feghali-Bostwick, Gadgil *et al.* 2008), as well as antibody dependent cell-mediated cytotoxicity (ADCC) (Gadgil, Duncan 2008).

#### **1.6.4 Eosinophils**

Eosinophilic inflammation is a hallmark of airway inflammation in asthma. Conversely, it is a feature of subgroups of patients with a variety of other chronic lung diseases that are not typically considered eosinophilic including COPD and idiopathic pulmonary fibrosis. Eosinophilic inflammation is also a cardinal feature of some airway diseases and is necessary for their diagnosis such as non-asthmatic eosinophilic bronchitis. Therefore, eosinophilic inflammation cannot be assumed to be present or absent dependent on the chronic lung disease, but needs to be measured as part of the assessment and phenotyping of a disease. This is important as identification of eosinophilic inflammation predicts response to therapy and future risk of exacerbations and disease progression.

#### **1.6.4.1 Eosinophil Biology**

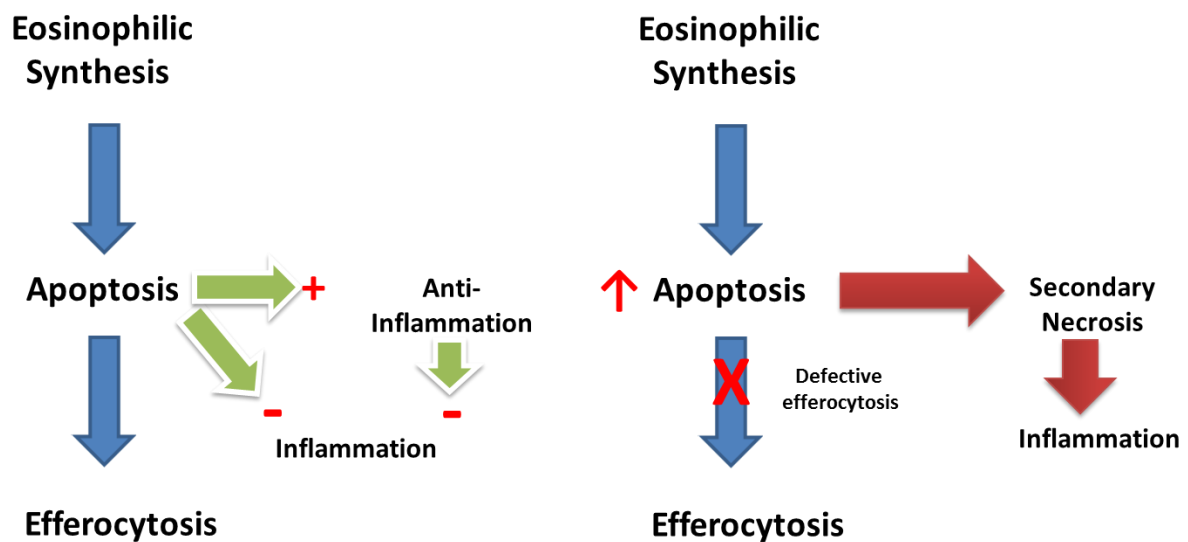
##### **1.6.4.1.1 Synthesis and cell trafficking**

Eosinophils are derived from CD34(+) stem cells in the bone marrow in response to stimulation by the cytokines interleukin (IL)-3, IL-5, and granulocyte macrophage colony-stimulating factor (GM-CSF) cytokines (Wardlaw, Brightling *et al.* 2000, Denburg 1999). After differentiation, eosinophils enter the blood stream, where they can live for 8-12 hours to constitute 1-3% of total white blood cell count, before recruitment into the lungs under the influence of CCR3 chemokines such as chemokine (C-C motif) ligand 11 (CCL11) and other eotaxins (Uhm, Kim *et al.* 2012). T-helper-2 lymphocytes direct the complex process of eosinophil recruitment into the airways, which starts by release of IL-5 and eotaxins. Next, IL-4 and IL-13 are released by T-helper-2 cells, which results in increasing eosinophils adhesion to endothelial tissues in the airways vessels mediated by locally secreted P-selectin, vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4). Consequently, eosinophils are bound with 10 times greater affinity than neutrophils (Wardlaw 1999, Saha, Brightling 2006, Symon, Lawrence *et al.* 1996, Woltmann, McNulty *et al.* 2000). IL-5, GM-CSF, and IL-3, which are found to be high in asthmatics airways, prevent eosinophils undergoing apoptosis and thus survive longer in tissues (Wardlaw 1999).

##### **1.6.4.1.2 Eosinophil clearance**

Normally, there is a balance between synthesis and clearance of cells. Any aged cells undergo apoptosis or programmed cell death (Woolley, Gibson *et al.* 1996, Blank, Shiloh 2007, Henson, Tuder 2008, Hallett, Leitch *et al.* 2008) and are subsequently engulfed and eliminated by macrophages in a process similar to phagocytosis called ‘efferocytosis’ (“‘effero’ means to take to the grave or to bury”) (Yun, Henson *et al.* 2008), which is an

essential “non-inflammatory” mechanism by which granulocytes, including eosinophils and neutrophils are cleared (Matsumoto, Terakawa *et al.* 2007, Mahajan, Madan *et al.* 2008) to maintain tissue homeostasis. Apoptosis and clearance of apoptotic cells inhibit inflammatory and stimulate anti-inflammatory responses (Yun, Henson *et al.* 2008), whereas necrosis of cells eventually results in release of toxic intracellular pro-inflammatory mediators (Ward, Dransfield *et al.* 1999), (Figure 1-1). The pathogenesises of many chronic inflammatory diseases, including COPD, asthma and autoimmune diseases such as inflammatory bowel disease and rheumatoid arthritis (Hallett, Leitch *et al.* 2008), are believed to be related to either excessive apoptosis or impaired efferocytosis with subsequent secondary necrosis or both. Evidences suggest that the mechanism of COPD is beyond the concept of protease/anti-protease concept as increased apoptotic alveolar septal cells were found in emphysematous lungs (Tuder, Petrache *et al.* 2003). Impaired phagocytic function of macrophages is persistently seen in COPD (Ferrara, D'Adda *et al.* 1996, Hodge, Hodge *et al.* 2003, Berenson, Garlipp *et al.* 2006, Taylor, Finney-Hayward *et al.* 2010, Donnelly, Barnes 2012, Berenson, Kruzel *et al.* 2013) and asthma (Simpson, Gibson *et al.* 2013). Various targets were used to assess the phagocytosis in previous studies including: opsonized yeast (Alexis, Soukup *et al.* 2001); *Escherichia coli* (Prieto, Reyes *et al.* 2001); *Candida albicans* (Ferrara, D'Adda *et al.* 1996); epithelial cells (Hodge, Hodge *et al.* 2003); *Haemophilus influenza* and *Streptococcus pneumonia* (Taylor, Finney-Hayward *et al.* 2010) and zymosan (Damon, Godard *et al.* 1981, Godard, Chaintreuil *et al.* 1982), which raises important critical questions; whether this is also applied to altered efferocytotic ability of macrophages to efferocytose eosinophils in COPD, whether it is related to persistence of eosinophilic inflammation, its extent, clinical implications especially with regards to COPD exacerbation and its usefulness to be used as a potential therapeutic target to improve COPD treatment. This is not yet known.



**Figure 1-1 Schematic diagram illustrating the eosinophil homeostasis**

The normal pathway is represented in the left side and abnormal pathway with impaired efferocytosis and/or increased apoptosis is in the right.

#### 1.6.4.1.3 Eosinophils proteins

Eosinophils have four main specific basic proteins which are harmful to the epithelium of bronchial tree. These include: major basic protein (MBP); eosinophil cationic protein (ECP); eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN). All of these proteins are stored in the eosinophils secondary granules. The release of these proteins has been linked to many pathological conditions. For instance, ECP is associated with cytotoxicity of asthma and other allergic diseases (Swaminathan, Myszkowski *et al.* 2005), although the mechanism of cell toxicity is not clear (Navarro, Aleu *et al.* 2008). ECP also crosslinks with immune cells, coagulation and complement system and contributes to the exacerbations of asthma (Gibson,

Woolley *et al.* 1998, Pizzichini, Pizzichini *et al.* 1997) and COPD (Woolley *et al.* 1998, Fujimoto, Yasuo *et al.* 2005, Rohde, Gevaert *et al.* 2004, Fiorini, Crespi *et al.* 2000, Gibson) as well. Likewise, EPO is associated with pathogenesis of asthma through oxidative tissue injury (van Dalen, Winterbourn *et al.* 2006). Similarly, MBP is implicated in pathophysiology of eosinophilic pulmonary disorders including asthma. One possible mechanism of MBP cytotoxicity is lysis of alveolar epithelium which results in disruption of pulmonary epithelial barrier and penetration of inhaled antigens that triggers immunological response against them (Ayars, Altman *et al.* 1985). In addition, eosinophils are associated with apoptosis of airways epithelial cells (Trautmann, Schmid-Grendelmeier *et al.* 2002), most likely through release of ECP (Chang, Lo *et al.* 2010). Eosinophil is also an important source of cytokines and contributes to the ongoing inflammatory response (Wardlaw, Brightling *et al.* 2000). Thus, eosinophilic inflammation contributes to the persistent inflammation observed in airways disease and its presence is associated with future risk of exacerbations (Pizzichini, Pizzichini *et al.* 1997, Saetta, Di Stefano *et al.* 1994) and lung function decline in both asthma (Jatakanon, Lim *et al.* 2000, Fujimoto, Kubo *et al.* 1997, Virchow, Holscher *et al.* 1992, Pizzichini, Pizzichini *et al.* 1996) and COPD (Gibson, Woolley *et al.* 1998, Gursel, Turktas *et al.* 1997, Balzano, Stefanelli *et al.* 1999, Amici, Moratti *et al.* 2006). The mechanisms whereby eosinophilic inflammation contributes to exacerbations is unclear, but is likely to be the synergistic effects upon airway geometry due to airway inflammation (Bathoorn, Kerstjens *et al.* 2008), oedema and airway remodelling (Fahy, Corry *et al.* 2000, Holgate, Arshad *et al.* 2009), as well as mucus plugging as a consequence of stimulation of mucus production (Messina, O'Riordan *et al.* 1991) or changes in its composition (Innes, Carrington *et al.* 2009).

#### **1.6.4.2 Eosinophilic inflammation in chronic obstructive pulmonary disease**

In COPD, the airways inflammation is typically neutrophilic. However, there have been a significant numbers of COPD subjects between 20-40% with sputum eosinophilia more than 3% (Saha, Brightling 2006). These subjects cannot be distinguished based on clinical features and lung function from those without sputum eosinophilia. Eosinophils are also found to be raised in bronchial biopsies, mainly in lamina propria in COPD subjects (Rutgers, Timens *et al.* 2000, Pesci, Majori *et al.* 1998). In addition, eosinophils and ECP are demonstrated to be higher in BAL in patients with COPD, compared to non-smoker controls (Pesci, Balbi *et al.* 1998), as well as in current smokers with mild to moderate COPD, in contrast to ex-smoker COPD subjects with similar degree of severity (Wen, Reid *et al.* 2010). Moreover, compared to normal people, subjects with COPD are found to have higher levels of MPO and ECP, but similar to those in asthma (Yamamoto, Yoneda *et al.* 1997, Balzano, Stefanelli *et al.* 1999). Additionally, there was an association between sputum ECP and ECP in the bronchial wash (Rutgers, Timens *et al.* 2000). Sputum eosinophils and macrophages counts are positively correlated with the pack-years of smoking in asymptomatic smokers, in comparison to normal controls (Dippolito, Foresi *et al.* 2001) and serum eosinophils  $\geq 275$  cells/mm<sup>3</sup> is associated with increased all-cause mortality (Hospers, Schouten *et al.* 1999, Hospers, Schouten *et al.* 2000). FEV<sub>1</sub> is negatively correlated with the activated eosinophils to total eosinophil ratio in endobronchial biopsies from COPD patients (Lams, Sousa *et al.* 2000). Balzano *et al.*, demonstrated a negative association between neutrophils, eosinophils and ECP and FEV<sub>1</sub> and FEV<sub>1</sub>/FVC ratio in COPD subjects (Balzano, Stefanelli *et al.* 1999). Rutgers and colleagues also found an inverse correlation between sputum eosinophils and FEV<sub>1</sub>/FVC (Rutgers, Postma *et al.* 2000).



As described above, increased eosinophils in bronchial mucosa of COPD subjects has been reported by many investigators. However, the main focus of most of the prior studies was on the intraluminal differences in inflammatory cells between asthma, COPD and healthy subjects and also the correlations between lung function decline, amount of smoking and the inflammatory profiles of these cells in different airway compartments, but few investigated, although not fully, the relationship between eosinophils in various airway compartments as well as peripheral blood (Maestrelli, Saetta *et al.* 1995, Rutgers, Timens *et al.* 2000), (Figure 1-2), with controversial finding reported from these studies. Also to the best of my knowledge, no study explored the relationship between sputum or peripheral blood eosinophils and remodelling in COPD.

Corticosteroids are the most frequently anti-inflammatory medications used in asthma and COPD. Their efficacy in COPD is controversial, but they are recommended in those who have moderate to severe disease with frequent exacerbations. Short term studies of oral (Brightling, Monteiro *et al.* 2000, Pizzichini, Pizzichini *et al.* 1998, Fujimoto, Kubo *et al.* 1999) and inhaled corticosteroid treatment (Brightling, McKenna *et al.* 2005) suggest that a sputum eosinophilia predicts response to therapy in COPD. In one of these studies, oral prednisolone was administered daily for 14 days to COPD subjects and there was a correlation between the substantial reduction in sputum eosinophilia secondary to corticosteroid therapy and improvement of lung function, health status and tolerance to exercise (Brightling, Monteiro *et al.* 2000). However, neutrophilic inflammatory markers in sputum were not affected. On the other hand, sputum eosinophil count can predict exacerbation following withdrawal of inhaled corticosteroids (Liesker, Bathoorn *et al.* 2011). So, findings of the previous studies highlight the significance of eosinophilic airway inflammation in subgroup of COPD patients and suggest that corticosteroid treatment works

through adjustment of the eosinophilic airway inflammation to exert its beneficial actions on these subjects.

Based on the beneficial effects of use of sputum eosinophil count as a guide for treatment of asthmatics, implementation of this practice has been applied in a 1 year trial involving 80 COPD subjects, where corticosteroids were utilised in a management strategy to normalise the sputum eosinophil count below 3% in comparison with conventional treatment based on symptoms. The result was 62% fall in severe COPD exacerbations that warrant hospitalisation (Siva, Green *et al.* 2007). As observed with asthma, there was no overall increment in corticosteroid use associated with this beneficial effect. Thus, similar to asthma, sputum eosinophil measurement might be of a clinical use to recognise those COPD subjects who are more likely to benefit from corticosteroid treatment and then monitor their response to therapy. However, it is still precocious to implement this practice as a recommendation for the routine COPD management as long-term studies are required to confirm the potential value of induced sputum in COPD management and whether this will be applicable to specific patient's subgroups.

IL-5 is critical in survival of eosinophils (Saha, Brightling 2006). Sputum IL-5 is associated with a sputum eosinophilia and can be reduced by oral corticosteroids (Bafadhel, Saha *et al.* 2009). Whether measures to target IL-5 in COPD will be effective, it is still under research. In a recent randomised, double-blind, placebo-controlled, phase-2a study, peripheral blood eosinophil count was useful in identifying COPD subjects that had the greatest improvement in lung function, health status and exacerbations in response to Benralizumab an anti-IL-5 receptor (IL-5R), although the overall rate of exacerbation was not decreased in treated subjects in comparison to control (Brightling, Bleecker *et al.* 2014).

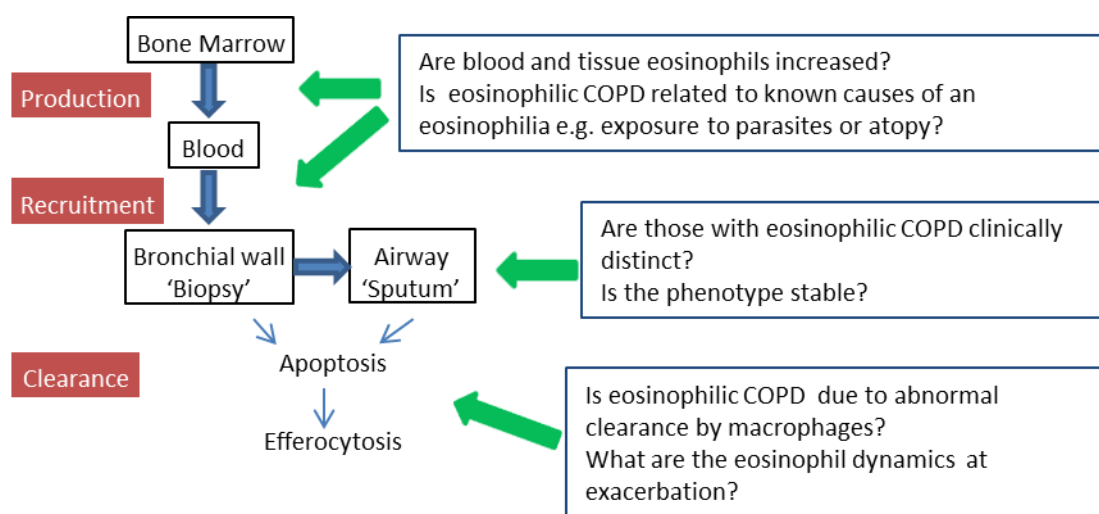
Eosinophilic airway inflammation is also important at exacerbation events. Recently, Bafadhel *et al.* have identified 4 COPD exacerbation biological clusters: bacterial; viral; eosinophilic and pauci-inflammatory (Bafadhel, McKenna *et al.* 2011). Interestingly, in each group the biological profile was similar, although less intensive at stable state such those measures at stable state were predictive of the exacerbation phenotype. The best predictor of a sputum eosinophilia in this study was a peripheral blood eosinophil count. In a subsequent study, Bafadhel *et al.* applied the peripheral blood eosinophil count at the exacerbation event to direct oral corticosteroid therapy in a randomised double blind placebo-controlled trial (Bafadhel, McKenna *et al.* 2012). It has been found that patients without a peripheral blood eosinophilia were able to withhold oral corticosteroid therapy without causing any harm and indeed those subjects without a peripheral blood eosinophilia treated with oral corticosteroids had the poorest outcomes. This suggests that eosinophilic inflammation can identify both COPD patients that will benefit from oral corticosteroids at an exacerbation as well as those that will have worst outcomes as a consequence of the therapy.

Taken together, several studies emphasise the importance of eosinophilic airway inflammation in a subgroup of COPD patients. However, the clinical characteristics of this subgroup in stable and exacerbation events, its differences with other COPD phenotypes are not very clear. Approaches to re-classify asthma and COPD using statistical models have further validated the importance of the eosinophilic phenotype, but have not fully explored the stability of this phenotype over time or its temporal relationship with other clinical parameters. Indeed, in reviewing the literature little information has been found about phenotypic stability of the eosinophilic sub-phenotype in COPD (Beeh, Beier *et al.* 2003, Brightling, Monterio *et al.* 2001), despite the presence of several studies with controversial results in asthma (In 't Veen, de Gouw *et al.* 1996, Pizzichini, Pizzichini *et al.* 1996,

Spanevello, Migliori *et al.* 1997, Reining, Mattes *et al.* 2000, Fahy, Boushey *et al.* 2001, Bacci, Cianchetti *et al.* 2002, Van Veen, Ten Brinke *et al.* 2009, Al-Samri, Benedetti *et al.* 2010, Sohani, Strinich *et al.* 2011, McGrath, Icitovic *et al.* 2012).

In addition, little or no data are found in the literature with regards to the aetiology of eosinophilic airway inflammation in COPD and whether atopy or parasitic infestation/exposure may play a role is still unknown and needs to be scouted.

Figure 1-2 sums up the unanswered questions in relation to the eosinophilic airway inflammation in COPD that need to be addressed in this thesis.



**Figure 1-2 Schematic diagram illustrating the unanswered questions related to the eosinophil trafficking in COPD**

## 1.7 Biomarkers in COPD

Biological marker (biomarker) as defined by the National Institutes of Health (NIH) is “A characteristic that is objectively measured and evaluated as an indicator of normal biological

processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group. 2001). There have been an increasing need and extensive approaches to identify robust biomarkers which aid in diagnosis, direct treatment, monitor response and predict the outcome in chronic respiratory diseases including COPD and asthma. To achieve this, various procedures have been used to obtain representative specimens including: exhaled volatile gases; exhaled breath condensates; sputum induction; bronchial biopsies and BAL (Sin, Vestbo 2009). Apart from lung function test, to date no single biomarker can be effectively utilised to validate the efficacy of any new medication in COPD. Furthermore, pulmonary function is not the ideal biomarker for short clinical trials as it is not specific for COPD, does not reflect the inflammatory and complex pathological heterogeneity of the disease, cannot discriminate between different COPD phenotypes and is not responsive to treatments that increase survival (Sin, Vestbo 2009).

### **1.7.1 Biomarkers in induced sputum**

Induced sputum is one of the commonly used techniques to study inflammation in airway disease as it is simple and safe. Several inflammatory cells and mediator have been widely studied in induced sputum in COPD. Sputum neutrophils percentage correlates with FEV<sub>1</sub> (Stanescu, Sanna *et al.* 1996). IL-8 found to be higher in sputum of COPD patients than smoker controls. It is correlated with predicted FEV<sub>1</sub> and raised in exacerbations (Barnes, Chowdhury *et al.* 2006). TNF- $\alpha$  also was reported to be increased in COPD in contrast with smokers. In addition, TNF- $\alpha$ , IL-8, and IL-6 are associated with the COPD severity (Barnes, Chowdhury *et al.* 2006). As discussed before, sputum eosinophilia is found in 20-40% of subjects with COPD (Saha, Brightling 2006) and the evidence to use eosinophils as a tool for studying, directing and monitoring the treatment in asthma and COPD (Green, Brightling *et*

*al.* 2002, Siva, Green *et al.* 2007) is well known. Despite this, there is an increasing demand to discover alternative simpler biomarkers and validate the existing ones such as peripheral blood eosinophils. Interestingly, Kulkarni and colleagues demonstrated that the red colour (hue) of sputum macrophages corresponds to the amount of ingested eosinophilic proteins and the red hue increased after oral corticosteroid therapy in severe asthmatic subjects. Therefore, measuring the red hue of macrophages in these patients can be potentially utilised as a novel non-invasive biomarker for eosinophilic inflammation over time to differentiate between true non-eosinophilic individuals with asthma and those who are under control with negative sputum eosinophilia, but with high red hue. Thus, this approach may provide an important complimentary tool to the sputum differential count (Kulkarni, Hollins *et al.* 2010). Whether this can also be applied to study eosinophilic airway inflammatory profile particularly the macrophage's efferocytosis of eosinophils in COPD is yet not known.

### **1.7.2 Biomarkers in exhaled breath**

A number of exhaled gases have been also investigated in COPD. Fractional exhaled nitric oxide (FeNO) is probably the most heavily studied marker in the exhaled air. Although, FeNO is very useful as surrogate of eosinophilic airway inflammation in asthma, its beneficial role in COPD is not well-established despite some reports denoted its significant increase in ex-smoker COPD patients compared to non-smoker controls, smoker COPD, versus healthy smokers (Corradi, Majori *et al.* 1999) and in non-smoker COPD, but less than the levels in asthmatics (Beg, Alzoghaibi *et al.* 2009) and smokers with severe COPD, compared to smokers with chronic bronchitis and despite also its inverse correlation with predicted FEV<sub>1</sub> and FEV<sub>1</sub>/FVC (Maziak, Loukides *et al.* 1998, Beg, Alzoghaibi *et al.* 2009). On the other hand, other studies did not support its role (Rutgers, Timens *et al.* 2000, Agusti,

Villaverde *et al.* 1999) and some reported positive correlation with FEV<sub>1</sub> (Corradi, Majori *et al.* 1999). However, FeNO still correlates with eosinophilic inflammation, as well as to bronchodilator reversibility in COPD (Papi, Romagnoli *et al.* 2000). Barnes and co-workers suggest that the presence of FeNO in COPD is related to oxidative stress especially at exacerbations (Barnes, Chowdhury *et al.* 2006). However, cigarette smoking is associated with reduction in FeNO level (Barnes, Dweik *et al.* 2010), probably through inhibition of the inducible Nitric Oxide synthase (iNOS) enzyme (Kharitonov, Robbins *et al.* 1995).

### **1.7.3 Biomarkers in exhaled breath condensate**

Many substances have been tested in the exhaled breath condensate including: hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>); 8-isoprostane and other proinflammatory cytokines such as LT-B<sub>4</sub>, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (Barnes, Chowdhury *et al.* 2006), but they lack specificity (Cazzola, Novelli 2010) and reproducibility, need very sensitive techniques to measure them and their usage is impeded by the absence of larger studies to prove their efficacy (Angelis, Porpodis *et al.* 2014).

### **1.7.4 Biomarkers in Blood**

Since COPD is an inflammatory disease with inflammatory mediators that can spread to the systemic circulation, researchers attempted to focus on finding a blood biomarker that provides an insight into the inflammatory nature of the disease. Although, markers in the plasma are easy to obtain using standardised equipments, they might be affected by other comorbidities. Serum C-reactive protein (CRP) has been found to be raised in COPD (Thomsen, Ingebrigtsen *et al.* 2013), including mild COPD (Piehl-Aulin, Jones *et al.* 2009), independent from smoking and comorbidities and decreased by inhaled corticosteroids

(Pinto-Plata, Mullerova *et al.* 2006). It is related to IL-6, which is one of the important cytokines that are involved in the inflammatory process in COPD. It is correlated with all-cause, cardiovascular and cancer specific causes of mortality (Cazzola, Novelli 2010). Serum CRP and MMP-9 are good predictors of a rapid FEV<sub>1</sub> fall (Man, Connett *et al.* 2006, Higashimoto, Iwata *et al.* 2009). Chronic rise of CRP was associated with decrease of FEV<sub>1</sub> over time (Shaaban, Kony *et al.* 2006) and it can also predict hospitalization and death (Dahl, Vestbo *et al.* 2007). However, the lack of specificity is a major obstacle to use CRP as a biomarker in COPD since it can be increased in many other chronic and acute conditions. Similarly, fibrinogen which is another acute-phase reactant protein, is also a common biomarkers that has gained a large attention in COPD as it showed promising results in terms of predicting the risk of exacerbations, hospitalization and mortality in COPD, as well as association with decrease in FEV<sub>1</sub> (Dahl, Tybjaerg-Hansen *et al.* 2001, Rosenberg, Kalhan 2012, Duvoix, Dickens *et al.* 2013, Thomsen, Ingebrigtsen *et al.* 2013). It was correlated with exacerbations that had been associated with cough and purulent sputum (Wedzicha, Seemungal *et al.* 2000) and predicted rapid FEV<sub>1</sub> decline (Donaldson, Seemungal *et al.* 2005). However, its predictive value has been reported to be low (Cazzola, Novelli 2010). Surfactant protein D (SP-D), which is produced by type II pneumocytes, is another serum biomarker that has the potential to be used in the future (Leung, Sin 2013). In large cohort of COPD patients, SP-D was greatly higher in COPD than normal controls, its levels decreased following four weeks therapy with prednisolone without affecting the FEV<sub>1</sub> and at its highest levels can predict exacerbations risk in the next one year (Lomas, Silverman *et al.* 2009). It has a good correlation with health status over 3 months (Sin, Leung *et al.* 2007). However, it did not show any correlation with COPD severity or radiological evidence of emphysema (Cazzola, Novelli 2010).



### **1.7.5 Biomarkers in broncho-alveolar lavage**

BAL is one of the invasive techniques to obtain representative samples from the lung peripheries. In COPD, alveolar macrophages are the most abundant cells in the BAL fluid, whilst neutrophils and CD8<sup>+</sup> T lymphocytes are the second commonest cells in smokers and ex-smokers respectively with eosinophils and mast cells reported in some patients compared to control (Barnes, Chowdhury *et al.* 2006, Cazzola, Novelli 2010). CD8<sup>+</sup> T cells and their cytokines are negatively correlated with FEV<sub>1</sub> (Hodge, Nairn *et al.* 2007). It has been revealed that alveolar macrophages in COPD have altered function in terms of over-secretion of some pro-inflammatory mediators and under-expression of anti-inflammatory molecules such as histone deacetylase 2 (HDAC2) (Ito, Ito *et al.* 2005). With regards to inflammatory mediators, ECP, myeloperoxidase and IL-8 dominate in COPD and control smokers compared to non-smoker controls (Barnes, Chowdhury *et al.* 2006, Cazzola, Novelli 2010). IL-8 in BAL is associated with fall in FEV<sub>1</sub> (Soler, Ewig *et al.* 1999), whereas ECP and eotaxin-1 are associated with a favourable response to bronchodilators (Miller, Ramsdell *et al.* 2007). Histamine and tryptase have been also reported to be increased in BAL of COPD subjects, but not compared with healthy control, which may suggest an association with smoking (Kalendarian, Raju *et al.* 1988, Pesci, Balbi *et al.* 1998).

### **1.7.6 Biomarkers in bronchial biopsies**

Despite endobronchial biopsies can afford comprehensive information about bronchial tissue and the beneath structures from epithelium to smooth muscles, but due to its invasiveness nature it cannot be used for every patient, especially in those with severe disease or comorbidities (Cazzola, Novelli 2010). In addition to the lack of availability of sufficient biopsy size in some occasions, there is a marked intra- and inter-subject variability of findings

(Sullivan, Stephens *et al.* 1998, Gamble, Qiu *et al.* 2006). Macrophages and CD8<sup>+</sup> T-lymphocytes are the main cells populations found in bronchial biopsies from COPD subjects in stable condition, with the expression of interferon gamma, CXCL10 and IL-9 and type-1 response associated chemokine receptors, e.g. CXCR3 (Barnes, Chowdhury *et al.* 2006). However, unlike severe COPD (Di Stefano, Capelli *et al.* 1998) and COPD exacerbation (Qiu, Zhu *et al.* 2003), in mild-to-moderate COPD, neutrophils are mostly found in the epithelium in contrast to eosinophils which are primarily demonstrated in lamina propria (Pesci, Majori *et al.* 1998), with a tendency to have decreased numbers of eosinophils in biopsies and increased percentages of eosinophils in sputum of COPD individuals with chronic bronchitis (Snoeck-Stroband, Lapperre *et al.* 2008). T-lymphocytes are correlated with the level of alveolar damage and the extent of airway obstruction (Barnes, Chowdhury *et al.* 2006). The mechanism of alveolar destruction is thought to be through lysis and apoptosis of alveolar epithelial cells via release of perforins, granzyme-B and TNF- $\alpha$  by CD8<sup>+</sup> cells (Angelis, Porpodis *et al.* 2014).

### **1.7.7 Biomarkers in COPD exacerbations**

Bafadhel *et al* have recognised various biomarkers for COPD exacerbations for different biological COPD phenotypes. In this study, it has been demonstrated that the best biomarkers for bacterial associated exacerbations were sputum IL-1 $\beta$  and serum CRP with 90% sensitivity and 80% specificity for IL-1 $\beta$  sputum count of 125 pg/mL and 60% sensitivity and 70% specificity for CRP serum level of 10 mg/L or above. Blood eosinophils of 2% of the total white cell count was the best surrogate biomarker of sputum eosinophilia of 3% or above during exacerbation with 90% sensitivity and 60% specificity (Bafadhel, McKenna *et al.* 2011). Subsequently, baseline blood eosinophil count was shown that it can predict the

blood eosinophil biomarker status at exacerbation with an odds ratio 5.5. It also predicts the response to corticosteroid therapy in the eosinophilic subtype of COPD and treatment failure in the non-eosinophilic group as categorised by peripheral blood eosinophil count of equal to or higher than 2% versus those with <2% respectively (Bafadhel, McKenna *et al.* 2012). Furthermore, serum CXCL10 was the best to predict viral associated exacerbation with a 75% sensitivity 65% and specificity for a level of 56 pg/mL (Bafadhel, McKenna *et al.* 2011).

In a systemic review that was recently conducted by Koutsokera and co-workers, many biomarkers have been evaluated in COPD exacerbations and some were associated with encouraging results including: FeNO in exhaled breath; IL-8; TNF- $\alpha$ ; neutrophil elastase and IL-1 $\beta$  in spontaneous sputum; IL-6; IL-8; TNF $\alpha$ ; LTB4 and myeloperoxidase in induced sputum. The best combination was sputum IL-8 and TNF- $\alpha$ , which correlate with bacterial infection, symptoms and resolution. The authors concluded that there was limited data to support the role of these biomarkers, none of the conducted researches was double blinded, controlled or multi-center trial and therefore no single biomarker can be extensively used in clinical practice (Koutsokera, Kostikas *et al.* 2013). Procalcitonin (PCT), a peptide that increases in many bacterial infections in response to endotoxins, is another biomarker that has been used to identify bacteria-associated COPD acute exacerbations and guide antibiotic treatment in those patients (Brightling 2013).

To summarise, all of the available biomarkers in COPD need further large scale studies to validate their clinical utility before they can be widely applied in clinical practice. This also warrants the need to find alternative simpler biomarkers that can be easily validated. Some suggested combining more than one biomarker and use multi-score system or genomic and

proteomic analyses (Leung, Sin 2013), while others proposed using computerised models in order to incorporate them in the future (Koutsokera, Kostikas *et al.* 2013). One of the best examples is using BODE (**B**MI, **O**airflow **O**bststruction, **D**yspnea, and **E**xercise capacity) as a prognostic index, where combined data in multidimensional grading system can predict all-cause mortality and respiratory mortality than FEV<sub>1</sub> alone (Celli, Cote *et al.* 2004). Other examples include the ADO index (age, dyspnea, FEV<sub>1</sub>), and the DOSE index (dyspnea, FEV<sub>1</sub>, smoking status, and exacerbation frequency). However, all of these tools do not incorporate the inflammatory or other components of disease (Agusti, Sobradillo *et al.* 2011), which means that they are not sufficient to reflect the wide spectrum of complexity and phenotypic heterogeneity of COPD.

A typical biomarker should fulfill the following criteria: reflects the ongoing pathophysiological process of the disease; is specific and sensitive; is capable to discriminate particular phenotype from others; reflects disease activity; able to respond to therapy; can be measured easily and feasibly; minimally invasive; repeatable and well validated by a standard protocol (Taylor, Pavord 2008, Angelis, Porpodis *et al.* 2014). Additional suggested requirement are that there should be a robust correlation between the biomarker and important clinical outcomes such as mortality and hospitalisation and also modification of the biomarker can lead to an improvement of clinical outcomes such as FEV<sub>1</sub>, exacerbation rate, quality of life and death rate (Cazzola, Novelli 2010). Currently, it is extremely hard to have a single biomarker that has all of these qualities since COPD is very heterogeneous, has complex pathogenesis, encompasses various components and is not a single disease entity. Therefore, the best approach will be clustering the disease into various phenotypes and identifying biomarkers that fit with each cluster or phenotype.

## 1.8 COPD Phenotypes

Despite the effectiveness of existing COPD treatment in reducing symptoms, exercise intolerance and exacerbations rate, its benefit in an individual level is minimal and the extent of its beneficiary effects does not reflect on all the COPD patients (GOLD 2011). Therefore, it is becoming progressively apparent that there is a demanding need to use innovative tools to understand COPD better and develop new therapies to improve the current situation.

Phenotype is defined as a physical or biochemical characteristics of an organism or disease that results from interaction between environmental and genetic factors (Han, Agusti *et al.* 2010). The concept of phenotype began earlier when COPD was subdivided into emphysema and chronic bronchitis (Burgel, Paillasseur *et al.* 2014). Over the last decade, there has been a growing interest in clustering COPD and other airways diseases into various subgroups or phenotypes. However, stratifying a disease according to characteristics or severity does not in its own serve the purposes of phenotyping from the clinical and research points of view (Han, Agusti *et al.* 2010). Ideally, the best classification should be based on a unique prognostic or therapeutic characteristics with identification of common clinical, physiologic, inflammatory, radiological or genetic biomarker that is associated with clinical meaningful and measurable outcome, such as quality of life, exacerbations, clinical response to specific treatment, disease progression rate or mortality, instead of using a single physiological parameter to classify the disease as in the current staging system of COPD (Han, Agusti *et al.* 2010, Carolan, Sutherland 2013). This may aid in comprehension of the mechanism, improvement of the management and prediction of prognosis of the disease more accurately and ultimately facilitates guided and personalized therapy thereafter (Agusti 2013), as well as helps in a better disease classification for clinical and research purposes. Moreover, any phenotyping

system has to be validated against the related outcome parameter before it is widely used (Han, Agusti *et al.* 2010). Importantly, despite that phenotyping may reveal an aetiology of a disease in some instances, but identification of a phenotype does not necessarily imply the mechanistic process behind it as the most important is the beneficial effect to the patients, even if the mechanism is not known or unclear (Han, Agusti *et al.* 2010).

One of the commonest approaches to perform phenotyping is using cluster analysis technique in which clinical, physiologic and radiologic variables are utilised to categorise COPD subjects into subsets that have related features with no prepared hypothesis. In other word, the diversities between heterogeneous subjects groups are diminished and they are converted into more similar clusters (Wardlaw, Silverman *et al.* 2005). This is usually performed by utilising data from studies with large number of patient's population (Carolan, Sutherland 2013).

It is widely accepted that spirometry alone does not sufficiently address the complexity of COPD and other chronic airway diseases, nor is appropriate to phenotype COPD according to the severity, which might be a consequence of other phenotypes, but does not in itself denote a single phenotype (Agusti, Sobradillo *et al.* 2011). Thus alternative approaches to reclassify COPD have been sought. There are numerous proposed clinical phenotyping methods based on various variables (Miravittles, Calle *et al.* 2012), leading to development of a great phenotypic heterogeneity in COPD. The most common are: the “frequent exacerbators” (Hurst 2011); “persistent systemic inflammation” (Agusti, Edwards *et al.* 2012) and radiological phenotypes (Carolan, Sutherland 2013). The exacerbation phenotype, which was derived from “Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points” (ECLIPSE) cohort (Hurst, Vestbo *et al.* 2010), categorises patients into frequent

exacerbators for subjects who had two or more exacerbations in the last year against infrequent for those who did not. The “persistent systemic inflammation” is another important phenotype that was also derived from ECLIPSE dataset and it describes a group of COPD patients who persistently have systemic inflammation and experience greater numbers of exacerbation with higher rates of all-cause mortality than “non-inflamed” group, despite similarly impaired lung function in both clusters. The radiological phenotyping uses the bronchial wall thickness, total lung emphysema percentage and other measures in computed tomography (CT) as an index of frequency of exacerbations. Other phenotypes were revealed by Bafadhel and colleagues using cluster analysis, where subjects were divided according to inflammatory or biological biomarkers and include: bacterial (neutrophilic); eosinophilic; viral and pauci-inflammatory phenotypes (Bafadhel, McKenna *et al.* 2011). The results of this study was implemented clinically by the same author in a in a single centre double-blind randomised controlled trial as a phenotype-specific treatment of exacerbations with corticosteroids in subjects with evidence of eosinophilic COPD inflammation, using peripheral blood eosinophil count as a biomarker guide (Bafadhel, McKenna *et al.* 2012). The beneficial effect of using phenotype-specific or biomarker-driven management in this study was to give the therapy to those who do need it and at the same time avoid treatment failure, delayed recovery and adverse effects as a consequence of use of the medication unnecessarily in those who do not require it (Brightling 2013), despite a little evidence of reducing treatment failure was shown when this study was replicated with larger numbers of subjects using data shared from randomised clinical trials from other centres (Bafadhel, Davies *et al.* 2014).

There are several other suggested phenotypes. However, most of these phenotyping methodologies focus on a single scale of the disease and none combines the clinical and

biological properties of the disease together, nor uses various scales from gene to organ level (Barker, Brightling 2013). Ahn and colleagues described the major disadvantage of the “reductionism” principle, which is based on dividing the chronic and complex diseases into smaller and simpler elements, as ignoring the interplay between these small disease components as well as the outcome secondary to this interrelationship, unlike in acute conditions where a single pathological process prevails and therefore can be easily interpreted and targeted (Ahn, Tewari *et al.* 2006a, Ahn, Tewari *et al.* 2006b). Furthermore, many issues arise as a result of the overlaps between different “single scope phenotypes” which make it sometimes hard to sort, for instance an individual who may have airway wall thickening, frequent exacerbations and high BMI at the same time in a single unique phenotype. (Bon, Liao *et al.* 2013). Another challenge might arise due to an exclusion of some patients that do not meet the inclusion criteria. For example, those who have radiological evidence of emphysema or normal FEV<sub>1</sub>/FVC ratio due to fibrotic element, yet may not be considered as having COPD since they do not fit the spirometry-based GOLD definition of the disease (Bon, Liao *et al.* 2013). Altogether, these limitations of the current discipline of a phenotyping underscore the needs to adopt a broader view of the concept of phenotyping (Bon, Liao *et al.* 2013). New research perspective emphasises on evolving from standard “reductionist” towards a wider scope of “system biology or integrated approach” (Kitano 2002, Ahn, Tewari *et al.* 2006b, Auffray, Adcock *et al.* 2010, Agusti, Sobradillo *et al.* 2011). This strategy, which is gaining a considerable interest, involves studying the disease “as a complex biological system” in multiple aspects rather than focusing on single spectrum or disease entity (Longtin 2005). It requires a high-throughput biotechnology and system biology to gather biological information for subjects from genome to organ level and generate a large multi-scale database (Agusti, Sobradillo *et al.* 2011). These dataset are utilised to predict mathematical models that can be implemented for creating and assessing



hypotheses on the mechanisms and pathogenesis of complex diseases, which may eventually aid in better diagnosis and treatment (Agusti, Sobradillo *et al.* 2011).

## **1.9 COPD Exacerbations**

### **1.9.1 Definition and classification of COPD exacerbation**

In 1987, COPD exacerbation was defined by Anthonisen (Anthonisen, Manfreda *et al.* 1987) as having 2 out of the 3 following symptoms: increased breathlessness; production of sputum or sputum purulence. It has been suggested to use either two major (increasing breathlessness, wheezing or cough) or one major and one minor symptom for at least 2 consecutive days from the following minor symptoms: fever with no known cause; respiratory or heart rate rise by 20% above the baseline or any symptom suggestive of common cold within the last 5 days including sore throat nasal congestion or discharge (Anthonisen, Manfreda *et al.* 1987). All of these symptoms are suggestive and no conclusive test is available to confirm exacerbation. However, it is important to exclude any other condition which may mimic an acute exacerbation. e.g. pneumonia, pneumothorax, congestive cardiac failure, etc. So, performing chest radiograph (CXR) might be helpful to rule out them. Based on Anthonisen, exacerbation severity can be classified to three degrees: type III or mild exacerbation, with one of the 3 major symptoms is present and the patient usually has increased need for medication, but can be managed at home; type II or moderate exacerbation, where 2 out of the 3 symptoms concur and the patient has higher need for medication and often requires medical treatment with antibiotics and/or corticosteroids and finally type I or severe exacerbation, where all the symptoms coexist and hospitalization is usually necessary (Balter, La Forge *et al.* 2003).

Rodriguez-Roisin defined COPD exacerbation as ‘a sustained worsening of the patient's condition from the stable state and beyond normal day-to-day variations, that is acute in onset and necessitates a change in regular medication in a patient with underlying COPD’ (Rodriguez-Roisin 2000). The GOLD definition is based on Rodriguez-Roisin description. However, there is a wide variability of reporting exacerbation as the definition is very subjective and depends on patients’ threshold of perception of symptoms, as well as the decision of the health care provider to prescribe additional treatment. The definition is also influenced by the disease severity, presence of comorbidities and socio-psychological impacts.

### **1.9.2 Burden of COPD exacerbations**

10-15% of all acute admissions in the UK are sequels of COPD exacerbations. More than 50% of the treatment cost of COPD is due to exacerbations (Seemungal, Hurst *et al.* 2009) that carry a high mortality (Anzueto 2010). Exacerbation of COPD is increasing both in the U.K and globally and is significantly higher if the FEV<sub>1</sub> is below 50% predicted (Seemungal, Hurst *et al.* 2009). In a systemic review for 11 studies from 1998 to 2008 that explored healthcare costs related to COPD exacerbations, each exacerbation was estimated to account for a direct cost of \$88 to \$7,757 (as per 2007 US dollars). The author reported large diversities in the studies due to demographic, geographical, treatment and methodological variabilities as well as lack of standard definition, classification and tool to measure exacerbation (Toy, Gallagher *et al.* 2010). The frequency of exacerbations in COPD has a significant negative impact on health related quality of life (Miravittles, Ferrer *et al.* 2004, Seemungal, Donaldson *et al.* 1998, Anzueto 2010). In addition, it leads to a rapid deterioration of lung function (Donaldson, Seemungal *et al.* 2002, Anzueto 2010) and

reduced exercise tolerance (Anzueto 2010). Furthermore, it has been demonstrated that frequent exacerbations are associated with recurrent and worse exacerbations (Hurst, Vestbo *et al.* 2010, Anzueto 2010), increased mortality rate (Cote, Dordelly *et al.* 2007) and reduced five years survival to 30% for those who had three or more exacerbations, compared to 80% survival for patients without exacerbations (Soler-Cataluna, Martinez-Garcia *et al.* 2005).

### **1.9.3 Aetiology of COPD exacerbation**

#### **1.9.3.1 Bacteria**

50-55% of COPD exacerbations are associated with bacterial colonisations (Patel, Seemungal *et al.* 2002, Bafadhel, McKenna *et al.* 2011), which were related to frequency and severity of exacerbations (Patel, Seemungal *et al.* 2002). There has been some controversy about whether bacterial infection is a primary cause or epiphenomenon in COPD exacerbation (Hurst 2011). The reason behind this conflict is that several studies reported that the incidence of pathogenic bacterial colonisation in the sputum was not different between stable and COPD exacerbation (Sethi 2004). Additionally, randomized double-blind clinical trials have shown minimal or no role of antibiotics in COPD exacerbations (Saint, Bent *et al.* 1995). However, the methods used to detect or culture bacteria are not standardised and have some limitations such as oral flora contamination, which makes it challenging to define a causal relationship between bacterial infection and exacerbation. Besides, the lack of firm objective definition of COPD exacerbation adds further to these obstacles. In addition, the absence of a strong evidence for benefit of antibiotics does not rule out the bacteria as a causative pathogen (Sethi 2004). Sethi and Murphy proposed that the bacterial strain rather than the load is responsible for COPD exacerbation (Sethi, Murphy 2008). Despite these issues,

bacterial culture and colony forming units (CFU) quantification are the most common methods used to detect and quantify bacteria in the sputum.

### **1.9.3.2 Viruses**

With the innovation of modern techniques to detect viruses such as reverse transcriptase polymerase chain reaction (RT-PCR), viruses are frequently reported in COPD exacerbations. The incidence varies greatly (Wu, Chen *et al.* 2014), which may reflect seasonal fluctuations, variation in sampling time and methods of detection of viruses. It has been revealed that viral infections are associated with severity (Wedzicha 2004) and frequency of exacerbations (Seemungal, Harper-Owen *et al.* 2001). The most common virus detected in acute COPD exacerbation is rhinovirus. Other reported viruses are influenza, adenovirus and respiratory syncytial virus (RCV) (Seemungal, Harper-Owen *et al.* 2001).

## **1.9.4 Inflammatory cells in exacerbation**

### **1.9.4.1 Neutrophilic inflammation in COPD exacerbation**

As with stable disease, COPD exacerbation is commonly associated with neutrophilic inflammation, but higher than in stable state particularly at submucosal and subepithelial tissue (Bathoorn, Kerstjens *et al.* 2008). Neutrophilic airway inflammation is associated with fall in FEV<sub>1</sub> (Papi, Luppi *et al.* 2006, Wilkinson, Hurst *et al.* 2006) and increased bacterial colonization (Gompertz, O'Brien *et al.* 2001). In the same way, treatment of bacterial infection in exacerbation is related to reduction in neutrophilic inflammation in airways (White, Gompertz *et al.* 2003). However, neutrophils increase is not only present in exacerbations associated with bacteria, but also in viral exacerbations and exacerbations with no detected pathogens (Papi, Bellettato *et al.* 2006). Neutrophilic airways inflammatory

markers and chemo-attractants such as TNF-alpha, IL-8, LT-B4, neutrophil elastase, epithelial-derived neutrophil attractant-78 and myeloperoxidase are also significantly increased in exacerbations in contrast to stable state (Aaron, Angel *et al.* 2001, Bathoorn, Kerstjens *et al.* 2008). Additionally, myeloperoxidase has been proposed to be responsible for the purulence and greenish colour of sputum (Stockley, O'Brien *et al.* 2000, Stockley 2002).

#### **1.9.4.2 Eosinophilic inflammation in COPD exacerbation**

In addition to the presence of sputum eosinophilia in great number of COPD patients in stable times, eosinophil count is raised even further in the sputum, BAL and lung tissue during exacerbations compared to baseline (Saetta, Di Stefano *et al.* 1994, Fujimoto, Yasuo *et al.* 2005, Mercer, Shute *et al.* 2005, Bathoorn, Kerstjens *et al.* 2008). Interestingly, Saetta *et al.* reported that eosinophil count increases 30 times in the airway mucosa during exacerbation than in baseline (Saetta, Stefano *et al.* 1994). Eosinophil rise in exacerbation is also accompanied with elevation in RANTES (regulated upon activation normal T cell-expressed and secreted) (Zhu, Qiu *et al.* 2001, Fujimoto, Yasuo *et al.* 2005, Papi, Bellettato *et al.* 2006), eotaxin (Bocchino, Bertorelli *et al.* 2002) and interleukin-5 (IL-5) (Bathoorn, Kerstjens *et al.* 2008), in addition to sputum and serum ECP and MPO (Fiorini, Crespi *et al.* 2000, Fujimoto, Yasuo *et al.* 2005), which suggests that eosinophils are not only increased in numbers, but also are in an activated form. Additionally, Papi and co-workers proposed that sputum eosinophilia can predict viral infections during COPD exacerbations (Papi, Bellettato *et al.* 2006) and reduction of soluble interleukin-5 receptor alpha during recovery from viral exacerbation has been accompanied by increase in FEV<sub>1</sub> (Rohde, Gevaert *et al.* 2004).

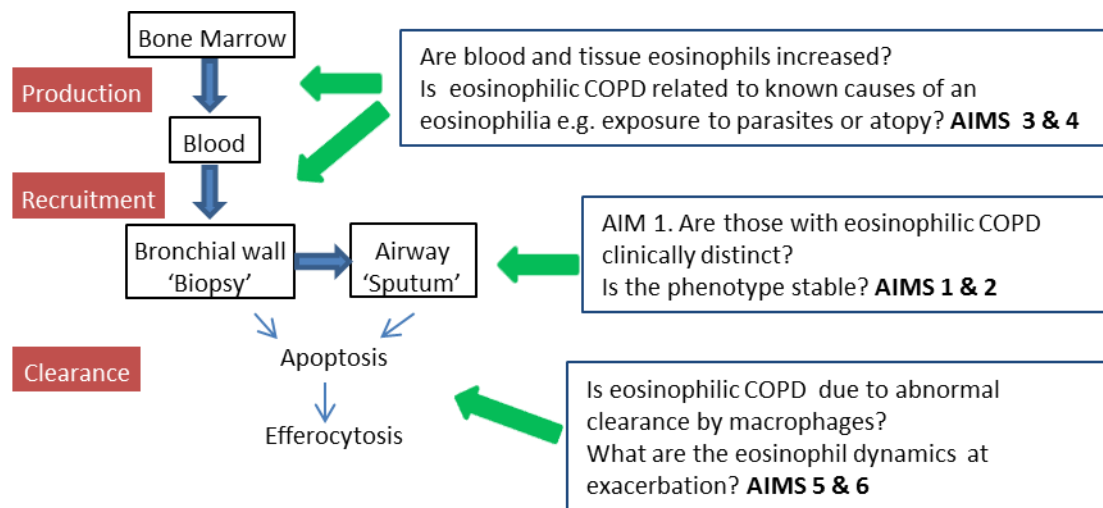
## **1.1 Hypotheses**

I hypothesise that eosinophilic COPD subgroup is a distinct and stable phenotype that persists in blood, sputum and lungs, partly related to atopy or parasite exposure and its persistence could be partly due to impaired macrophage efferocytosis of eosinophils/inadequate clearance of eosinophils, which could be related to exacerbation severity and frequency.

## **1.2 Aims**

- 1- Investigate the heterogeneity of the inflammatory profile of COPD in an existing cohort to define the characteristics of eosinophilic sub-phenotype.
- 2- Define the phenotypic stability of the eosinophilic sub-phenotype of COPD.
- 3- Measure the burden of eosinophils in bronchial mucosa in COPD compared to normal subjects and assess the relationship between blood as a surrogate biomarker of sputum eosinophils, tissue eosinophils and reticular basement membrane thickening.
- 4- Find out whether there is any relationship between eosinophilic COPD and exposure to parasites and/or atopy.
- 5- Scrutinise whether eosinophilic COPD is partly due to abnormal macrophage efferocytosis/poor eosinophil clearance and its relation with severity and frequency of COPD exacerbation.
- 6- Explore the dynamics of eosinophil clearance in an exacerbation event.

Figure 1-3 summarises the aims of my thesis.



**Figure 1-3 Summary diagram illustrating the aims and questions of the thesis**

## **Chapter 2 MATERIALS AND METHODS**



## **2.1 Questionnaires**

### **2.1.1 St George's Respiratory Questionnaire**

This questionnaire has been developed at St George's University of London as a tool of assessing quality of life and was validated in asthma and COPD, as well as in some other respiratory diseases. In COPD, it was a good predictor of survival (Domingo-Salvany, Lamarca *et al.* 2002). It contains 50 items with 76 weighted responses. The questionnaire is composed of three components. The first part measures the frequency and severity of symptoms; the second section examines the relationship between various activities and dyspnoea and the third one assesses disease impact on the socio-psychological functioning of the patient. Each component is scored from 0 to 100. The higher the score the more symptoms or limitations are (Jones, 1992; ATS, 2014). The minimum change in total SGRQ score, which is considered as clinically significant difference, is 4 units (Jones 2002).

### **2.1.2 Chronic Respiratory Disease Questionnaire**

The Chronic Respiratory Disease Questionnaire is another well-established test to evaluate quality of life, which has been validated in research and clinical trials in COPD (Guyatt 1988, Reda, Kotz *et al.* 2010). The questionnaire is administered by an interviewer, although there is a validated self-reported version of the CRQ (Williams, Singh *et al.* 2001). The CRQ has 20 questions which are related to four different domains: dyspnoea; fatigue; emotional functioning and mastery. Each question has a score from one to seven, with 1 is the worst and 7 is the best quality of life. The score of each domain is taking by measuring the average score of the questions related to that domain. The calculation of the total score is made by taking the mean of the score of the 4 domains. It has been recognised that the minimum

accepted score as clinically significant difference is 0.5 (Jones 2002, Williams, Singh *et al.* 2003).

### **2.1.3 Visual analogue scale (VAS)**

VAS is a measurement instrument for subjective characteristics that cannot be easily measured (Gould *et al.* 2001). The measurement is made by requesting the subject to draw a mark on 100 mm line, where a “0” means no symptoms and “100” denotes the worst symptoms. The VAS for the domains of cough, breathlessness, sputum production and sputum purulence was demonstrated to be repeatable outcome measures in COPD by Brightling *et al* (Brightling, Monterio *et al.* 2001).

### **2.1.4 Medical Research Council (MRC) dyspnoea scale**

The MRC dyspnoea scale is a well known grading measure for the magnitude of breathlessness that has been found to be a valid and useful tool for grading dyspnoea in COPD (Bestall, Paul *et al.* 1999). It has 5 grades, where 1 has no breathlessness at rest and 5 has the worst dyspnoea. It is a better predictor of mortality than FEV<sub>1</sub> (Nishimura, Izumi *et al.* 2002). In the modified version of the test, the grades start from 0 to 4 instead.

## **2.2 Spirometry**

Spirometry was performed according to joint American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines (Brusasco, Crapo *et al.* 2005, Miller, Crapo *et al.* 2005). Readings were taken pre and post 400 µg salbutamol bronchodilatation. From three consecutive readings for FEV<sub>1</sub> and FVC, the best result was utilised.

### **2.3 Sputum induction protocol**

Sputum induction has been demonstrated to be safe in COPD subjects with a FEV<sub>1</sub> of equal or greater than 0.5 L (Brightling, Monterio *et al.* 2001). 5 mL nebulised saline was given via an ultra-sonic nebuliser (UltraNeb, DeVilbiss, Sunrise Medical, USA) for 5 minutes in the following concentrations and order: 3; 4; and 5%. Subjects were asked to blow their nose and rinse their mouth before coughing and expectoration of sputum, following each inhalation. FEV<sub>1</sub> was measured prior and following administration of 400 µg inhaled salbutamol and then after each inhalation to assess for any bronchoconstriction due to saline inhalation and to ensure procedure safety before carrying on. Any fall of FEV<sub>1</sub> more than 20% or significant symptoms results in termination of the procedure and supplementation of the subject with 400 µg inhaled salbutamol. If the subject is able to successfully expectorate sputum at any point, the process is also ended up. The produced sputum was sent to the laboratory to be processed within 2 hours as will be shown below.

### **2.4 Sputum samples processing**

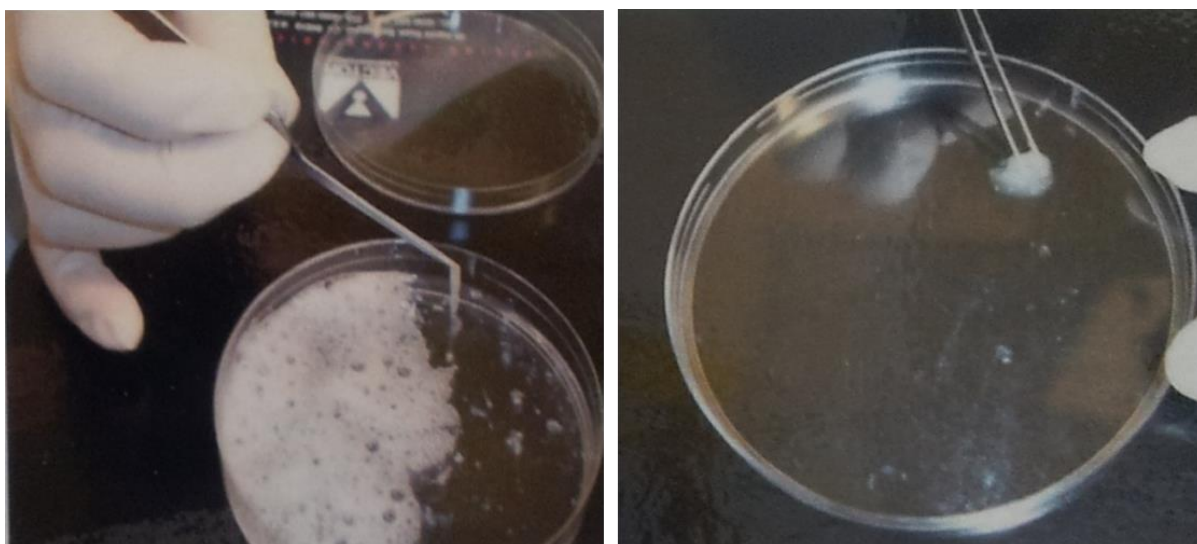
Spontaneous or induced sputum was collected on ice and processed at 4 °C. Sputum samples were processed in a 2 step method with a Dulbecco's phosphate buffered saline (D-PBS) initial wash step before the dithiothreitol (DTT) step and cytopins. In a petri dish with a dark background, a sputum sample was examined by naked eye and described as mucoid (colourless or white in appearance), purulent (yellowish to green) or muco-purulent (for colour in between). Differentiation between saliva and sputum plugs was then performed using larger blunt ended forceps (Figure 2-1). Following selection of sputum plugs, they were assembled in one mass by moving around the petri dish with small circular motions to get rid of as much saliva as possible and reduce squamous cell contamination. Apart from some

plugs that were sent to analyse for bacteria, virus and fungi, the rest of the mass was transferred to a pre-weighed falcon tube, weighed and incubated with 8x volume (sputum weight) of Dulbecco's phosphate buffered saline (D-PBS) (Sigma-Aldrich, Dorset, UK), before dispersing by gentle aspiration into a Pasteur pipette, vortexing for 15 seconds and rocking with ice on a bench rocker (ThermoFisher Scientific Basingstoke, UK) for 15 minutes. Next, the filtrate was centrifuged for 10 minutes at 4 °C with 2000 rpm (790 g), then 4 volumes of the supernatant were removed, aliquoted in 300 µL and stored at -80 °C for additional mediator analysis later on; this was called the D-PBS supernatant. Following this, 4 volumes of 0.2 % sputolysin or dithiothreitol (DTT) (#560000, Sigma-Aldrich) was added to the rest of the sample to break down the protein disulfide bonds between cells (Cleland 1964, Hirsch, Zastrow *et al.* 1969, Voynow 2002), vortexed for 15 seconds and rocked on a bench rocker for 15 minutes on ice before filtering through 48 µm nylon gauze (#03-48/31, Serofar, Lancashire, UK), that was pre-wet with D-PBS. Then, total cell count and viability count was performed using 10 µL of the filtrate added to 10 µL of 0.4% trypan blue (#T - 8154, Sigma-Aldrich) and visualised in a Neubauer haemocytometer under the light microscope. For colony forming units counting, 100 µL of the remaining filtrate was taken. For bacteria quantitative real time PCR (qPCR) another 500 µL was kept aside.

The following formula was used to quantify the total number of cells and total cell counts:

Total number of cells ( $\times 10^6$ ) = [mean number of cells counted/square  $\times 2 \times$  filtrate volume (mL)] / 100

Total cell count ( $\times 10^6$ /g sputum) = [mean number of cells counted/square  $\times 2 \times$  filtrate volume (mL)] / 100  $\times$  selected sputum weight.

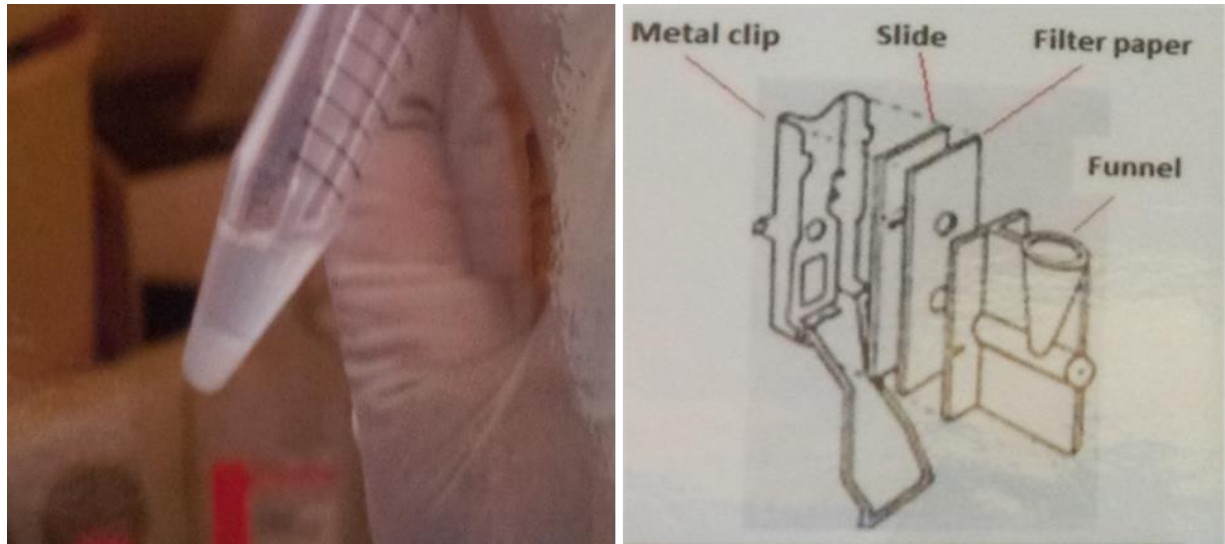


**Figure 2-1 Sputum plug selection**

## **2.5 Cytospin preparation and staining procedure**

Using a small volume of D-PBS, cell pellet was re-suspended and adjusted to make a  $0.5$  to  $0.75 \times 10^6$  cell/mL at  $75 \mu\text{L}$  of cell suspension (Figure 2-2). The slides were centrifuged at  $450$  rpm for  $6$  minutes using a cytocentrifuge (ThermoFisher Scientific). Following this, the slides were air dried for  $15$  minutes at room temperature and stained with Rowanowski stain (#130832, Medion Diagnostics, Dudingon, Germany). Smears were immersed  $5$  times (one second each) in a fixative solution (Fast green in methanol) and excess fluid was eliminated by placing the slides on tissue following each dip. Then the same step was repeated twice, but using Eosin G., which is an anionic dye that stains cytoplasmic proteins with red colour in PBS pH  $6.6$ , once and then Thiazine, a cationic dye which stains negatively charged nuclear material with blue colour, in PBS pH  $6.6$ . Slides were washed with distilled water or Weise's buffer, pH  $7.2$ , blotted before allowing to dry in air.

A differential cell count was then performed by counting >400 non squamous cells on the prepared slide under the light microscope.



**Figure 2-2 Cytospin preparation**

## **2.6 Analysis for bacteria**

### **2.6.1 Bacterial culture**

Sputum sample was incubated with equal volume of 0.1% of DTT for 15 minutes at 37 °C following gentle mixing. Next, 10 µL of the sputum samples homogenised with dithiothreitol was added to 5 mL of sterile distilled water before adding 10 µL of the diluted sample, via a sterile loop using the streaking method, to a chocolate and blood agar media plate, which were primed with a bacitracin and optochin disc respectively. They were then incubated in for 48 hours with 10% CO<sub>2</sub> before assessing the results. The following species of bacteria were tested: *Haemophilus influenzae*; *Streptococcus pneumoniae*; *Moraxella catarrhalis*; *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The results were described as either

“no significant growth”, mild, moderate or heavy growth of one of the above mentioned species.

### **2.6.2 Colony forming units (CFU)**

CFU was measured semi-quantitatively using previously published method (Pye, Stockley *et al.* 1995). The 100  $\mu$ L DTT filtrate, which was kept aside during the sputum processing, was diluted in a series from  $10^{-1}$  to  $10^{-5}$  by adding 900  $\mu$ L of sterile D-PBS solution. Following this, three 20  $\mu$ L droplets from each dilution set were inoculated onto chocolate and blood agar media and the plates were then incubated in 5%  $\text{CO}_2$  at 37  $^{\circ}\text{C}$  for 24 hours. After incubation, counting of viable bacterial numbers was performed and presented as CFU/mL of sputum (Pye, Stockley *et al.* 1995).

### **2.6.3 Quantitative assay for real time polymerase chain reaction (q PCR) for bacteria**

Using the commercial QIAmp DNA Mini Kit (Qiagen Ltd, Hilden Germany), which binds specifically to DNA, the total bacterial DNA was measured in 500  $\mu$ L of homogenised sputum. The procedure was performed in two wash steps to purify pure DNA after eliminating PCR inhibitors including divalent cations and proteins. The first stage implicates lysis using 20 mg/mL lysozyme before incubation for 30 minutes at 37  $^{\circ}\text{C}$ . Following this, the sample was incubated with proteinase K (600 mAU/mL solution) for 30 minutes at 55 $^{\circ}\text{C}$  and 15 minutes at 95 $^{\circ}\text{C}$ . Next, was adding the wash buffers and loading onto the QIAamp spin column to further purify the DNA before short centrifuging. This was followed by three additional wash steps before eluting the pure DNA in 200  $\mu$ L of DNase and RNase free distilled water and storing at -20  $^{\circ}\text{C}$ .

Overnight cultures of *E. coli*, *H. influenzae*, *S. pneumonia*, *S. aureus* and *M. catarrhalis* was carried out for extraction of pure culture DNA, which was then used as DNA standards for RT-PCR, with 10 times dilutions from  $10^2$  to  $10^7$ . SYBR Green assay (PE Applied Biosystems, Warrington, UK) was utilised to count the total bacterial load for *H. influenzae* and *S. aureus* using fluorescent binding to the minor groove of the DNA double helix prior to polymerisation. Quantification of *M. catarrhalis* and *S. pneumoniae* was performed using the TaqMan (Applied BioSystems, UK). Two negative controls (distilled RNase DNase water) were utilised in each assay session and assessment of all sputum samples was performed in duplicate.

## **2.7 Measuring eosinophils in lung tissue**

### **2.7.1 Bronchial tissues immunohistochemistry staining protocol**

#### **2.7.1.1 Cutting GMA sections**

Bronchial sections were cut at 2  $\mu$ m thickness using Leica RM 2155 microtome (Figure 2-3) and placed on a 0.2% ammonia water bath (1 mL ammonia per 500 mL distilled water) for 1 minute. Excess resin from block was trimmed using hacksaw and vice. They were then floated on to a positive charged slide (2 sections per slide) and left to dry at room temperature for 1-4 hours. Tissue sections were adhered to positively charged glass slides (#9991009, Shandon™ ColorFrost™ Plus Slides, Thermo scientific).





**Figure 2-3 Photograph of Leica microtome**

#### **2.7.1.2 GMA-embedded tissue staining**

EnVision™ FLEX+, Mouse, High pH kit (#K8002, Dako, Glostrup, Denmark) was used for the GMA-embedded staining.

##### **Step 1: Envision Block**

50 µL of EnVision™ FLEX Peroxidase-Blocking Reagent (SM801) was applied to the sections to block any nonspecific bindings by endogenous peroxidase and incubated for 5 minutes. Following this, the sections were rinsed with wash buffer for 5 minutes.

## **Step 2: Primary antibody number 1 or negative control reagent number 1**

Excess wash buffer was tapped off before adding 50  $\mu$ L of primary antibody or isotype control to the respective slides and incubated for 30 minutes. After 30 minutes, the sections were rinsed twice with wash buffer for 5 minutes each.

## **Step 3: Polymer/HRP detection reagent**

Excess wash buffer was tapped off before adding 50  $\mu$ L of the Dako EnVision™ FLEX /HRP detection reagent complex (#SM802, Dako) to each section and incubated for 30 minutes. The complex contains a dextran backbone coupled with large number of peroxidase (HRP) molecules and secondary antibody molecules. Following this, the sections were rinsed three times with wash buffer for 5 minutes each.

## **Step 4: DAB+ working solution for colour reaction**

The substrate system consists of Dako EnVision™ FLEX DAB+ Chromogen (#DM827, Dako), a concentrated 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution, and EnVision™ FLEX substrate buffer (SM803) containing hydrogen peroxide. The produced colour varies between strong violet to colorless. DAB+ working solution was prepared by adding 1 drop of DAB+ Chromogen to 1 mL of DAB substrate buffer. Excess wash buffer was tapped off before adding 50  $\mu$ L of the DAB working solution to each section and incubated for 5 minutes. Next, the slides were rinsed with distilled water and then with running tap water for 5 minutes. Slides were then placed in Mayers Haematoxylin (#MHS1-100ML, Sigma-Aldrich) for 30 minutes, after which the slides were placed in running tap water for further 5 minutes to allow to blue. Slides were then placed on a flat tray and the sections covered with SuperMount® Permanent Mounting Medium (Biogenex, California, USA), before leaving to dry overnight on a cupboard. The slides were then cover slipped with

(DPX) mountant for histology (#44581-500 mL, Sigma-Aldrich) and left to dry in fume hood and analysed later on.

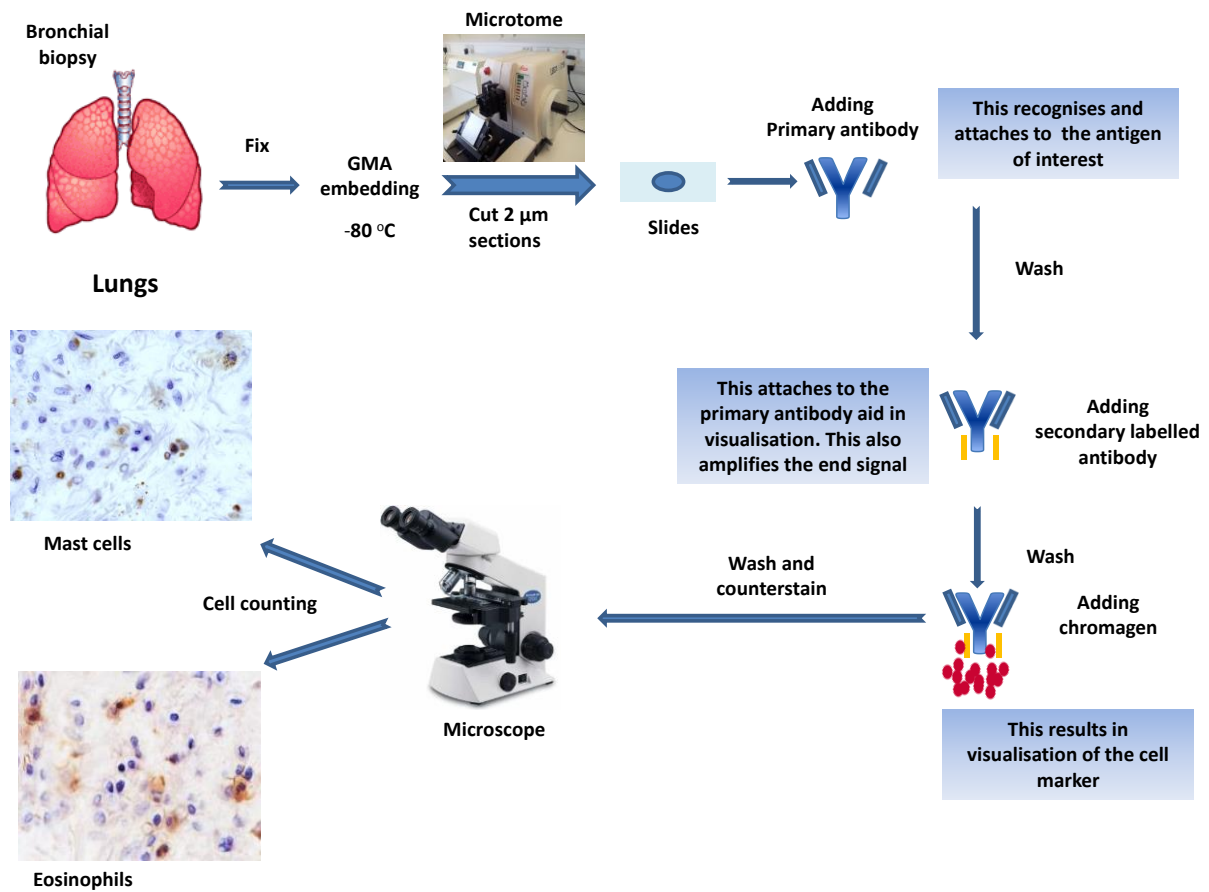
### **2.7.1.3 Haematoxylin and eosin staining**

Bronchial sections were stained by haematoxylin and eosin to view the morphology clearly and measure the reticular basement membrane (RBM). Slides were stained by the histology department at University Hospitals of Leicester using an automated Leica Stainer. In summary, slides were immersed in xylene for 1 minute, fresh xylene for 30 seconds, 2X 99% industrial methylated spirit (IMS) for 20 seconds, distilled water for 20 seconds, 2X haematoxylin for 2.15 minutes each, 2X distilled water for 1 minute, eosin for 40 seconds, water for 30 seconds, 4X IMS for 20 seconds each and 5X xylene for 30 seconds.

### **2.7.1.4 Assessment and quantification of immunohistochemical staining**

Using a computer analysis system (analySIS docu. Olympus, Germany), epithelial, submucosal and airway smooth muscle (ASM) bundle areas were identified and measured in  $\text{mm}^2$  in both upper and lower sections of each slide. Then, the numbers of positively stained nucleated cells in epithelial, sub mucosal areas were quantified in upper and lower sections and the average reading was expressed per  $\text{mm}^2$ . Areas of lamina propria  $<0.1 \text{ mm}^2$  in both sections combined were excluded as it was considered insufficient area for measurements. The reticular basement membrane was measured as the mean of 50 measurements at 20  $\mu\text{m}$  intervals in both sections and the average reading of the two is taken.

Figure 2-4 shows an overview of the method of the immunohistochemical staining.

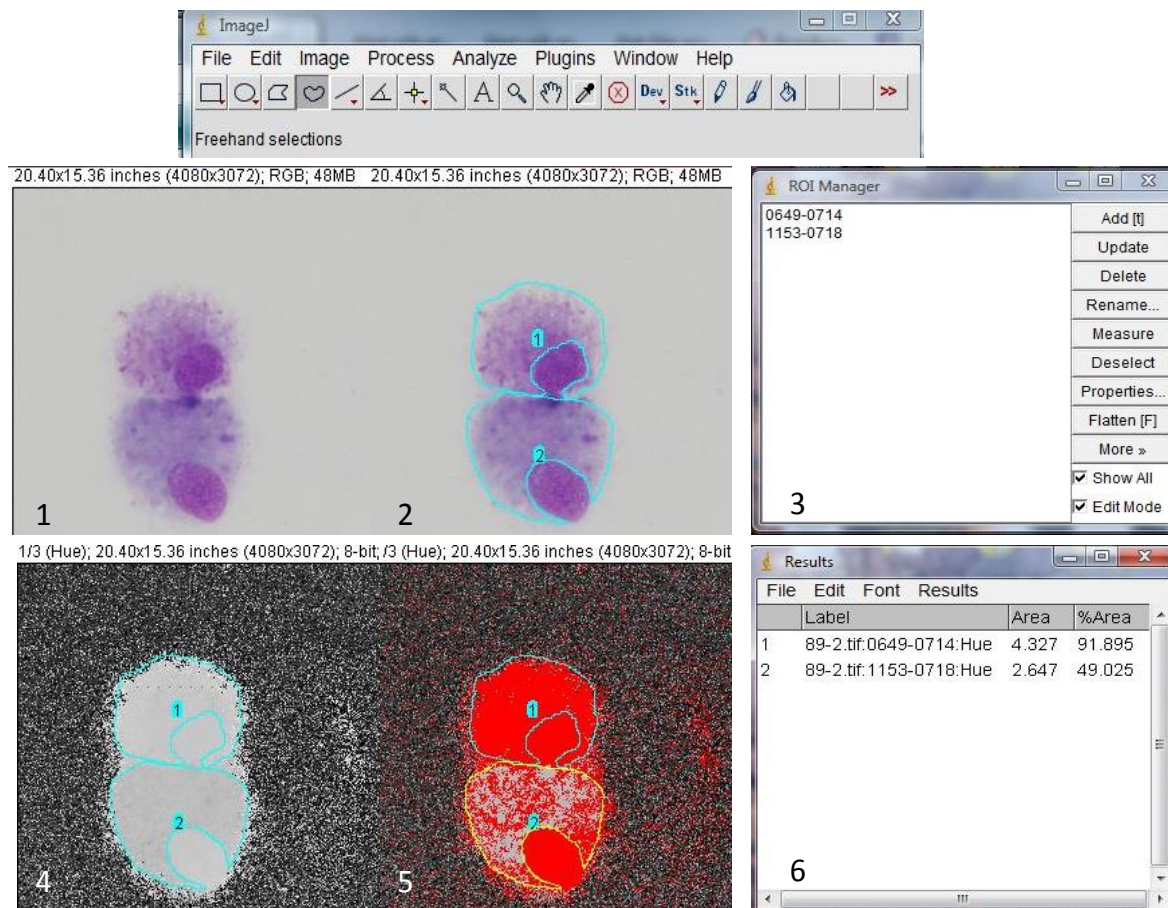


**Figure 2-4 Summary flow diagram illustrating the process of bronchial tissues immunohistochemical staining.**

## **2.8 Measuring red hue% cytoplasmic area in macrophages**

### **2.8.1 Imaging and colour analysis of images**

The median of the percentage area of red/purple hue of macrophages' cytoplasm, which represents eosinophil protein content, was calculated using a previously published method by Kulkarni and colleagues (Kulkarni, Hollins *et al.* 2010). Using Olympus DP70 digital camera, with ultrahigh resolution (4080 x 3072) (AnalySIS, Soft Imaging System GmbH, Munster, Germany), 100 macrophages were imaged per cytospin slide. Then the percentage area of cytoplasm with a red hue was measured for all of them using "Image J" software (ImageJ 1.40g/java 1.6.0\_05, National Institutes of Health Image) and the median of the percentage area of cytoplasm with a red hue was calculated as illustrated (Figure 2-5).



**Figure 2-5 Method of measuring the percentage of red hue in the macrophages by thresholding using image J software**

1, Using Image J program, a saved image of airway macrophage (TIFF file) was opened. 2, The cytoplasmic area of the macrophage was determined using the free hand-drawing tool (the fourth option in the tools) as a single area without the nucleus). 3, the selection was then added to the region of interest (ROI) manager by pressing on “T” in the keyboard. In the same way the cytoplasmic areas of all the other identified macrophages in the same image were added for batch analysis (as in the shown example). 4, the plugins tool was selected, then “Macro” is chosen before clicking on “run”. The red/green/blue image was converted to a hue/saturation/brightness stack. The hue image (which was seen as a gray scale image in the shown example), was utilised to threshold for red-purple hue. 5, all the pixels with red-

purple hue (those between 190-256) were identified by thresholding (shown as red-colored areas in the example, with the nucleus excluded from measurement). 6, the red hue of this area was expressed as percentage of the total measured macrophage cytoplasmic area. The same analysis was repeated for the rest of the macrophages up to one hundred macrophages per subject and results were copied into an Excel sheet. The percentage area of red/purple hue of airway macrophage for a subject was derived by calculating the median of the red hue percentage areas of all the measured macrophages.

The macro used for analysis of percentage area of red hue in macrophages (steps 4-6) was as follows:

```
run("HSB Stack");
n = roiManager("count");
for (i=0; i<n; i++) {
    roiManager("select", i);
    setThreshold(190, 255);
    run("Set Measurements...", "area area_fraction limit display redirect=None decimal=3");
    run("Measure");
    updateResults();
}
roiManager("deselect");
roiManager("Delete");
run("Open Next");
roiManager("Delete"). (Eltboli, Bafadhel et al. 2014).
```

## **2.9 Assessing the efferocytosis of eosinophils by blood monocyte-derived macrophages (MDM)**

### **2.9.1 Blood collection and processing for full blood and differential cell count and CRP**

A 10 mL of venous blood was withdrawn by venipuncture, aliquoted and collected into EDTA plasma prepared container (coated with K2 to prevent clotting) and serum gel activator containers (coated with silica particles to enable clotting). Following collection, the containers were kept to stand upright for 1 hour and before centrifuging at 1700 rpm for 10 minutes at room temperature. Serum and plasma were then separated and following a further centrifugation step (2300 rpm for 10 minutes at room temperature) were divided into serum and plasma aliquots for biomarker analysis. Serum was also sent to measure full blood count, differential cell count and CRP.

### **2.9.2 Purification of blood monocyte-derived macrophages**

20 to 90 mL of whole blood was collected (from a COPD or normal subject) in a heparinised syringe using 10 units/mL of Heparin-Sodium. Blood was then diluted with an equal volume of calcium and magnesium-free Hank's Balanced Salt Solution (HBSS) (#H9394-500ML, Sigma-Aldrich) at room temperature under sterile conditions and mixed by pipette, before layering gently over Ficoll-Paque (GE Healthcare, Uppsala, Sweden) at 1:1 ratio. The mixture was then centrifuged at 1500 rpm for 30 minutes at 22 °C, with a break off to allow a gradual slow down and prevent disruption of the buffy layer. During the time of centrifugation, a buffer containing 25 mL of sterile HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Sigma-Aldrich), 1% foetal bovine serum (FBS) and a 2 mM Ethylenediaminetetraacetic acid (EDTA) (#AM9260G, Sigma-Aldrich), was prepared and labeled as 'MACS buffer' before



incubating in ice to be used in the forthcoming positive selection process of CD14<sup>+</sup> monocytes.

After centrifugation, the buffy layer containing the peripheral blood mononuclear cells (PBMC), which consists of monocytes, lymphocytes and platelets was pooled into a fresh sterile tube and then HBSS was added up, before centrifuging again at 1500 rpm for another 10 minutes at 22 °C. Next, the cell pellet was resuspended and topped up with 50 mL of HBSS. Centrifugation was then repeated at 1200 rpm for 10 minutes at 4 °C and topped up with 40 mL HBSS.

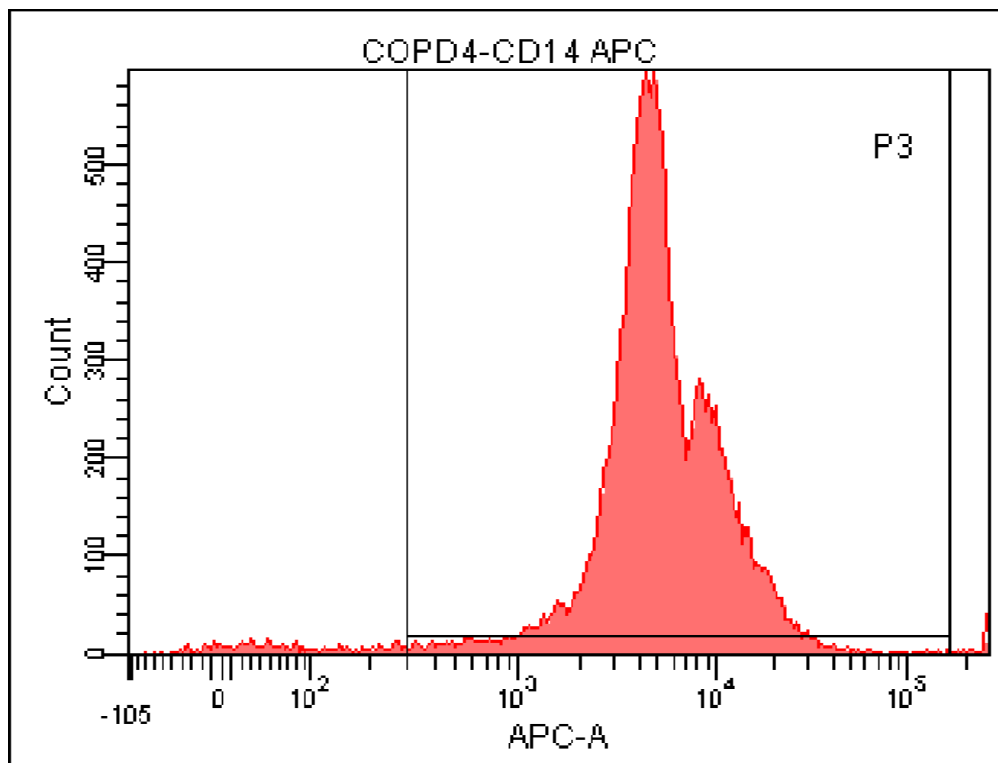
Following this, the cell pellet was resuspended in 5-20 mL (depending on the pellet size) of HBSS and the PBMCs were counted, taking the average count in 4 large squares, using Trypan blue stain (#T8154-100ML, Sigma-Aldrich) and Neubauer haemocytometer. During counting, cell suspension was centrifuged at 1500 rpm for 5 minutes at 4 °C. After that, cells pellet was resuspended in 80 µL of the previously prepared 'MACS buffer' and 20 µL of CD14 microbeads (#130-045-701, Miltenyi Biotec) for each 10<sup>7</sup> cells, before incubating in ice for 15 minutes. Subsequently, 1-2 mL of 'MACS buffer' were added to cells and centrifuged at 1500 rpm for 5 minutes at °C, then resuspended in 500 µL of 'MACS buffer' for each 10<sup>8</sup> cells and kept on ice. Thereafter, the single use, sterile Mini Separation MS columns (#130-042-201, Miltenyi Biotec) with the MiniMACS magnetic unit (#130-042-102 Miltenyi Biotec) was prepared and the column was primed twice with 500 µL of 'MACS buffer' and allowed to run through into a waste pot. Before the second 500 µL of 'MACS buffer' passed completely, the PBMCs containing CD14 microbeads were added into the column. Any remaining cells in the tube were subsequently washed twice with 500 µL

‘MACS buffer’ and added to the column. Next, the column was washed twice with 500 µL ‘MACS buffer’ (in order to get rid of lymphocytes and platelets, whilst keep monocytes attached to the column). After the last wash passes completely, MS column containing the CD14 positive cells was removed from the MiniMACS magnetic, placed over a 15 mL centrifuge tube and flushed four times using 1 mL of ‘MACS buffer’ by forcefully applying a plunger. Cells were recounted as described previously to determine purity microscopically. Finally, cells were centrifuged at 1500 rpm for 10 minutes at 22 °C, before plating them out as required in petri dish or T75 flask in Dulbecco modified Eagle medium (DMEM) with high glucose, L-glutamine, D-glucose, HEPES without sodium pyruvate, phenol red (#VX21063029, Fisher Scientific, UK), supplemented with 10% foetal calf serum (FBS), 1% Non-essential amino acids (NEAA), 1% antibiotics and antifungal (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin and 25 µg/mL of amphotericin B) and 1% Sodium pyruvate and 100 ng/mL recombinant Human M-CSF (#CBR-160-008R, R&D Systems Europe). Monocytes were then incubated at 37 °C, 5% CO<sub>2</sub> in Petri dishes for 6 nights.

The advantage of positive selection of CD14 positive monocytes using MACs separation technique is that pure population of cells is collected and incubated. Therefore, less volume of media and M-CSF, which is very expensive, is used in the experiment. In addition, no need to rely on the adherence of monocytes to flask, which is not constant each time. Additionally, following washing of the adherent cells in the adherence method, a great proportion of them were lost, leaving only small number of cell population for the phagocytosis assay.

### 2.9.3 Determination of monocytes purity

$10^5$  cells of CD14 positive purified monocytes were divided into two parts. The first was mixed with 5  $\mu$ L CD14-Alexa Fluor®647 (#HCD14) conjugated antibody (Biolegend, London, UK) on a Becton Dickinson FACS Canto II in one 100  $\mu$ L tube and the other part was kept without staining in another tube. Both tubes were incubated on ice for 15 minutes, before adding 1000  $\mu$ L Phosphate buffered saline (PBS) to them and centrifugation at 1500 rpm for 5 minutes at 4 °C. Following this, pellets were resuspended with 500  $\mu$ L flurofix buffer, before performing flow cytometry (Figure 2-6).



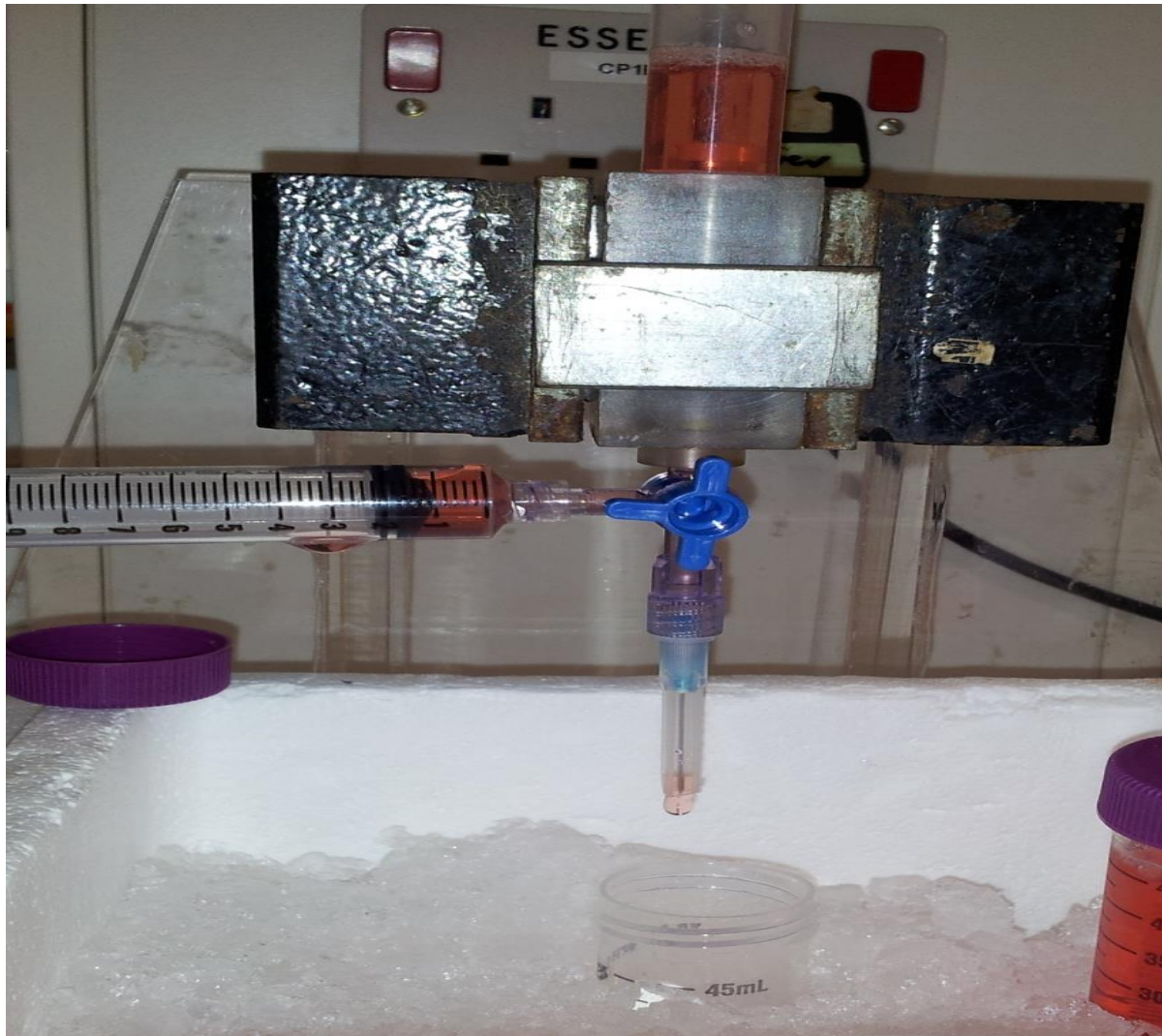
**Figure 2-6 Purity of monocytes as assessed by flow cytometry using CD14-Alexa Fluor®647 conjugated antibody on a Becton Dickinson FACS Canto II. 97% of the cells were CD14<sup>+</sup> monocytes.**

#### **2.9.4 Immunomagnetic isolation of eosinophils from peripheral blood**

On day 5 (after 4 nights), 50 to 100 mL of a whole blood was collected (from an asthmatic or atopic subject) in a heparinised syringe using 10 units/mL of Heparin-Sodium. Then, using a filling tube, 10 mL of 6% filter sterilised dextran (at room temperature) was added to each 50 mL blood in a syringe. Dextran solution was prepared by adding 3 g of dextran (Sigma-Aldrich) to 50 mL wash buffer (containing autoclaved 50 mL 10× HBSS (#H4641-500ML, Sigma-Aldrich); 5 mL 0.5 M EDTA, pH 8.0 (5 mM) (#AM9260G, Invitrogen, Paisley, UK); 15 mL 1M HEPES Buffer Solution (30 mM) (#15630080, Invitrogen) and 430 mL of distilled water), before filter sterilized and stored at 4 °C. Syringes were clamped at a 45 degree angle (with tip up) for 45 minutes. By this technique, majority of the erythrocytes are removed. After 45 minutes, the clear plasma layer was removed via the filling tube, collected into a fresh 50 mL tube and spun at 1000 rpm for 10 minutes at 22 °C without brake. Following this, each pellet was resuspended in 10-15 mL of cold-iced wash buffer supplemented with 2% FBS, before layering gently over 10 mL Histopaque 10830 (#10831-6X100ML, Sigma-Aldrich) and then centrifuged at 1400 rpm for 25 minutes, at 22 °C without brake. Next, the two cell pellets were wiped dry using cotton buds after removing the supernatant, subsequently pooled together into a fresh 50 mL tube and washed with 50 mL ice-cold wash buffer, prior to centrifuging at 1200 rpm for 8 minutes at 4 °C. Following this, the cell pellet was resuspended in 20 mL ice-cold distilled water for 30 seconds. Then a mixture of 4 mL of 10× HBSS, 600 µL 1M Hepes, 400µL 0.5M EDTA and 15 mL distilled water was added before spinning at 1200 rpm for 8 minutes at 4 °C. Afterwards, cell pellet was resuspended in 30 ml wash buffer and total cell count was performed using 10 µL for a Kimura stain and Neubauer haemocytometer to calculate the volume of anti-CD16 immunomagnetic microbeads (#130-045-701, Miltenyi Biotec) to be added.

$$\text{Volume of beads to add in } \mu\text{L} = \frac{\text{Total cell number in } \times 10^7 (\times 50)}{2 \times 10^7}$$

During counting, cell suspension was centrifuged as before and then cells were incubated with the calculated volume of anti-CD16 beads to remove contaminating neutrophils and an equal amount of wash buffer on ice for 40 minutes. Cells were resuspended every 10 minutes by gently vortexing using a bench rocker. If the cells were overly infiltrated with monocytes as visualized by microscope, 35-50  $\mu\text{L}$  of anti-CD3 microbeads (#130-050-101, Miltenyi Biotec) and CD14 microbeads each, then added at approximately 30 minutes after the start of incubation. During the incubation period, CS MACS columns (#130-041-305, Miltenyi Biotec) were set up and placed in a holder with magnet (Figure 2-7). Cells mixed with microbeads were then resuspended in 4 mL wash buffer, loaded onto the column and the effluent containing eosinophils was collected using a tube surrounded by ice beneath the needle. Columns were then washed through with 4 column volumes of wash buffer (about 16 mL), before they run dry. The CD16, CD14 and CD3 positive cells were negatively trapped and depleted in the column, whilst eosinophils passed through and collected in a tube underneath (Figure 2-7).



**Figure 2-7 Photograph showing the CS MACS columns set up.** The columns are placed in a holder with two magnets in each side. The CD16, CD14 and CD3 positive cells were negatively trapped and depleted in the column, whilst eosinophils passed through and collected in a tube underneath

Eosinophils were spun down at 1200 rpm for 8 minutes at 4 °C, resuspended in a 6 mL of culture media and final cell count was measured using Kimura stain as described previously. Thereafter, eosinophils were incubated at 37 °C, 5% CO<sub>2</sub> in a T25 flask in the usual (DMEM) Dulbecco modified Eagle medium supplemented (#61965-059, Invitrogen), with 10 % FBS

(#10500-064, Invitrogen), 1% antibiotic and antimycotic (#15240-062, Invitrogen), 1% sodium pyruvate (SP) (S8636-100ML, Sigma-Aldrich) and 1% NEAA, but without cytokines to for 48 hours to induce aging. Before incubation, a small aliquot of cell suspension was taken, from which cytopsin was made and stained with “Diff Quick” stain, a Romanowsky stain variant, to determine the purity of eosinophils preparation microscopically. Percentage of apoptotic/dead cells before and after 48 hours were determined using Annexin V and Propidium iodide staining (#556547, BD Biosciences, USA) as will be described below.

#### **2.9.5 Determination of percentage of apoptotic/necrotic eosinophils using Annexin V and Propidium iodide staining**

$10^5$  eosinophils were added to 5 (100  $\mu$ L flow tubes) and labelled from 1 to 5. All the tubes were centrifuged at 1500 rpm for 5 minutes at  $^{\circ}$ C, before discarding the supernatants. Next, cell pellets were resuspended in either 100  $\mu$ L of Annexin V binding buffer (#556454, BD Biosciences, USA), made up with distilled water as per instructions or 100  $\mu$ L HBSS (Calcium and magnesium free to be used as a control since Annexin V reaction is calcium dependent), according to the table below (Table 2-1). Following this, 5  $\mu$ L of Annexin V conjugated to FITC (#560931, BD Biosciences) and 5  $\mu$ L of Propidium iodide (#556463, BD Biosciences), were added to each tube according to the table below, gently vortexed and incubated in the dark at room temperature for 15 minutes. After 15 minutes, 400  $\mu$ L of binding buffer (or HBSS) were added to each corresponding tube and analysed within 1 hour.

**Table 2-1 Reagents combinations used to determine the percentage of apoptotic/necrotic eosinophils**

	<b>Tube 1</b>	<b>Tube 2</b>	<b>Tube 3</b>	<b>Tube 4</b>	<b>Tube 5</b>
<b>Binding</b>	Binding	Binding	Binding	HBSS	Binding
<b>Buffer/HBSS</b>	buffer	buffer	buffer		buffer
<b>Annexin V</b>	Yes	No	Yes	Yes	No
<b>PI</b>	Yes	Yes	No	Yes	No

### **2.9.6 Harvesting of monocyte-derived macrophages (MDM)**

MDMs were subsequently harvested in the morning of day 7 using a cell lifter (#CLS3008-100EA, Sigma-Aldrich) and vigorous pipetting with warm HBSS after aspirating the non-adherent cells and prior to centrifugation at 1200 rpm for 8 minutes at 4 °C. Following this, they were resuspended and counted as described previously. They were then re-plated in a Petri dish and kept in an incubator pending feeding with apoptotic eosinophils. Before this step, 1 million of macrophages was set apart and kept in ice to perform cytospin and stain by “Diff Quick” stain to measure the red hue before phagocytosis.

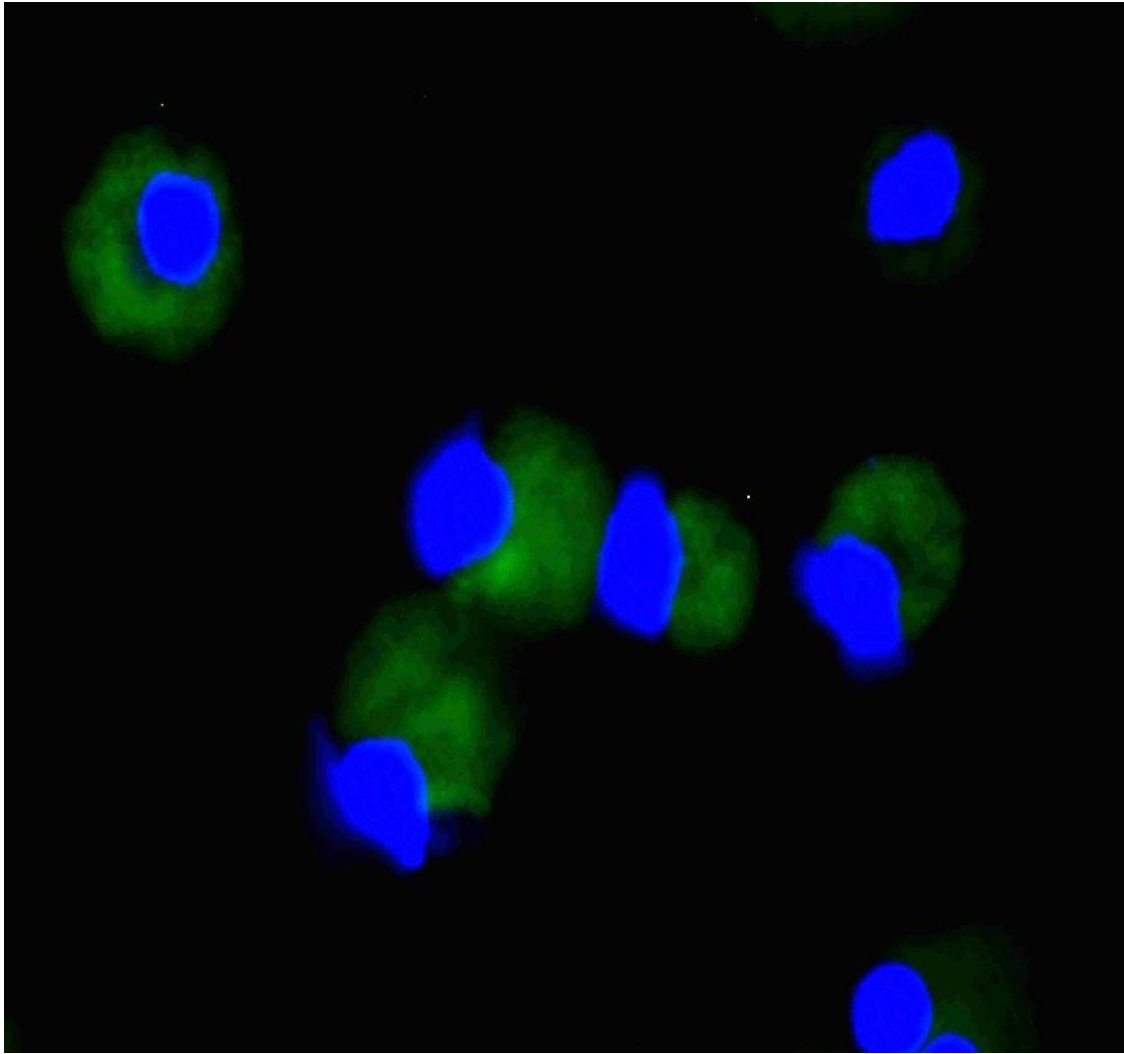
### **2.9.7 Phagocytosis assay**

After harvesting macrophages as described above, on day 7 (after 48 hours of incubating eosinophils), eosinophils were retrieved using vigorous pipetting with warm HBSS and tapping on the flask. Next to this, cells were centrifuged at 1200 rpm for 8 minutes at 4 °C, counted using Trypan blue and kept in ice during counting. Then the numbers of eosinophils to be added to macrophages in a 5:1 (eosinophils: macrophage) ratio was calculated.



Subsequently, the desired amounts of eosinophils were added to the harvested MDMs. Following 2 hours, the media with non-adherent cells were aspirated after gentle shaking and kept in separate tubes at 4 °C, before harvesting the adherent macrophages using a cell lifter and vigorously pipetting the dishes and washing 3 times with warm HBSS and then centrifugation at 1200 rpm for 8 minutes at 4 °C. Finally, cell pellet was resuspended in a small amount of cold HBSS stained with Trypan blue and macrophages were counted as before, prior to adjusting the cell number to  $5-10 \times 10^5/\text{mL}$ .

The reason of choosing eosinophils: macrophage ratio of 5:1 is when 10:1 was used; large numbers of eosinophils on the expense of no or very few macrophages per slide were viewed under the florescent microscope. On the other hand, when 1:1 ratio was used; no or very few eosinophils/eosinophilic protein were seen per slide (Figure 2-8).



**Figure 2-8 Representative immunofluorescence staining of macrophages cultured with apoptotic eosinophils at 1:1 ratio.** Macrophages were stained with CD68-FITC (green) and ECP-PE (red) and DAPI (blue). No eosinophils or eosinophilic protein can be seen.

### **2.9.8 Cytospin preparation and fixation of slides**

At least 8 cytopsin slides were prepared using  $37.5 \times 10^3$  cells (adjusted to 75  $\mu$ L volume) per cytopsin slide. The slides were centrifuged at 450 rpm for 6 minutes, as was described in section 2.5. Slides were labelled as Eosinophils, Macrophages: Eosinophils and Macrophages alone). Then, half of them were labelled with primary antibodies and half with negative

control. All the cytopins were circled with Dako pen (#S200230, Dako), then fixed and permeabilised within 24 hours with paraformaldehyde (#P6148-500G, Sigma-Aldrich) with 0.1% saponin (#S7900, Sigma-Aldrich) for 20 minutes, before leaving them to air dry for 10 minutes and storing at cold for future staining.

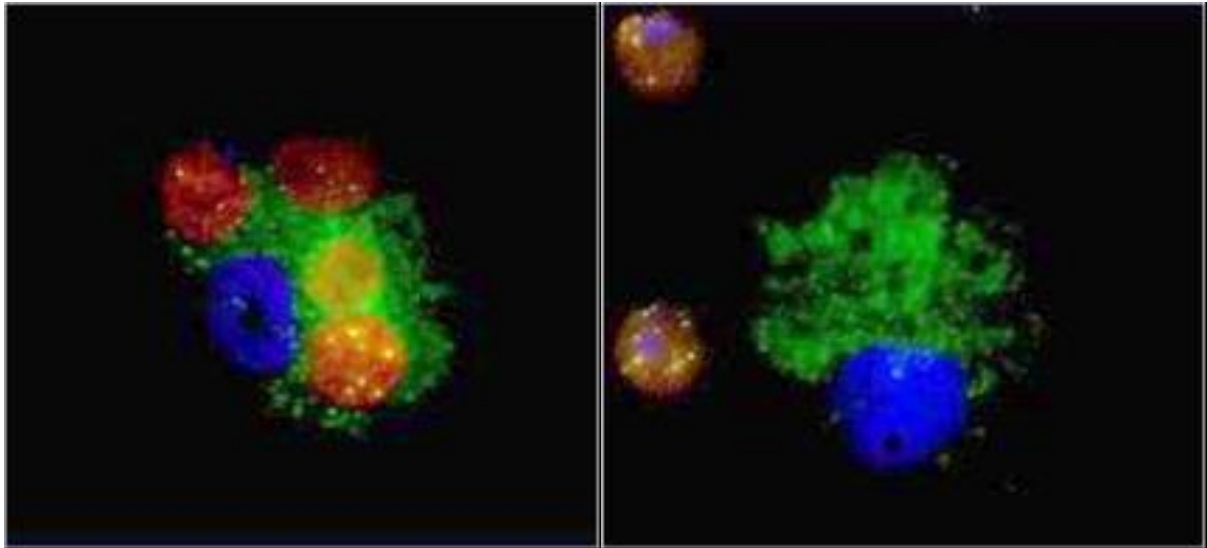
### **2.9.9 Immunofluorescence staining**

200 µL of PBS, 3% bovine serum albumin (BSA) (#A7906, sigma-Aldrich), was added to each slide and incubated for 30 minutes before removing the buffer and adding 200 µL of the first primary antibody (Mouse monoclonal anti-ECP) (#EG2, Diagnostics Development, Sweden), diluted to 1:50 with PBS, 0.5% BSA and 0.1% saponin, which was labeled as “Anibody buffer”, to each slide that contain eosinophils and labelled a primary antibody. At the same time 200 µL of Mouse Ig-G1 negative control (#YYX093101, Dako), diluted to 1:5000 with “Anibody buffer”, was added to each slide containing eosinophils and labelled as isotope control. “Anibody buffer” was also added to the remaining slides to prevent drying, before incubating all the slides in cold room at 4 °C for 90 minutes. After 90 minutes, slides incubated with antibodies were rinsed 3 times with “Anibody buffer”, before adding 200 µL of secondary antibody (Polyclonal Rabbit Anti-Mouse Immunoglobulin RPE. (#R043901, Dako), diluted to 1:10 with “Anibody buffer”, in the dark, prior to covering with foil and incubation in cold for another 90 minutes. Following this, slides were rinsed 3 times with “Antibody buffer” as described, before adding 200 µL of the second primary antibody (CD68-FITC Monoclonal Mouse Anti-Human) (#YYF713501, Dako), diluted to 1:10 with “Anibody buffer”, to each slide that contains macrophages and labelled as primary antibody. Similarly, 200 µL of isotype control (Mouse IgG1 FITC Control) (#X092701, Dako), diluted to 1:10 with “Anibody buffer”, was added to each slide containing macrophages, but labelled

as isotope control, whilst “Anibody buffer” was added to the remaining slides to prevent drying, before incubating all the slides in cold, covering with foil for 90 minutes and rinsing 3 times with “Antibody buffer” as described above. Next, 200  $\mu$ L of 4',6-diamidino-2-phenylindole (DAPI) (diluted to 1:1000 with “Anibody buffer”) was added to all previous slides and incubated for 50 seconds, before washing 6 times with D-PBS and gently wiping the extra fluids in all slides with soft tissue. Finally, one drop of prolonged gold antifade (#P36930, Invitrogen), was added to each slide before mounting them with cover slips (VWR international, Lutterworth, UK), covering by foil and incubating overnight in the dark at 4 °C. All the slides were then viewed within the 48 hours using fluorescence microscope.

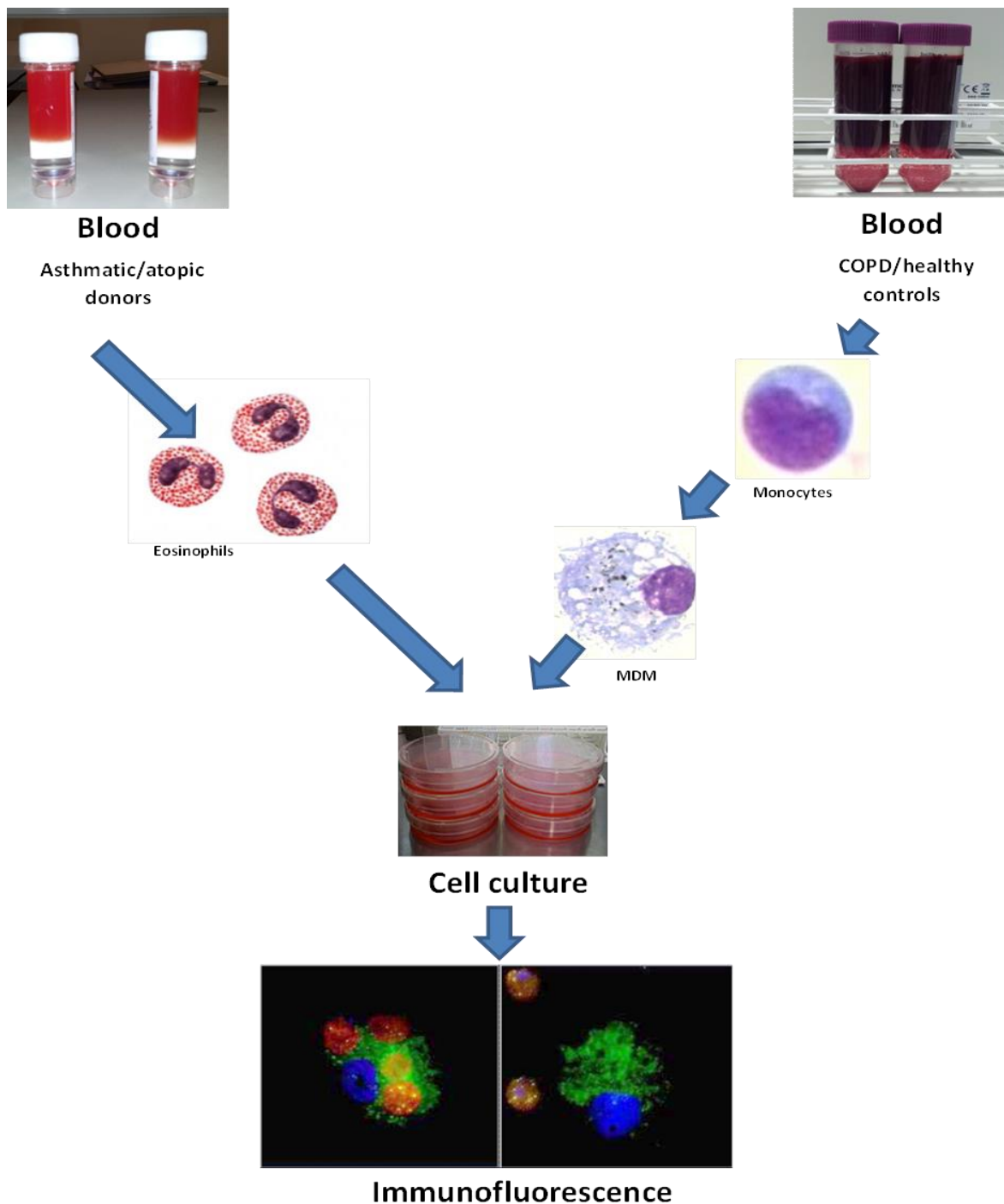
#### **2.9.10 Phagocytosis quantification**

Phagocytosis was quantified using fluorescent microscope, where 100 macrophages were imaged and the percentage of cells that have ingested or starting to engulf eosinophils (attached to macrophage) were counted (Figure 2-9).



**Figure 2-9 Representative immunofluorescence staining of macrophages cultured with apoptotic eosinophils.** Macrophages were stained with CD68-FITC (green), ECP-PE (red) and DAPI (blue). Left panel shows efferocytosis of eosinophils by a macrophage positively stained with ECP and the right panel shows ECP positive eosinophils and a macrophage with no evidence of efferocytosis.

Figure 2-10 briefly summarises the steps of efferocytosis assay.



**Figure 2-10** Schematic figure showing the summary of efferocytosis assay: Blood collected from COPD and healthy controls for purification of MDM and from asthmatic/atopic donors for eosinophils purifications, eosinophils were then fed to MDMs and cultured together for 2 hours before performing immunofluorescence staining. MDM: monocyte-derived macrophage.

## 2.10 Statistical analysis

GraphPad Prism version 6 software (GraphPad software, San Diego, CA) and SPSS version 20 (SPSS, Inc. Chicago, IL), were used to perform statistical analysis. Mean (standard error of the mean) was used to present parametric data, whilst median (interquartile range) was used for non-parametric data. Geometric mean (95% confidence interval) was utilised to express Log transformed exponential data. To check whether the data are normally distributed, D'Agostino & Pearson omnibus, Shapiro-Wilk and Kolmogorov-Smirnov (with Dallal-Wilkinson-Lilliefors p value) normality tests were used. The Student unpaired T-test and Paired T-test was used to compare unpaired and paired parametric data respectively, whilst Mann-Whitney test and Wilcoxon matched pairs test was used for comparison of unpaired and paired non-parametric variables. Chi-square or Fisher's exact test as appropriate was used to assess categorical data. Comparisons across three groups or greater were assessed by one-way analysis of variance (ANOVA) with Tukey pair-wise multiple comparison test for parametric and "Kruskal-Wallis test with Dunn's multiple comparison test for non-parametric data. The repeatability of data was measured using intraclass correlation coefficient and expressed as Cronbach's alpha reliability coefficient ( $R_i$ ). Spearman's rank and Pearson correlation coefficient were used to check the correlation between variables and expressed as Spearman  $r$  for nonparametric and Pearson  $r$  for parametric variables. For all the statistical tests used, p value was considered statistically significant when it is less than 0.05.

## **Chapter 3    What is eosinophilic COPD phenotype? Is it a distinct entity?**



### 3.1 Cross-sectional study for clinical description of eosinophilic COPD phenotype

#### 3.1.1 Abstract

**Background:** Sputum eosinophilia is persistently present in 10-30% of subjects of COPD.

The role of the eosinophilic inflammation in COPD is not very clear.

**Methods:** 196 subjects that had participated in previous studies of COPD exacerbations were divided into 2 groups based on sputum eosinophils count with a cut-off of 3%.

**Results:** There were no differences in baseline lung function, health status or exacerbation frequency between the 2 groups. Bacterial colonisation of the sputum and neutrophilic inflammation at baseline were significantly lower in the eosinophilic (36%), compared to non-eosinophilic group (12.5%); ( $p=0.03$ ). Sputum eosinophils and neutrophils were inversely correlated ( $r=-0.35$ ;  $p<0.0001$ ). There was also a strong positive correlation between sputum and blood eosinophils ( $r=0.40$ ;  $p<0.0001$ ), weak inverse correlation of sputum eosinophilia with CRP ( $r=-0.18$ ;  $p=0.001$ ), CFU ( $r=-0.25$ ;  $p=0.0007$ ) and VAS cough ( $r=-0.17$ ;  $p=0.016$ ) and no correlation with FEV<sub>1</sub>, FEV<sub>1</sub>% predicted or FEV<sub>1</sub>/FVC%, nor with pack-years of smoking. Baseline sputum neutrophils% correlated negatively with FEV<sub>1</sub> ( $r=-0.26$ ;  $p=0.0002$ ) and FEV<sub>1</sub>/FVC% ( $r=-0.16$ ;  $p=0.027$ ), and positively with CRP ( $r=0.22$ ;  $p=0.0019$ ) and CFU ( $r=0.25$ ;  $p=0.0006$ ). The fall in FEV<sub>1</sub> from stable to exacerbation was not different between eosinophilic versus non-eosinophilic group although the FEV<sub>1</sub> and FEV<sub>1</sub>% predicted were slightly lower in the eosinophilic compared to the non- eosinophils.

**Conclusion:** Eosinophilic COPD is a distinct phenotype that rarely coexists with bacterial or neutrophilic phenotype. Blood eosinophil is a strong potential biomarker of sputum eosinophils. Sputum eosinophil is not related to degree of the outflow obstruction or pack-years of smoking in COPD.

### **3.1.2 Introduction**

Chronic obstructive pulmonary disease is a heterogeneous condition exemplified by the identification of a subgroup of COPD subjects with eosinophilic airway inflammation (Eltboli, Brightling 2013, Barker, Brightling 2013). Eosinophilic airway inflammation plays a role mainly in asthma, but its role in COPD remains controversial, although it is consistently reported in 10-30% of COPD subjects and is associated with a better response to both inhaled and oral corticosteroids (Brightling, Monteiro *et al.* 2000, Brightling, McKenna *et al.* 2005). Whether the phenotypes are similar between the 2 diseases or represent distinct entities is unknown. I postulated that COPD subjects categorised into subgroups determined by their sputum eosinophils count (3%) will identify important differences in terms of their clinical characteristics and eosinophilic subgroup is a unique COPD phenotype.

### **3.1.3 Methods**

#### **3.1.3.1 Subjects and study design**

Clinical data and sputum cytopins were available from 196 subjects that had participated in two clinical studies of COPD exacerbations (Bafadhel, McKenna *et al.* 2011, Bafadhel, McKenna *et al.* 2012). Subjects had undergone extensive clinical characterisation including clinical history, demographics, visual analogue symptom (VAS) scores, health status assessment, using the chronic respiratory questionnaire (CRQ) and St George's Respiratory questionnaires (SGRQ), spirometry before and after administration of a short-acting bronchodilator, sputum analysis for cellular profiles and microbiological assessment as described (chapter 2) at baseline, 3 monthly stable follow-up visits and exacerbations for at least one year. Subjects were divided into two groups based on  $\geq$  or  $<3\%$  baseline sputum eosinophil count.

### **3.1.3.2 Statistical analysis**

GraphPad Prism version 6 (GraphPad, San Diego) and IBM SPSS version 20 (SPSS, Inc. Chicago) were used to perform statistical analysis. Mean (standard error of the mean [SEM]) was used to present parametric data, whilst median (interquartile ranges [IQR]) was used for non-parametric data and geometric mean (95% confidence interval) for data that was log normally distributed. Comparisons between groups used unpaired T-test or Mann-Whitney for parametric and non-parametric data respectively. Comparisons across groups were assessed by one-way analysis of variance (ANOVA) with Tukey pair-wise comparisons or Kruskal-Wallis test with Dunn's multiple pair-wise comparisons for parametric and non-parametric data respectively. Chi-square or Fisher's exact test as appropriate was used to assess categorical data. Spearman or Pearson rank correlation coefficient was used to assess the correlations for non-parametric and parametric variables respectively. A p value less than 0.05 was considered statistically significant.

### **3.1.4 Results**

Table 3-1 shows a comparison of baseline clinical characteristics of the 196 COPD subjects included in the study after dividing them into eosinophilic and non-eosinophilic group. 51 out of the 196 subjects were eosinophilic (26%). No significant differences were observed between the groups with regard to age, gender, race, BMI, smoking history, lung function, use of oral and/or antibiotics or hospital admissions in the last 12 months.

The sputum neutrophils percentage was raised in non-eosinophilic subjects than eosinophilics (median [IQR] 78 [56-93], compared to 65 [40-80];  $p=0.0008$ ). Blood neutrophils count was also increased in non-eosinophilics 5.1 (4-6.1), than eosinophilics 5.5 (4.5-6.6), but to a lesser

extent ( $p=0.02$ ). C-reactive protein (CRP) was mildly higher in non-eosinophilic COPD (6 [5-12], than eosinophilics  $<5$  [ $<5-7$ ];  $p=0.02$ ), although was not clinically significant. Serum ECP was not different between the two COPD groups (median [IQR] 35 g/L [28-58] in the eosinophilic, versus non-eosinophilic 33 [21-50];  $p=0.11$ ). Cough and purulent sputum were almost twice as high in the non-eosinophilic group as eosinophilic (Visual analogue scale (VAS) for cough (median [IQR]) 36.5 mm [18-62]) for the non-eosinophilics, versus (20 mm [12-45]) in the eosinophilic; ( $p=0.0098$ ) and VAS for sputum purulence (median [IQR]), 29 mm [11-54] in the non-eosinophilics, compared to eosinophilics (14 mm [3.8-48];  $p=0.03$ ). In addition, Chronic respiratory health Questionnaire (CRQ) total score was worse in the non-eosinophilics (median [IQR] 2.3 [1.9-3.6]), compared to eosinophilic subjects (3.8 units [3.3-4.2];  $p<0.0001$ ).

**Table 3-1 Baseline characteristics of all the COPD subjects based on their baseline sputum eosinophils count.**

	<b>Eosinophilic (n=51)</b>	<b>Non-eosinophilic (n=145)</b>	<b>p value</b>
<b>Male, n (%)</b>	31 (60.8)	96 (66.2)	0.49
<b>Race (Caucasian), n (%)</b>	50 (98)	145 (100)	0.26
<b>Age (years) *</b>	68 (1.2)	68 (0.74)	0.84
<b>Body mass index kg/m<sup>2</sup></b>	25 (23-29)	26 (23-30)	0.33
<b>Current smokers, n (%)</b>	15 (29)	55 (38)	0.26
<b>Ex-smokers smokers, n (%)</b>	35 (69)	89 (61)	0.36
<b>COPD duration (years)</b>	5.1 (1.9-11)	5.2 (2.7-9.6)	0.69
<b>Number of hospital admissions in the last year</b>	0 (0-1)	0 (0-1)	0.70
<b>Number of steroid and/or antibiotics in the last</b>	3 (1-6)	3 (1-5)	0.10
<b>ICS dose (BDP equivalent) (mcg/day)</b>	2.0 (1.6-2.0)	2.0 (0.8-2.0)	<b>0.02</b>
<b>Sputum total cell count x10<sup>6</sup>/g</b>	3.3 (1.5-6.7)	3.6 (1.3-7.8)	0.73
<b>Sputum neutrophils (%)</b>	65 (40-80)	78 (56-93)	<b>0.0008</b>
<b>Sputum macrophages (%)</b>	19 (11-33)	18 (6.4-37)	0.59
<b>Peripheral blood total cell count (x10<sup>9</sup>/L)</b>	8.3 (6.7-9.7)	8.6 (7.1-10)	0.22
<b>Peripheral blood neutrophils cell count (x10<sup>9</sup>/L)</b>	5.1 (4-6.1)	5.5 (4.5-6.6)	<b>0.02</b>
<b>Peripheral blood eosinophils cell count (x10<sup>9</sup>/L)</b>	0.34 (0.20-0.48)	0.19 (0.12-0.29)	<b>&lt;0.0001</b>
<b>Serum ECP (µg/L)</b>	35 (29-58)	33 (21-50)	0.08
<b>Peripheral monocytes cell count (x10<sup>9</sup>/L)</b>	0.55 (0.43-0.73)	0.60 (0.50-0.72)	0.28
<b>Peripheral blood basophils cell count (x10<sup>9</sup>/L)</b>	0.05 (0.03-0.07)	0.04 (0.03-0.05)	<b>0.002</b>
<b>CRP (mg %)</b>	<5 (<5-7)	6 (<5-12)	<b>0.02</b>
<b>FEV<sub>1</sub> post bronchodilator (L)</b>	1.1 (0.79-1.7)	1.3 (0.86-1.7)	0.05
<b>FEV<sub>1</sub>% predicted, (%)</b>	45 (29-61)	49 (35-65)	0.05
<b>Post FEV<sub>1</sub>/FVC (%)*</b>	50 (2.2)	51 (1.1)	0.70
<b>Reversibility, mL</b>	50 (0-100)	30 (-20-100)	0.67
<b>Reversibility, %</b>	5.2 (0- 9.9)	3.0 (-1.8- 9.7)	0.64
<b>GOLD 1, n (%)</b>	5 (10)	15 (10)	0.91
<b>GOLD 2, n (%)</b>	19 (37)	56 (39)	0.86
<b>GOLD 3, n (%)</b>	17 (33)	50 (35)	0.88
<b>GOLD 4, n (%)</b>	10 (20)	24 (17)	0.62
<b>MRC total score (unit)</b>	3 (2-4)	3 (2-4)	0.96
<b>SGRQ total score (unit)*</b>	50 (2.5)	54 (1.4)	0.17
<b>CRQ total score (unit)</b>	3.8 (3.3-4.2)	2.3 (1.9-3.6)	<b>&lt;0.0001</b>
<b>VAS Cough (mm)</b>	20 (12-45)	37 (18-62)	<b>0.009</b>
<b>VAS Dyspnoea (mm)</b>	46 (26-70)	50 (30-70)	0.81
<b>VAS Sputum Production (mm)</b>	24 (9-45)	36 (12-60)	0.05
<b>VAS Sputum Purulence (mm)</b>	14 (3.8-48)	29 (11-54)	<b>0.03</b>

Data presented as median (inter-quartile range) unless stated, \*mean (SEM), SEM: standard error of mean, **Abbreviations:** BDP: Beclometasone Dipropionate; CRQ: Chronic respiratory health Questionnaire; CRP: C-Reactive Protein; ECP: Eosinophil cationic protein; FEV<sub>1</sub>: Forced expiratory volume in 1 second; FVC: Forced vital capacity; GOLD: Global Initiative for chronic Obstructive Lung disease; ICS: inhaled corticosteroids, MRC: Medical Research Council; SGRQ: St George's Respiratory Questionnaire; VAS: Visual analogue score.

There was a higher proportion of positive sputum bacterial culture in the sputum of the non-eosinophilic group (n. [%], 50 [36%], versus 6 [12.5 %] in the eosinophilics (Chi-squared  $p=0.03$ ) (Table 3-2) and also colony forming unit (CFU) for bacteria (geometric mean [95% confidence interval]) (6.4 [6.3-6.6], versus 6.0 [5.8-6.3]); respectively ( $p=0.01$ ). Among these, there was also increased number of quantitative PCR (qPCR) for *staphylococcus aureus* in non-eosinophilic, in contrast to eosinophilic subjects (median [IQR] 2.4 [2.2-2.6], compared to 2.1 [2-2.3];  $p=0.04$ ). The sputum colonisation for viruses and fungi were similar between the 2 groups.

**Table 3-2 Sputum microbiology in the two groups at baseline**

	Eosinophilic (n= 51)	Non-eosinophilic (n= 145)	p value
<b>Sputum colonisation for bacteria, n (%)</b>	6 (12.8)	50 (35.7)	<b>0.003</b>
<b>CFU unit/mL</b>	6 (5.8-6.3)	6.4 (6.3-6.6)	<b>0.01</b>
<b>16s (gene copies <math>\times 10^6</math>/mL)</b>	8.4 (8.1-8.7)	8.4 (8.2-8.5)	0.52
<b>qPCR <i>Haemophilus influenza</i></b>	4.4 (3.8-5.1)	4.2 (3.8-4.6)	0.76
<b>qPCR <i>Staphylococcus aureus</i></b>	2.1 (2-2.3)	2.4 (2.2-2.6)	<b>0.04</b>
<b>qPCR <i>Streptococcus pneumonia</i></b>	2.9 (2.5-3.4)	2.9 (2.6-3.1)	0.74
<b>qPCR <i>Morexella catarrhalis</i></b>	3.3 (2.8-4)	3.6 (3.2-3.9)	0.51
<b>Sputum colonisation for <i>Aspergillus fumigatus</i>, n (%)</b>	9 (27.3)	39 (41.9)	0.14
<b>Picorna Virus PCR, n (%)</b>	1 (5.3)	1 (1.5)	0.38

Data presented as Geometric mean of Log (95% CI). unless stated, CI: confidence interval. **Abbreviations:** CFU: colony forming unit; qPCR: quantitative polymerase chain reaction.

Similarly, inflammatory cytokines associated with neutrophilic inflammation or bacterial infection including: IL8; TNF- $\alpha$ ; TNF1a; TNF1b; MM7; MMP8; MMP9; CSF2; IL1b and IFN  $\gamma$ , were higher in the non-eosinophilics (Table 3-3), whilst some markers of eosinophilic inflammation such as CCL26 were increased in the eosinophilics. On the other hand, no significant differences were found between the 2 groups with regards to serum cytokines (Table 3-4).

**Table 3-3 Sputum biomarkers in the two groups at baseline**

	<b>Eosinophilic (n= 51)</b>	<b>Non-eosinophilic (n= 145)</b>	<b>p value</b>
<b>CCL2 (pg/mL)</b>	453 (316-1064)	533 (271-1185)	0.80
<b>CCL3 (pg/mL)</b>	39 (22-129)	70 (34-166)	0.07
<b>CCL4 (pg/mL)</b>	738 (394-1716)	949 (473-2171)	0.50
<b>CCL5 (pg/mL)</b>	2 (1-6)	3.8 (1.7-6.3)	0.09
<b>CCL13 (pg/mL)</b>	43 (22-62)	31 (14-48)	0.17
<b>CCL17 (pg/mL)</b>	34 (9.7-116)	20 (10-36)	0.20
<b>CCL26 (pg/mL)</b>	3.8 (2.1-14)	1.5 (0.49-6)	<b>0.03</b>
<b>CXCL10 (pg/mL)</b>	183 (96-546)	254 (83-941)	0.74
<b>CXCL11 (pg/mL)</b>	10 (3.1-38)	12 (3.4-45)	0.87
<b>CSF2 (pg/mL)</b>	0.015 (0-0.14)	0.12 (0.04-0.36)	<b>0.005</b>
<b>IFN <math>\gamma</math> (pg/mL)</b>	0 (0-0.05)	0.03 (0-0.63)	<b>0.03</b>
<b>IL1B pg/mL</b>	21 (8.7-99)	73 (18-628)	<b>0.02</b>
<b>IL4 (pg/mL)</b>	0 (0-0.005)	0 (0-0.07)	0.15
<b>IL5 (pg/mL)</b>	1.5 (0.43-8.3)	0.98 (0.35-2)	0.13
<b>IL6 (pg/mL)</b>	381 (114-705)	579 (209-1550)	0.09
<b>IL6R (pg/mL)</b>	105 (58-180)	157 (181-340)	0.12
<b>IL8 (pg/mL) <math>\times 10^3</math></b>	1.53 (1.16-5.32)	6.53 (2.04-16.66)	<b>0.007</b>
<b>IL10 (pg/mL)</b>	0.62 (0.08-1.8)	1.4 (0.25-5.5)	<b>0.049</b>
<b>IL13 (pg/mL)</b>	3.7 (0.96-11)	2.6 (1.4-7.7)	0.80
<b>IL17 A (pg/mL)</b>	0 (0-0.18)	0 (0-0.09)	0.52
<b>MMP1 (pg/mL)</b>	242 (205-516)	464 (197-1097)	0.19
<b>MMP2 (pg/mL) <math>\times 10^3</math></b>	1.45 (0.73-1.82)	2.3 (1.27-5)	<b>0.02</b>
<b>MMP3 (pg/mL)</b>	106 (61-179)	168 (103-706)	<b>0.03</b>
<b>MMP7 (pg/mL) <math>\times 10^3</math></b>	16.1 (6.83-29)	32.8 (16.66-	<b>0.01</b>
<b>MMP8 (pg/mL) <math>\times 10^3</math></b>	113 (61-317)	234 (109-822)	<b>0.03</b>
<b>MMP9 (pg/mL) <math>\times 10^3</math></b>	203 (105-588)	345 (153-918)	0.08
<b>MMP13 (pg/mL)</b>	13 (0-89)	24 (0-176)	0.52
<b>MMP12 (pg/mL)</b>	162 (78-195)	202 (131-394)	0.06
<b>TNF-<math>\alpha</math>- (pg/mL)</b>	0.9 (0.34-5.7)	6.6 (1.7-46)	<b>0.002</b>
<b>TNF1A (pg/mL) <math>\times 10^3</math></b>	0.83 (0.51-1.22)	1.28 (0.6-2.77)	0.07
<b>TNF1B (pg/mL)</b>	223 (124-523)	403 (205-964)	<b>0.04</b>
<b>VEGFA (pg/mL) <math>\times 10^3</math></b>	1.26 (0.61-1.85)	1.26 (0.93-2.15)	0.49

Data presented as median (inter-quartile range) unless stated.

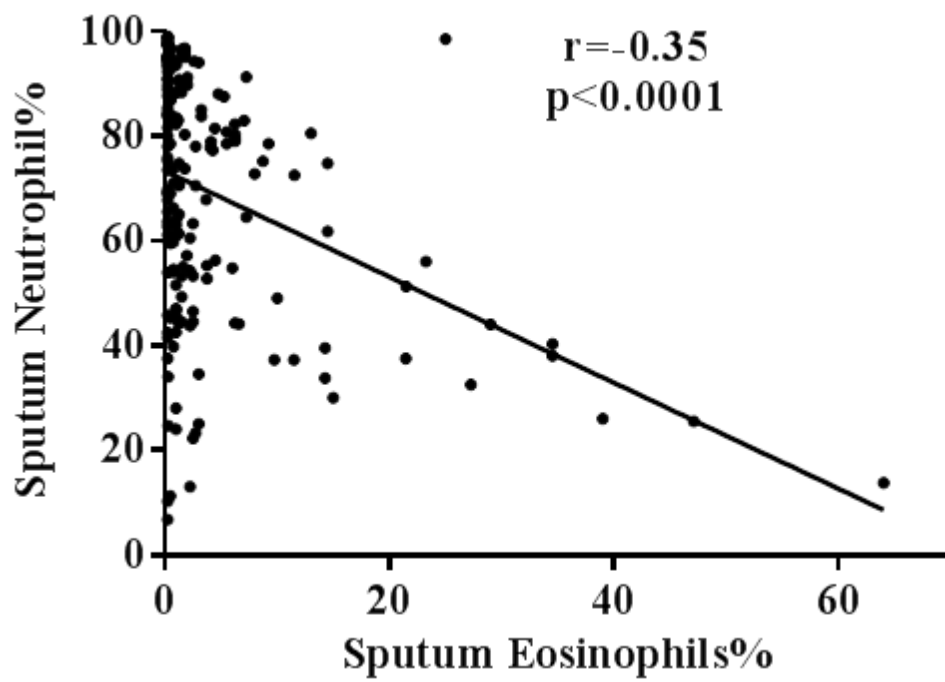
**Table 3-4 Serum biomarkers in the two groups at baseline**

	<b>Eosinophilic (n= 51)</b>	<b>Non-eosinophilic (n= 145)</b>	<b>p value</b>
<b>CCL2 (pg/mL)*</b>	642 (56)	695 (29)	0.36
<b>CCL3 (pg/mL)</b>	22 (18-30)	20 (14-30)	0.33
<b>CCL4 (pg/mL)</b>	439 (339-536)	425 (319-544)	0.62
<b>CCL13 (pg/mL)</b>	661 (388-953)	715 (580-931)	0.56
<b>CCL17 (pg/mL)</b>	403 (250-954)	429.830 (331-	0.71
<b>CCL26 (pg/mL)</b>	11 (6.9-14)	11 (7.8-14)	0.93
<b>CK-MB (ng/mL)</b>	4.4 (2.8-5)	4.1 (3.1-6.6)	0.58
<b>CXCL10 (pg/mL)</b>	53 (35-66)	56 (40-73)	0.40
<b>CXCL11 (pg/mL)</b>	60 (39-85)	59 (45-83)	0.63
<b>CSF2 (pg/mL)</b>	0.17 (0.05-0.52)	0.25 (0.02-1.18)	0.55
<b>EPO (mIU/mL)</b>	4.9 (2.1-6.9)	5.1 (3.2-6.8)	0.58
<b>IGFBP1 (pg/mL) x10<sup>3</sup></b>	1.2 (0.5-1.9)	2.1 (0.5-4.6)	0.18
<b>IL1β (pg/mL)</b>	7.5 (5.9-10)	7.0 (5.4-8.4)	0.28
<b>IL4 (pg/mL)</b>	0.05 (0-0.12)	0.03 (0-0.2)	0.80
<b>IL5 (pg/mL)</b>	2.3 (1.4-3.4)	2.3 (1.8-3)	1.0
<b>IL6 (pg/mL)</b>	10 (8.3-17)	14 (9.9-22)	0.06
<b>IL8 (pg/mL)</b>	6.3 (4.4-7.9)	5.7 (4.6-8.7)	0.69
<b>IL10 (pg/mL)</b>	0.54 (0.38-1.4)	1.1 (0.42-2.5)	0.09
<b>IL13 (pg/mL)</b>	26 (18-40)	29 (20-40)	0.75
<b>IL17 A (pg/mL)</b>	0.005 (0-0.08)	0 (0-0.06)	0.87
<b>IFN γ (pg/mL)</b>	0.11 (0.1-0.16)	0.15 (0.08-0.35)	0.41
<b>MMP1 (pg/mL) x10<sup>3</sup></b>	3.3 (2.5-534.9)	3.3 (1.7-5.4)	0.70
<b>MMP2 (pg/mL) x10<sup>3</sup></b>	151 (108-168)	131 (113-160)	0.62
<b>MMP3 (pg/mL) x10<sup>3</sup></b>	9.6 (5.8-12.5)	10.5 (6.9-15.3)	0.30
<b>MMP7 (pg/mL)</b>	827 (564-1373)	864 (628.4-1152)	0.87
<b>MMP8 (pg/mL) x10<sup>3</sup></b>	7.0 (4.9-8.5)	7.3 (4.7-12.8)	0.21
<b>MMP9 (pg/mL) x10<sup>3</sup></b>	214 (172-275)	247 (183-358)	0.09
<b>MMP12 (pg/mL)</b>	0 (0-0)	0 (0-0)	0.83
<b>MMP13 (pg/mL)</b>	297 (64-377)	306 (0-359)	0.72
<b>Myoglobin (ng/mL)</b>	6.1 (5.2-6.4)	6.3 (5.1-7.7)	0.41
<b>TNF-α (pg/mL)</b>	3.4 (2.6-4)	3.3 (2.8-4.6)	0.38
<b>TNF1A (pg/mL) x10<sup>3</sup></b>	7.8 (6.6-9.9)	8.0 (6.7-10.9)	0.55
<b>TNF1B (pg/mL) x10<sup>3</sup></b>	4.8 (4.2-5.7)	5.2 (4.1-6.3)	0.56
<b>Troponin-I (ng/mL)</b>	0.12 (0.10-0.13)	0.12 (0.09-0.14)	0.73
<b>SAA1 (ng/mL) x10<sup>3</sup></b>	3.7 (2.1-8.2)	4.8 (2.0-10.7)	0.61
<b>sICAM1 (ng/mL)</b>	211 (165-247)	221 (168-272)	0.44
<b>sVCAM1 (ng/mL)</b>	393 (310-512)	356 (301-471)	0.38
<b>VEGFA (pg/mL)</b>	666 (464-1029)	756 (574-1043)	0.39

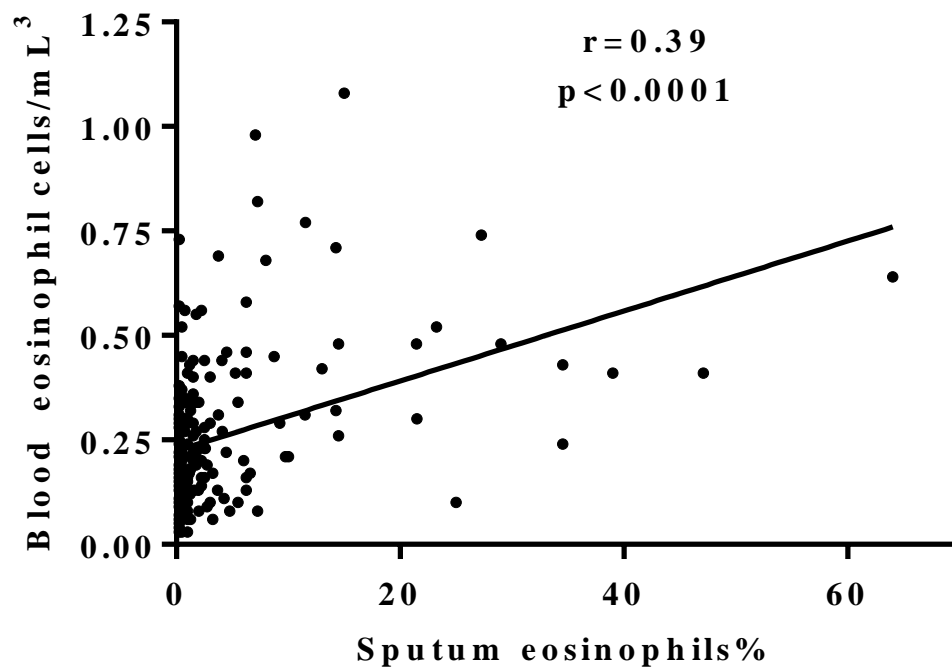
Data presented as median (inter-quartile range) unless stated, \*mean (standard error of mean).

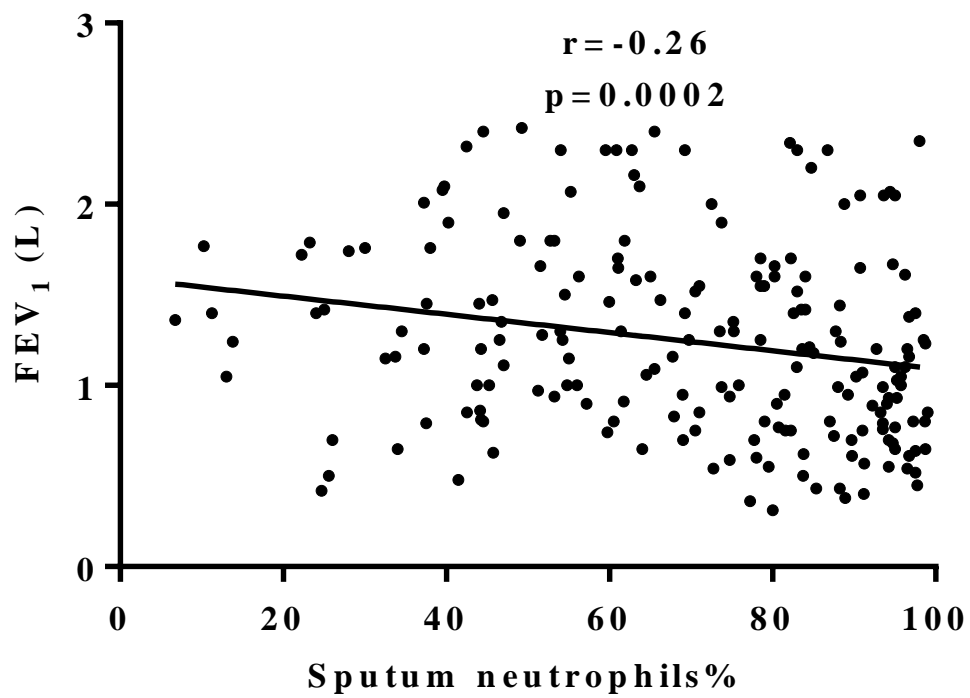


In the whole cohort, there was a strong inverse correlation between sputum eosinophils% and sputum neutrophils% (Spearman  $r=-0.35$ ;  $p<0.0001$ ), (Figure 3-1). Moreover, blood and sputum eosinophils were significantly correlated (Spearman  $r=0.40$ ;  $p<0.0001$ ), (Figure 3-2). Serum ECP was also positively correlated with blood (Spearman  $r=0.57$ ;  $p<0.0001$ ), but not sputum eosinophils. There was a weak inverse association between sputum eosinophils count and CRP (Spearman  $r=-0.18$ ;  $p=0.001$ ), log sputum CFU (Spearman  $r=-0.25$ ;  $p=0.0007$ ) and VAS cough (Spearman  $r=-0.17$ ;  $p=0.016$ ). No correlation existed between sputum eosinophils count and FEV<sub>1</sub>, FEV<sub>1</sub>% predicted or FEV<sub>1</sub>/FVC%. Likewise, no correlation found between sputum eosinophils and pack-years of smoking, despite the presence of a weak association between the serum ECP and duration of smoking (Spearman  $r=0.19$ ;  $p=0.03$ ), but not with the pack-years of smoking. On the other hand sputum neutrophils% correlated positively with blood neutrophils (Spearman  $r=0.23$ ;  $p=0.0012$ ), serum CRP (Spearman  $r=0.22$ ;  $p=0.0019$ ) and log CFU (Spearman  $r=0.25$ ;  $p=0.0006$ ), but not with VAS cough. In addition, there was an inverse relationship between sputum neutrophils count and FEV<sub>1</sub> (Spearman  $r=-0.26$ ;  $p=0.0002$ ), (Figure 3-3) and to a lesser degree FEV<sub>1</sub>/FVC% (Pearson  $r=-0.16$ ;  $p=0.027$ ), (Figure 3-4), but not with pack-years of smoking,

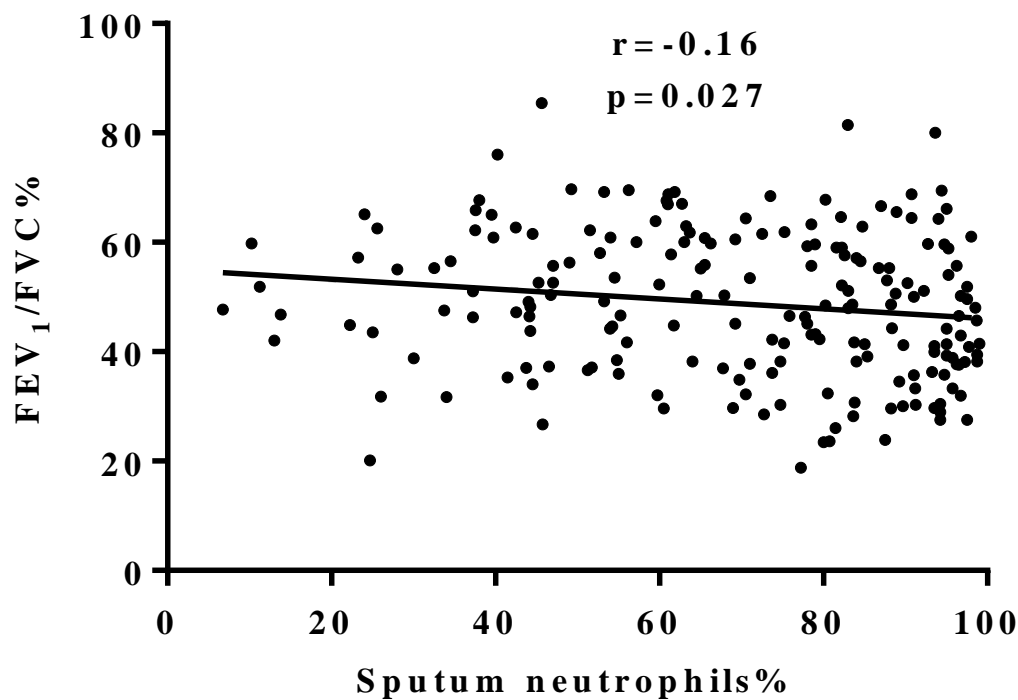


**Figure 3-1** Scatter-plot demonstrating the negative correlation between the sputum eosinophils and neutrophils count.





**Figure 3-3** Scatter-plot demonstrating the inverse correlation between the sputum neutrophils% and FEV<sub>1</sub>.



**Figure 3-4 Scatter-plot illustrating the inverse correlation between the sputum neutrophils% and FEV<sub>1</sub>/FVC%.**

Table 3-5 describes the clinical characteristics of eosinophilic and non-eosinophilic subjects during the first exacerbation in the study. No great differences in the numbers of exacerbations (median number [IQR]), 0 [0-2] in the eosinophilic, versus 1 [0-2] in the non-eosinophilic group; ( $p=0.52$ )) and percentage of subjects who had exacerbations between the two groups (number [%] 31 [61%] in the eosinophilic, versus 106 [73%] in the non-eosinophilic group; ( $p=0.10$ )). However, there was an increase in the use of ICS by the non-eosinophilic subjects during exacerbations, compared to eosinophilics (n [%], 22 [20.8], versus 1 [3.2]; Chi-squared  $p=0.02$ ), although no differences in the use of oral corticosteroids and antibiotics and the length of the treatment between the two groups. There were also no differences in the sputum or blood differential cell count apart from eosinophils. Chronic

respiratory health Questionnaire (CRQ) score was again worse in the non-eosinophils, against eosinophilic subjects (median [IQR], 1.8 [1.3-2.7] units, versus 2.8 [2.2-3.3];  $p=0.0002$ ). However, other health status scores and delta change of health (data are not shown) were not significantly different. Unlike the baseline results, the bacterial colonisation of sputum at exacerbation was not statistically different between the 2 groups at exacerbation (n. [%], 38 [40%] in non-eosinophilic, versus 9 [31%] in eosinophilic patients; (Chi-squared  $p=0.41$ )), although the bacterial load was still greater in the neutrophilic (log of geometric mean CFU [95% confidence interval] 6.1 [5.6-6.6]), compared to eosinophilic arm (5.6 [4.9-6.4]); ( $p=0.02$ ). However, the serum CRP was not statistically different, although the median was almost twice in the non-eosinophilic 10 (5-28) versus eosinophilic 5.5 (5-32); ( $p=0.47$ ).

FEV<sub>1</sub> and percentage of predicted FEV<sub>1</sub> were significantly lower in the eosinophilic group, (median FEV<sub>1</sub> [IQR]) 0.84 L [0.59-1]), compared to (1 L [0.74-1.4]) the non-eosinophils ( $p=0.008$ ), (mean of FEV<sub>1</sub>% predicted [SEM] 35% [24-48]) in the eosinophils, compared to (41% [29-58]) in the non-eosinophils;  $p=0.04$ ), as was FVC (mean [SEM] 1.8 L [0.1] versus 2.3 L [0.1] respectively;  $p=0.001$ ), but the FEV<sub>1</sub>/FVC was the same. However, the absolute and percentage of FEV<sub>1</sub> change between stable and first exacerbation visits were similar between the two groups ( $\Delta$ FEV<sub>1</sub> median [IQR] -150 mL [-350 to -10]) for the eosinophilic, in comparison to -107 mL [-241 to -7.5] in the non-eosinophilic group, ( $p=0.35$ ) and mean (SEM) -14% [3.7] in the eosinophilic versus -10.9% [1.5] for the non-eosinophils; ( $p=0.35$ )) respectively, although there were significant falls of FEV<sub>1</sub> in both groups equally during exacerbations,  $p<0.05$  (Table 3-6).

**Table 3-5 Comparison of exacerbations between the two COPD groups.**

	<b>Eosinophilic (n=31)</b>	<b>Non-eosinophilic (n=106)</b>	<b>p value</b>
<b>Number of exacerbation(s) per year</b>	1 (0-2)	1 (0-2)	0.52
<b>ICS dose increase, n (%)</b>	1 (3.2)	22 (20.8)	<b>0.02</b>
<b>Use of oral corticosteroids treatment, n (%)</b>	19 (82.6)	50 (84.8)	0.81
<b>Length of oral corticosteroids course</b>	10 (9.3-14)	10 (7-12)	0.22
<b>Use of antibiotics, n (%)</b>	7 (77.8)	42 (85.7)	0.56
<b>Antibiotics course length</b>	7 (7-7)	7 (7-7)	0.73
<b>Hospitalisation due to COPD, n (%)</b>	2 (6.25)	21 (19.8)	0.07
<b>Sputum total cell count x10<sup>6</sup>/g</b>	6.8 (2.7-17)	6.8 (1.8-18)	0.87
<b>Sputum neutrophils (%)</b>	84 (70-95)	86 (66-95)	0.70
<b>Sputum macrophages (%)</b>	6.8 (2.9-18)	11 (2.8-25)	0.70
<b>Sputum eosinophils (%)</b>	2.3 (0.88-12)	0.25 (0.25-1)	<b>&lt;0.0001</b>
<b>Peripheral blood total cell count (x10<sup>9</sup>/L)</b>	9.1 (7.8-11)	9.1 (7.8-11)	0.85
<b>Peripheral blood neutrophils cell count (x10<sup>9</sup>/L)</b>	5.6 (4.8-7.3)	6.1 (4.9-8.1)	0.52
<b>Peripheral blood eosinophils cell count (x10<sup>9</sup>/L)</b>	0.25 (0.19-0.48)	0.17 (0.11-0.28)	<b>0.0045</b>
<b>Peripheral monocytes cell count (x10<sup>9</sup>/L)</b>	0.6 (0.5-0.73)	0.6 (0.5-0.7)	0.59
<b>Peripheral blood basophils cell count (x10<sup>9</sup>/L)</b>	0.05 (0.03-0.07)	0.04 (0.02-0.05)	0.08
<b>CRP (mg%)</b>	5.5 (5-32)	10 (5-28)	0.47
<b>FEV<sub>1</sub> post bronchodilator (L)</b>	0.84 (0.59-1)	1 (0.74-1.4)	<b>0.008</b>
<b>FEV<sub>1</sub>% predicted, (%)</b>	35 (24-48)	41 (29-58)	<b>0.04</b>
<b>Post FEV<sub>1</sub>/FVC (%)*</b>	55 (39-63)	50 (39-63)	0.75
<b>Delta FEV<sub>1</sub> (mL) §</b>	-150	-106.7	0.43
<b>Delta FEV<sub>1</sub> % § *</b>	-14.28 (3.6)	-10.87 (1.5)	0.32
<b>GOLD 1, n (%)</b>	1 (3)	5 (5)	0.68
<b>GOLD 2, n (%)</b>	4 (13)	31 (31)	0.05
<b>GOLD 3, n (%)</b>	12 (39)	37 (38)	0.91
<b>GOLD 4, n (%)</b>	14 (45)	27 (27)	0.05
<b>Sputum colonisation for bacteria, n (%)</b>	9 (31.03)	38 (39.6)	0.41
<b>CFU x10<sup>6</sup>/mL**</b>	5.6 (4.9-6.4)	6.1 (5.6-6.6)	<b>0.02</b>
<b>16s (gene copies x10<sup>6</sup>/mL)**</b>	8.0 (7.7-8.3)	8.4 (8.2-8.7)	0.05
<b>qPCR <i>Haemophilus influenza</i>**</b>	15 (53.56)	46 (56.10)	0.96
<b>qPCR <i>Staphylococcus aureus</i>**</b>	1 (03.57)	5 (06.10)	0.6
<b>qPCR <i>Streptococcus pneumonia</i>**</b>	12 (42.86)	37 (45.12)	0.84
<b>qPCR <i>Morexella catarrhalis</i>**</b>	9 (32.14)	33 (40.24)	0.45
<b>mCRQ total score (unit)</b>	2.8 (2.2-3.3)	1.8 (1.3-2.7)	<b>0.0002</b>
<b>VAS Cough (mm)</b>	68 (39-90)	65 (44-74)	0.36
<b>VAS Dyspnoea (mm)</b>	78 (61-95)	73 (57-83)	0.07
<b>VAS Sputum Production (mm)</b>	52 (24-83)	56 (37-73)	0.75
<b>VAS Sputum Purulence (mm)</b>	47 (21-76)	52 (30-70)	0.91

Data presented as median (ranges) unless stated, \*mean (SEM), \*\*Geometric mean of Log (95% CI). SEM: standard error of mean, CI: confidence interval. § Delta represents the difference between the 1<sup>st</sup> exacerbation and means of previous FEV<sub>1</sub>. **Abbreviations:** CFU: colony forming unit; **mCRQ:** Modified chronic respiratory health Questionnaire; **CRP:** C-Reactive Protein; **FEV<sub>1</sub>:** Forced expiratory volume in 1 second; **FVC:** Forced vital capacity; **GOLD:** Global Initiative for chronic Obstructive Lung disease; **ICS:** inhaled corticosteroids; **VAS:** Visual analogue score; **TCC:** total cell count.

**Table 3-6 Comparison of FEV<sub>1</sub> between stable and first exacerbation visit in the 2 groups**

Group	Stable FEV <sub>1</sub> (mL)	First exacerbation FEV <sub>1</sub> (mL)	P value
Eosinophilic COPD	0.90 (0.79-1.4)	0.78 (0.55-1.1)	<b>0.0002</b>
Non-eosinophilic COPD	1.21 (0.86-1.61)	1.03 (0.75-1.46)	<b>&lt;0.0001</b>

Data presented as median (IQR).

### 3.1.5 Discussion

In this chapter, I have shown the characteristics of the eosinophilic COPD phenotype and the difference between it and the non-eosinophilic (neutrophilic) subgroup. As expected, eosinophilic subjects represent 26% of the studied cohort in agreement with the previous reports (Brightling 2006, Saha, Brightling 2006), including what is noted in older population (McDonald, Simpson *et al.* 2011), although there was some variabilities between various studies, that might be partly due to the inclusion criteria used (Balzano, Stefanelli *et al.* 1999), especially the different cut-off levels of sputum eosinophil%. In the current study, although the eosinophilic COPD subjects were not different from non-eosinophilics with regard to clinical features and lung function (Brightling 2006), but in line with a previous studies (Gao, Zhang *et al.* 2013), they have distinct features with reduced sputum bacterial colonisation, decreased markers of bacterial infection and lower neutrophilic airways inflammation, in contrast to non-eosinophilic group, in which neutrophilic inflammation with bacterial colonisation, as well as symptoms of infection predominate. These findings were supported by the negative association between the sputum eosinophils and CRP, CFU and VAS cough, albeit weak, and also the positive correlation between the sputum neutrophils and CRP and CFU in the whole cohort. Interestingly, it was observed that *staphylococcus aureus* was significantly higher in the non-eosinophilic group, versus the eosinophilic and in



comparison to other bacterial species studied. However, this observation needs to be investigated and validated further in the future.

Importantly, this study demonstrated that sputum and blood eosinophils are strongly correlated, which confirms the previous results from the same cohort that baseline blood eosinophil count is the best predictor of eosinophilic exacerbations (Bafadhel, McKenna *et al.* 2011, Bafadhel, McKenna *et al.* 2012). However, contrary to what Balzano's group (Balzano, Stefanelli *et al.* 1999) described before as a positive relationship between sputum eosinophils and neutrophils, the present study has shown that they are negatively correlated. Moreover, unlike prior studies in COPD (Balzano, Stefanelli *et al.* 1999) and asthma (Ronchi, Piragino *et al.* 1996), there was no association between sputum eosinophils and FEV<sub>1</sub> or FEV<sub>1</sub>/FVC% (Rutgers, Postma *et al.* 2000) in this study despite the inverse correlation between sputum neutrophils% with both FEV<sub>1</sub> and FEV<sub>1</sub>/FVC%, in agreement with previous results (Stanescu, Sanna *et al.* 1996, Balzano, Stefanelli *et al.* 1999, Thompson, Daughton *et al.* 1989). Furthermore, although some investigators reported positive association between sputum eosinophils and pack-years of smoking, (Dippolito, Foresi *et al.* 2001), whilst others showed negative relationship (Telenga, Kerstjens *et al.* 2013, Thomsen, Ingebrigtsen *et al.* 2013) and a third exhibited none (Westergaard, Porsbjerg *et al.* 2014), there was no relationship observed between them in the current study. These discrepancies between the findings in the instant research and past studies need to be investigated further in the future.

Interestingly, despite the FEV<sub>1</sub> and FEV<sub>1</sub>% predicted were marginally lower in the eosinophilic, in comparison to the non-eosinophilic subjects at exacerbation, the delta fall in FEV<sub>1</sub> from stable to exacerbation was not statistically significant between the two groups. Another surprising observation is the lack of statistical significant differences of sputum

bacterial colonisation, CRP and symptoms suggestive of infection between the eosinophilic and non-eosinophilic groups during exacerbation event, despite the increased bacterial load in the non-eosinophilic subjects compared to eosinophilic group. It is difficult to explain this result, but it could be that the routine bacterial culture was not sensitive enough to include all bacterial colonisation, compared to the bacterial load especially with the lack of a firm definition of exacerbation. These findings need to be interpreted with caution and investigated further using larger studies in the future to assess whether they were consistent at all the exacerbations or only at the first exacerbation event that was studied here.

There are two potential limitations of this study. Firstly, the study was retrospective. Although this might be a weakness, it could also be considered a strength to utilise information from two clinical studies of COPD subjects, extensively investigated in stable state and at exacerbations, to generate novel findings. Second obstacle is that the comparisons were made between one stable and one exacerbation visits and was not replicated in all the other visits. However, this is because a lot of subjects in the study had one exacerbation and some have none at all, in addition to lack of produced sputum in several occasions which made it difficult to measure repeatability of the measures accurately. This also needs to be addressed in larger prospective studies in the future.

### **3.1.6 Conclusion**

In summary, the main distinguishable feature between the eosinophilic and non-eosinophilic phenotype is the reduced bacterial colonisation in the former. Blood eosinophil count is a potential surrogate biomarker of sputum eosinophil. There is no correlation between eosinophils and the extent of outflow obstruction or pack-years of smoking in COPD.

## **Chapter 4 Is the eosinophilic COPD a stable phenotype?**

## 4.1 Stability of sputum eosinophilia

### 4.1.1 Abstract

**Background:** Eosinophilic airways inflammation plays an important role in COPD. There are controversial data on the reproducibility of sputum eosinophils in COPD and asthma and most of the available studies were focusing on short term repeatability.

**Methods:** Sputum eosinophils measurements from 123 COPD subjects were analysed on 4 individual visits, 3 months apart. Repeatability was measured using intraclass correlation coefficient and expressed as Cronbach's alpha reliability coefficient (Ri).

**Results:** The reproducibility of sputum eosinophils count between the first 2 stable visits was (0.60) ( $p < 0.001$ ) and was even higher when adding more visits (0.79 and 0.81) respectively. The mean of the log differences of sputum eosinophils measures was nearly zero and the majority of the measurements lie between 2 standard deviation (SD) of the log differences in most visits.

**Conclusion:** Sputum eosinophil count is stable over time in COPD.

### 4.1.2 Introduction

It has been recognised that eosinophilic inflammation is consistently noted in 10-30% of COPD population. In the previous chapter, I have shown that eosinophilic COPD phenotype is a unique phenotype with its own clinical characteristics. However, little information was found about the stability of this phenotype. Despite the presence of several studies with controversial results in asthma (In 't Veen, de Gouw *et al.* 1996, Pizzichini, Pizzichini *et al.* 1996, Spanevello, Migliori *et al.* 1997, Reining, Mattes *et al.* 2000, Fahy, Boushey *et al.* 2001, Bacci, Cianchetti *et al.* 2002, Van Veen, Ten Brinke *et al.* 2009, Al-Samri, Benedetti *et al.* 2010, Sohani, Strinich *et al.* 2011, McGrath, Icitovic *et al.* 2012), but only few authors

have explored the repeatability of the this phenotype in COPD (Beeh, Beier *et al.* 2003, Brightling, Monterio *et al.* 2001).

#### **4.1.3 Method**

Clinical data for 196 COPD subjects that had participated in two longitudinal studies of COPD exacerbations (Bafadhel, McKenna *et al.* 2011, Bafadhel, McKenna *et al.* 2012) for at least one year were available. Sputum eosinophils measurements of one hundred and twenty three subjects that met the inclusion criteria of having  $\geq 2$  stable visits were analysed for reproducibility. Repeatability was initially calculated between the first two visits with 3 months intervals and then further visits were subsequently added until 4 visits. The repeatability of data was measured using intraclass correlation coefficient and expressed as Cronbach's alpha reliability coefficient (Ri). It was also expressed as proposed by Bland and Altman model (Bland, Altman 1986). GraphPad Prism version 6 (GraphPad, San Diego) and IBM SPSS version 20 (SPSS, Inc. Chicago) were used to perform statistical analysis. Mean (standard error of the mean [SEM]) was used to present parametric data, whilst median (interquartile ranges [IQR]) was used for non-parametric data and geometric mean (95% confidence interval) for data that was log normally distributed. Comparison between groups was performed using unpaired T-test or Mann-Whitney for parametric or non- parametric data respectively. Spearman or Pearson rank correlation coefficient was used to assess the correlations for parametric and non-parametric data respectively. A p value less than 0.05 was considered statistically significant.

#### 4.1.4 Results

Reproducibility of sputum eosinophils count between the first 2 stable visits was high (0.60) ( $p < 0.001$ ) and was even greater when adding more visits, despite the reduction of number of subjects after each subsequent visits since patients were not attending all the visits (Table 4-1 and Figure 4-1) shows a very good reproducibility of sputum eosinophils% between the first two visits (V2 and V1) for the 123 subjects as the mean of the log differences (0.0045) was almost zero and the majority of the measurements 119/123 (96.7%) lie between 2 standard deviation (SD) ( $\pm 1.33$ ) of the log differences.

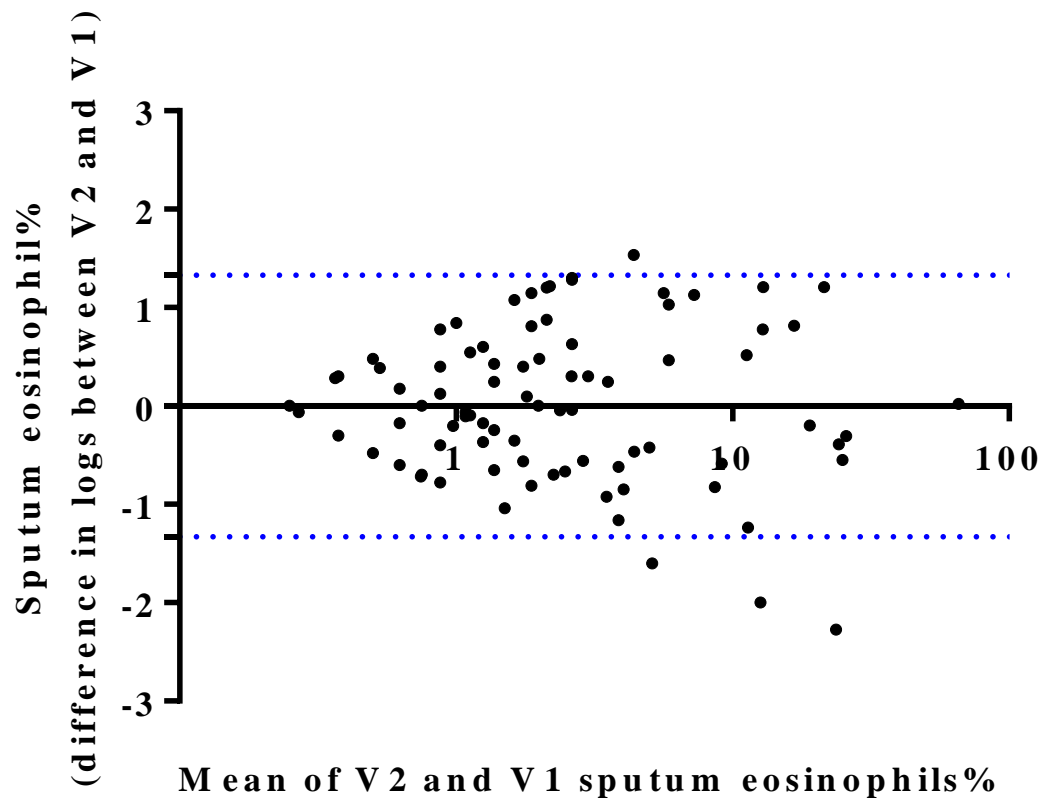
**Table 4-1 Repeatability of sputum eosinophils% between all the stable visits**

Number of included visits	Number of subjects	Ri	p-value
2 (visit 1 and 2)	123	0.60	<0.001
3 (visit 1, 2, and 3)	88	0.79	<0.001
4 (visit 1, 2, 3 and 4)	48	0.81	<0.001
2 (visit 1 and 3)	113	0.70	<0.001
2 (visit 1 and 4)	63	0.58	<0.001
2 (visit 2 and 3)	90	0.72	0.001
2 (visit 2 and 4)	63	0.71	<0.001
2 (visit 3 and 4)	57	0.61	<0.001

**Ri:** intraclass correlation coefficient between logged data (cronbach alpha).

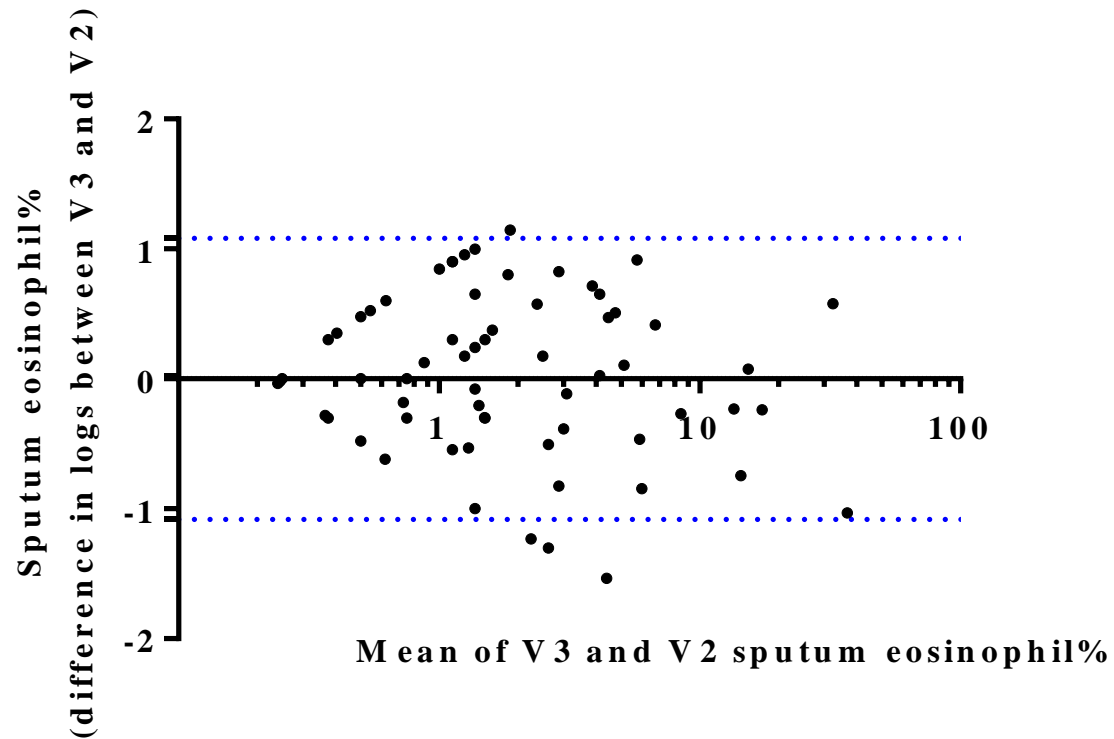
Likewise, most of the sputum eosinophils% measurements 87/90 (96.7%) between visit 3 and 2 (V3 and V2) were between the 2 standard deviation ( $\pm 1.1$ ) of the log differences with a mean of the log differences of 0.019 (Figure 4-2). Similarly, when the readings between the third and first visits (V3 and V1) were compared, 109 out of 113 subjects (95.6%) had their measurements between 2 standard deviation ( $\pm 1.2$ ) of the log differences with a mean of the log differences of 0.016 (Figure 4-3). In the same way, using the third and first visits (V4 and

V1), 60/63 (95%) of the subjects measurements fall between 2 standard deviation ( $\pm 1.3$ ) of the log differences, with a mean of the log differences of 0.03 (Figure 4-4).



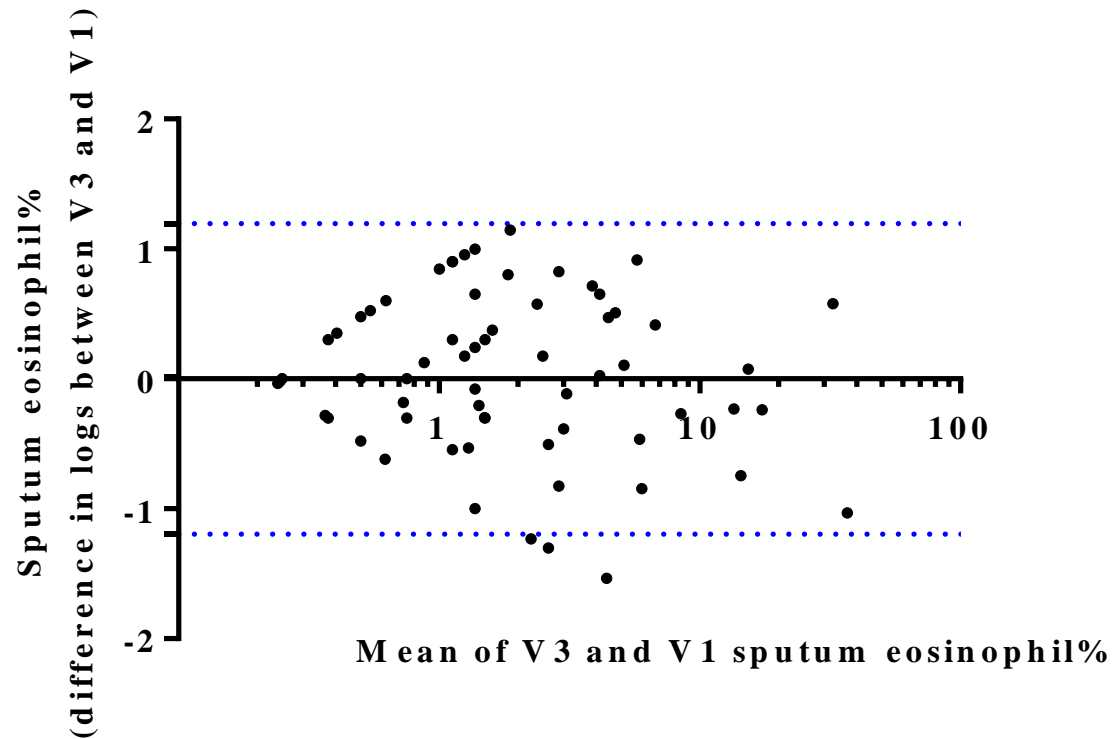
**Figure 4-1 Repeatability of sputum eosinophils between the first 2 stable visits.**

Repeatability is presented as suggested by Bland and Altman (Bland, Altman 1986). It is expected that 95% of the measures are less than 2 SD. “Y axis” represents the differences in logs between the V2 (the 2<sup>nd</sup> visit) and V1 (the 1<sup>st</sup> visit), whilst means of the 2 visits are plotted in the “X axis”. The solid line represents mean of the log differences (0.0045). The upper and lower scattered blue lines are the  $2 \pm \text{SD}$  of the means of log differences ( $\pm 1.33$ ).



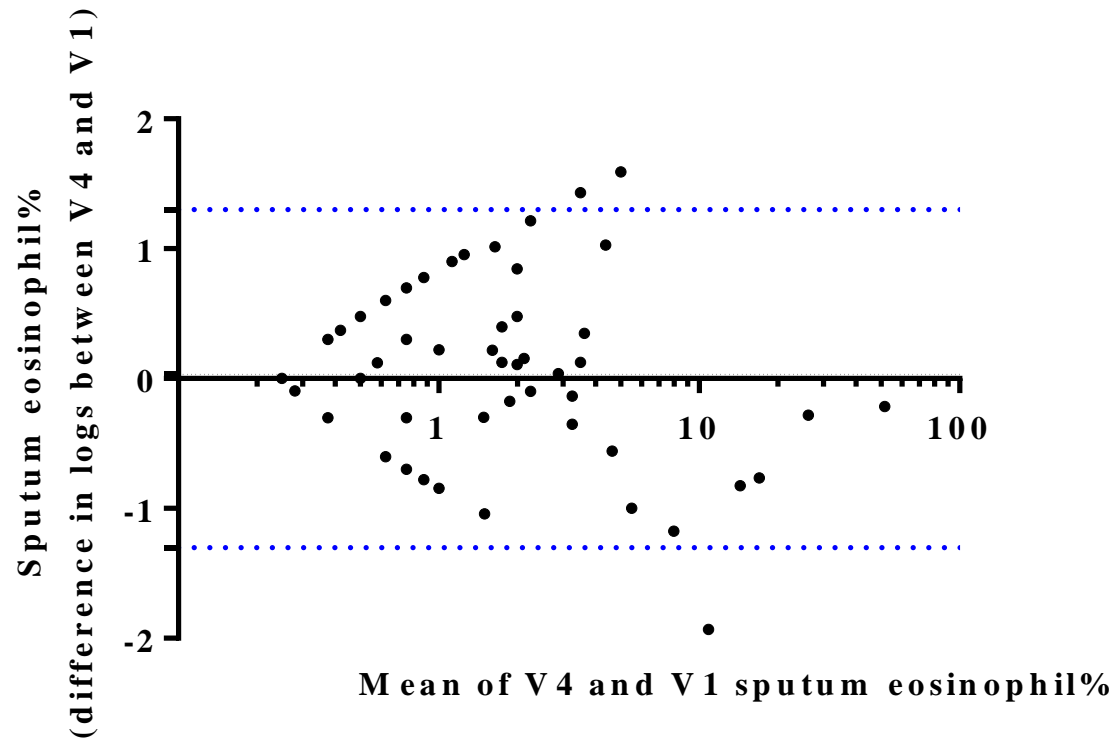
**Figure 4-2 Repeatability of sputum eosinophils between the second and third stable visits.** Repeatability is presented as suggested by Bland and Altman (Bland, Altman 1986). It is expected that 95% of the measures are less than 2 SD. “Y axis” represents the differences in logs between the V3 (the 3rd visit) and V2 (the 2nd visit), whilst means of the 2 visits are plotted in the “X axis”. The solid line represents mean of the log differences (0.019). The upper and lower scattered blue lines are the  $2 \pm \text{SD}$  of the means of log differences ( $\pm 1.1$ ).





**Figure 4-3 Repeatability of sputum eosinophils between the first and third stable visits.**

Repeatability is presented as suggested by Bland and Altman (Bland, Altman 1986). It is expected that 95% of the measures are less than 2 SD. “Y axis” represents the differences in logs between the V3 (the 3rd visit) and V1 (the 1st visit), whilst means of the 2 visits are plotted in the “X axis”. The solid line represents mean of the log differences (0.016). The upper and lower scattered blue lines are the  $2 \pm \text{SD}$  of the means of log differences ( $\pm 1.2$ ).



**Figure 4-4 Repeatability of sputum eosinophils between the first and forth stable visits**

Repeatability is presented as suggested by Bland and Altman (Bland, Altman 1986). It is expected that 95% of the measures are less than 2 SD. “Y axis” represents the differences in logs between the V4 (the 4th visit) and V1 (the 1st visit), whilst means of the 2 visits are plotted in the “X axis”. The solid line represents mean of the log differences (0.03). The upper and lower scattered blue lines are the  $2 \pm \text{SD}$  of the means of log differences ( $\pm 1.3$ ).

Table 4-2 summarises the average measurements of sputum eosinophils counts for all subjects in different visits. There were no significant statistical differences between the means of measurements in each two individual visits.

**Table 4-2 Repeatability of sputum eosinophilia between the stable visits**

	Sputum eosinophils%	Subjects numbers	p-value
<b>Visit 1</b>	1.0 (0.25-2.5)	123	0.94
<b>Visit 2</b>	0.75 (0.25-2.8)		
<b>Visit 2</b>	0.75 (0.25-2.1)	90	0.76
<b>Visit 3</b>	1.0 (0.25-2.5)		
<b>Visit 1</b>	1.0 (0.25-2.7)	112	0.85
<b>Visit 3</b>	1.0 (0.25-3.7)		
<b>Visit 1</b>	0.75 (0.25-2.25)	63	0.86
<b>Visit 4</b>	1.0 (0.25-2.5)		

Data presented as median (IQR) unless stated. The average time between 2 subsequent visits is 3 months.

#### **4.1.5 Discussion**

This is the largest study up to date to evaluate the sputum eosinophils stability in COPD. The reproducibility of measures between two first visits in this study was good (0.60), which corroborates the findings from previous work in adults with COPD (Brightling, Monterio *et al.* 2001, Beeh, Beier *et al.* 2003), asthma (Pin, Gibson *et al.* 1992, Pizzichini, Pizzichini *et al.* 1996, Spanevello, Migliori *et al.* 1997, Fahy, Boushey *et al.* 2001, Bacci, Cianchetti *et al.* 2002, Van Veen, Ten Brinke *et al.* 2009, Simpson, McElduff *et al.* 2010, McGrath, Icitovic *et al.* 2012) and to a lesser extent children with asthma (Reining, Mattes *et al.* 2000). Chinn suggested intraclass correlation coefficient (Ri) value of 0.60 to be used as the minimal acceptable level of repeatability (Chinn 1991), despite values of 0.54 (Brightling, Monterio *et al.* 2001) and 0.47 (Beeh, Beier *et al.* 2003) were still considered acceptable or high by other investigators.

The repeatability was still great and even higher when 3 or 4 visits were included in the statistical analysis. In addition, after applying the Bland and Altman model (Bland, Altman 1986), the current observations were similar to previously published reports (Beeh, Beier *et al.* 2003, Brightling, Monterio *et al.* 2001, Pizzichini, Pizzichini *et al.* 1996), as majority of the readings were between 2 standard deviation of the log difference, despite lower intraclass correlation coefficient in this study compared to Pizzichini and colleagues (0.94) (Pizzichini *et al.* 1996) and In 't Veen *et al.* (0.84) (In 't Veen, de Gouw *et al.* 1996) studies. On the other hand, very few articles demonstrated that the stability of eosinophils is variable in adult (Al-Samri, Benedetti *et al.* 2010, Kupczyk, Dahlen *et al.* 2014) and children subjects with asthma (Fleming, Tsartsali *et al.* 2012).

One potential criticism here is that the study was not extended to measure repeatability over a longer period of time and the number of subjects in different visits is variable. However, with the exception of three studies (Van Veen, Ten Brinke *et al.* 2009, McGrath, Icitovic *et al.* 2012, Kupczyk, Dahlen *et al.* 2014) in asthma, the results of all the other previous articles were intended to measure the short-term repeatability within days or weeks, whilst in the present study it was calculated over longer period of time (one year) which is probably sufficient and makes these findings robust. Furthermore, the number of subjects used in this study (123 for 2 visits, 90 for 3 visits and 48 for 4 visits) was relatively high compared to those used in the previous studies (34, 21, 39, 61, 29, 12, 26 and 11) (Pin, Gibson *et al.* 1992, In 't Veen, de Gouw *et al.* 1996, Pizzichini, Pizzichini *et al.* 1996, Brightling, Monterio *et al.* 2001, Bacci, Cianchetti *et al.* 2002, Beeh, Beier *et al.* 2003, Simpson, McElduff *et al.* 2010, Sohani, Strinich *et al.* 2011) respectively, which make it stronger as a valid evidence of reproducibility of sputum eosinophils in COPD. Nonetheless, it would be more robust if the study is replicated over a longer period of time using larger number of subjects.

#### **4.1.6 Conclusion**

Taken together and in accordance with most previous reports, sputum eosinophil count in COPD is stable biomarker over time, but further prospective and long term studies are needed to validate and replicate these findings.

**Chapter 5 Does the eosinophilic airway inflammation  
reflect tissue as well as sputum?**

## **5.1 Clinical cross-sectional description of the relationship between blood and bronchial submucosal eosinophilia and reticular basement membrane thickening in chronic obstructive pulmonary disease.**

### **5.1.1 Abstract**

**Background:** Eosinophilic airway inflammation, defined by a sputum eosinophilia, is observed in 10-30% of COPD subjects. The peripheral blood eosinophil count has been proposed as a surrogate biomarker of airway eosinophilic inflammation. Whether the peripheral blood eosinophil count is associated with increased eosinophils in the bronchial submucosa and features of airway remodelling is unclear.

**Method:** In 20 subjects with COPD and 21 controls, the number of eosinophils and reticular basement membrane thickening were assessed. The subjects were stratified into eosinophil<sup>high</sup> and eosinophil<sup>low</sup> determined by the group's median peripheral blood eosinophil percentage (2.3%).

**Results:** The submucosal eosinophil count and reticular basement membrane thickening was greatest in the eosinophil<sup>high</sup> COPD subjects. The peripheral blood eosinophil percentage was correlated with the bronchial eosinophil count ( $r=0.57$ ;  $p=0.009$ ) and the reticular basement thickness ( $r=0.59$ ;  $p=0.006$ ).

**Conclusion:** In COPD, peripheral blood eosinophil count is a good biomarker of an airway eosinophilia and remodelling.

### 5.1.2 Introduction

There is an increasing need to find non-invasive reliable biomarkers in asthma and COPD to identify different phenotypes and direct treatment. A sputum eosinophilia (>3%) is present in 10-30% of COPD subjects (Eltboli, Brightling 2013). This biomarker and the intensity of eosinophilic inflammation in the bronchial submucosa predict the response to corticosteroid therapy in COPD (Chanez, Vignola *et al.* 1997, Brightling, Monteiro *et al.* 2000, Eltboli, Brightling 2013). Alternative simpler biomarkers of eosinophilic inflammation are required. The peripheral blood eosinophil count and percentage are associated with a sputum eosinophilia (Bafadhel, McKenna *et al.* 2011, Eltboli, Brightling 2013) and can be successfully used to direct corticosteroid therapy in COPD (Bafadhel, McKenna *et al.* 2012). There are few reports with conflicting results about the relationship between sputum and bronchial tissue eosinophils in asthma (Grootendorst, Sont *et al.* 1997) and COPD (Maestrelli, Saetta *et al.* 1995, Rutgers, Timens *et al.* 2000). However, to date studies have not explored the relationship between peripheral blood eosinophil count and the degree of submucosal eosinophilia and remodelling in COPD. I hypothesised that the bronchial submucosal (lamina propria) eosinophil count and thickening of the reticular basement membrane and lamina reticularis (RBM) are related to the peripheral blood eosinophil percent as a surrogate biomarker of sputum eosinophilia in COPD. To test my hypothesis, I analysed large airway bronchial tissue samples from COPD subjects and controls to determine the association between blood and bronchial eosinophil counts and the airway remodelling.



### **5.1.3 Materials and Methods**

#### **5.1.3.1 Subjects and study design**

A single-centre observational study was undertaken at Glenfield Hospital, Leicester, UK. Subjects with or without COPD undergoing surgical lung resection for cancer or suspected cancer were recruited. COPD subjects (n=20) were all ex- or current smokers with spirometric evidence of airflow obstruction according to the GOLD criteria for COPD and those with a history of asthma were excluded. Control subjects (n=21) included those with and without a smoking history and with no airflow obstruction. All subjects gave written informed consent. The study was approved by the Leicestershire, Northamptonshire and Rutland local ethics committee.

Bronchial tissue was dissected from the lung resection material as described (section 2.7.1), embedded in glycol-methacrylate (GMA) and stored at -80 °C. 2 µm sections were stained with monoclonal antibodies directed to major basic protein (MBP) (#MON6008-1, Monosan; Newmarket Scientific, UK) and tryptase (#M7052, Dako, Ely, UK) for eosinophils and mast cells respectively and corresponding isotype controls (Dako) (Table 5-1). The number of eosinophils and mast cells were enumerated per mm<sup>2</sup> submucosa (lamina propria between the RBM and airway smooth muscle bundle) and the RBM was measured as the mean of 50 measurements at 20 µm intervals. All measurements were undertaken by an observer blinded to the clinical characteristics. The interobserver and intraobserver variability for measurements were low.

**Table 5-1 Primary antibodies and their isotype control**

Primary antibody	Clone	Working Concentration	Manufacturer	Isotype control	Manufacturer
Mast cell tryptase	AA1	0.1 µg/mL	Dako	Mouse Ig-G1	Dako
Major Basic Protein (MBP)	BMK-13	0.2 µg/mL	Monosan	Mouse Ig-G2	Dako

Ig: Immunoglobulin

### 5.1.3.2 Statistical analysis

Statistical analysis was performed using Prism version 6 (GraphPad, San Diego). In addition to comparisons between COPD and controls, the subjects were stratified into eosinophil<sup>high</sup> and eosinophil<sup>low</sup> determined by the group median peripheral blood eosinophil percentage (2.3%), as well as using the peripheral blood percentage cut-off of 2% (Bafadhel, McKenna *et al.* 2011, Eltboli, Brightling 2013) and an absolute peripheral blood eosinophil count of  $0.3 \times 10^9/L$ . Group comparisons were made using t-tests and Analysis of variance (ANOVA) or Mann-Whitney and Kruskal-Wallis for parametric and non-parametric analyses as appropriate and correlations by Pearson and Spearman rank tests respectively. A p value of <0.05 was considered statistically significant. Comparisons were made initially between COPD and healthy, then among the groups after combining all the subjects and categorising into eosinophil<sup>high</sup> and eosinophil<sup>low</sup> determined by the median peripheral blood eosinophil percentage (2.3%).

### 5.1.4 Results

The clinical characteristics of COPD and normal subjects are shown in Table 5-2. There were no differences in gender between those with COPD and without COPD. COPD subjects were older and had a greater pack-years smoking history and poorer lung function. The use of

corticosteroids was similar between the eosinophil<sup>low</sup> (3/10) and eosinophil<sup>high</sup> (4/10) COPD groups (Fisher's Exact Test p=1.0). In the COPD eosinophil<sup>high</sup> group, 7/10 (70%) of the subjects have no reversibility and in 3/10 (30%), post FEV<sub>1</sub> was not performed. In the Eosinophil<sup>low</sup> COPD, post FEV<sub>1</sub> was carried out in one subject out of 10, who has no reversibility, but was not tested in the other 9/10. There were no significant differences in the blood or submucosal eosinophil counts between COPD and controls, nor in submucosal mast cell counts, although the RBM thickness was increased in COPD (mean [SEM] 9.7  $\mu$ m [0.6], versus 7.1 [0.4]; p<0.001), (Table 5-2). The submucosal eosinophil count was increased in the COPD eosinophil<sup>high</sup> group, (mean [SEM] 3.6 [10.8], compared to 0 [0.6], 0 [2.1] and 0 [0.35] in COPD eosinophil<sup>low</sup>, control eosinophil<sup>high</sup> and control eosinophil<sup>low</sup> groups respectively (Kruskal-Wallis p=0.009), (Table 5-3 and Figure 5-1). RBM thickness was also significantly increased in the COPD eosinophil<sup>high</sup> group, (mean [SEM] 10.5 [0.9]), compared to 8.9 [0.7], 7.9 [0.5] and 6.2 [0.5] in COPD eosinophil<sup>low</sup>, control eosinophil<sup>high</sup> and control eosinophil<sup>low</sup> groups respectively; (ANOVA p=0.0007), (Table 5-3 and Figure 5-1).

Similar findings were observed with a cut-off of peripheral blood eosinophils percent >2% and absolute count >0.3 x10<sup>9</sup>/L (data are not shown). Examples of bronchial tissues with thickened RBM and increased submucosal eosinophil counts from eosinophil<sup>high</sup> compared to eosinophil<sup>low</sup> COPD groups and isotpe control are shown in (Figure 5-2).

**Table 5-2 Clinical characteristics of the COPD and control.**

	<b>COPD (n=20)</b>	<b>Control (n=21)</b>	<b>p value</b>
<b>Gender (Male)</b>	17	18	1.0
<b>Age (years)</b>	67 (1.7)	58 (1.8)	<b>&lt;0.001</b>
<b>Smoking (packs/year)*</b>	40 (20-50)	4 (0-28)	<b>&lt;0.001</b>
<b>FEV<sub>1</sub></b>	1.94 (0.14)	2.71 (0.12)	0.001
<b>FEV<sub>1</sub>% predicted</b>	66.5 (3.8)	83.8 (2.2)	0.002
<b>FEV<sub>1</sub>/FVC%</b>	57.9 (2.9)	76.5 (1.2)	<b>&lt;0.001</b>
<b>Blood Eosinophils (%)</b>	2.7 (0.4)	2.9 (0.4)	0.65
<b>Blood Eosinophils x10<sup>9</sup>/L</b>	0.23 (0.03)	0.22 (0.03)	0.90
<b>Eosinophils/mm<sup>2</sup> submucosa*</b>	0.75 (3.9)	0 (1.6)	0.09
<b>Mast cells/mm<sup>2</sup> submucosa*</b>	18 (37)	21 (32)	0.70
<b>RBM <math>\mu</math>m</b>	9.7 (0.6)	7.1 (0.4)	<b>&lt;0.001</b>

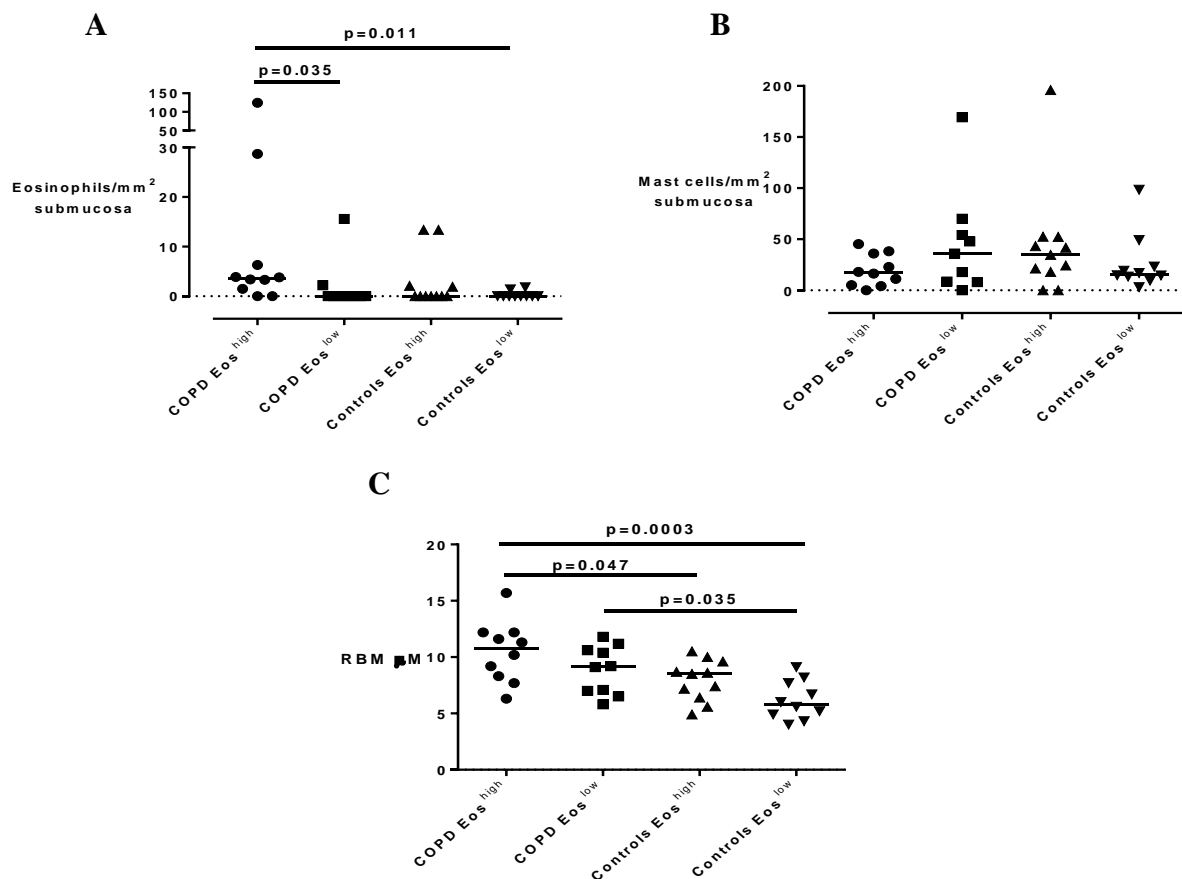
Data presented as mean (SEM), unless otherwise stated. \*Median (IQR). **Abbreviations:** **SEM:** standard error of the mean; **FEV<sub>1</sub>:** Forced expiratory volume in 1 second; **FVC:** Forced vital capacity; **RBM:** reticular basement membrane and lamina reticularis.

**Table 5-3 Clinical characteristics of the COPD and control subjects stratified by the group median peripheral blood eosinophil count percentage.**

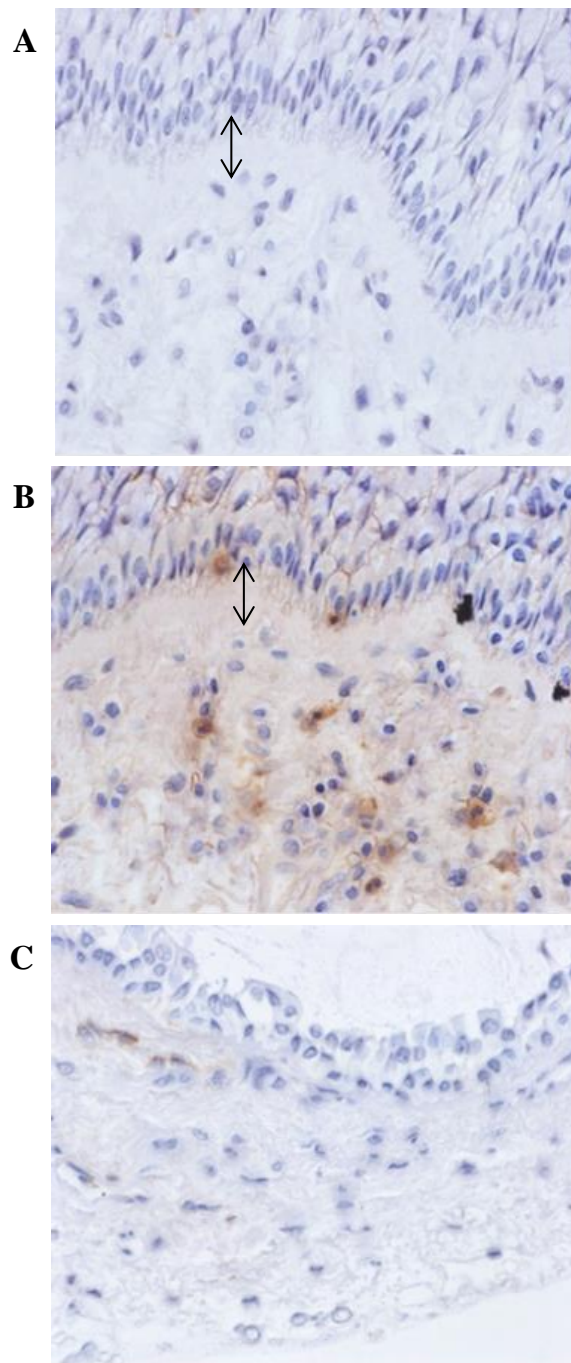
	<b>COPD Eosinophil<sup>high</sup> (n=10)</b>	<b>COPD Eosinophil<sup>low</sup> (n=10)</b>	<b>Control Eosinophil<sup>high</sup> (n=11)</b>	<b>Control Eosinophil<sup>low</sup> (n=10)</b>	<b>p value</b>
<b>Gender (Male)</b>	9	8	10	8	0.82
<b>Age (years)</b>	69 (2.2)	65 (2.5)	55 (2.7)	61 (2.0)	<b>0.003</b>
<b>Smoking (packs/year)*</b>	45 (20-50)	40 (18-53)	0 (0-34)	8 (0-25)	<b>0.009</b>
<b>FEV<sub>1</sub></b>	2.06 (0.22)	1.81 (0.18)	2.84 (0.18)	2.56 (0.16)	<b>0.001</b>
<b>FEV<sub>1</sub>% predicted</b>	70.6 (4.7)	62.4 (5.9)	84.7 (3.3)	82.9 (3.2)	<b>0.002</b>
<b>FEV<sub>1</sub>/FVC%</b>	59.2 (3.9)	56.6 (4.5)	77.6 (1.7)	75.3 (1.7)	<b>&lt;0.001</b>
<b>Blood Eosinophils (%)</b>	4.0 (0.42)	1.4 (0.25)	4.3 (0.5)	1.4 (0.12)	<b>&lt;0.001</b>
<b>Blood Eosinophils x10<sup>9</sup>/L</b>	0.32 (0.05)	0.14 (0.03)	0.31 (0.04)	0.13 (0.01)	<b>&lt;0.001</b>
<b>Eosinophils/mm<sup>2</sup> submucosa*</b>	3.6 (10.8)	0.0 (0.6)	0.0 (2.1)	0.0 (0.35)	<b>0.009</b>
<b>Mast cells/mm<sup>2</sup> submucosa*</b>	17 (34)	36 (54)	35 (34)	16 (17)	0.45
<b>RBM <math>\mu</math>m</b>	10.5 (0.9)	8.9 (0.7)	7.9 (0.5)	6.2 (0.5)	<b>&lt;0.001</b>

Data presented as mean (SEM), unless otherwise stated. \*Median (IQR). **Abbreviations:** **SEM:** standard error of the mean; **FEV<sub>1</sub>:** Forced expiratory volume in 1 second; **FVC:** Forced vital capacity; **RBM:** reticular basement membrane and lamina reticularis.

Post hoc pairwise comparisons p<0.05: 1- COPD eosinophil<sup>high</sup> versus COPD eosinophil<sup>low</sup>, 2- COPD eosinophil<sup>high</sup> versus control eosinophil<sup>high</sup>, 3- COPD eosinophil<sup>high</sup> versus control eosinophil<sup>low</sup>, 4- COPD eosinophil<sup>low</sup> versus control eosinophil<sup>high</sup>, 5- COPD eosinophil<sup>low</sup> versus control eosinophil<sup>low</sup>, and 6- control eosinophil<sup>high</sup> versus control eosinophil<sup>low</sup>.

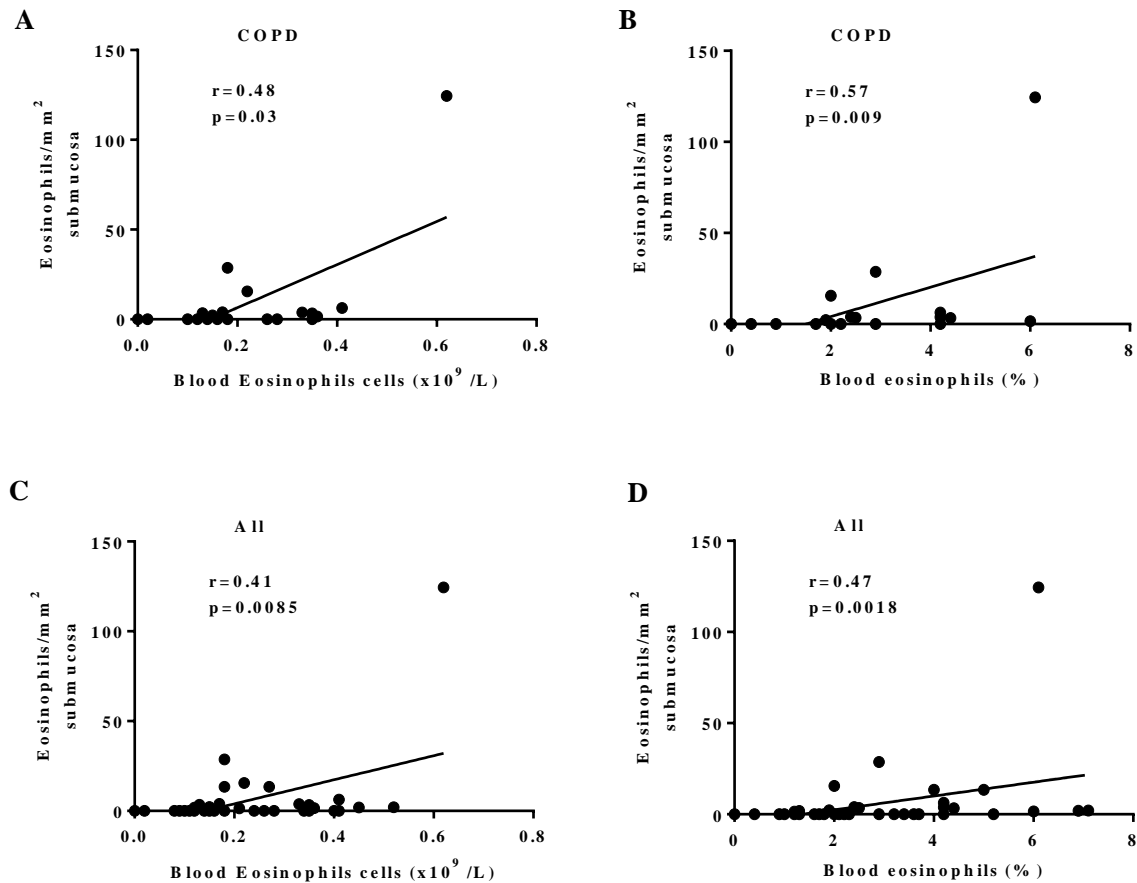


**Figure 5-1** Dot-plots of (A) the submucosal eosinophil counts, (B) submucosal mast cells and (C) RBM thickness in the 2 COPD and 2 control groups, categorised by the median peripheral blood eosinophil count percentage. The horizontal bars represent the median comparisons by ANOVA (parametric and non-parametric as appropriate) and p values for post hoc tests as shown.



**Figure 5-2 Representative bronchial tissue section stained with (A) isotype control, (B) MBP illustrating eosinophilic inflammation and RBM thickening and (C) MBP showing absence of eosinophilic inflammation and no RBM thickening (x400). The arrows show the RBM thickness.**

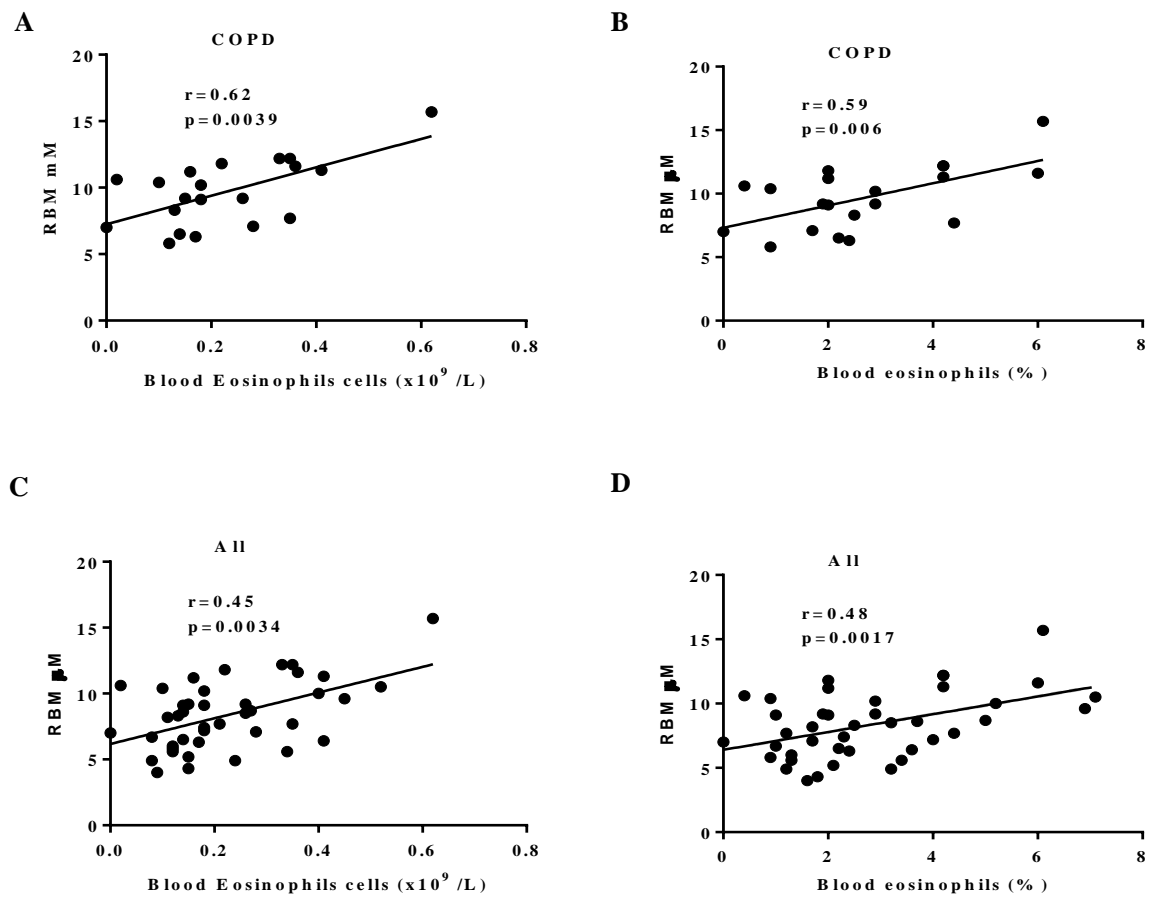
In the COPD subjects, there was a strong correlation between the peripheral blood eosinophil percent and the submucosal eosinophil count (Spearman  $r=0.57$ ;  $p=0.009$ ), (Figure 5-3a) and to a lesser extent between the peripheral blood eosinophil absolute count and the submucosal eosinophil count (Spearman  $r=0.48$ ;  $p=0.03$ ), (Figure 5-3b). This was also observed, but less significantly in both COPD and control subjects combined (Spearman  $r=0.47$ ;  $p=0.0018$  for peripheral blood eosinophil percent and Spearman  $r=0.41$ ;  $p=0.0085$  for the absolute count), (Figure 5-3c and Figure 5-3d). Moreover, the correlation is still significant even after excluding the highest value of submucosal eosinophil count as the Z score of this value is less than 8 (6.29), which means that this is not statistically different from the mean of the group, therefore it is not an outlier.



**Figure 5-3 Scatter-plot demonstrating the positive correlation between the peripheral blood eosinophil and submucosal eosinophil count.**

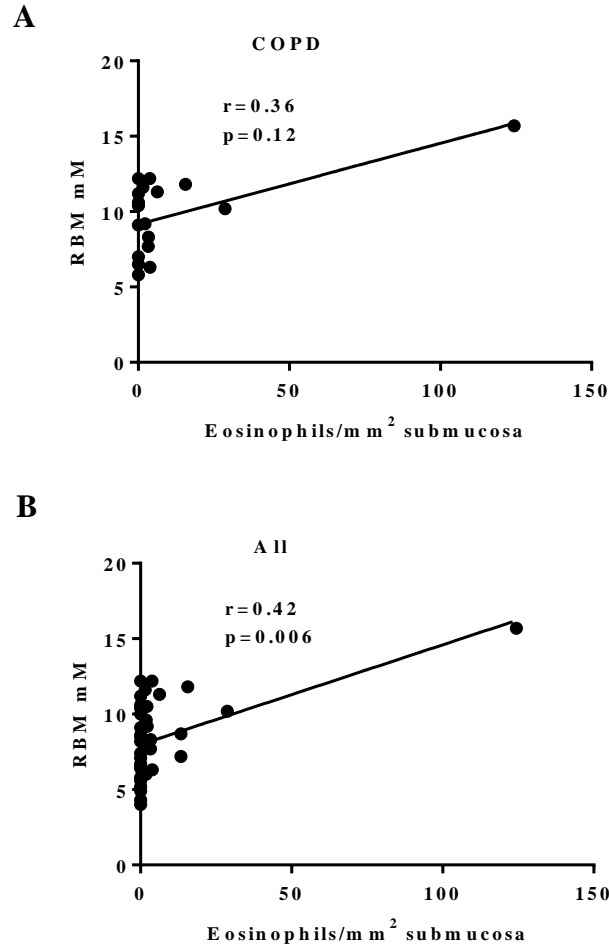
In addition, both peripheral blood eosinophil absolute count and percent were significantly correlated with RBM thickness in COPD (Spearman  $r=0.62$ ;  $p=0.0039$ ) and (Spearman  $r=0.59$ ;  $p=0.006$ ) respectively, with similar, but less significant findings in COPD and control subjects combined together (Spearman  $r=0.48$ ;  $p=0.0017$  for peripheral blood eosinophil percent and Spearman  $r=0.45$ ;  $p=0.0034$  for the absolute count), (Figure 5-4).





**Figure 5-4 Scatter-plot demonstrating the positive correlation between the peripheral blood eosinophil and RBM thickness.**

Although, there was a weak association between bronchial submucosal eosinophil count and RBM thickness in COPD and control subjects combined (Spearman  $r=0.42$ ;  $p=0.006$ ), there was no significant correlation between the two in COPD alone (Spearman  $r=0.36$ ;  $p=0.12$ ), (Figure 5-5).



**Figure 5-5 Scatter-plot showing the weak correlation between the submucosal eosinophil count and RBM thickness.**

There was no correlation between the peripheral blood eosinophil count or percentage and submucosal mast cells, FEV<sub>1</sub>, FEV<sub>1</sub>% predicted, FEV<sub>1</sub>/FVC ratio or pack/years of smoking. There were no correlations between the submucosal eosinophils and mast cells, FEV<sub>1</sub>, FEV<sub>1</sub>% predicted, FEV<sub>1</sub>/FVC ratio or pack/years of smoking either (data are not shown).

### 5.1.5 Discussion

This study sheds a light on the association between peripheral blood and submucosal eosinophils and airway remodelling in COPD. Although in asthma (Maestrelli, Sietta *et al.* 1995, Grootendorst, Sont *et al.* 1997) and exacerbated, but not stable chronic bronchitis (Maestrelli, Sietta *et al.* 1995), there was a strong correlation between eosinophils% in sputum and their corresponded values in BAL and bronchial wash. However, there was a weak (Grootendorst, Sont *et al.* 1997), or no relationship between the sputum and submucosal eosinophils, neither in asthma (Maestrelli, Sietta *et al.* 1995), nor in COPD and healthy controls (Rutgers, Timens *et al.* 2000), despite the robust association between ECP in the sputum and bronchial wash. Additionally, Maestrelli and colleagues found no relationship either between eosinophils in peripheral blood and submucosal tissue (Maestrelli, Sietta *et al.* 1995). Thus, the current study, with a higher number of subjects than previously reported, shows for the first time a strong correlation between peripheral blood and bronchial eosinophils and RBM thickening in COPD and normal subjects. Moreover, both the submucosal eosinophil count and RBM thickening were significantly increased in the COPD eosinophil<sup>high</sup> group suggesting that the peripheral blood eosinophil count, as a surrogate marker of sputum eosinophils, does identify COPD subjects with a greater tissue eosinophilia and remodelling.

Peripheral blood eosinophil count is emerging as a valuable biomarker to phenotype airways disease and direct therapy. In the Dose Ranging Efficacy And safety with Mepolizumab (DREAM) study, the peripheral blood eosinophil count correlated with response to the anti-interleukin (IL)-5 monoclonal antibody mepolizumab (Pavord, Korn *et al.* 2012). The success of anti-IL5 in severe asthmatics with evidence of eosinophilic inflammation (Eltboli, Brightling 2013) raises the possibility that blood eosinophils might be an appropriate

biomarker to direct eosinophil-specific therapy in COPD. Indeed, a recent report does suggest that the peripheral blood eosinophil count identifies a subgroup of COPD subjects that have the greatest benefit in terms of lung function, health status and exacerbations in response to anti-IL-5R, Benralizumab (Brightling, Bleecker *et al.* 2014).

There were some potential drawbacks in this study. Baseline differences in age and smoking history between COPD and control groups might affect the differences observed in inflammation and remodelling. Additionally, the data were collected retrospectively and it was not formally investigated whether subjects had atopy, although subjects with asthma were excluded, but no assessment for asthma-COPD overlap syndrome (ACOS) was made and this might have been different between groups, although most of the COPD eosinophil<sup>high</sup> group had no bronchodilator reversibility. The study was retrospective and contemporaneous sputum analysis was not performed to validate the results of peripheral blood eosinophils. However the relationship previously reported between blood and sputum eosinophils (Bafadhel, McKenna *et al.* 2011), the results in chapter 3 and the prior reports of the clinical usefulness of targeting peripheral blood eosinophils (Bafadhel, McKenna *et al.* 2012, Brightling, Bleecker *et al.* 2014), suggest that the findings from the current study are likely to be valid. The number of subjects included was relatively small, especially when they were subdivided into four groups based on blood eosinophil levels; although the correlations in the whole group were strong suggesting that the findings were robust. Despite the lack of association between this biomarker and lung function or pack/years of smoking, unlike sputum eosinophils, yet it has a promising role in predicting the degree of airways eosinophilia and remodelling. However, further large scale prospective studies are warranted in order to validate and reproduce these findings.

### **5.1.6 Conclusion**

In summary, COPD peripheral blood eosinophils are associated with submucosal eosinophils and airway remodelling. Therefore, consistent with previous reports of the peripheral blood eosinophil count as a biomarker of a sputum eosinophilia, it is also related to an airway tissue eosinophilia. These data further support the peripheral blood eosinophil count as an important biomarker of an airway eosinophilia and is likely to be important in targeting current and future anti-eosinophil therapies for COPD.

**Chapter 6    What is the aetiology of eosinophilic inflammation in COPD? Is it due to parasites exposure or related to atopy?**

## **6.1 Eosinophilic chronic obstructive pulmonary disease is not associated with helminth infection or exposure**

### **6.1.1 Abstract**

Eosinophilic airway inflammation is observed in 10-30% of COPD subjects, but its cause is unclear. I have investigated whether sputum eosinophilia is associated with previous helminth exposure in 150 subjects with COPD, 14 of them had positive serology for parasites. Positive serology was typically associated with previous travel to endemic areas. The clinical and sputum characteristics were not significantly different in those with and without positive parasite serology. In conclusion, eosinophilic COPD is not associated with parasite infection or exposure.

### **6.1.2 Introduction**

A sputum eosinophilia (>3%) is present in 10-30% of COPD subjects (Eltboli, Brightling 2013, Barker, Brightling 2013). The underlying cause is poorly understood. I hypothesised that eosinophilic airway inflammation in COPD is associated with prior exposure to parasites. To test my hypothesis, I analysed serum samples from 150 COPD subjects that had participated in two longitudinal studies of COPD exacerbations (Bafadhel, McKenna *et al.* 2011, Bafadhel, McKenna *et al.* 2012).

### **6.1.3 Materials and Methods**

150 serum samples were analysed in the Parasitology Reference Laboratory at the Hospital for Tropical Diseases, London, to test for antibodies to; *Strongyloides stercoralis*, cysticercosis (*Taenia solium*), *Toxocara canis* and *Echinococcus granulosus* (hydatid cyst) (Collier, Manser *et al.* 2010, Kunst, Mack *et al.* 2011). All of the patients were white British.

The sputum eosinophil count was assessed 3 monthly for 1 year at visits when subjects were exacerbation-free for at least 6 weeks. The sputum eosinophil area under the curve (AUC) was derived from the sputum samples collected at stable visits and expressed as sputum eosinophil %/year. An extensive travel history was obtained retrospectively in those subjects with positive serology.

GraphPad Prism version 6 (GraphPad, San Diego) and IBM SPSS version 20 (SPSS, Inc. Chicago) were used to perform statistical analysis. Mean (standard error of the mean [SEM]) was used to present parametric data, whilst median (interquartile ranges [IQR]) was used for non-parametric data and geometric mean (95% confidence interval) for data that was log normally distributed. For comparisons between groups, unpaired T-test or Mann-Whitney was used for parametric or non-parametric data respectively. Spearman or Pearson rank correlation coefficient was used to assess the correlations for parametric and non-parametric data respectively. A p value less than 0.05 was considered statistically significant.

#### **6.1.4 Results**

There were 16 positive results for 14 patients and two of them had positive results for 2 parasites (*Toxocara* with *Strongyloides* and *Hydatid* with *Strongyloides*). None of the subjects had symptoms suggestive of active parasitic infection. There were 11 positive results for *Strongyloides stercoralis*, 2 for *Echinococcus granulosus*, 1 for *Taenia solium* and 2 for *Toxocara canis*. Among the 11 cases who were positive for antibodies to *Strongyloides stercoralis*; 6 had travelled abroad to South-East Asia (n=3), Tunisia (n=3) and among these six, one travelled also to Israel and Syria and another to South Africa. Two subjects had never travelled outside Europe, 2 were lost to follow-up and 1 had died. Of the two subjects with



positive *Toxocara canis* serology; 1 had traveled to South-East Asia, Australia, New Zealand, Israel and Syria and the other had died. Of the two subjects positive for antibodies to *Echinococcus granulosus*; 1 lived for 3 years in Egypt during childhood and the other travelled to South-East Asia and South Africa. The patient with positive cysticercosis serology had travelled to Vietnam and South Africa. Among the eosinophilic COPD subjects, 3/28 had positive serology for parasites, whilst 11/122 of non-eosinophilic patients were seropositive. There were no significant differences in clinical or sputum characteristics between those with positive and negative serum results (Table 6.1).

**Table 6-1 Baseline characteristics of parasite serology positive versus negative COPD subjects**

	Parasite serology negative (n=136)	Parasite serology positive (n=14)	p value
Male, n (%)	94 (69)	11 (79)	0.46
Age (years)*	69 (0.8)	64.5 (3)	0.24
Body mass index (kg/m <sup>2</sup> )	26 (0.4)	27 (1.7)	0.73
Packs year history (packs/year)	46 (30-60)	39 (28-79)	0.89
Exacerbations in last year	3 (1-5)	2 (0-7.5)	0.68
ICS (BDP equivalent) (mcg/day)	2000 (900-2000)	2000 (800-2000)	0.86
FEV <sub>1</sub> % predicted, (%)	49 (1.7)	45.7 (4.8)	0.69
FEV <sub>1</sub> /FVC (%)*	49 (1.1)	50 (3.2)	0.80
Reversibility, %	3.7(-1.3- 10)	0.69(-6.2- 6.7)	0.10
Sputum TCC x10 <sup>6</sup> /g	3.6 (1.5-7.9)	1.8 (0.75-4.5)	0.06
Sputum neutrophils (%)	73 (53-89)	60 (37-77)	0.11
Sputum eosinophils (%)	1.0 (0.25-3)	0.75 (0.44 - 2.1)	0.89
AUC sputum eosinophils %/year	0.79 (0.27 - 1.1)	0.41 (0.25 - 2.2)	0.30
PB. eosinophils (x10 <sup>9</sup> /L)	0.22 (0.13 - 0.34)	0.17 (0.09 - 0.29)	0.22
MRC Dyspnoea Scale	3 (2-4)	3 (2-3)	0.44
SGRQ total score (unit)*	51 (1.6)	56 (4.4)	0.34
CRQ total score (unit)*	16 (0.41)	17 (0.95)	0.47
VAS Cough (mm)	33 (13 - 54)	30 (13 - 72)	0.93
VAS Dyspnoea (mm)	46 (23 - 67)	50 (24 - 69)	0.79
VAS Sputum Production (mm)	31 (11 - 55)	37 (6 - 55)	0.96
VAS Sputum Purulence (mm)	21 (8 - 49)	26 (10 - 51)	0.82

Data presented as median (IQR) unless stated, \*mean (SEM), \*\*Geometric mean of Log data (95% CI). SEM: standard error of mean, CI: confidence interval. **Abbreviations:** **AUC:** area under the curve; **BDP:** Beclomethasone Dipropionate; **CRQ:** Chronic respiratory health Questionnaire; **FEV<sub>1</sub>:** Forced expiratory volume in 1 second; **FVC:** Forced vital capacity; **ICS:** inhaled corticosteroids; **MRC:** Medical Research Council; **PB:** peripheral blood; **SGRQ:** St George's Respiratory Questionnaire; **TCC:** total cell count; **VAS:** Visual analogue score.

### 6.1.5 Discussion and conclusion

It is reported here for the first time helminth serology in a group of subjects with COPD. The proportion of COPD subjects with positive parasite serology was 9%, which was associated in most cases with a relevant travel history to endemic areas. The proportion of positive serum parasites was similar between eosinophilic and non-eosinophilic COPD subjects (3/28 [10.7 %], versus 11/122 [9%] respectively; p=1.0 [Fisher's Exact test]).

Critically, these findings do not support a role of helminth exposure or infection in eosinophilic COPD. Nevertheless, as for any patient due to receive corticosteroid or immunosuppressive therapy, *Strongyloides* infection must be sought in those with a relevant travel history and treated if found, to remove the risk of developing *Strongyloides* hyperinfection.

One criticism to this study is that the sera of only four species of parasites were tested, which may not be sufficient to exclude parasitic exposure as a possible causative factor for raised eosinophils in the sputum. However, selecting these 4 species was based on preliminary screening data from a clinical trial on targeted treatment in subjects with sputum eosinophilia COPD (Brightling et al. 2014). Perhaps, these were the most commonly tested species in the clinical trials, although some such as *Ascaris lumbricoides* was not tested here as it was not available in the reference laboratories in the UK, despite few positive results were observed in the trial. Importantly, the absence of blood eosinophilia ( $\geq 0.40 \times 10^9/L$ ) in the subjects with sputum eosinophilia may also support the conclusion that parasites are unlikely to have a role of helminth exposure or infection in eosinophilic COPD. However, further studies are needed to replicate and validate these observations.

## 6.2 Relationship between atopy and sputum eosinophilia

### 6.2.1 Abstract

**Background:** The aetiology of sputum eosinophilia in COPD is not known and it is not clear whether atopy is related to sputum eosinophilia or contributes to the pathogenesis of eosinophilic COPD phenotype.

**Methods:** 65 COPD subjects were tested for serum total Ig-E, skin prick test for common aeroallergens and categorized into two groups differentiated by the area under the curve (AUC) sputum eosinophil count %/year ( $\geq 3\%$ /year) and then by the upper limit of normal of total serum Ig-E.

**Results:** The total serum Ig-E was significantly higher in the eosinophilic group (135 [78.4-268.5], compared to the non-eosinophilic (37.1 [14.7-121.5];  $p=0.002$ ). Baseline sputum eosinophils% and AUC sputum eosinophil count were higher in the Ig-E<sup>high</sup> compared to Ig-E<sup>low</sup> group (3.21 [1.25-19.7], versus 1 [0.28-2.9];  $p=0.0016$ ) and (2.99 [0.42-6] versus 1.1 [0.45-1.9];  $p=0.03$ ) respectively. The total serum Ig-E and baseline sputum eosinophils levels were positively correlated ( $r=0.39$ ;  $p=0.0015$ ). Skin prick test to various aeroallergens was similarly negative in the all the groups.

**Conclusion:** Sputum eosinophilia in COPD could be related to allergy.

### 6.2.2 Introduction

Asthma is typically eosinophilic with significant number of asthmatic patients have atopy, whilst COPD is generally considered non-atopic disease. The cause of sputum eosinophilia in COPD is still unknown and it is not recognized whether atopy plays an important role or related to sputum eosinophilia. Atopy is an immediate type II hypersensitivity reaction and is clinically defined as increase in total or specific Ig-E or positive skin test to common aeroallergens (Weiss 2000). There are numerous reports about the relationship between atopy and decline in lung function (Vollmer, Buist *et al.* 1986, Dow, Coggon *et al.* 1992, Omenaas, Bakke *et al.* 1995, Tracey, Villar *et al.* 1995), but few studied the correlation between atopy and sputum eosinophilia in COPD, despite some reported association with blood eosinophils (Lewis, Pavord *et al.* 2001, Sitkauskienė, Sakalauskas *et al.* 2003). I hypothesised that eosinophilic airway inflammation in COPD is associated with atopy. To test my hypothesis, data available from 65 COPD subjects that had participated in two longitudinal studies of COPD exacerbations (Bafadhel, McKenna *et al.* 2011, Bafadhel, McKenna *et al.* 2012), were analysed.

### 6.2.3 Materials and Methods

Subjects were divided into two groups, eosinophilic (n=17) and non-eosinophilics (n=48), determined by the area under the curve (AUC) sputum eosinophil count ( $\geq 3\%$ /year). The sputum eosinophil area under the curve was derived from the sputum samples collected at stable visits as described (6.1.3). Serum samples were analysed for serum total Ig-E using the ImmunoCap 250 system (Phadia, UK) with detection limits 2-5000 kU/L and normal reference values 0–114 kU/mL). Subjects were then categorized into 2 categories based on the total serum Ig-E upper limit of normal (114 kU/mL). They were also tested with skin

pricking for allergy for the following agents; cat, dog, grass pollen, house dust mite, *Aspergillus fumigatus*, *Alternaria alternata*, *Cladosporium herbarum*, *Penicillium* and *Botrytis cinerea* (Alk-Abello, Denmark) against negative and histamine controls. Wheal larger than 3 mm compared to the negative control was considered positive. In addition, sputum cultures were performed for yeast, *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium/Paecilomyces* and other fungi.

GraphPad Prism version 6 (GraphPad, San Diego) and IBM SPSS version 20 (SPSS, Inc. Chicago) were used to perform statistical analysis. Mean (standard error of the mean [SEM]) was used to present parametric data, whilst median (interquartile ranges [IQR]) was used for non-parametric data and geometric mean (95% confidence interval) for data that was log normally distributed. Spearman or Pearson rank correlation coefficient was used to assess the correlations for parametric and non-parametric data respectively. A p value less than 0.05 was considered statistically significant.

#### **6.2.4 Results**

The baseline clinical characteristics of the eosinophilic and non-eosinophilic groups are as shown (Table 6-2 and Table 6-3). There were no significant differences in age, gender, smoking history, pack-years of smoking, health symptoms, frequency of hospital admissions or lung function between the 2 groups. All of the subjects were current or ex-smokers. The eosinophilic COPD group has smaller BMI (mean [SEM] 23.2 [1.03]), than the non-eosinophilic (27.24 [0.68];  $p=0.003$ ) and the use of inhaled corticosteroids was less in non-eosinophilics (median [IQR] 1000 [800-2000]), than in eosinophilics (2000 [1400-2000];  $p=0.028$ ). The median [IQR] of the total serum Ig-E was significantly higher in the

eosinophilic group (135 [78.4-268.5]), compared to the non-eosinophilic (37.1 [14.7-121.5];  $p=0.003$ ), (Figure 6-1). However, Skin hypersensitivity for various reagents was similarly negative in the two clusters. There were no differences in bacterial or fungal colonisation of the sputum between the two categories apart from small increase in number of colonisations with *Penicillium/Paecilomyces* fungi in the sputum of the eosinophilic (n [%], 3 [33]), in contrast with non-eosinophilics COPD (1 [4];  $p=0.038$ ). There was a positive correlation between the total serum Ig-E and sputum eosinophils at baseline ( $r=0.39$ ;  $p=0.0015$ ), (Figure 6-2), but not with AUC sputum eosinophil%.

**Table 6-2 Baseline clinical characteristics of the eosinophilic and non-eosinophilic groups**

	<b>Eosinophilic (n=17)</b>	<b>Non-Eosinophilic (n=48)</b>	<b>p value</b>
Male, n (%)	14 (82)	28 (58)	0.08
Age (years)*	66.54 (2.11)	68.52 (1.31)	0.44
Body mass index kg/m <sup>2</sup> *	23.2 (1.03)	27.24 (0.68)	<b>0.003</b>
Current smokers, n (%)	5 (29)	24 (50)	0.14
Ex-smokers smokers, n (%)	12 (71)	24 (50)	0.14
Packs year history (packs/year)	39 (33-62.75)	47 (33-65)	0.91
COPD duration (years)	4.65 (1.83-8.69)	4.16 (2.51-7.76)	0.79
Number of hospital admissions in the last year	0 (0-1.5)	0 (0-1)	0.51
Number of steroid and/or antibiotics in the year	4.5 (2-7.25)	3 (1.25-5)	0.08
ICS dose (BDP equivalent) (mcg/day)	2000 (1400-2000)	1000 (800-2000)	<b>0.028</b>
FEV <sub>1</sub> % predicted, (%)*	47.46 (4.3)	55.36 (3.24)	0.20
FEV <sub>1</sub> post bronchodilator (L)*	1.37 (0.13)	1.38 (0.08)	0.99
Post FEV <sub>1</sub> /FVC (%)*	51.56 (3.76)	50.87 (1.91)	0.86
Reversibility, %	3.4 (-2.4-9.5)	2.3 (-3.1-7.7)	0.86
GOLD 1, n (%)	0 (0)	8 (17)	<b>0.02</b>
GOLD 2, n (%)	9 (53)	21 (44)	0.51
GOLD 3, n (%)	6 (35)	10 (21)	0.25
GOLD 4, n (%)	2 (12)	9 (48)	0.50
Sputum TCC x10 <sup>6</sup> /g	4.47 (1.2-7.95)	2.78 (1.31-4.9)	0.48
Sputum neutrophils (%)*	53.15 (5.61)	63.62 (3.1)	0.1
Blood total cell count (x10 <sup>9</sup> /L)*	7.94 (0.61)	7.86 (0.24)	0.89
Blood eosinophils cell count (x10 <sup>9</sup> /L)	0.34 (0.23-0.5)	0.17 (0.1-0.25)	<0.0001
Blood neutrophils cell count (x10 <sup>9</sup> /L)	4.67 (3.43-5.86)	5.10 (4.0-6.12)	0.3922
CRP (mg %)	7 (4.38-21.5)	9.5 (5.25-13)	0.76
Serum total Ig E (kU/mL)	135 (78.4-268.5)	37.1 (14.7-121.5)	<b>0.002</b>
Skin hypersensitivity to cat (mm)	0 (0-0)	0 (0-0)	0.49
Skin hypersensitivity to dog (mm)	0 (0-0)	0 (0-0)	0.85
Skin hypersensitivity to HDM (mm)	0 (0-0)	0 (0-0)	0.77
Skin hypersensitivity to Aspergillus fumigatus (mm)	0 (0-0)	0 (0-0)	>0.9999
Skin hypersensitivity to grass (mm)	0 (0-0)	0 (0-0)	0.73
Skin hypersensitivity to Alternaria alternata (mm)	0 (0-0)	0 (0-0)	0.55
Skin hypersensitivity to Cladosporium herbarum (mm)	0 (0-0)	0 (0-0)	0.58
Skin hypersensitivity to Penicillium (mm)	0 (0-0)	0 (0-0)	0.55
Skin hypersensitivity to Botrytis (mm)	0 (0-0)	0 (0-0)	0.58

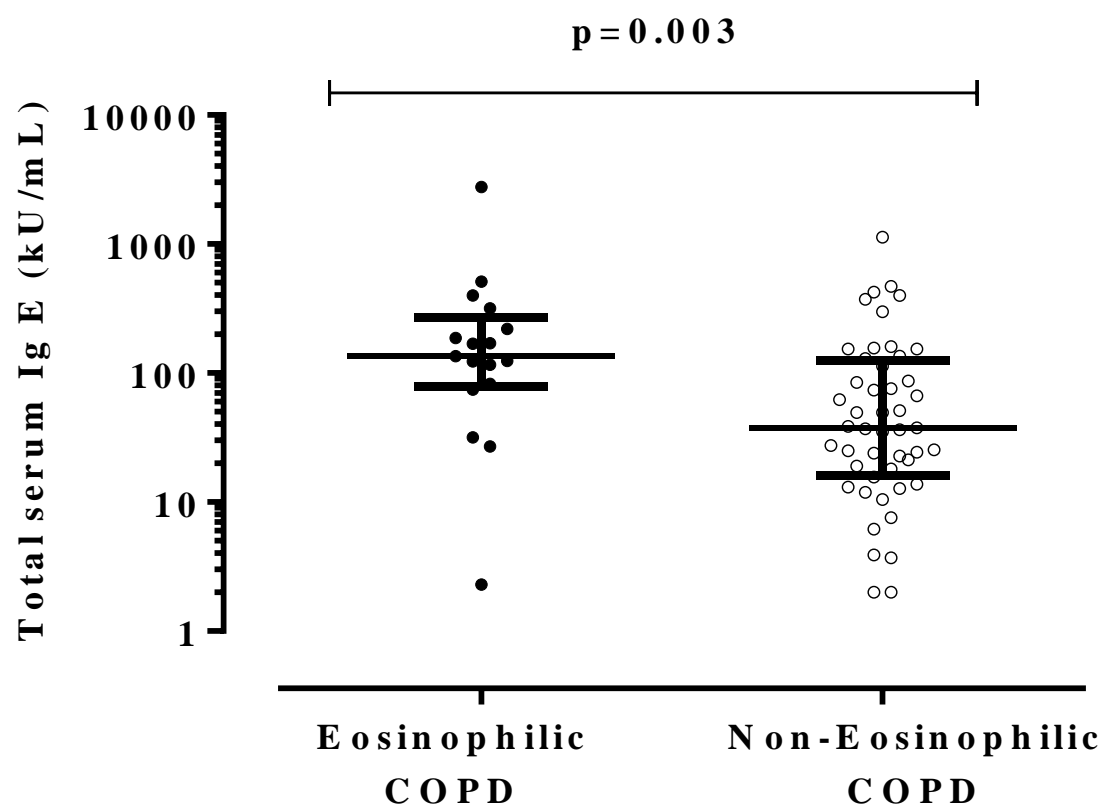
Data presented as median (IQR) unless stated, \*mean (SEM), SEM: standard error of mean. **Abbreviations:** AUC: area under the curve; BDP: Beclometasone Dipropionate; CRQ: Chronic respiratory health Questionnaire; CRP: C-Reactive Protein; HDM: House dust mite; FEV<sub>1</sub>: Forced expiratory volume in 1 second; FVC: Forced vital capacity; GOLD: Global Initiative for chronic Obstructive Lung disease; ICS: inhaled corticosteroids.



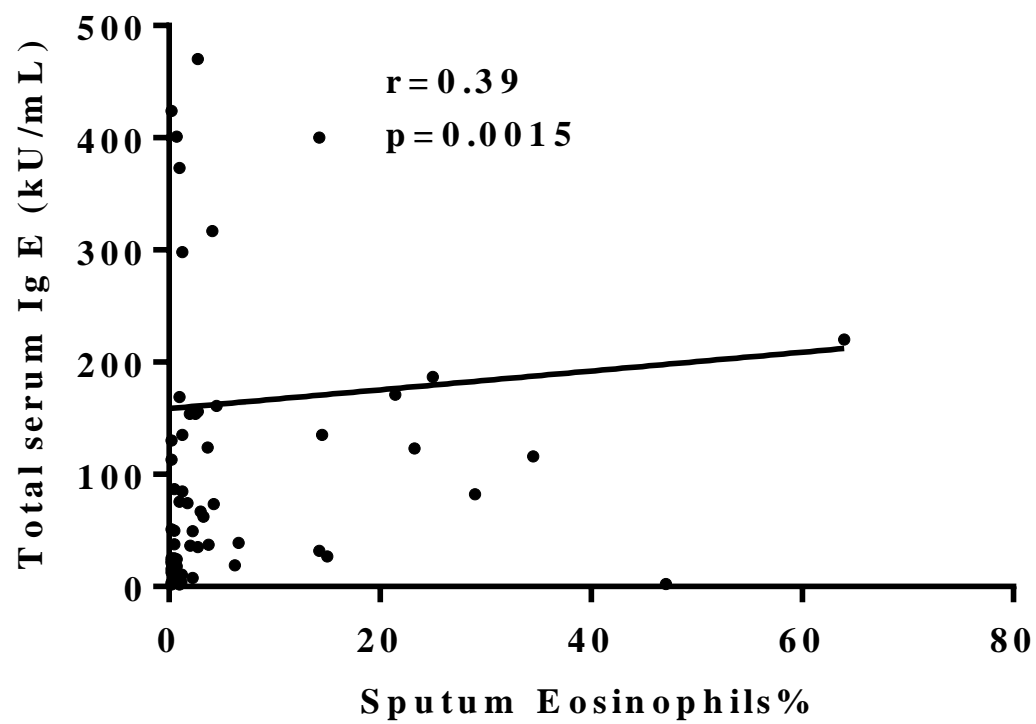
**Table 6-3 Baseline microbiology and health status of the eosinophilic and non-eosinophilic groups**

	<b>Eosinophilic (n=17)</b>	<b>Non-Eosinophilic (n=48)</b>	<b>p value</b>
<b>Sputum bacterial colonisation, n (%)</b>	1 (6)	12 (48)	0.06
<b>Sputum CFU/mL**</b>	6.2 (5.7-6.8)	6.4 (6.1-6.6)	0.50
<b>Sputum 16s (gene copies x10<sup>6</sup> /mL)**</b>	8.2 (7.6-8.9)	8.3 (8-8.5)	0.48
<b>Sputum colonisation for Yeast, n (%)</b>	14 (88)	39 (83)	0.66
<b>Sputum colonisation for Aspergillus fumigatus, n (%)</b>	6 (60)	22 (46)	0.62
<b>Sputum colonisation for Aspergillus nigar, n (%)</b>	1 (11)	4 (14)	0.81
<b>Sputum colonisation for Penicillium/Paecilomyces, n (%)</b>	3 (33)	1 (4)	<b>0.038</b>
<b>Sputum colonisation for other fungi, n (%)</b>	5 (29)	8 (17)	0.38
<b>MRC total score (unit)</b>	3 (2-4)	3 (2-4)	0.7
<b>SGRQ Symptoms (unit)*</b>	58.0 (7.3)	64.8 (2.6)	0.28
<b>SGRQ Activity (unit)</b>	72.9 (49.3-83.7)	66.1 (47.9-79.8)	0.61
<b>SGRQ Impacts (unit)</b>	32.2 (17.6-47.8)	32.7 (23.8-49.2)	0.9
<b>SGRQ total score (unit)*</b>	49.2 (5.6)	49.4 (2.6)	0.97
<b>CRQ Emotional Functioning (unit)*</b>	5.11 (0.35)	4.5 (0.20)	0.13
<b>CRQ Fatigue (unit)*</b>	4.1 (0.35)	3.7 (0.21)	0.36
<b>CRQ Dyspnoea (unit)</b>	3.7 (2.7-4.7)	3.4 (2.2-4.4)	0.22
<b>CRQ Mastery (unit)</b>	5.5 (4-6.6)	5.3 (3.8-6.3)	0.54
<b>CRQ total score (unit)*</b>	4.5 (0.3)	4.1 (0.2)	0.22
<b>VAS Cough (mm)*</b>	34.6 (6.8)	40.7 (3.9)	0.43
<b>VAS Dyspnoea (mm)*</b>	44.8 (7.2)	47.7 (3.3)	0.68
<b>VAS Sputum Production (mm)</b>	30 (11.5-57.5)	36.5 (12.3-64)	0.63
<b>VAS Sputum Purulence (mm)</b>	12 (1-44.5)	27 (12-43.5)	0.21

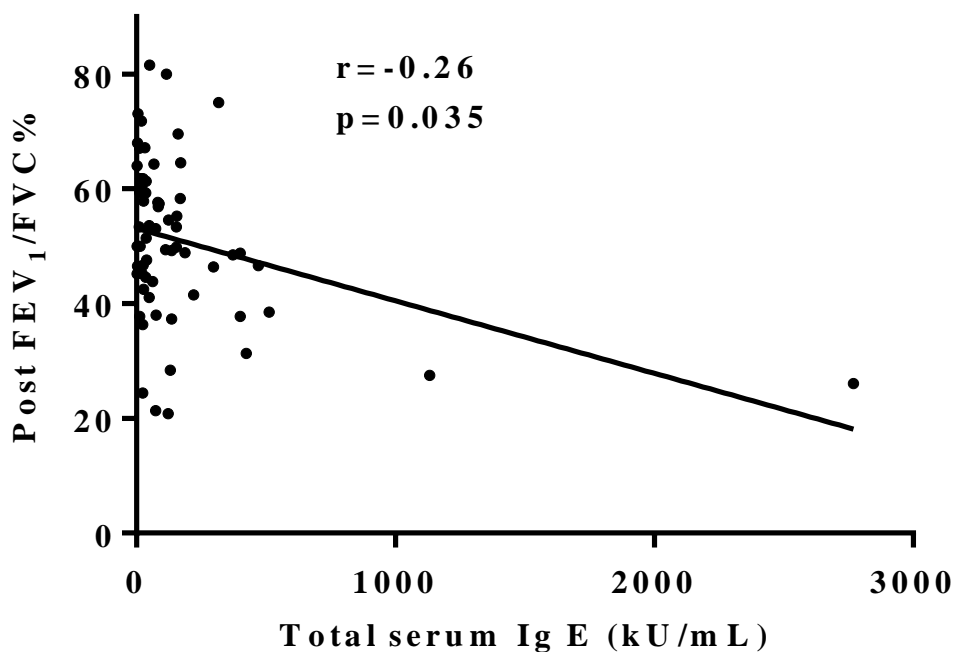
Data presented as median (IQR) unless stated, \*mean (SEM), \*\*Geometric mean of Log data (95% CI). SEM: standard error of mean, CI: confidence interval. **Abbreviations:** CFU: colony forming unit; CRQ: Chronic respiratory health Questionnaire; MRC: Medical Research Council; SGRQ: St George's Respiratory Questionnaire; VAS: Visual analogue score.



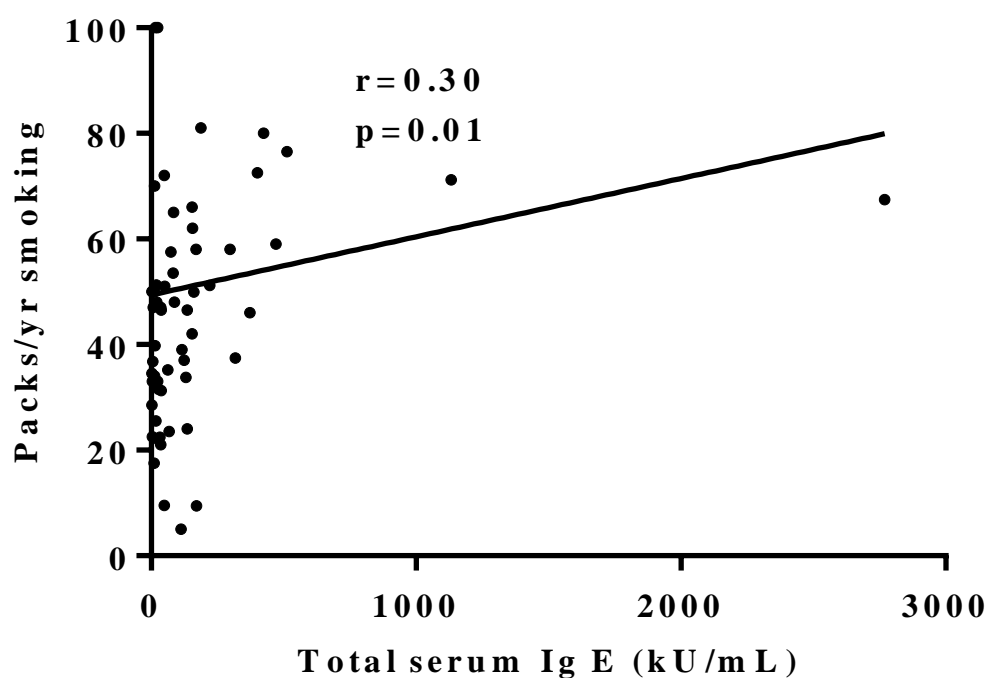
**Figure 6-1** Dot-plot showing the total serum Ig-E in the eosinophilic and non-eosinophilic COPD groups. Horizontal bars are medians (IQR).



There was also a trend for a negative association between the total serum Ig-E and post FEV<sub>1</sub>/FVC ratio (Spearman  $r=-0.26$ ;  $p=0.035$ ), (Figure 6-3), but not with post bronchodilator FEV<sub>1</sub> or FEV<sub>1</sub>% predicted, nor with blood eosinophils count or BMI (data is not shown). Additionally, total serum Ig-E and pack-years of smoking were positively correlated (Spearman  $r=0.30$ ;  $p=0.01$ ), although the relationship was not strong (Figure 6-4). There was no correlation to health status either, except for a weak positive correlation with SGRQ<sub>activity</sub> domain (Spearman  $r=0.26$ ;  $p=0.036$ ).



**Figure 6-3** Scatter plot showing the negative correlation between the total serum Ig-E and FEV<sub>1</sub>/FEC ratio.



**Figure 6-4 Scatter plot showing the positive correlation between the total serum Ig-E and pack-years of smoking.**

However, when performing a linear regression model between the log of sputum eosinophils%, pack-years of smoking as predictors and the log total blood Ig-E, the R squared value was 0.152 with log sputum eosinophils% as a significant predictor (beta=0.326 (0.159);  $p=0.006$ ). Whilst pack-years history being a predictor was not quite at the significant level (beta=0.005 (0.003);  $p=0.075$ ). This suggests that log sputum eosinophils% is a better predictor for log total blood Ig-E than pack-years history.

The baseline clinical characteristics of the two groups which were divided into Ig-E<sup>high</sup> and Ig-E<sup>low</sup> serum total Ig-E levels are presented in (Table 6-4 and Table 6-5). There were no marked differences between the two groups in age, sex, BMI, smoking history, pack-years of

smoking, health symptoms, frequency of hospital admissions, use of inhaled corticosteroids, sputum microbiology or lung function. Both the baseline sputum eosinophils% and AUC sputum eosinophil count were significantly higher in the Ig-E<sup>high</sup>, in comparison to Ig-E<sup>low</sup> group (Median [IQR] 3.21 [1.25-19.7], versus 1 [0.28-2.9]; p=0.0016) and (2.99 [0.42-6], versus 1.1 [0.45-1.9]; p=0.03) respectively. There was also more frequent use of oral corticosteroids in the previous 12 months in the Ig-E<sup>high</sup> (5 [2-7.25], compared to Ig-E<sup>low</sup> group 3 [1-4]; p=0.028).

**Table 6-4 Baseline clinical characteristics of the Ig-E<sup>high</sup> and Ig-E<sup>low</sup> groups**

	<b>Ig-E<sup>High</sup> (n=24)</b>	<b>Ig-E<sup>Low</sup> (n=41)</b>	<b>p value</b>
<b>Male, n (%)</b>	19 (79)	23 (56)	0.06
<b>Age (years)*</b>	69.4 (1.5)	67.8 (1.5)	0.48
<b>Body mass index kg/m<sup>2</sup>*</b>	26.2 (21.7-28.8)	25.3 (23.3-30.2)	0.35
<b>Current smokers, n (%)</b>	10 (42)	19 (46)	0.71
<b>Ex- smokers, n (%)no</b>	14 (58)	22 (54)	0.71
<b>Pack year history (packs/year)</b>	51.2 (37.5-67.5)	46.5 (29.9-55.5)	0.12
<b>COPD duration (years)</b>	4.4 (1.7-8.4)	4.3 (2.6-7.6)	0.99
<b>Number of hospital admissions in the last year</b>	0.5 (0-1)	0 (0-1)	0.13
<b>Number of steroid and/or antibiotics in the year</b>	5 (2-7.3)	3 (1-4)	<b>0.028</b>
<b>ICS dose (BDP equivalent) (mcg/day)</b>	2000 (850-2000)	1500 (950-2000)	0.65
<b>FEV<sub>1</sub>% predicted, (%)*</b>	46.7 (4.3)	57.1 (3.3)	0.06
<b>FEV<sub>1</sub> post bronchodilator (L)*</b>	1.2 (0.1)	1.5 (0.09)	0.07
<b>Post FEV<sub>1</sub>/FVC (%)*</b>	47.4 (3.1)	53.2 (1.9)	0.1
<b>Reversibility, %</b>	2.1 (-3.2-6.2)	3.0 (-2.4-10.2)	0.48
<b>GOLD 1, n (%)</b>	1 (4)	7 (17)	0.1
<b>GOLD 2, n (%)</b>	9 (38)	22 (54)	0.21
<b>GOLD 3, n (%)</b>	8 (33)	8 (20)	0.21
<b>GOLD 4, n (%)</b>	6 (25)	5 (12)	0.19
<b>Sputum TCC x10<sup>6</sup>/g</b>	3.2 (1.3-7.3)	2.9 (1.4-5.3)	0.9
<b>Sputum neutrophils (%)*</b>	57.8 (4.7)	62.7 (3.4)	0.39
<b>Sputum eosinophils (%)</b>	3.2 (1.3-19.7)	1 (0.3-2.9)	<b>0.0016</b>
<b>AUC sputum eosinophils (%)</b>	2.99 (0.42-6)	1.1 (0.45-1.9)	<b>0.03</b>
<b>Blood total cell count (x10<sup>9</sup>/L)*</b>	7.94 (0.61)	7.86 (0.24)	0.89
<b>Blood eosinophils cell count (x10<sup>9</sup>/L)</b>	7.7 (0.5)	8 (0.3)	0.47
<b>Blood neutrophils cell count (x10<sup>9</sup>/L)</b>	0.24 (0.11-0.34)	0.18 (0.13-0.33)	0.71
<b>CRP (mg %)*</b>	4.9 (0.32)	5 (0.18)	0.67
<b>Serum total Ig-E (kU/mL)</b>	179 (139.8-	25.4 (12.9-50.4)	<0.0001
<b>Skin hypersensitivity to cat (mm)</b>	0 (0-0)	0 (0-0)	0.38
<b>Skin hypersensitivity to dog (mm)</b>	0 (0-0)	0 (0-0)	0.38
<b>Skin hypersensitivity to HDM (mm)</b>	0 (0-0)	0 (0-0)	0.24
<b>Skin hypersensitivity to Aspergillus fumigatus (mm)</b>	0 (0-0)	0 (0-0)	0.34
<b>Skin hypersensitivity to grass (mm)</b>	0 (0-0)	0 (0-0)	0.76
<b>Skin hypersensitivity to Alternaria alternata (mm)</b>	0 (0-0)	0 (0-0)	>0.9999
<b>Skin hypersensitivity to Cladosporium herbarum (mm)</b>	0 (0-0)	0 (0-0)	>0.9999
<b>Skin hypersensitivity to Penicillium (mm)</b>	0 (0-0)	0 (0-0)	0.37
<b>Skin hypersensitivity to Botrytis (mm)</b>	0 (0-0)	0 (0-0)	>0.9999

Data presented as median (IQR) unless stated, \*mean (SEM), SEM: standard error of mean. **Abbreviations:** **AUC:** area under the curve; **BDP:** Beclometasone Dipropionate; **CRP:** C-Reactive Protein; **HDM:** House dust mite; **FEV<sub>1</sub>:** Forced expiratory volume in 1 second; **FVC:** Forced vital capacity; **GOLD:** Global Initiative for chronic Obstructive Lung disease; **ICS:** inhaled corticosteroids; **TCC:** total cell count.

**Table 6-5 Baseline microbiology and health status of the Ig-E<sup>high</sup> and Ig-E<sup>low</sup> groups**

	Ig-E <sup>High</sup> (n=24)	Ig-E <sup>Low</sup> (n=41)	p value
<b>Sputum bacterial colonisation, n (%)</b>	6 (25)	7 (17)	0.44
<b>Sputum CFU unit/mL**</b>	6.3 (6.8-5.9)	6.3 (6.0-6.6)	0.98
<b>Sputum 16s (gene copies x10<sup>6</sup> /mL)**</b>	8.0 (7.6-8.4)	8.4 (8.1-8.7)	0.14
<b>Sputum colonisation for Yeast, n (%)</b>	20 (83)	33 (85)	0.89
<b>Sputum colonisation for Aspergillus fumigatus, n (%)</b>	10 (42)	18 (46)	0.73
<b>Sputum colonisation for Aspergillus nigar, n (%)</b>	2 (14)	3 (13)	0.92
<b>Sputum colonisation for Penicillium/Paecilomyces, n (%)</b>	3 (21)	1 (4)	0.11
<b>Sputum colonisation for other fungi, n (%)</b>	6 (25)	7 (18)	0.51
<b>MRC total score (unit)</b>	3 (3-4)	3 (2-4)	0.06
<b>SGRQ Symptoms (unit)*</b>	66.5 (5)	61.0 (3.2)	0.34
<b>SGRQ Activity (unit)*</b>	70.4 (4.5)	59.9 (3.7)	0.08
<b>SGRQ Impacts (unit)</b>	31.6 (26.5-47.8)	33.5 (17.5-48.6)	0.43
<b>SGRQ total score (unit)*</b>	53.3 (3.7)	47 (3.1)	0.21
<b>CRQ Emotional Functioning (unit)*</b>	4.6 (0.32)	4.7 (0.20)	0.62
<b>CRQ Fatigue (unit)*</b>	3.5 (0.30)	4.0 (0.22)	0.18
<b>CRQ Dyspnoea (unit)*</b>	3.1 (0.28)	3.7 (0.22)	0.11
<b>CRQ Mastery (unit)</b>	5.1 (3.8-6.1)	5.5 (4-6.3)	0.65
<b>CRQ total score (unit)*</b>	4.0 (0.27)	4.2 (0.21)	0.46
<b>VAS Cough (mm)</b>	41 (21-61.5)	33 (13-61)	0.60
<b>VAS Dyspnoea (mm)*</b>	54.3 (5.1)	42.7 (3.7)	0.07
<b>VAS Sputum Production (mm)*</b>	41.4 (6.3)	36.5 (4.1)	0.50
<b>VAS Sputum Purulence (mm)*</b>	28.7 (5.3)	30.0 (3.6)	0.83

Data presented as median (IQR) unless stated, \*mean (SEM), \*\*Geometric mean of Log data (95% CI). SEM: standard error of mean, CI: confidence interval. **Abbreviations:** **CFU:** colony forming unit; **CRQ:** Chronic respiratory health Questionnaire; **MRC:** Medical Research Council; **SGRQ:** St George's Respiratory Questionnaire; **VAS:** Visual analogue score.

## 6.2.5 Discussion

This is the first study to elucidate the relationship between atopy and sputum eosinophilia in COPD. Although the skin sensitivity was not different between eosinophilic and non-eosinophilic COPD subjects, but higher total serum Ig-E with more specificity (Saarinen, Juntunen *et al.* 1982), along with raised sputum eosinophil count in the Ig-E<sup>high</sup>, in contrast to Ig-E<sup>low</sup> group, as well as positive correlation between sputum eosinophilia and serum Ig-E give a strong indicator that a relationship between allergy and sputum eosinophilia in COPD is highly possible. However, previous studies report that current or prior smoking may be a



contributing factor for atopy (Venables, Topping *et al.* 1985, Goel, Singh *et al.* 2008) and since all the subjects included in the current study were current or ex-smokers and there was some association between pack-years of smoking and total serum Ig-E in the current study, the possibility of smoking being a confounding variable cannot be eliminated, although the relationship was weak and further statistical analysis did not show a significant correlation. This needs to be studied further in the future using larger number of smoker and non-smoker subjects.

Additionally, Burrows *et al* reported that skin test reactivity was negatively correlated with FEV<sub>1</sub>/FVC in current or ex-smokers subjects aged between 15 and 54 years (Burrows, Lebowitz *et al.* 1976), which is consistent with the current findings. However, this relationship did not persist when subjects with eosinophilia (Burrows, Lebowitz *et al.* 1976) and asthma (Sherrill, Lebowitz *et al.* 1995) were excluded.

In contrast with earlier studies, which revealed that BMI is higher in atopic than non-atopic subjects (Fattahi, ten Hacken *et al.* 2013), this study has not shown any significant difference. This might be explained by the fact that the average value of total serum Ig-E in the eosinophilic arm is quite lower than previous studies (Subrahmanyam, Srikantaiah *et al.* 2011), despite some variations in defining the normal ranges of total serum Ig-E (Laurent, Noirot *et al.* 1985).

A potential criticism in the present study is that total Ig-E instead of specific Ig-E was used and the skin prick test to common aeroallergens was not significantly different between the groups. This was mainly due to the retrospective nature of this study and it would be

interesting to measure specific Ig-E and include more allergens in prospective studies in the future.

#### **6.2.6 Summary**

To conclude, sputum eosinophilia is associated with atopy in COPD, although it might be a synergetic rather than causal relationship. These results therefore need to be interpreted with caution and further research is required to validate and reproduce these findings.

**Chapter 7 Is the eosinophilic airway inflammation in COPD partly due to poor eosinophil clearance or abnormal macrophage efferocytosis?**

## 7.1 COPD exacerbation severity and frequency is associated with impaired macrophage efferocytosis of eosinophils

### 7.1.1 Abstract

**Background:** Sputum eosinophilia is seen in 10-30% of COPD subjects. Whether its persistence is related to increased production of eosinophils or poor eosinophil clearance and whether impairment in their clearance by macrophages is associated with the severity and frequency of exacerbations is unknown.

**Methods:** 103 COPD subjects were categorised into 4 groups determined by the upper limit of normal for their cytoplasmic macrophage red hue (<6%), an indirect measure of macrophage efferocytosis of eosinophils, and area under the curve sputum eosinophil count ( $\geq 3\%$ /year). Eosinophil efferocytosis by monocyte-derived macrophages was studied in 17 COPD subjects and 8 normal controls.

**Results:** There were no differences in baseline lung function, health status or exacerbation frequency between the groups; A-low red hue, high sputum eosinophils (n=10), B-high red hue, high sputum eosinophils (n=16), C-low red hue, low sputum eosinophils (n=19) and D-high red hue, low sputum eosinophils (n=58). Positive bacterial culture was lower in groups A (10%) and B (6%), compared to C (44%) and D (21%) ( $p=0.01$ ). The fall in FEV<sub>1</sub> from stable to exacerbation was greatest in group A ( $\Delta$  FEV<sub>1</sub> [95% CI] -0.41 L [-0.65 to -0.17]), versus group B (-0.16 L [-0.32 to -0.011]), C (-0.11 L [-0.23 to -0.002]) and D (-0.16 L [-0.22 to -0.10];  $p=0.02$ ). Macrophage efferocytosis of eosinophils was impaired in COPD, versus controls (86 [75 to 92]%, versus 93 [88 to 96]%;  $p=0.028$ ); was most marked in group A (71 [70 to 84]%;  $p<0.05$ ) and inversely correlated with exacerbation frequency ( $r=-0.63$ ;  $p=0.006$ ).

**Conclusions:** Macrophage efferocytosis of eosinophils is impaired in COPD and is related to the severity and frequency of COPD exacerbations.

### 7.1.2 Introduction

The relationship between eosinophilic airway inflammation, clearance of these cells and clinical outcomes in COPD is poorly understood. Apoptosis and subsequent removal of dead cells by phagocytes is a critical mechanism for the non-inflammatory clearance of granulocytes, including eosinophils (Woolley, Gibson *et al.* 1996, Hallett, Leitch *et al.* 2008, Matsumoto, Terakawa *et al.* 2007, Mahajan, Madan *et al.* 2008). Failure of phagocytosis and efferocytosis, the clearance of apoptotic cells, leads to secondary necrosis of these cells and release of toxic intracellular pro-inflammatory mediators (Ward, Dransfield *et al.* 1999), which is thought to be linked to pathogenesis of many chronic inflammatory diseases including COPD and asthma (Hallett, Leitch *et al.* 2008). Impaired phagocytic ability of macrophages is consistently observed in COPD (Ferrara, D'Adda *et al.* 1996, Hodge, Hodge *et al.* 2003, Berenson, Garlipp *et al.* 2006, Taylor, Finney-Hayward *et al.* 2010, Donnelly, Barnes 2012, Berenson, Kruzel *et al.* 2013) and asthma (Simpson, Gibson *et al.* 2013), but whether this extends to abnormal efferocytosis of eosinophils in COPD, its clinical implications and usefulness to improve COPD treatment needs to be determined.

Acceleration of the excretion process of eosinophils and other inflammatory cells by stimulating apoptosis or enhancement of efferocytosis can be a potential target to pharmacological intervention to treat conditions related to ineffective removal of these cells such as COPD. Several agents thought to provoke apoptosis in granulocytes (Stern, Meagher *et al.* 1992, Morita, Lamkhioed *et al.* 1996, Ward, Dransfield *et al.* 1999, Lai, Wang *et al.*

2001, Matsumoto, Terakawa *et al.* 2007, Hallett, Leitch *et al.* 2008, Mahajan, Madan *et al.* 2008). Corticosteroids are shown to be not only efficient in halting inflammatory response, but also in motivating apoptosis of eosinophils and retarding neutrophils apoptosis, as well as promoting the phagocytic ability of macrophages (Heasman, Giles *et al.* 2003, McColl, Michlewska *et al.* 2007, Kulkarni, Hollins *et al.* 2010).

Efferocytosis of eosinophils by macrophages can be measured directly *in vitro* and indirectly *in vivo* by the assessment of macrophage cytoplasmic red hue analysed on stained sputum cytopins, which represents eosinophilic protein ingested by airway macrophages (Kulkarni, Hollins *et al.* 2010). In asthma, increased macrophage cytoplasmic red hue predicts future risk of the emergence of a sputum eosinophilia and poor asthma control following corticosteroid withdrawal (Kulkarni, Hollins *et al.* 2010). Whether this biomarker can identify clinically important subgroups with impaired eosinophil efferocytosis in COPD is unknown.

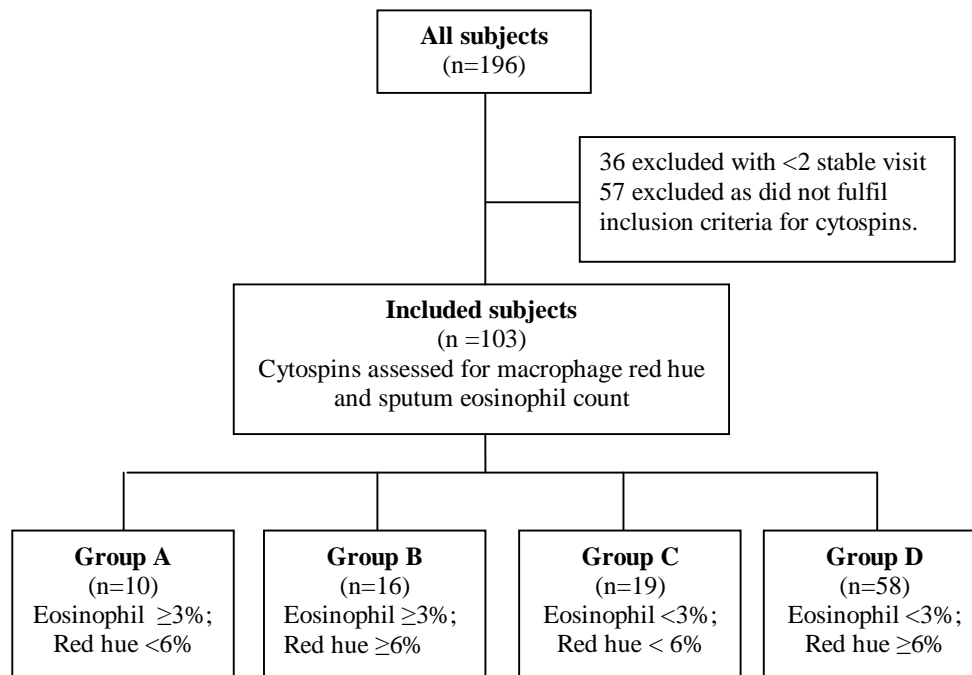
I hypothesised that i) COPD subjects categorised into subgroups determined by their sputum eosinophilia and sputum macrophage red hue will identify important differences in terms of their clinical characteristics, exacerbation frequency and severity. ii) Macrophage efferocytosis of eosinophils in COPD will be impaired; directly related to the sputum macrophage cytoplasmic red hue and indirectly associated with exacerbation frequency and severity. iii) Persistence of eosinophilic inflammation in COPD could be partly due to phagocytic dysfunction of macrophages. To test my hypotheses, I have examined sputum cytopins available from earlier studies (Bafadhel, McKenna *et al.* 2011, Bafadhel, McKenna *et al.* 2012) and prospectively assessed macrophage efferocytosis in subjects that participated in this study.

### 7.1.3 Materials and Methods

#### 7.1.3.1 Subjects and study design

Clinical data and sputum cytospins were available from 196 subjects that had participated in two observational studies of COPD exacerbations (Bafadhel, McKenna *et al.* 2011, Bafadhel, McKenna *et al.* 2012). Subjects had undergone extensive clinical characterisation as described (chapter 3.1.3.1). All COPD included subjects were either ex- or current smokers. Subjects assessed at  $\geq 2$  stable visits, with sputum cytospins of adequate quality to assess the cytoplasmic red hue of  $\geq 50$  macrophages, were included.

From the original cohort of 196 subjects, 103 subjects met the inclusion criteria (Figure 7-1). These subjects were not significantly different from the 196 in terms of lung function, symptoms or health status. I then imaged between 70-100 macrophages for each subject in Romanowsky-stained sputum cytospin slides, except in 15 subjects that had fewer macrophages in which at least 50 were imaged. The percentage area of cytoplasm with red hue was determined by thresholding. Using “Image J” software, the cytoplasmic area of macrophages is selected in saved (tiff) images (Figure 2-5). After defining the suitable threshold, the software calculates the number of red pixels, which correspond to eosinophilic staining, and the median percentage area of cytoplasm was derived as previously described (Kulkarni, Hollins *et al.* 2010). The sputum eosinophil area under the curve (AUC) was derived from the sputum samples collected at stable visits as described (6.1.3). Subjects were stratified into 4 groups based on cut-offs for the sputum eosinophil count ( $\geq 3\%$ ) and the upper limit of the normal range for %area macrophage red hue ( $>6\%$ ) (Kulkarni, Hollins *et al.* 2010), (Figure 7-1).



**Figure 7-1 Consort diagram showing the design of the study**

To study macrophage efferocytosis, 17 of the 103 COPD subjects and 8 healthy controls were prospectively recruited. These subjects underwent clinical characterisation and donated blood to generate monocyte derived macrophages (MDM). Of the remaining subjects from the 103, 19 had died, 8 were too unwell, 20 were participating in other studies and 39 were lost to follow-up. A further 17 subjects were recruited as peripheral blood eosinophil donors and these included 4 healthy atopic subjects and 13 subjects with asthma. All subjects gave written informed consent and the study was approved by the Leicestershire, Northamptonshire and Rutland local ethics committee.



### **7.1.3.2 Generation of monocyte-derived macrophages (MDM)**

Purification of monocytes from peripheral blood was performed as described (Section 2.9.2) using Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and immunomagnetic positive selection of CD14<sup>+</sup> monocytes using MS columns and CD14 microbeads (Miltenyi Biotec, Surrey, UK). The % median [IQR] purity was 97 [88-97]%) as assessed by flow cytometry as described (Section 2.9.3), using CD14-Alexa Fluor®647 conjugated antibody (Biolegend, London, UK) on a Becton Dickinson FACS Canto II (Figure 2-6) and was 98-99% as examined under the light microscope.

The monocytes were incubated for 6 days at 37 °C and 5% CO<sub>2</sub> with Dulbecco modified Eagle medium (DMEM) with high glucose, L-glutamine, D-glucose, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), without sodium pyruvate, phenol red (Fisher Scientific, UK) and supplemented with 10% foetal calf serum, 1% Non-essential amino acids, 1% mixture of antibiotics and antifungal and 1% Sodium pyruvate in the presence of recombinant human macrophage colony-stimulating factor (100 ng/mL) (R&D Systems, Europe) as described (Section 2.9.2).

### **7.1.3.3 Eosinophils purification and induction of apoptosis**

Eosinophils were immunomagnetically purified from peripheral blood 2 days before co-culture with MDM as described previously (Kulkarni, Hollins *et al.* 2010) (Section 2.9.4) (purity % median [IQR] was 98.1 [98.0-98.3]%). Apoptosis was induced by aging in culture deprived of cytokines for 48 hours at 37°C and 5% CO<sub>2</sub>. Percentages of apoptotic/dead cells were determined using Annexin V and Propidium iodide staining (BD Biosciences) and flow

cytometry as described (Section 2.9.5). The percentage of apoptosis within the first 2 hours after purification was <5% and after 48 hours, it was 97% [85-99] (% median [IQR]).

#### **7.1.3.4 MDM efferocytosis of eosinophils**

Apoptotic eosinophils were added to MDM in 1:5 ratio and incubated for 120 minutes in the same medium and conditions used to culture MDM as described (Kulkarni, Hollins *et al.* 2010) (Section 2.9.6 and 2.9.7). Cells were then fixed and permeabilised with 4% paraformaldehyde and 0.1% saponin. Immunofluorescence staining was carried out as previously described (Kulkarni, Hollins *et al.* 2010) (Section 2.9.9) with mouse monoclonal anti-human ECP (Diagnostic Development, Sweden) indirectly conjugated with RPE (Dako) and directly conjugated CD68-FITC (Dako). Efferocytosis was quantified in 100 macrophages per donor as described (Section 2.9.10) and the percentage of MDM that had ingested fully or were engulfing eosinophils was recorded. Cytospins were prepared from the efferocytosis experiments and stained with “Diff Quick” stain as per the sputum slides as described (Section 2.9.8) and %area of MDM red hue was measured prior and following feeding with eosinophils as described (Section 2.7), in order to validate the concept that red hue increases after ingestion of eosinophils (Kulkarni, Hollins *et al.* 2010) in COPD as well and verify the results of efferocytosis assay. All the slides were assessed by a single blinded observer. The observer was blinded during the counting of MDM efferocytosis from the captured images.

#### **7.1.3.5 Statistical Analysis**

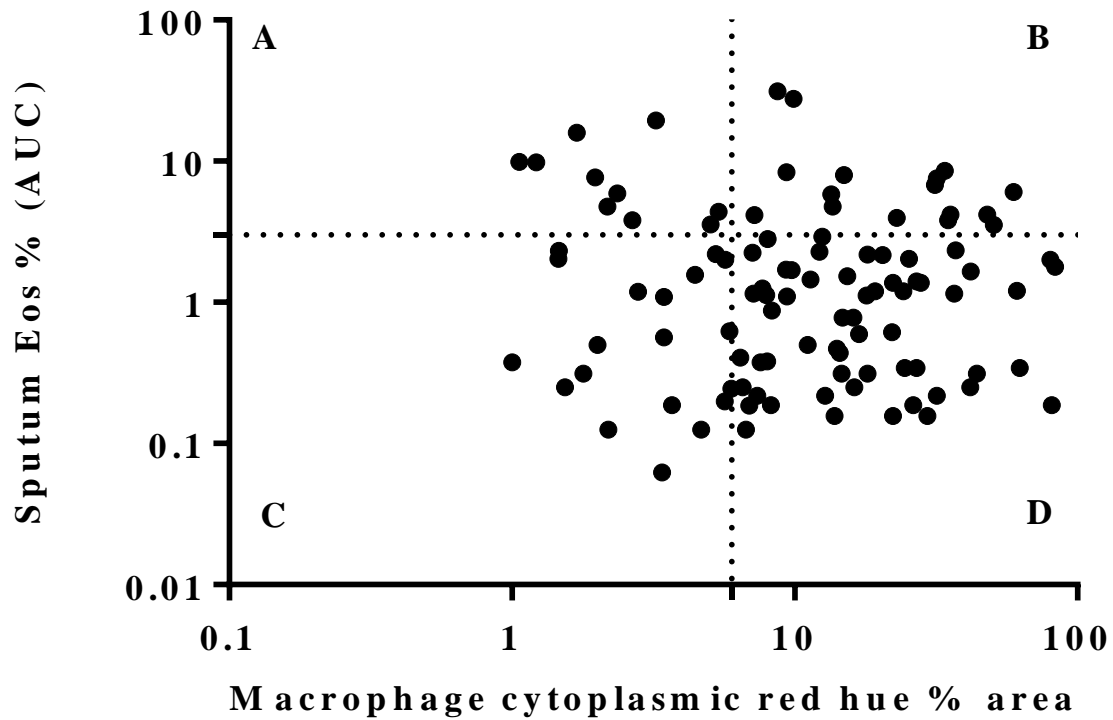
GraphPad Prism version 6 (GraphPad, San Diego) and IBM SPSS version 20 (SPSS, Inc. Chicago) were used to perform statistical analysis. Mean (standard error of the mean [SEM])

was used to present parametric data, whilst median (interquartile ranges [IQR]) was used for non-parametric data and geometric mean (95% confidence interval) for data that was log normally distributed. For comparisons between groups, unpaired T-test or Mann-Whitney was used for parametric or non- parametric data respectively. Comparisons across groups were assessed by one-way analysis of variance (ANOVA) with Tukey pair-wise comparisons or Kruskal-Wallis test with Dunn's multiple pair-wise comparisons for parametric and non-parametric data respectively. Chi-square or Fisher's exact test, as appropriate, were used to assess categorical data. Spearman or Pearson rank correlation coefficient was used to assess the correlations for parametric and non-parametric data respectively. A p value less than 0.05 was considered statistically significant.

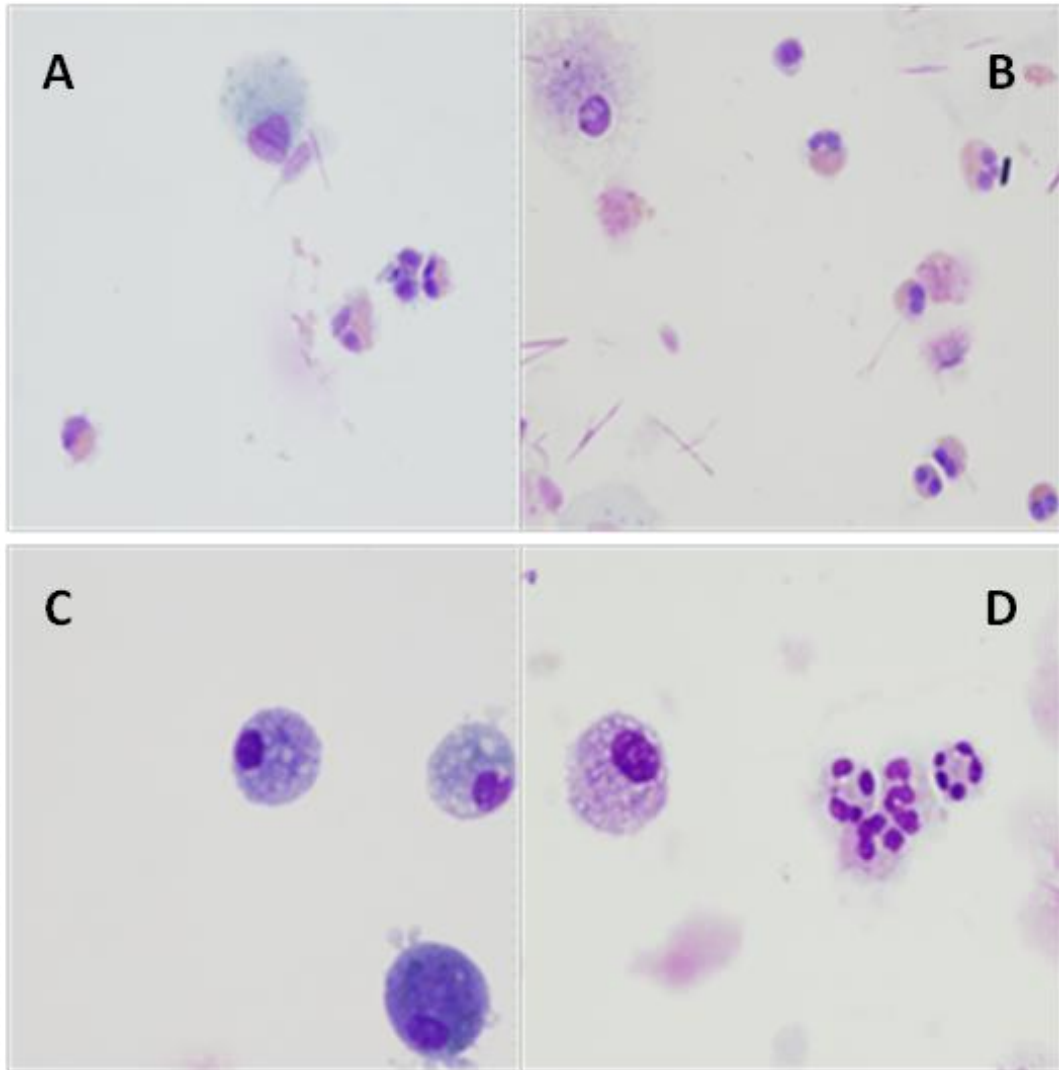
#### **7.1.4 Results**

The 103 COPD subjects were categorised into four groups; A-low red hue, high area under the curve sputum eosinophil count (n=10), B-high red hue, high sputum eosinophils (n=16), C-low red hue, low sputum eosinophil (n=19) and D-high red hue, low sputum eosinophils (n=58), (Figure 7-1). The distributions of the macrophage red hue and sputum eosinophils for the 4 groups are as shown (Figure 7-2) and example cytopins for each group are as illustrated (Figure 7-3). The baseline clinical characteristics of the groups are as shown (Table 7-1) and (Table 7-2). There were no significant differences in age, gender, health status, symptoms, use of inhaled corticosteroids or exacerbation frequency between the 4 groups. Lung function was not significantly different between groups. However, group A had a greater proportion of subjects with GOLD stage 2 and lower GOLD stage 3 than the other groups. The peripheral blood eosinophil count was elevated in groups A and B. Positive bacterial culture was lower in groups A (10%) and B (6%) (eosinophilic groups), compared

to non-eosinophilic groups) C (44%) and D (21%) ( $p=0.04$ ), although there was no difference in total bacterial colony forming units (CFU).



**Figure 7-2** Percentage area of sputum macrophage cytoplasmic red hue in COPD subjects against sputum eosinophil area under the curve (AUC) %/year. The cut-off point for the horizontal and vertical axis is 6 and 3% respectively.



**Figure 7-3 Representative images of sputum macrophages** A: Subjects with high eosinophils  $\geq 3\%$  and low red hue  $<6\%$ ; B: high eosinophils  $\geq 3\%$  and high red hue  $\geq 6\%$ ; C: low eosinophils  $<3\%$  and low red hue  $<6\%$ ; D: low eosinophils  $<3\%$  and high red hue  $>6\%$ . Group B and D subjects have purple coloured cytoplasm in their macrophages. Group A and C have light blue cytoplasm.

**Table 7-1 Baseline characteristics of the 4 groups of COPD patients**

	<b>A (n=10)</b>	<b>B (n=16)</b>	<b>C (n=19)</b>	<b>D (n=58)</b>	<b>p value</b>
<b>Male, n (%)</b>	6 (60)	13 (81.25)	8 (42.1)	42 (72.4)	0.06
<b>Age (years)*</b>	64 (3.3)	67 (2.3)	65 (2.4)	70 (1.2)	0.17
<b>Body mass index (kg/m<sup>2</sup>)</b>	26 (23-28)	23 (20-26)	25 (23-33)	27 (23-30)	0.09
<b>Packs/year smoked</b>	49 (36-58)	47 (25-66)	47 (34-60)	48 (32-64)	0.96
<b>COPD duration (years)</b>	4.7 (2.9-10)	8.5 (2.0-12)	4.8 (2.1-6.7)	4.9 (2.8-8.1)	0.67
<b>Exacerbations/yr</b>	0.7 (0-1.8)	0.8 (0.1-1.4)	1.2 (0-1.9)	0.8 (0-1.6)	0.51
<b>Frequent exacerbators, n (%)</b>	4 (40)	6 (38)	9 (47)	21 (36)	0.86
<b>ICS dose (BDP equivalent) (mcg/day)</b>	2000 (2000-2000)	2000 (1000-2000)	2000 (800-2000)	1300 (800-2000)	0.26
<b>FEV<sub>1</sub>% predicted, (%)</b>	62 (43-75)	44 (32-58)	43 (26-67)	55 (42-71)	0.16
<b>FEV<sub>1</sub> post BD (L)</b>	1.7 (1.2-1.9)	1.1 (0.91-1.6)	0.87 (0.73-	1.4 (1-1.9)	0.13
<b>Post FEV<sub>1</sub>/FVC (%)*</b>	55 (5.4)	50 (3.3)	51 (4.2)	53 (1.6)	0.73
<b>Reversibility, %</b>	3.5 (-7.1- 8)	4.3 (1.5- 13)	4.5 (-2.2- 14)	1.5 (-4.4-	0.33
<b>GOLD 1, n (%)</b>	1 (10)	1 (6)	5 (26)	8 (14)	0.38
<b>GOLD 2, n (%)</b>	7 (70)	6 (38)	3 (16)	26 (45)	<b>0.02</b>
<b>GOLD 3, n (%)</b>	0 (0)	7 (44)	4 (21)	19 (33)	<b>0.02</b>
<b>GOLD 4, n (%)</b>	2 (20)	2 (13)	7 (37)	5 (9)	<b>0.05</b>
<b>Sputum TCC x10<sup>6</sup>/g</b>	1.2 (0.41-2)	5.3 (2.1-8.2)	1.6 (0.94-4.5)	3.4 (1.7-7.6)	<b>0.002</b>
<b>Sputum neutrophils (%)</b>	45 (6.3)	59 (6.1)	66 (5.3)	63 (2.7)	0.07
<b>Sputum eosinophils (%)</b>	9 (1.6-35)	14 (4.9-24)	0.5 (0.25-2.8)	1 (0.3-2.1)	<b>&lt;0.0001</b>
<b>Sputum eosinophil AUC</b>	6.8 (4.2-11)	5.9 (4.2-8.3)	0.5 (0.2-1.6)	0.78 (0.3-1.5)	<b>&lt;0.0001</b>
<b>% of red hue of macrophages</b>	2.3 (1.6-3.7)	27 (11-35)	3.4 (1.8-5.3)	16 (9.1-27)	<b>&lt;0.0001</b>
<b>PB neutrophils (x10<sup>9</sup>/L)</b>	5.1 (3.5-5.8)	4.8 (3.8-5.9)	5.9 (4.7-6.3)	5.3 (3.9-6.1)	0.13
<b>PB eosinophils (x10<sup>9</sup>/L)</b>	0.38	0.41	0.16	0.18	<b>&lt;0.0001</b>
<b>CRP (mg/dl)</b>	5 (2.5-5.3)	5 (<5-7)	5 (2.5-11)	5 (<5-11)	0.08
<b>Bacterial colonisation, n (%)</b>	1 (10)	1 (6)	8 (44)	12 (21)	<b>0.04</b>
<b>CFU unit/mL**</b>	6.2 (5.8-6.6)	6.1 (5.5-6.7)	6.6 (6.3-7)	6.2 (5.9-6.4)	0.19

Data presented as median (interquartile range) unless stated, \*mean (SEM), \*\*Geometric mean of Log data (95% CI). SEM: standard error of mean, CI: confidence interval. **Abbreviations:** AUC: area under the curve; **BDP:** Beclometasone Dipropionate; **CFU:** colony forming unit; **CRP:** C-Reactive Protein; **FEV<sub>1</sub>:** Forced expiratory volume in 1 second; **FVC:** Forced vital capacity; **ICS:** inhaled corticosteroids, **MRC:** Medical Research Council; **PB:** peripheral blood; **TCC:** total cell count; **yr:** Year.

**Table 7-2 Health status and symptom scores of 4 the groups of COPD patients at stable visit**

	<b>A (n=10)</b>	<b>B (n=16)</b>	<b>C (n=19)</b>	<b>D (n=58)</b>	<b>p value</b>
<b>MRC Dyspnoea Scale</b>	3 (2-4)	3 (2-4)	3 (2.5-4.5)	3 (2-4)	0.82
<b>SGRQ Symptoms (unit)</b>	61 (37-80)	65 (29-82)	73 (55-84)	62 (50-77)	0.34
<b>SGRQ Activity(unit)</b>	76 (35-89)	73 (48-85)	73 (59-92)	67 (48-86)	0.55
<b>SGRQ Impacts (unit)</b>	31 (16-58)	44 (20-66)	42 (29-47)	32 (23-51)	0.86
<b>SGRQ total score (unit)</b>	50 (30-67)	55 (32-71)	57 (49-65)	50 (37-65)	0.75
<b>CRQ Emotional Functioning (unit)</b>	4.7 (3.4-5.8)	4.3 (3.9-6.3)	4.4 (3.9-5.3)	4.5 (3.6-5.6)	0.83
<b>CRQ Fatigue (unit)</b>	3.8 (3.4-4.9)	3.3 (2.8-5.3)	4.0 (2.3-4.8)	3.5 (2.5-4.8)	0.51
<b>CRQ Dyspnoea (unit)</b>	4 (3-4.9)	3.4 (2.6-4.2)	3 (2.2-4)	3.4 (2.2-4.4)	0.28
<b>CRQ Mastery (unit)</b>	5.4 (4.4-6.4)	5.0 (3.8-6.3)	4.0 (3.8-5.5)	5.3 (3.7-6.1)	0.63
<b>CRQ total score (unit)</b>	4.4 (3.7-5.2)	4.1 (3.4-4.9)	4.0 (3.1-4.6)	4.2 (3.1-5.0)	0.50
<b>VAS Cough (mm)</b>	17 (10-43)	35 (14-52)	47 (18-62)	34 (13-54)	0.23
<b>VAS Dyspnoea (mm)</b>	40 (4.8-59)	43 (32-81)	51 (35-62)	46 (22-65)	0.40
<b>VAS Sputum Production (mm)</b>	15 (4.5-42)	36 (19-52)	38 (93-66)	28 (12-63)	0.40
<b>VAS Sputum Purulence (mm)</b>	14 (2-61)	17 (1-45)	38 (15-62)	21 (9.5-41)	0.41

Data presented as median (interquartile range) unless stated. **Abbreviations:** **CRQ:** Chronic respiratory health Questionnaire; **GOLD:** Global Initiative for chronic Obstructive Lung disease; **MRC:** Medical Research Council; **SGRQ:** St George's Respiratory Questionnaire; **VAS:** Visual analogue score.

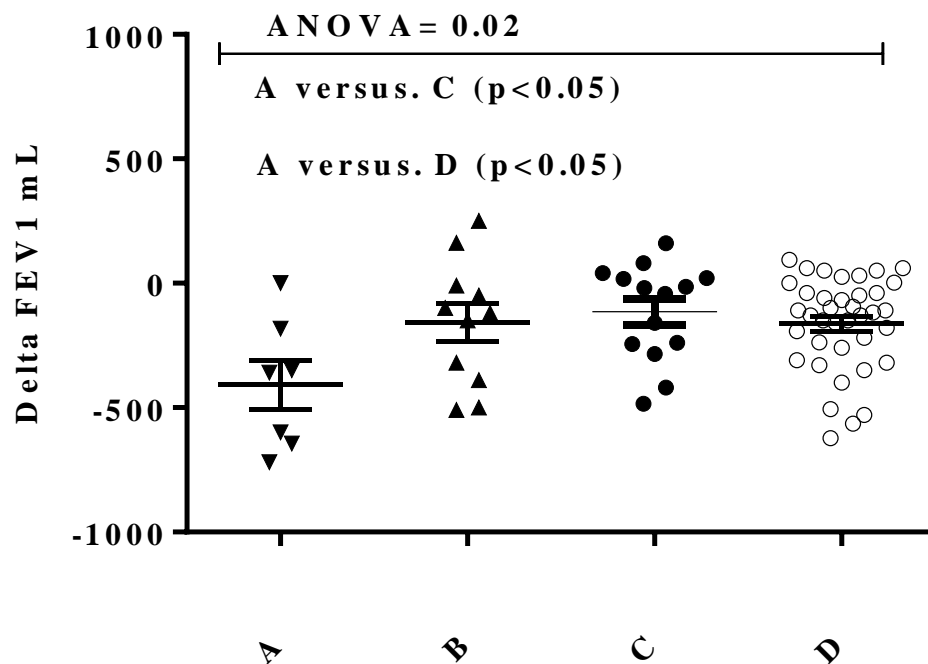
The change in lung function, health status and symptoms at the first exacerbation was compared to the mean of the prior stable visits. The change in FEV<sub>1</sub> was greatest in group A ( $\Delta$  FEV<sub>1</sub> [95% CI] -0.41 L [-0.65 to -0.17]) in group A, compared to group B (-0.16 L [-0.33 to -0.011]), C (-0.11 L [-0.23 to -0.002]) and D (-0.16 L [-0.22 to -0.10]; p=0.02), (Figure 7-4). Health status and symptoms worsened at exacerbation in all groups, but there were no differences between groups (Table 7-3).

**Table 7-3 Change in health status and symptom scores of 4 the groups of COPD patients at exacerbations compared to stable visits**

	<b>A (n=10)</b>	<b>B (n=16)</b>	<b>C (n=19)</b>	<b>D (n=58)</b>	<b>p value</b>
<b>Δ CRQ Fatigue (unit)</b>	-1.36 (-2.61 to -0.12)*	-1.33 (-2.35 to -0.31)*	-1.11 (-1.76 to -0.45)*	-1.00 (-2.61 to -0.12)*	0.78
<b>Δ CRQ Dyspnoea (unit)</b>	-1.49 (-2.92 to -0.05)*	-1.05 (-2.32 to 0.21)*	-1.04 (-1.41 to -0.67)*	-0.57 (-0.98 to -0.15)*	0.34
<b>Δ CRQ Emotion (unit)</b>	-1.22 (-1.52 to -0.91)*	-1.07 (-2.00 to -0.14)*	-0.62 (-1.27 to 0.02)*	-0.89 (-1.31 to -0.46)*	0.67
<b>Δ CRQ Mastery (unit)</b>	-0.99 (-2.33 to 0.36)*	-0.97 (-2.28 to 0.34)*	-1.10 (-1.99 to -0.21)*	-0.75 (-1.09 to -0.41)*	0.59
<b>Δ CRQ total (unit)</b>	-1.26 (-2.21 to -0.32)*	-1.12 (-1.99 to -0.22)*	-0.97 (-1.49 to -0.44)*	-0.79 (-1.04 to -0.55)*	0.54
<b>Δ VAS cough (mm)</b>	22 (3 to 42)*	35 (21 to 48)*	7 (3 to 42)*	27 (18 to 36)*	0.06
<b>ΔVAS dyspnoea (mm)</b>	34 (3 to 65)*	35 (20 to 51)*	17 (-2 to 35)	25 (17 to 33)*	0.31
<b>Δ VAS sputum production (mm)</b>	8 (-32 to 49)	24 (-2 to 50)	9 (-2 to 21)	21 (12 to 29)*	0.45
<b>ΔVAS sputum purulence (mm)</b>	28 (-13 to 68)	18 (-5 to 42)	31(17 to 45)*	15 (5 to 24)*	0.36

Data presented as mean (95% CI) unless stated; \* p<0.05 stable versus exacerbation.





**Figure 7-4 Change of FEV<sub>1</sub> between stable and first exacerbation visits for the 4 groups.**

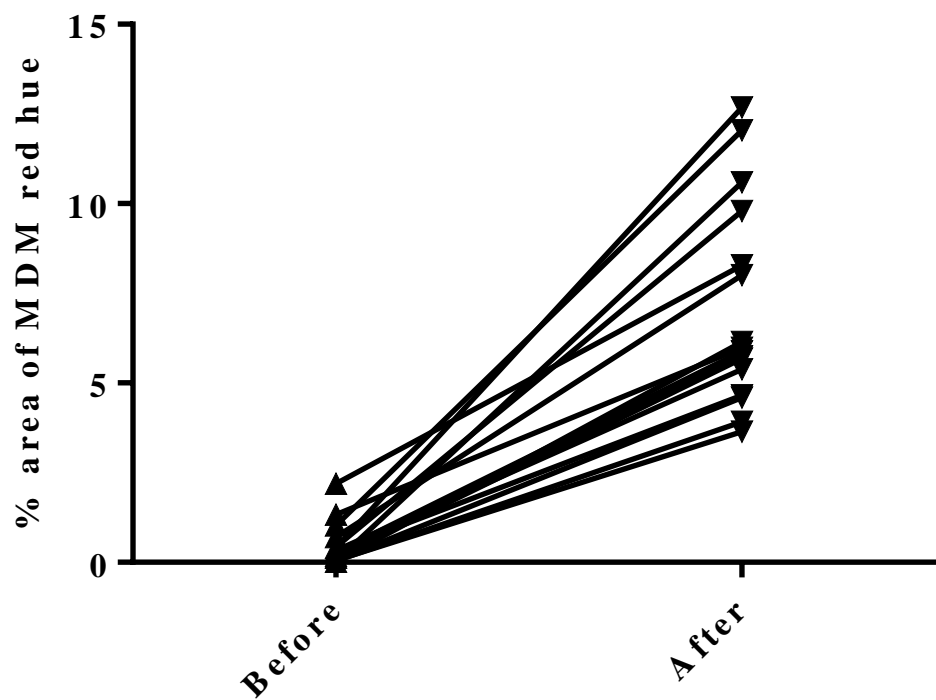
The lines represent mean (SEM).

The clinical characteristics of the COPD subjects and healthy volunteers that provided blood to generate MDM are as shown (Table 7-4). There were no significant differences in age, gender, lung function, or dose of inhaled corticosteroids between the 4 COPD groups (ANOVA  $p=0.26$ ). Examples of MDM either undergoing eosinophil efferocytosis or not are shown (Figure 2-9). As expected, MDM efferocytosis of eosinophils was significantly correlated with sputum macrophage cytoplasmic red hue (Spearman  $r=0.54$ ;  $p=0.027$ ). Furthermore, there was a significant increase in mean [SEM] % area of MDM red hue from 0.5 [0.2] before to 7.1 [0.7]% following COPD-derived MDM feeding with eosinophils ( $p<0.0001$ ), (Figure 7-5). The mean [SEM]% area of MDM red hue after feeding with eosinophils was significantly lower in COPD subjects compared to control (5.8 [0.6]%, versus 10.3 [1.5]% respectively;  $p=0.027$ ), (Figure 7-6).

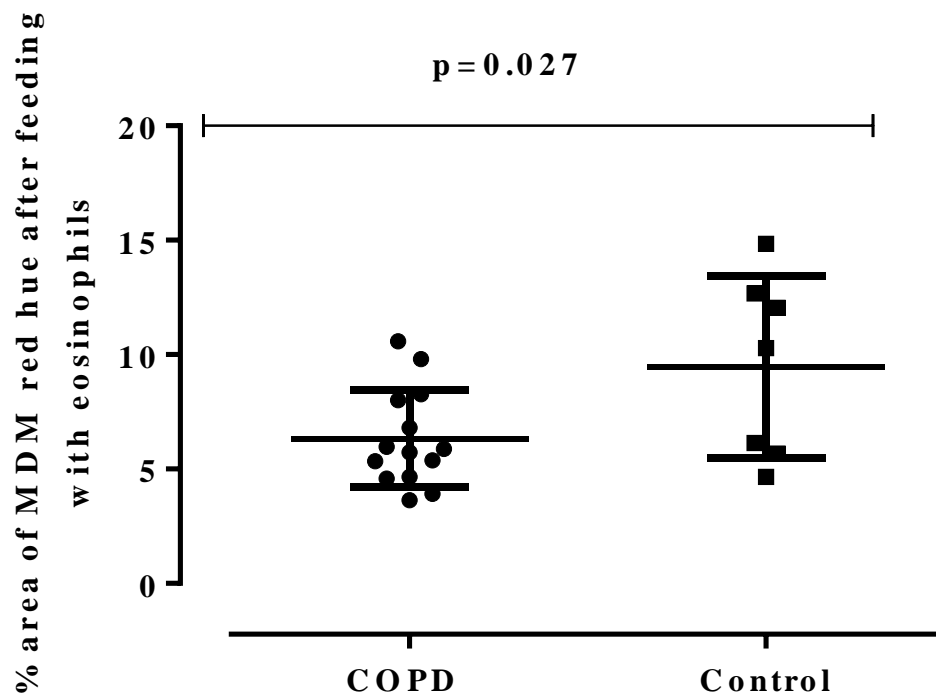
**Table 7-4 Baseline characteristics of the COPD and healthy subjects that donated blood for the MDM eosinophil efferocytosis assays**

	<b>COPD (n=17)</b>	<b>Control (n=8)</b>	<b>P value</b>
<b>Male, n (%)</b>	10 (58.8)	4 (50)	1.0
<b>Age (years)*</b>	68.05 (2.3)	54.1 (4.7)	<b>0.006</b>
<b>Body mass index (kg/m<sup>2</sup>)</b>	27.17 (1.36)	33 (29-34)	0.07
<b>Never-smokers n (%)</b>	0 (0)	6 (75)	<b>0.0002</b>
<b>Packs/year smoked</b>	42(31-70)	13 (5-22)	0.05
<b>COPD duration (years)</b>	9.6 (8-13)	NA	NA
<b>Exacerbations in last year</b>	1 (1-2)	NA	NA
<b>ICS dose (BDP equivalent) (mcg/day)</b>	2000 (1600-2000)	NA	NA
<b>FEV<sub>1</sub>% predicted, (%)</b>	43 (5.1)	95 (85-110)	<b>&lt;0.0001</b>
<b>FEV<sub>1</sub> post bronchodilator (L)</b>	1.13 (0.14)	2.8 (2.5-3.8)	<b>0.0004</b>
<b>Post FEV<sub>1</sub>/FVC (%)</b>	48.6 (4.23)	84.08 (1.7)	<b>&lt;0.0001</b>
<b>Reversibility, %</b>	5.67 (2.17)	0.57 (-1.9-9)	0.45
<b>Sputum Total cell count x10<sup>6</sup>/g</b>	3.3 (1.5-6.7)	NA	NA
<b>Sputum neutrophils (%)</b>	65 (40-80)	NA	NA
<b>Sputum eosinophils (%)</b>	7.3 (4.5-15)	NA	NA
<b>GOLD 1, n (%)</b>	0 (0)	NA	NA
<b>GOLD 2, n (%)</b>	7 (41.2)	NA	NA
<b>GOLD 3, n (%)</b>	5 (29.4)	NA	NA
<b>GOLD 4, n (%)</b>	5 (29.4)	NA	NA
<b>Blood neutrophils (x10<sup>9</sup>/L)</b>	4.66 (3.67-5.94)	3.5 (2.6-4.5)	<b>0.03</b>
<b>Blood eosinophils (x10<sup>9</sup>/L)</b>	0.21 (0.08-0.36)	0.15 (0.11-0.34)	0.86
<b>CRP (mg/dl)</b>	5 (<5-8)	5 (<5-5.75)	0.95
<b>Bacterial colonisation, n (%)</b>	3 (17.6)	NA	NA
<b>CFU unit/mL**</b>	6.5 (5.6-7)	NA	NA
<b>MRC Dyspnoea Scale</b>	3(2-4)	NA	NA
<b>SGRQ total score (unit)</b>	54 (41-66)	NA	NA
<b>CRQ total score (unit)</b>	4.1 (3.2-5.7)	NA	NA
<b>VAS Cough (mm)</b>	26 (11.5-67.5)	NA	NA
<b>VAS Dyspnoea (mm)</b>	50 (36-64)	NA	NA
<b>VAS Sputum Production (mm)</b>	23.7 (6-63)	NA	NA
<b>VAS Sputum Purulence (mm)</b>	12.4 (6-62)	NA	NA

Data presented as median (interquartile range) unless stated, \*mean (SEM), \*\*Geometric mean of Log data (95% CI). SEM: standard error of mean, CI: confidence interval. **Abbreviations:** **AUC:** area under the curve; **BDP:** Beclometasone Dipropionate; **CFU:** colony forming unit; **CRQ:** Chronic respiratory health Questionnaire; **CRP:** C-Reactive Protein; **FEV<sub>1</sub>:** Forced expiratory volume in 1 second; **FVC:** Forced vital capacity; **GOLD:** Global Initiative for chronic Obstructive Lung disease; **ICS:** inhaled corticosteroids, **MRC:** Medical Research Council; **NA:** Not available; **SGRQ:** St George's Respiratory Questionnaire; **TCC:** total cell count; **VAS:** Visual analogue score.

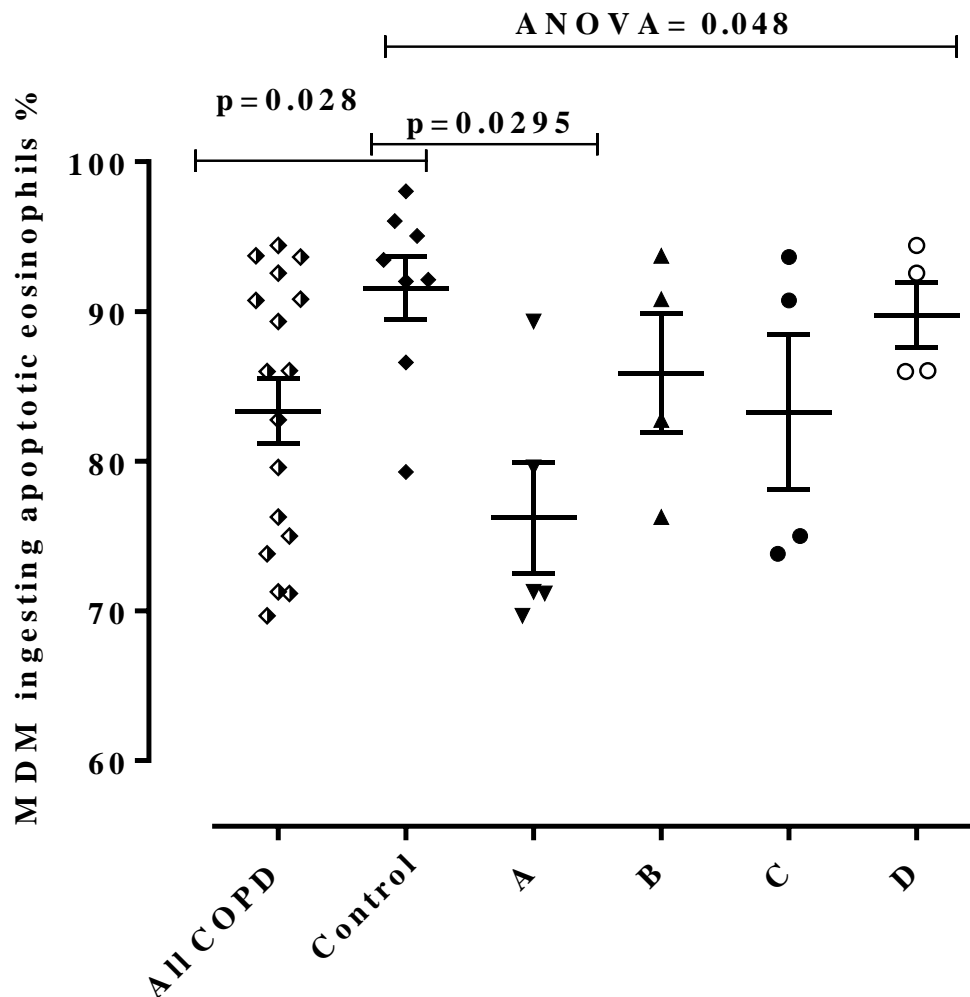


**Figure 7-5 MDM % red hue from stained cytopins before and after eosinophil feeding for both COPD and normal subjects. Arrows represent medians of percentage area of red hue for each subject.**



**Figure 7-6 Red hue % area of MDM following feeding with eosinophils in COPD versus control.** The horizontal bars represent the means (SEM).

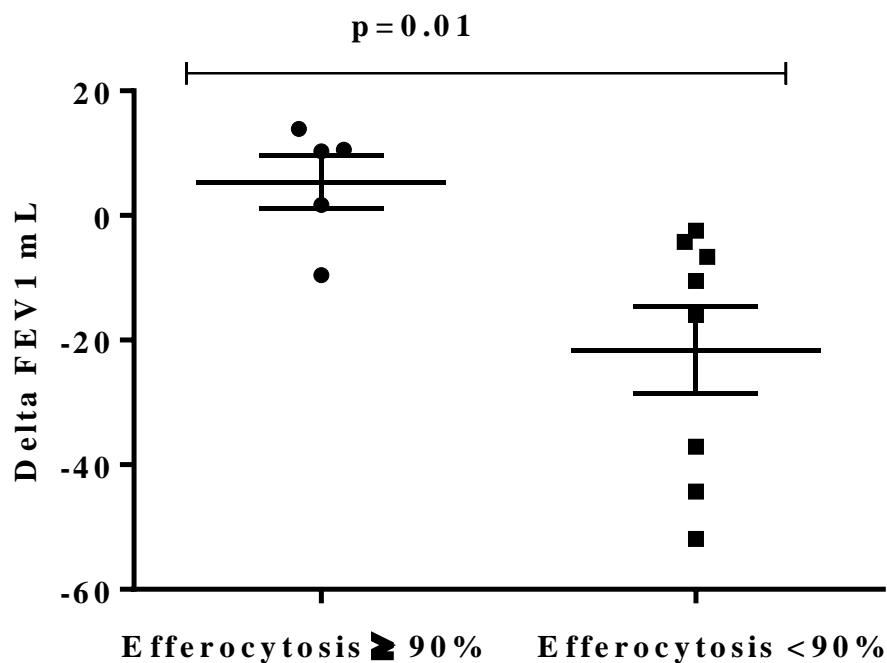
The median [IQR] proportion of MDM that efferocytosed eosinophils was impaired in COPD patients 86 [75 to 92]%, compared to controls 93 [88 to 96]%, ( $p=0.028$ ), (Figure 7-7).



**Figure 7-7 Comparison of macrophage efferocytosis of eosinophils in the 4 COPD groups and normal controls.** Horizontal bars represent the medians (IQR).

Even though the healthy controls were younger than the subjects with COPD, there was no significant difference in efferocytosis of eosinophils by macrophages between the 5 control subjects who were  $<60$  years old and the 3 subjects that were  $\geq 60$  years old ( $p=0.14$ ). In addition, there was no significant correlation between MDM efferocytosis of eosinophils and

age in the COPD subjects or COPD subjects and healthy controls combined. Likewise, there was no relationship between smoking status or pack-years of smoking and MDM efferocytosis of eosinophils in COPD subjects. There were no significant correlations between health status or symptoms and MDM efferocytosis of eosinophils. The fall in FEV<sub>1</sub> at exacerbation was greater in those COPD subjects with <90% (n=8), versus ≥90% MDM eosinophil efferocytosis (n=5), mean delta FEV<sub>1</sub> [95% confidence interval] (-22 [-5 to -38]%, versus 5 [18 to -6]%; p=0.016), (Figure 7-8).

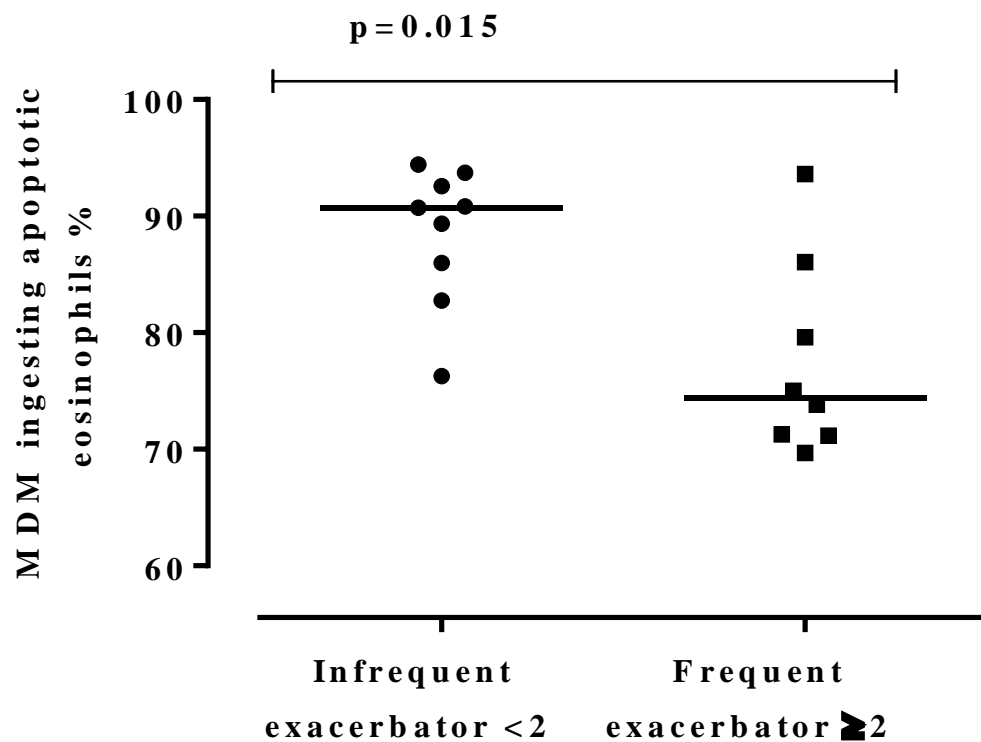


**Figure 7-8 Dot-plot of delta change of FEV<sub>1</sub> at exacerbation in those subjects with ≥90% and <90% MDM eosinophil efferocytosis. Horizontal bars are means (SEM).**

The MDM efferocytosis of eosinophils was significantly different between the 4 COPD subgroups and healthy controls (Kruskal-Wallis p=0.048). Post-hoc pairwise comparisons demonstrated that impairment of efferocytosis was greatest in group A, those subjects with

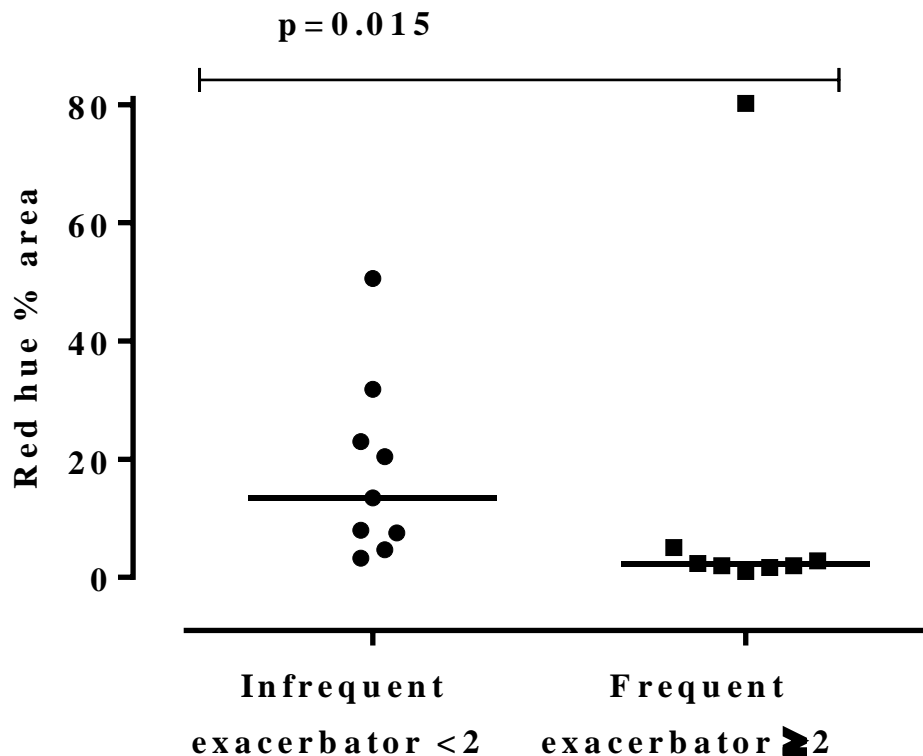
high sputum eosinophils and low red hue (71 [70 to 84]%) and was greatly lower than the healthy controls (93 [88 to 96]%;  $p=0.0295$ ), (Figure 7-7), but was not significantly different to the other COPD groups.

The median [IQR] proportion of macrophages that had efferocytosed eosinophils was decreased in those subjects with frequent exacerbations  $\geq 2$  compared to those with  $<2$  exacerbation/in the last year, 74 [71 to 84]%, versus 91 [84 to 93]%; ( $p=0.015$ ), (Figure 7-9).



**Figure 7-9** Dot-plot of macrophage efferocytosis of eosinophils in those subjects with or without frequent exacerbations ( $\geq 2$  exacerbations/year). Horizontal bars are medians.

The median [IQR] red hue of sputum macrophages was also significantly lower in those subjects with frequent exacerbations  $\geq 2$  compared to those with  $<2$  exacerbation/in the last 12 months, 2.2 [1.8 to 4.5]% versus 13.5 [6.1 to 27.4]%; ( $p=0.015$ ), (Figure 7-10).

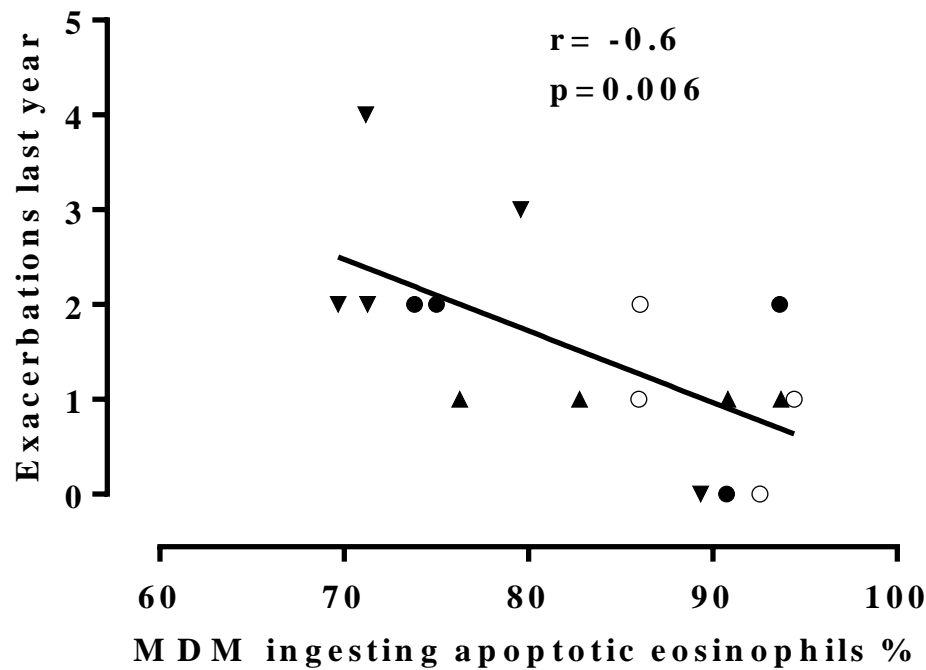


**Figure 7-10 Dot-plot of red hue % area of sputum macrophages in those subjects with or without frequent exacerbations ( $\geq 2$  exacerbations/year). Horizontal bars are medians.**

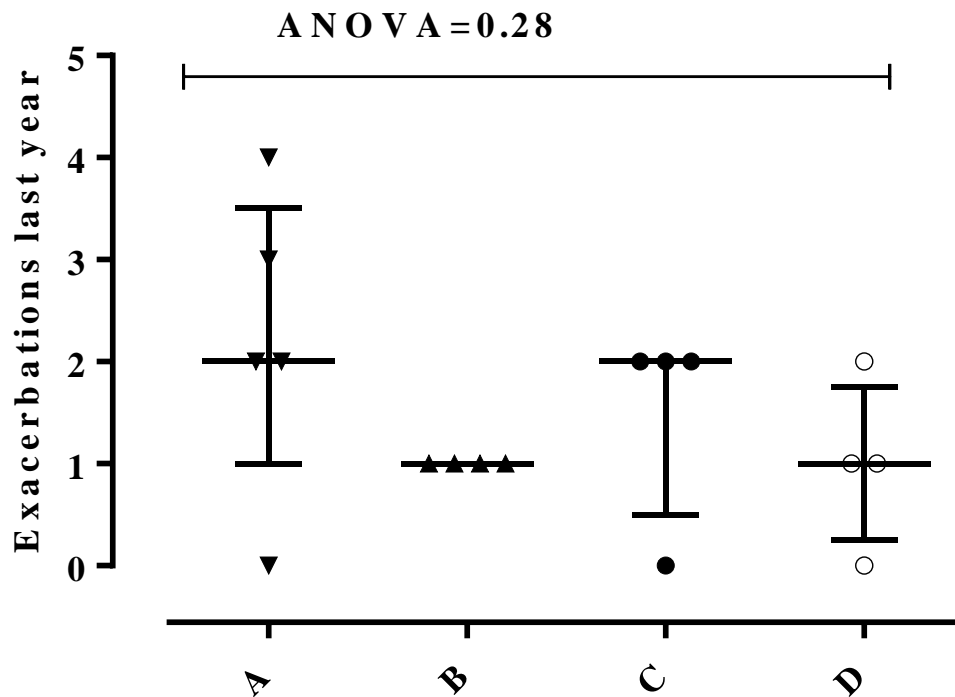
There was a strong inverse correlation between the exacerbation frequency in the last year and MDM efferocytosis of eosinophils (Spearman's  $r=-0.60$ ;  $p=0.006$ ), (Figure 7-11). However, with the inclusion of the total follow-up period available prior to the efferocytosis assessment (median [IQR] follow-up period 3.2 [2.1 to 3.5] years), this correlation was lost ( $r=-0.29$ ;  $p=0.26$ ). In addition, the frequency of exacerbations in the last 12 months was



slightly higher in group A and C (median [IQR] 2 [1 to 3.5], 2 [0.5 to 2]), in contrast to B and D (1 [1 to 1] and 1[0.25 to 1.75]) respectively, although the difference between them was not statistically significant (Kruskal-Wallis  $p=0.28$ ), (Figure 7-12).



**Figure 7-11 Scatter plot demonstrating the inverse correlation between the efferocytosis of eosinophils by macrophages and the frequency of exacerbations.** Group A: triangles pointed down, B: triangles pointed up, C: closed circles, D: open circles.



**Figure 7-12 Dot-plot of frequency of exacerbations across the 4 COPD groups.**

Horizontal bars are medians (IQR).

There was no correlation between GOLD stage and efferocytosis, nor with red hue in the 17 COPD patients in the sub-study.

### 7.1.5 Discussion

Here I report for the first time that macrophage efferocytosis of eosinophils is impaired in COPD and is related to increased exacerbation frequency and severity. MDM efferocytosis of eosinophils was assessed directly *in vitro* and indirectly *in vivo* by the assessment of the sputum macrophage cytoplasmic red hue. This index of macrophage function together with the sputum eosinophil count measured over time, allowed us to identify 4 subgroups of COPD segmented into those with high or low sputum eosinophil counts and high or low macrophage red hue. The group with high sputum eosinophil count and low red hue *in vivo* is predicted to represent those subjects with the greatest impairment in MDM efferocytosis of eosinophils, which was confirmed *in vitro*. This group had the greatest fall in lung function during exacerbations. Exacerbation frequency was associated with MDM efferocytosis of eosinophils and impairment was greatest in those with evidence of frequent exacerbations in the last year. Taken together, these findings suggest that macrophage dysfunction in COPD might play an important role in the persistence of eosinophilic inflammation in some subjects, which in turn is related to the severity and frequency of exacerbations.

This is the first study in COPD to apply the cytoplasmic macrophage red hue, an index validated in asthma as a specific biomarker of exposure to and efferocytosis of eosinophils, which had an excellent interobserver and intraobserver repeatability (Kulkarni, Hollins *et al.* 2010). A high cytoplasmic red hue suggests that the airway macrophages are both exposed to eosinophils and are able to competently efferocytose apoptotic cells (Kulkarni, Hollins *et al.* 2010). A low red hue suggests either lack of exposure over time or impaired eosinophil efferocytosis. Indeed, macrophage red hue was correlated with MDM efferocytosis and MDM red hue increased substantially following eosinophil efferocytosis. The application of this index reveals hitherto unrecognised features of COPD. Firstly, the majority of subjects

had high red hue and normal sputum eosinophil counts suggesting that the contribution of the eosinophil to the total inflammatory burden in COPD might be under-estimated. Applying the findings gained from chapter 5, that the blood and bronchial eosinophils are positively correlated, showed that 38/58 (58.5%) of these patients had blood eosinophils% equal or greater than 2% using the cut-off utilised in Bafadhel's clinical study (Bafadhel, McKenna *et al.* 2012) and 32/58 (54%) had 2.3%. using the group median employed in chapter 5, which suggests that these subjects may had a good reservoir of eosinophils in their bronchial tissue that resulted in high red hue. Indeed, only 18% of subjects had neither high sputum eosinophils nor high red hue. Secondly, some subjects that are exposed to eosinophils have impaired eosinophil clearance. This was confirmed by direct assessment of MDM efferocytosis of eosinophils suggesting that it is an intrinsic abnormality observed in peripheral blood derived cells rather than secondary to the airway environment. This failure of macrophage function in COPD adds to the growing evidence of impairment in macrophage efferocytosis and phagocytosis. To date, this has been considered a phenomenon that promotes bacterial colonisation (Ferrara, D'Adda *et al.* 1996, Berenson, Garlipp *et al.* 2006, Taylor, Finney-Hayward *et al.* 2010, Berenson, Kruzel *et al.* 2013). However, I have identified that impaired efferocytosis can occur in subjects with high sputum eosinophil counts and low bacterial colonisation. Therefore, a more plausible explanation is that impaired macrophage function acts as an amplification of the underlying abnormal innate immunity or inflammatory profile rather than being a primary event. This also suggests that strategies that promote macrophage function (Ferrara, D'Adda *et al.* 1996, Harvey, Thimmulappa *et al.* 2011) might be best targeted at those subjects with evidence of dysfunction rather than those with bacterial colonisation *per se*.

Interestingly, the subjects that had persistently high sputum eosinophilia with a low red hue were those that had the greatest impairment in MDM efferocytosis of eosinophils and the greatest fall in FEV<sub>1</sub> at exacerbations. Additionally, there was also a strong relationship between exacerbation frequency and MDM efferocytosis of eosinophils. Indeed, macrophage function was impaired in those subjects with frequent exacerbations ( $\geq 2$ /year) compared to those without (Hurst, Vestbo *et al.* 2010). However, this relationship did not persist retrospectively beyond the year prior to the assessment of the MDM efferocytosis, suggesting that this relationship is more variable. Future studies will need to evaluate the stability of this relationship. Notwithstanding this potential limitation, this group might represent those that are at greatest risk and would warrant further eosinophil-specific therapy such as anti-IL-5, which would likely reduce the overall eosinophil burden as observed in asthma (Halder, Brightling *et al.* 2009, Pavord, Korn *et al.* 2012).

On the other hand, the results of this study neither support the hypothesis, that persistence of eosinophilic inflammation in COPD is secondary to macrophage dysfunction, nor refute it, as the impaired efferocytosis is observed mainly in group A, with high eosinophils and low red hue, but not clear why (group D), those with low eosinophils count and high red hue, consistently have sputum eosinophilia despite their efferocytosis is not much different from normal control. However, the small number in the subgroups might be a plausible explanation for this and it is likely that if more subjects were included in group D, then it may have shown statistically significant difference in efferocytosis impairment in this group compared to control.

One of the strengths of this study was the ability to use data from a longitudinal observational study of COPD subjects extensively studied in stable state and at exacerbations. Although,

this was a strength for the comparison of macrophage cytoplasmic red hue and sputum eosinophil counts across groups, one important criticism is that the number of subjects in each of the COPD subgroups that were recruited to study MDM efferocytosis of eosinophils was small. This was a consequence of the inability to recall many of these subjects. Additionally, although the inter- and intra-observer variability of the sputum macrophage red hue is excellent, the reproducibility of this measure and how it varies dynamically with sputum eosinophil count overtime and in response to exacerbations is unknown and requires further study. Another potential drawback is that the normal controls in the current study were not well matched to the COPD subjects. The normal controls were younger and had a lower smoking pack years history than the COPD subjects. However, I have shown that age and smoking history, albeit in contrast to previous studies (Phipps, Aronoff *et al.* 2010) were not correlated with MDM eosinophil efferocytosis, Nevertheless, further comparisons in larger populations of healthy volunteers are required to explore the effects of age upon macrophage function. The effects observed in the COPD subjects was also independent of age, therefore although age might contribute to macrophage dysfunction, it is unlikely that it exerts a major influence upon the observations that have been made here. In addition, the normal ranges for macrophage red hue were derived from an earlier work (Kulkarni, Hollins *et al.* 2010). Although, it is agreed that this does not represent a large population study of the normal range of macrophage red hue, but does represent the largest study to date. Another minor potential critique is that eosinophils were obtained from subjects with asthma and or other allergic diseases. However, there was a strong correlation between red hue and efferocytosis in this study irrespective of the donor's diagnosis. Thus, the differences observed here between health and disease are likely to be real, but need to be interpreted with caution. Together these limitations underscore the need for a larger multi-centre study, including assessment of alveolar macrophages, to validate and replicate these findings.

Furthermore, the underlying mechanisms driving the macrophage dysfunction observed here have not been explored and this needs to be addressed in future studies.

#### **7.1.6 Conclusions**

In summary, it has been demonstrated using a combination of *in vitro* and *in vivo* approaches that macrophage efferocytosis of eosinophils is impaired in COPD. The severity and frequency of COPD exacerbations was related to impaired macrophage efferocytosis of eosinophils.

## **Chapter 8    What is the dynamic of eosinophil clearance in an exacerbation event?**



## **8.1 The dynamics of eosinophil clearance during exacerbations of chronic obstructive pulmonary disease**

### **8.1.1 Abstract**

**Background:** 10-30% of COPD subjects have eosinophilic airway inflammation. Airway macrophages cytoplasmic red hue is an indirect biomarker of eosinophils clearance (efferocytosis) that increases in response to corticosteroids in asthma. Macrophage efferocytosis of eosinophils is impaired in COPD patients with high sputum eosinophil count and low red hue. The dynamic of red hue% in COPD exacerbation was not explored before. Whether the response of the red hue to corticosteroid in COPD is similar to asthma and whether it is the same in all the COPD subgroups is not yet known.

**Methods:** Colour analysis was performed on digital images of macrophages from routinely stained sputum cytopsin slides of 30 COPD subjects at baseline, exacerbations, two weeks and six weeks visit after exacerbation (recovery). Corresponding sputum and blood eosinophils counts, lung function and health status were assessed concomitantly.

**Results:** There was a significant increase in red hue percentage area of macrophages, (median [IQR]%) from (9 [6-26.7]%) at baseline to (48.5 [25-60]%) during exacerbation with a peak at two weeks post treatment with prednisolone (53.5 [38-69]%) and a small decline at recovery period (46.9 [30-71]%;  $p < 0.0001$ ). Sputum eosinophils decreased, but less markedly over the same period of time. The rise of red hue was markedly less in group A subjects (with high sputum eosinophil count and low red hue%) and there was also a decline of red hue% in group C (with low sputum eosinophil count and low red hue%). There was no correlation between the red hue% and percentage of sputum eosinophils in exacerbations and follow up, nor with blood eosinophils, lung function or health status.

**Conclusions:** Red hue percentage area of macrophages increases in response to corticosteroids treatment in COPD, but less markedly in those with impaired efferocytosis to eosinophils. There might be an under-estimation of eosinophilic airways inflammation burden in COPD.

### 8.1.2 Introduction

Chronic obstructive pulmonary disease is a heterogeneous disease with identification of a subgroup of COPD subjects with sputum eosinophilia (Eltboli, Brightling 2013, Barker, Brightling 2013). There is a controversy about the role of eosinophilic inflammation in COPD, which is repeatedly observed in 10-30% of COPD subjects with several times increase during exacerbation (Brightling 2006, Saha, Brightling 2006) and a favourite response to inhaled (Brightling, McKenna *et al.* 2005) and oral corticosteroids (Brightling, Monteiro *et al.* 2000). Thus modifying eosinophilic inflammation is a potential therapeutic strategy by either targeting the eosinophils themselves (Green, Brightling *et al.* 2002) or IL-5 (Halдар, Brightling *et al.* 2009, Pavord, Korn *et al.* 2012).

Eosinophils take up the stain eosin and turn red (Woolley, Gibson *et al.* 1996). Airway macrophage's cytoplasm is shown to turn red/purple when fed with apoptotic eosinophils (efferocytosis) and also following steroid treatment in asthmatic patients (Kulkarni, Hollins *et al.* 2010). Thus, the degree of redness reflects the eosinophilic protein ingested by macrophages which can be used as an indirect measurement of efferocytosis. Kulkarni and colleagues developed a novel method to measure efferocytosis by quantification of the percentage area of red hue in the macrophages' cytoplasm in stained sputum cytopins (Kulkarni, Hollins *et al.* 2010). It has been also reported that increased macrophage cytoplasmic red hue in asthma following corticosteroid withdrawal predicts future risk of the

emergence of a sputum eosinophilia and subsequent poor asthma control (Kulkarni, Hollins *et al.* 2010). Yet, there is only one published work on the utilisation of this biomarker to study eosinophilic clearance in COPD (Eltboli, Bafadhel *et al.* 2014), but no data about the dynamics of red hue or clearance of eosinophils at exacerbations and follow up period. Whether the response of the red hue to corticosteroids is similar to what was described in asthma is unknown. In addition, macrophage efferocytosis of eosinophils, as assessed directly *in vitro* and indirectly *in vivo* by the measuring the sputum macrophage cytoplasmic red hue, is impaired in COPD, especially in those with high sputum eosinophil count and low red hue (chapter 7) (Eltboli, Bafadhel *et al.* 2014). Whether this extends also to exacerbations, needs to be determined. Corticosteroids induce apoptosis of eosinophils and promote the macrophages phagocytic function macrophages (Heasman, Giles *et al.* 2003, McColl, Michlewska *et al.* 2007, Kulkarni, Hollins *et al.* 2010). Therefore, it would be expected that the red hue will increase following treatment of corticosteroids as it is a reflection of efferocytosis of apoptotic eosinophils by macrophages. Thus, this can be also used indirectly to validate the red hue concept in COPD by demonstrating its rise after steroid treatment.

The aim of this study was to assess clearance of sputum eosinophils in COPD using red hue of alveolar macrophages as an indirect tool and evaluate its relationship with sputum eosinophils at various stages; baseline, exacerbations, two weeks and six weeks following commencement of exacerbation. Also to validate the concept of red hue in COPD by demonstrating that the red hue increases after steroid treatment. The hypothesis was to observe i) a significant increase in red hue percentage from baseline to 2 weeks post therapy with corticosteroids and concomitant fall of sputum eosinophils count, ii) less or no increase in subjects with impaired macrophage efferocytosis of eosinophils with high sputum eosinophil count and low red hue.

### **8.1.3 Methods**

#### **8.1.3.1 Subjects and study design**

Clinical data and sputum cytopins were available from 196 subjects that had participated in two longitudinal studies of COPD exacerbations (Bafadhel, McKenna *et al.* 2011, Bafadhel, McKenna *et al.* 2012). Subjects had undergone extensive clinical characterisation as described (chapter 3.1.3.1). Colour image analysis as described (Section 2.8) was performed on digital images of macrophages from sputum cytopsin slides, stained with “Diff Quick” stain, of 30 COPD subjects at baseline, exacerbations, two weeks and six weeks after exacerbation. 60-100 macrophages were imaged for each subject, apart from three slides of three different subjects that had macrophages count less than 50. However, when intra-class correlation coefficient was measured using 20 and 100 macrophages, the correlation between the median percentage area of cytoplasm of the two was very high (Cronbach's Alpha= 0.97;  $p < 0.05$ ). Also there was almost no difference between the median percentage area of cytoplasm when 20 cells or 100 cells are utilised (Kruskal-Wallis  $p > 0.9999$ ).

All of the slides were assessed by a single observer who was blinded to the clinical data of the patients. All subjects gave written informed consent and the study was approved by the Leicestershire, Northamptonshire and Rutland local ethics committee.

#### **8.1.3.2 Statistical Analysis**

GraphPad Prism version 6 (GraphPad, San Diego) was used to perform statistical analysis. Parametric data were expressed as mean (standard error of the mean [SEM]), whereas non-parametric data were presented as median (interquartile ranges [IQR]). Unpaired T-test or Mann-Whitney was used for comparisons between groups for parametric or non- parametric data respectively. One-way analysis of variance (ANOVA) with Tukey pair-wise

comparisons was used to perform comparisons across groups for parametric data and Kruskal-Wallis test with Dunn's multiple pair-wise comparisons for non-parametric ones. Correlations were assessed using Spearman or Pearson rank correlation coefficients for non-parametric and parametric data respectively. The Cronbach's Alpha was used to express intra-class correlation coefficient to measure reproducibility of the median percentage area of cytoplasm. A p value was considered statistically significant if it was less than 0.05.

The sputum eosinophil area under the curve (AUC) was derived from the sputum samples collected at stable visits as described (6.1.3) to stratify the subjects into eosinophilics and non-eosinophilics and presented as sputum eosinophil %/year. The correlations between red hue and sputum eosinophils percentage cell count, blood eosinophils, lung function and health status and symptoms were assessed across all the four visits. The correlations between the change in red hue (delta change) and proportions of sputum eosinophils count, lung function, health status and symptoms across all the 4 stages were also analysed. Analysis was also performed for delta change of red hue between the subjects after categorising them into four groups based on cut-offs for the sputum eosinophil count ( $\geq 3\%$ ) and the upper limit of the normal range for %area macrophage red hue ( $>6\%$ ) (Kulkarni, Hollins *et al.* 2010), as previously described (Figure 7-1, Figure 7-2 and Figure 7-3).

#### **8.1.4 Results**

Data was gathered from most of subjects (72%) at the four stages; baseline, exacerbations, post therapy (2 weeks) and recovery (6 weeks). Clinical characteristics of the 30 COPD patients are presented in Table 8-1.

**Table 8-1 Clinical characteristics of the 30 COPD subjects**

<b>COPD subjects (n=30)</b>	
<b>Male, n (%)</b>	16 (55)
<b>Age (years)*</b>	66 (1.8)
<b>Body mass index (kg/m<sup>2</sup>)</b>	25 (23-28)
<b>Current smokers, n (%)</b>	13 (44.8)
<b>Ex-smokers, n (%)</b>	16 (55)
<b>Packs/year smoked*</b>	41 (29-58)
<b>COPD duration (years)</b>	6.7 (2.1-10)
<b>Hospital Admin in last 12 months</b>	0 (0-1)
<b>Number of steroid and or antibiotics in last 12 months</b>	4.5 (2-7)
<b>ICS dose (BDP equivalent) (mcg/day)</b>	2000 (950-2000)

Data presented as median (IQR) unless stated, \*mean (SEM). SEM: standard error of mean. **Abbreviations:** **BDP:** Beclometasone Dipropionate; **ICS:** inhaled corticosteroids.

Table 8-2 shows the clinical characteristics of all the included subjects at various stages from baseline to recovery. There were no significant differences in the clinical parameters apart from the expected worsening of symptoms during exacerbation, particularly dyspnoea, fatigue, cough, sputum production and purulence.

**Table 8-2 Clinical characteristics of the COPD subjects during the 4 stages**

	<b>Stable</b>	<b>Exacerbation</b>	<b>2 weeks</b>	<b>6 weeks</b>	<b>P value</b>
<b>FEV<sub>1</sub>% predicted, (%)*</b>	44 (3.4)	38 (3.3)	45 (3.3)	42 (4.7)	0.62
<b>FEV<sub>1</sub> (L)*</b>	1.2 (0.10)	1.1 (0.10)	1.2 (0.11)	1.2 (0.11)	0.58
<b>FEV<sub>1</sub>/FVC (%)*</b>	47 (2.3)	48 (2.5)	48 (2.9)	50 (3.3)	0.89
<b>Reversibility (%)*</b>	8.3 (0.37-15)	3 (0-14)	0.05 (0-0.1)	0 (-0.05-0.1)	<b>0.0007</b>
<b>Sputum TCC x10<sup>6</sup>/g*</b>	3.2 (1.1-6.6)	5.3 (1.9-13)	3.6 (1.6-6.7)	3.0 (1.8-7.4)	0.33
<b>Sputum neutrophils (%)</b>	59 (3.8)	71 (4.5)	67 (4.3)	60 (4.8)	0.17
<b>Sputum eosinophils (%)</b>	2.00 (0.38-11)	0.88 (0.31-11)	0.71 (0.25-3.4)	1.25 (0.25-13.5)	0.42
<b>Red hue% area</b>	9.9 (6.1-26.7)	48.5 (25-60.3)	53.5 (38-69)	46.9 (30-71.4)	<0.0001
<b>Blood neutrophils (x10<sup>9</sup>/L)</b>	5.1 (3.8-5.7)	5.3 (4.5-6.8)	6.9 (4-10)	4.7 (3.6-6.1)	<b>0.047</b>
<b>Blood eosinophils (x10<sup>9</sup>/L)</b>	0.15 (0.10-0.41)	0.19 (0.07-0.27)	0.14 (0.1-0.24)	0.20 (0.1-0.42)	0.79
<b>CRP (mg/dl)</b>	8 (5-15)	14 (7-35)	12 (6.3-59)	8 (6-9)	0.19
<b>Bacterial colonisation, n</b>	4 (14)	5 (19)	3 (13)	4 (21)	0.85
<b>CFU unit/mL**</b>	6.3 (5.9-6.6)	6.4 (5.9-6.9)	ND	ND	0.84
<b>CRQ Emotional Functioning*</b>	4.7 (0.24)	3.8 (0.26)	4.2 (0.26)	4.4 (0.34)	0.12
<b>CRQ Fatigue (unit)*</b>	3.8 (0.25)	2.6 (0.2)	3.3 (0.27)	3.7 (0.35)	<b>0.01</b>
<b>CRQ Dyspnoea (unit)</b>	3.2 (2.2-4.2)	2.0 (1.6-2.8)	2.8 (2-4)	3.0 (2-4.2)	<b>0.008</b>
<b>CRQ Mastery (unit)</b>	5 (4-6.1)	3.9 (2.8-5.3)	4.1 (3.3-5.8)	5.5 (2.9-6)	0.07
<b>CRQ total score (unit)</b>	4.1 (3.3-5)	3.2 (2.3-3.8)	3.6 (2.9-4.6)	4.3 (2.7-5.3)	<b>0.0065</b>
<b>VAS Cough (mm)*</b>	36 (5)	60 (5.6)	35 (5.1)	34 (6.6)	<b>0.002</b>
<b>VAS Dyspnoea (mm)</b>	50 (29-61)	80 (62-92)	53 (29-67)	47 (14-78)	<b>0.0002</b>
<b>VAS Sputum Production (mm)</b>	29 (11-48)	59 (21-78)	25 (13-48)	17 (5-40)	<b>0.004</b>
<b>VAS Sputum Purulence (mm)</b>	25 (7-49)	54 (26-77)	25 (13-41)	14 (4-33)	<b>0.0007</b>

Data presented as median (IQR) unless stated, \*mean (SEM), \*\*Geometric mean of Log data (95% CI). SEM: standard error of mean, CI: confidence interval. **CFU**: colony forming unit; **CRQ**: Chronic respiratory health Questionnaire; **CRP**: C-Reactive Protein; **FEV<sub>1</sub>**: Forced expiratory volume in 1 second; **FVC**: Forced vital capacity; **GOLD**: Global Initiative for chronic Obstructive Lung disease; **ICS**: inhaled corticosteroids, **MRC**: Medical Research Council; **ND**: not done; **SGRQ**: St George's Respiratory Questionnaire; **TCC**: total cell count; **VAS**: Visual analogue score; **yr**: Year.

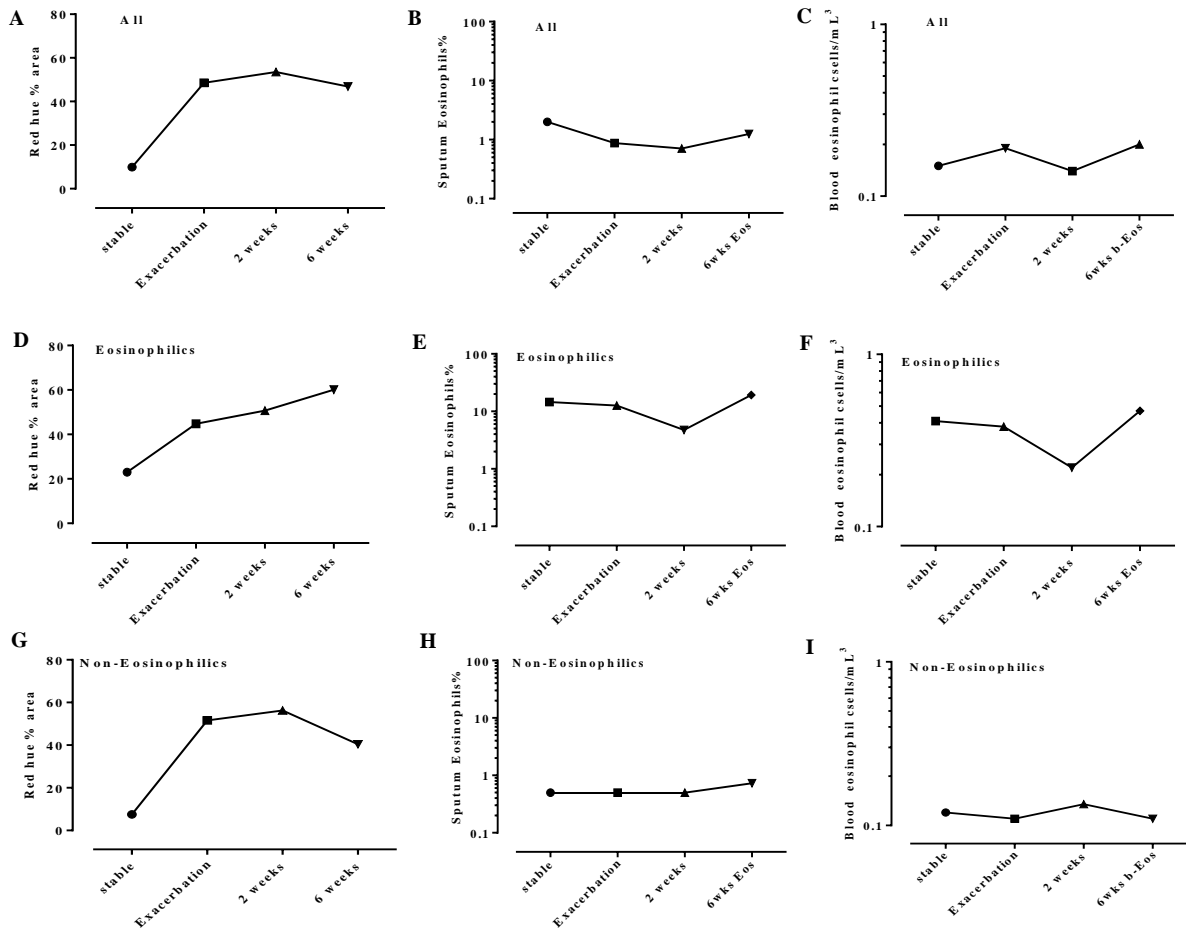
All subjects, except three received the same dose of oral prednisolone during exacerbations.

There was a small delay of time between the start of exacerbations and collection of samples and a similar lag between exacerbation onset and commencement of treatment (median [IQR], 2 [0.75-3.25] and 2 [2-3] respectively; (p=0.56)). This means that most samples were collected just before or at the onset of treatment. The median [IQR] length of treatment was

10 [7-10] days. There was no change of inhaled corticosteroids use during exacerbations, except for two subjects who had their dose increased.

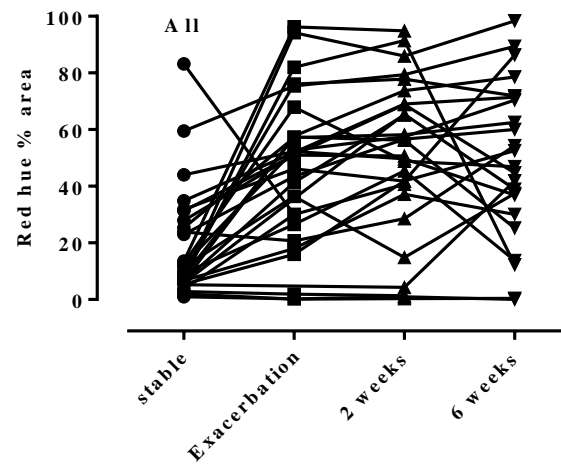
At exacerbations, majority of subjects (82.8%) have dramatic increase in median [IQR] proportion of cytoplasmic red hue percentage area of macrophages from 9.9% [6-26.7] at baseline to 48.5% [25-60] during exacerbation ( $p < 0.0001$ ), (Figure 8-1a and Figure 8-2). In five patients (17%), the red hue percentage area declined after 2 weeks post therapy (Figure 8-2).



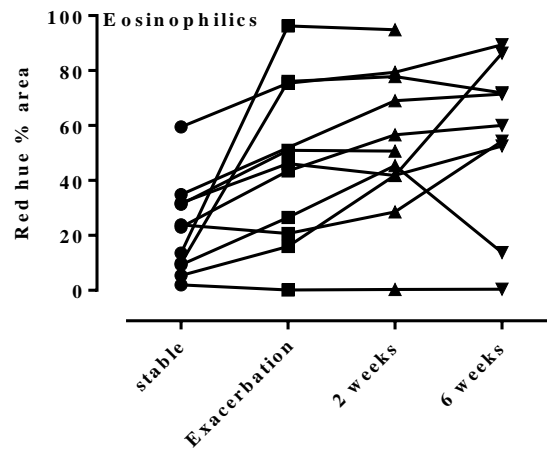


**Figure 8-1 Dynamics of red hue %area with corresponding sputum and blood eosinophils at stable exacerbation, 2 and 6 weeks after exacerbation.**

A



B



C

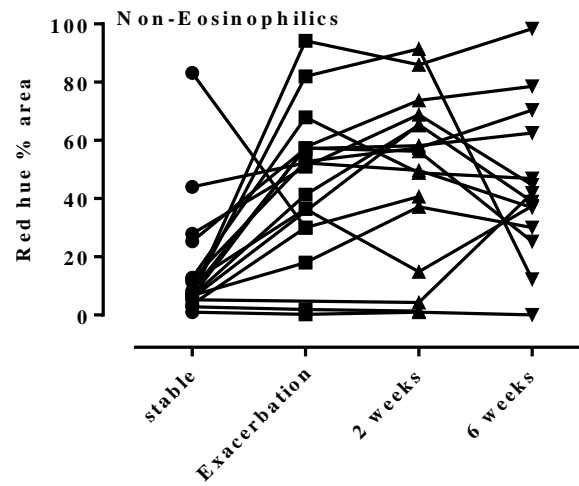
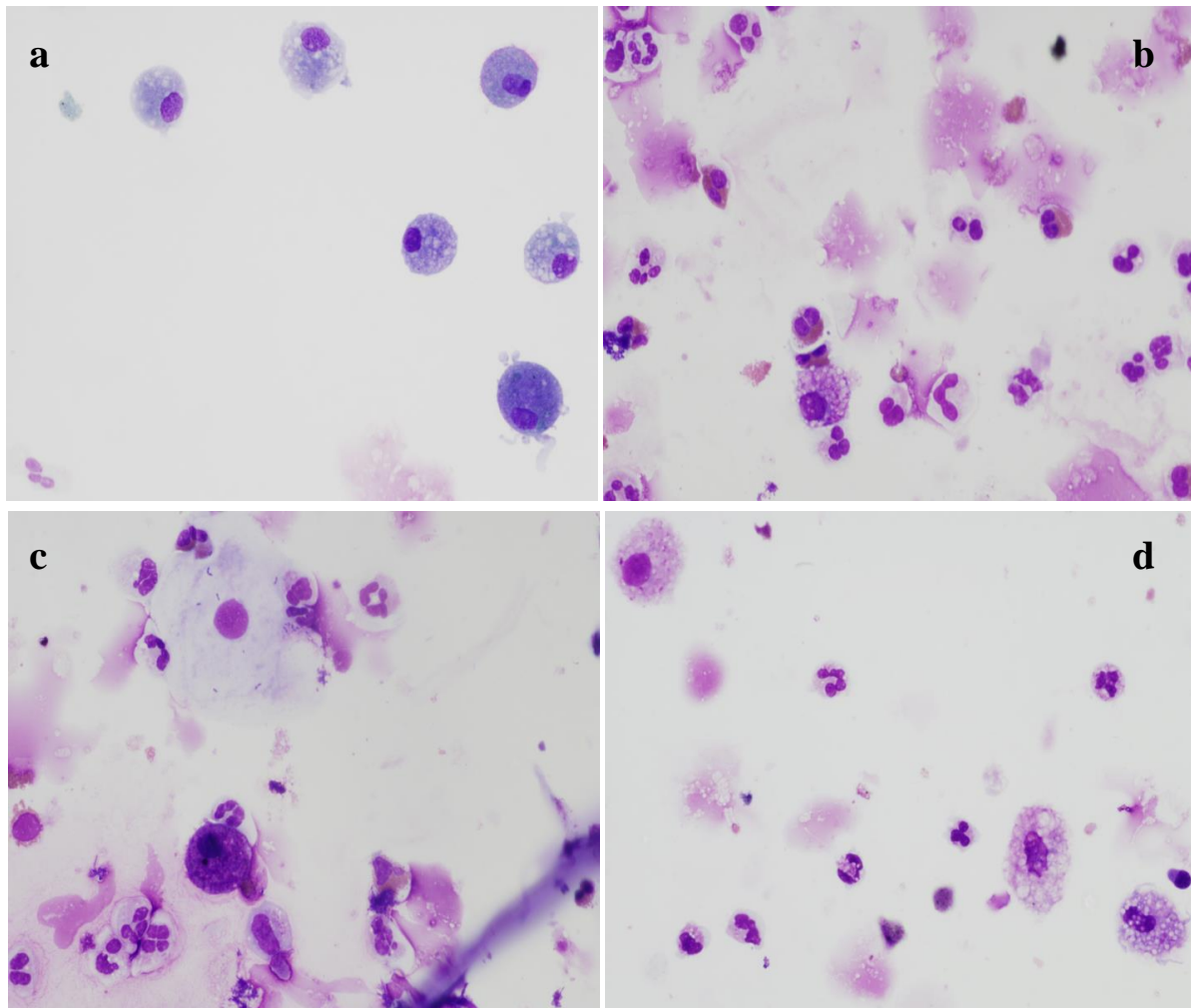


Figure 8-2 Red hue dynamics at exacerbations

The highest peak of red hue% [IQR] on the graph was at two weeks following treatment 53.5% [38-69]. This value decreased slightly at 6 weeks visit 46.9% [30-71] to similar levels at exacerbations, but not back to baseline levels (Kruskal-Wallis  $p < 0.0001$ ). Example cytopins for each group are as illustrated (Figure 8-3).



**Figure 8-3 Representative images of sputum macrophages during exacerbation:** a: Baseline; b: Exacerbation; c: Two weeks and d: Six weeks post exacerbation. The highest degree of red hue is at two weeks after exacerbation.

When stable values were compared with recovery ones, all (91%) but two subjects have shown an increased in red hue % area ( $p < 0.05$ ). The corresponding median [IQR] sputum eosinophils percentage fell over the same periods of time from stable (2% [0.38-11]) to exacerbation (0.88% [0.31-11]), with a further, but insignificant decline two weeks post treatment (0.71% [0.25-3.4]) and a minimal increase at recovery time (1.25% [0.25-13.5]), which was not statistically significant (Kruskal-Wallis  $p = 0.42$ ), (Figure 8-1b). However, when the non-eosinophilic subjects were excluded, the median (IQR) of red hue percentage area of the eosinophilic subjects ( $n$  [%]), 12 [40%]) exhibited more gradual rise from baseline to exacerbation, two weeks following administration of prednisolone and peaks at recovery. (23% [9.3-31.8], 44.8% [19.5-75.6], 50.7% [41.8-77.9], 60.1% [33.1-79.2]), respectively (One-way ANOVA  $p = 0.02$ ), (Figure 8-1d and Table 8-3). Whereas the corresponding sputum eosinophils percentage count decreased steadily from 14.5% [4.1-21.5] at baseline to 12.6% [7.3-21.4] during exacerbation and 4.8% [0.25-15.8] after 14 days of treatment with oral corticosteroids, but then soared again at the recovery time 27.9% [13.5-47.9]; (Kruskal-Wallis  $p = 0.06$ ), (Figure 8-1e). Similarly, cytoplasmic red hue in the non-eosinophilic subjects increased from 7.5% [5.7-16] at baseline, to 51.6% [30-58] at exacerbation, with a further increment to 56.3% [26-67] at two weeks post treatment, but declined after 6 weeks 40.4% [29-65]; (Kruskal-Wallis  $p = 0.002$ ), (Figure 8-1g). However, the sputum eosinophils counts remained stable at low levels of 0.5 or less all over this period of time (Kruskal-Wallis  $p = 0.94$ ), (Figure 8-1h and Table 8-3).

The average peripheral blood eosinophils count was fluctuating across the different 4 visits and was not corresponding to the red hue or sputum eosinophils curve, except in the eosinophilic subjects where the blood eosinophils count was going with sputum eosinophils percentage, despite the absence of statistically significant correlation between them (Figure

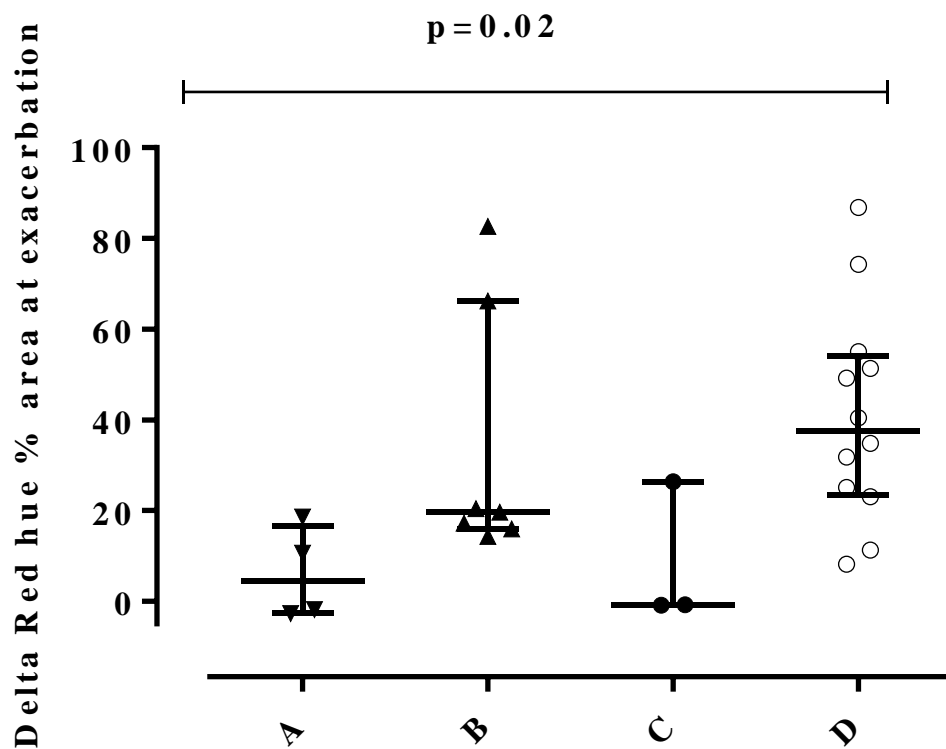
8-1 and Table 8-3). However, there was a strong positive correlation between the blood and sputum eosinophils percentage at all the four stages, (Spearman's  $r=0.71, 0.73, 0.39$  and  $0.81$ ;  $p<0.0001, <0.0001, 0.04$  and  $<0.0001$ ) respectively,

**Table 8-3 Red hue %area and corresponding sputum and blood eosinophils count for eosinophilic and non-eosinophilic COPD subjects during the 4 stages.**

		Stable	Exacerbation	2 weeks	6 weeks
<b>Eosinophilic COPD (n=12)</b>	<b>Red hue%</b>	23 (9.3-31.8)	44.8 (19.5-75.6)	50.7 (41.8-77.9)	60.1 (33.1-79.2)
	<b>Sputum eosinophils%</b>	14.5 (4.1 -21.5)	12.6 (7.3-21.4)	4.8 (0.25-15.8)	19.25 (10.6-37.5)
	<b>Blood eosinophils (cells/ mL<sup>3</sup>)</b>	0.41 (0.27-0.52)	0.38 (0.25-0.58)	0.22 (0.14-0.53)	0.47 (0.26-0.66)
<b>Non-Eosinophilic COPD (n=18)</b>	<b>Red hue%</b>	7.5 (5.7-16)	51.6 (30-57.7)	56.3 (26-67.1)	40.4 (28.8-64.5)
	<b>Sputum eosinophils%</b>	0.50 (0.3-2.3)	0.50 (0.3-1)	0.50 (0.3-1.3)	0.37 (0.3-1.4)
	<b>Blood eosinophils (cells/ mL<sup>3</sup>)</b>	0.12 (0.08-0.27)	0.11 (0.05-0.19)	0.14 (0.05-0.16)	0.11 (0.05-0.19)

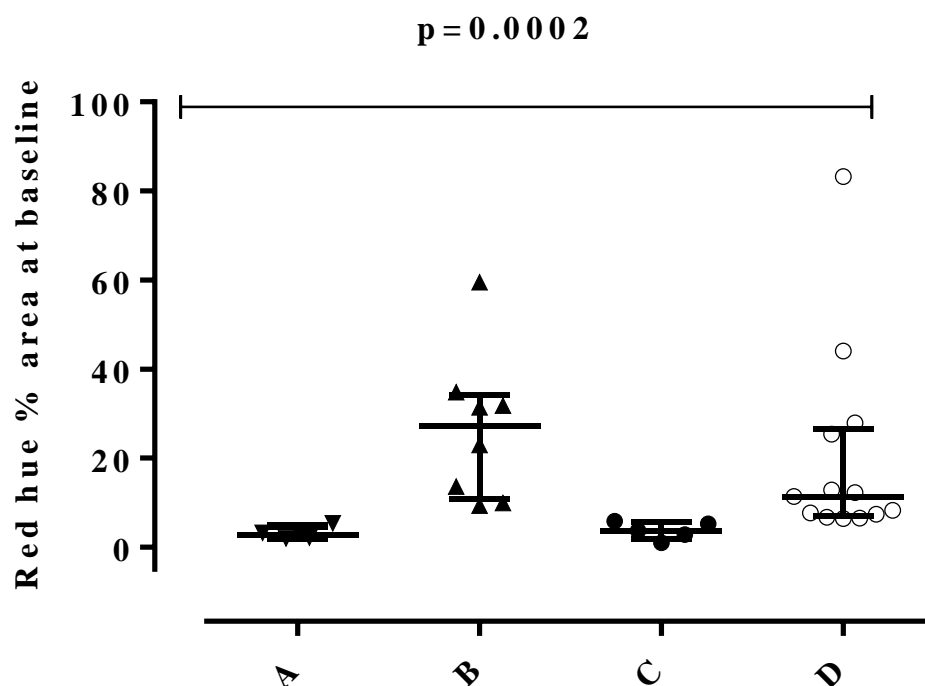
Data presented as median (inter-quartile range) unless stated.

The delta increase of red hue from baseline to exacerbation was markedly less in group A subjects (those who have greatest impairment in eosinophils efferocytosis with high sputum eosinophil count and low red hue%) (median delta red hue% [IQR] 4.4% [-2.5-16.5]), with a decline of red hue in group C (those with low sputum eosinophil count and low red hue% and impaired efferocytosis of eosinophils) (-0.78% [-0.91-26.4]), compared to groups B (19.7% [15.9-66.2]) and D (37.6% [23.5-54.1]) respectively; (Kruskal-Wallis  $p=0.02$ ), (Figure 8-4). These differences were very similar to what was observed at baseline (Figure 8-5); (Kruskal-Wallis  $p=0.0002$ ). However, the difference in delta red hue was lost after 2 weeks and in the recovery period (data are not shown).



**Figure 8-4 Delta Red hue% area at exacerbation across the four COPD groups.**

Horizontal bars are medians (IQR).

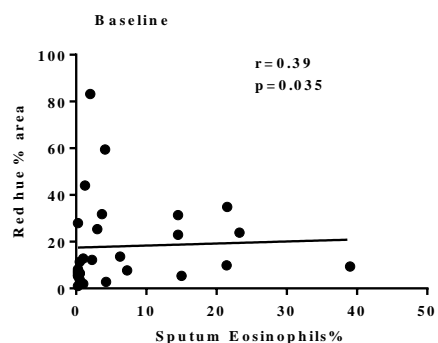


**Figure 8-5 Red hue %area at baseline across the four COPD groups.** Horizontal bars are medians (IQR).

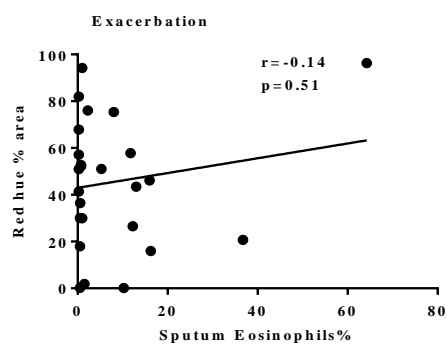
Apart from a weak positive correlation between the airway macrophage percentage red hue area and proportions of sputum eosinophils at stable visit, there were no statistically significant associations between the red hue% area or delta of red hue and sputum eosinophils count or its delta respectively at exacerbations or recovery time (Figure 8-6). Similarly, there were neither correlations between red hue% area or delta red hue% and FEV<sub>1</sub> or delta FEV<sub>1</sub> respectively, nor with health status or change in health status (data is not shown). Likewise, there were no statistically significant correlations between percentage of red hue and medications or length of treatment with oral corticosteroids either (data is not shown).

All

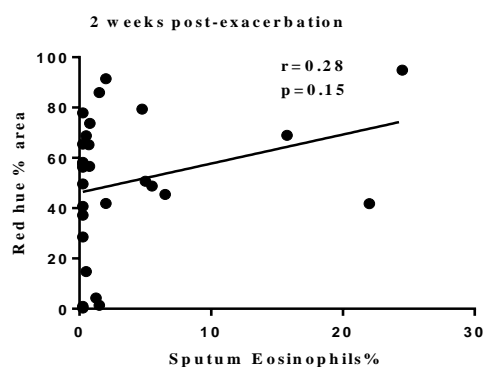
A



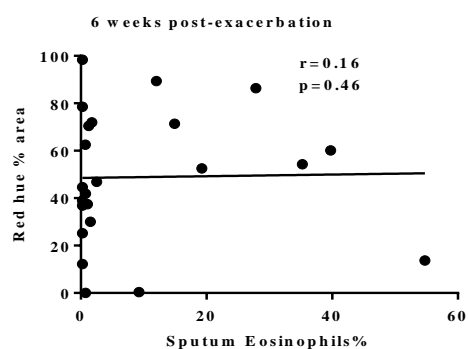
B



C

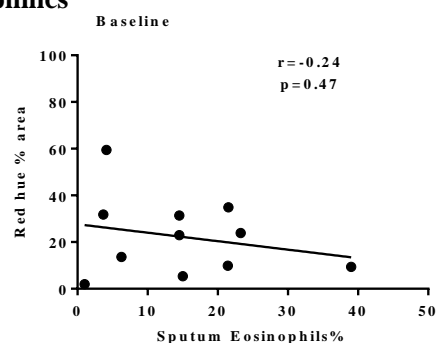


D

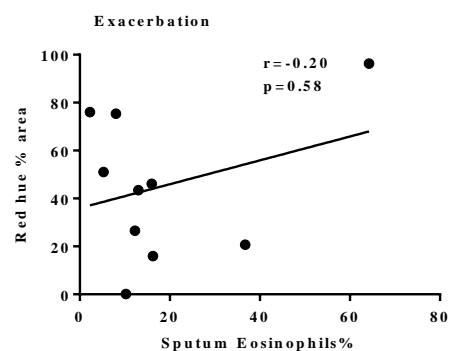


Eosinophils

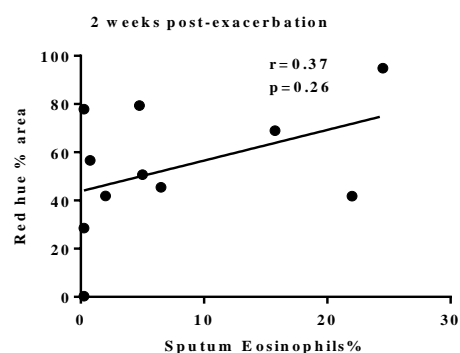
E



F



G



H

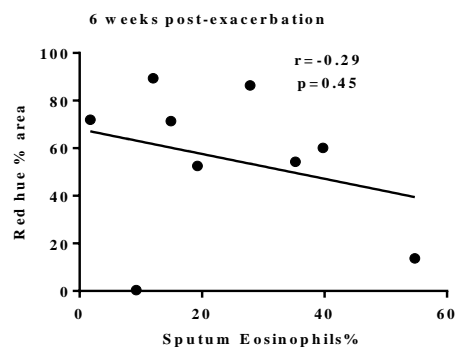


Figure 8-6 Scatter-plots showing no correlations between red hue% area and sputum eosinophils at the 4 stages in all the subjects combined except at baseline (A).



### 8.1.5 Discussion

Here I report for the first time a longitudinal assessment of stages of eosinophilic clearance during COPD exacerbations and therapy with corticosteroids. This study displayed a reciprocal relationship between eosinophils efferocytosis, as exemplified by red hue of airways macrophages, and sputum eosinophils count during exacerbations of COPD and follow up period and its response to treatment with corticosteroids, although the correlation was weak. As expected, majority of the subjects showed a marked increase in red hue and a corresponding decline of sputum eosinophils count in response to oral corticosteroids from baseline to recovery period, which was in line with the hypothesis. This percentage rise indicates an increase in eosinophilic proteins in macrophages as the macrophages are effectively clearing the airways of eosinophils that have carried out their function, the extracellular killing of large pathogens, before they release toxic substances that would be harmful to the body. This is mainly a consequence of treatment with corticosteroids as they were shown to be not only effective in reducing inflammatory response, but also in motivating apoptosis of eosinophils as well as promoting the phagocytic ability of macrophages (Heasman, Giles *et al.* 2003, McColl, Michlewska *et al.* 2007). These observations also support the previous findings of increased red hue in asthmatic patients following treatment of prednisolone (Kulkarni, Hollins *et al.* 2010) and further validate the concept of red hue in COPD. However, it might be difficult to distinguish whether the red hue response is due to exacerbations itself or a consequence of treatment with prednisolone. However, the corresponding reduction of sputum eosinophils count at exacerbations favours the second possibility.

Interestingly, in the current study five subjects have a decrease of their red hue at exacerbations, which does comply with the second part of my hypothesis as these patients

have either impairment of efferocytosis of eosinophils (2 subjects from group A), (Eltboli, Bafadhel *et al.* 2014), (chapter 7), or were persistently non-eosinophilic (2 subjects from group C and 1 from D), which probably explains their failure to have their red hue increased at exacerbation.

The sputum differential count appears to be a significant aspect in interpreting the results of red hue. If patients did not have large numbers of eosinophils at baseline, their red hue is not expected to rise significantly as that is dependent upon macrophages taking up eosinophils after treatment. Interestingly, non-eosinophilic subjects showed significantly increased red hue% at exacerbation despite their low sputum and blood eosinophil count at exacerbation, which suggests that lung eosinophils may contribute to this phenomenon. This may be supported by a previous observation that eosinophils increased in the lung tissue during exacerbation (Zhu, Qiu *et al.* 2001).

Importantly, these finding and previous data (Kulkarni, Hollins *et al.* 2010), which reports that red hue increases regardless of the sputum count of eosinophils, support the growing evidence that sputum eosinophilia in COPD and asthma is probably underestimated (Kulkarni, Hollins *et al.* 2010) and the scarcity of eosinophils in the sputum of non-eosinophilic COPD subjects is likely to be due to treatment with corticosteroids rather than true “non-eosinophilia”. Indeed, in this study the red hue increased more in non-eosinophilic patients than eosinophils in exacerbation as a response to treatment, which further supports the previous observations. However, the cut-off point for sputum eosinophilia of 3%, which was used in the current study, might be slightly high to include all the eosinophilic patients. In fact, some other studies demonstrated a clinical response to corticosteroids even if 2.5% or

greater sputum eosinophilia was used (Lex, Jenkins *et al.* 2007), which may suggest the need to revise the inclusion criteria for sputum eosinophilia.

The clinical implication of the current study is to show that by measuring the macrophages red hue over time, it may be possible to determine the success of current or even new medical treatments for COPD. For those that demonstrate high eosinophil counts, with a gradual increase in eosinophilic protein content over a period of time, it would indicate that the prescribed treatment is efficiently working. So by being able to track the progress of COPD via measuring percentages of eosinophilic protein in macrophages, it might be possible to modify medical treatments to patients individually and more effectively. However, the results of red hue over time should be interpreted with caution especially in those in whom red hue of sputum macrophages did not increase as it may indicate two possibilities; the first one is that the treatment is insufficient, not successful or there is a poor compliance, secondly it might be a consequence of an impairment of efferocytosis of eosinophils.

A potential limitation of the study is that the macrophage red hue area percentage does not measure the literal number of eosinophil proteins, but gives only the percentage, which might make it less precise. However, a previous study in asthma (Kulkarni, Hollins *et al.* 2010) has proven that it is reproducible, shows a good response to treatment with corticosteroid and can be potentially used to tailor treatment in the future. Another drawback was the fact that a relatively small number of patients were assessed, especially when considering the comparisons in the four groups. However, despite the small numbers of subjects in group A (n=4) and C (n=3) in this study, the differences in delta change of red hue observed at exacerbation between the 4 groups (Figure 8-4) are likely to be genuine as they are very similar to what was seen in the baseline (Figure 8-5) and also going with the previously

published findings about differences in these group (Eltboli, Bafadhel *et al.* 2014). Nevertheless, if follow up studies are to be carried out, increasing the number of population analysed would give more reliable and accurate results.

#### **8.1.6 Conclusions**

Red hue percentage area of macrophages increases in COPD exacerbations as a response to corticosteroids treatment, but less markedly in subjects with impaired macrophage efferocytosis of eosinophils. The contemporaneous sputum eosinophils count decreased concurrently, but less significantly. Sputum eosinophilia in COPD and Asthma might be underestimated.

## **Chapter 9   Final summary and conclusions**

## 9.1 Summary of findings

In this thesis the eosinophilic airway inflammation in COPD have been investigated in well-characterised subjects of COPD with varying severity. Firstly, I have defined this phenotype by determining its clinical characteristics, assessed its reproducibility, extent in bronchial airway and its indirect relationship with sputum eosinophils using blood eosinophils as a surrogate biomarker for sputum eosinophils. This has been achieved by measuring eosinophils in sputum and blood using differential cell count and in bronchial tissue from COPD and control subjects using immunohistology. In the second stage, I have looked for any possible aetiology of eosinophilia in COPD by ascertaining the relationship between sputum eosinophilia and previous parasite exposure or atopy using serology to specific helminthes antigens and via measuring total Ig-E and skin reactivity respectively. Thirdly, I have looked at the clearance of eosinophils and dynamic of eosinophil at exacerbation. This has been fulfilled indirectly *in vivo*, using colour analysis of macrophages images and directly *in vitro*, utilising immunohistochemistry technique on monocyte-derived macrophages fed with apoptotic eosinophils from COPD and normal individuals (Figure 9-1).

The key findings are:

Eosinophilic COPD is a unique subgroup that rarely coexists with bacterial or neutrophilic phenotype and blood eosinophil is a strong potential biomarker for this phenotype. This phenotype is stable over time. The airway tissue eosinophils and remodeling are positively correlated with peripheral blood eosinophil count as a biomarker of a sputum eosinophilia. The aetiology of eosinophilic chronic obstructive pulmonary disease is still unknown, but is not associated with helminthic infection or exposure and may be related to atopy. Macrophage efferocytosis of eosinophils is impaired in COPD compared to healthy controls

and is related to the severity and frequency of COPD exacerbations. I have also validated the red hue concept in COPD by firstly demonstrating that the cytoplasmic red hues of macrophages increases after feeding with apoptotic eosinophils (*in vitro*), secondly by showing that cytoplasmic red hue of macrophage and eosinophil efferocytosis by MDMs are correlated and finally through exhibiting an increase in red hue at exacerbation in response to oral corticosteroids treatment (*in vivo*).

The findings that the eosinophilic phenotype is unique, its persistence in the three compartments; sputum, blood and bronchial tissue and the interrelationship among them corporate, complement and expand the work from previous research. The association between the blood and lung eosinophils in particular is a novel finding that complete the missing chain in the circle in the relationship between these three compartments. The clinical implication of this is to provide attractive targets for the development of novel therapies (Figure 9-1). Although clinical trials with guided treatment against sputum (Brightling, Monteiro *et al.* 2000, Pizzichini, Pizzichini *et al.* 1998, Fujimoto, Kubo *et al.* 1999) and blood eosinophils (Bafadhel, McKenna *et al.* 2012) have already demonstrated favourable outcomes in subjects with eosinophilic COPD, but further efforts are probably required to target all the three levels at the same time. Oral corticosteroids can target at least two compartments, but there is still a need to have a more efficient novel treatment with further powerful results and minimum adverse effects.

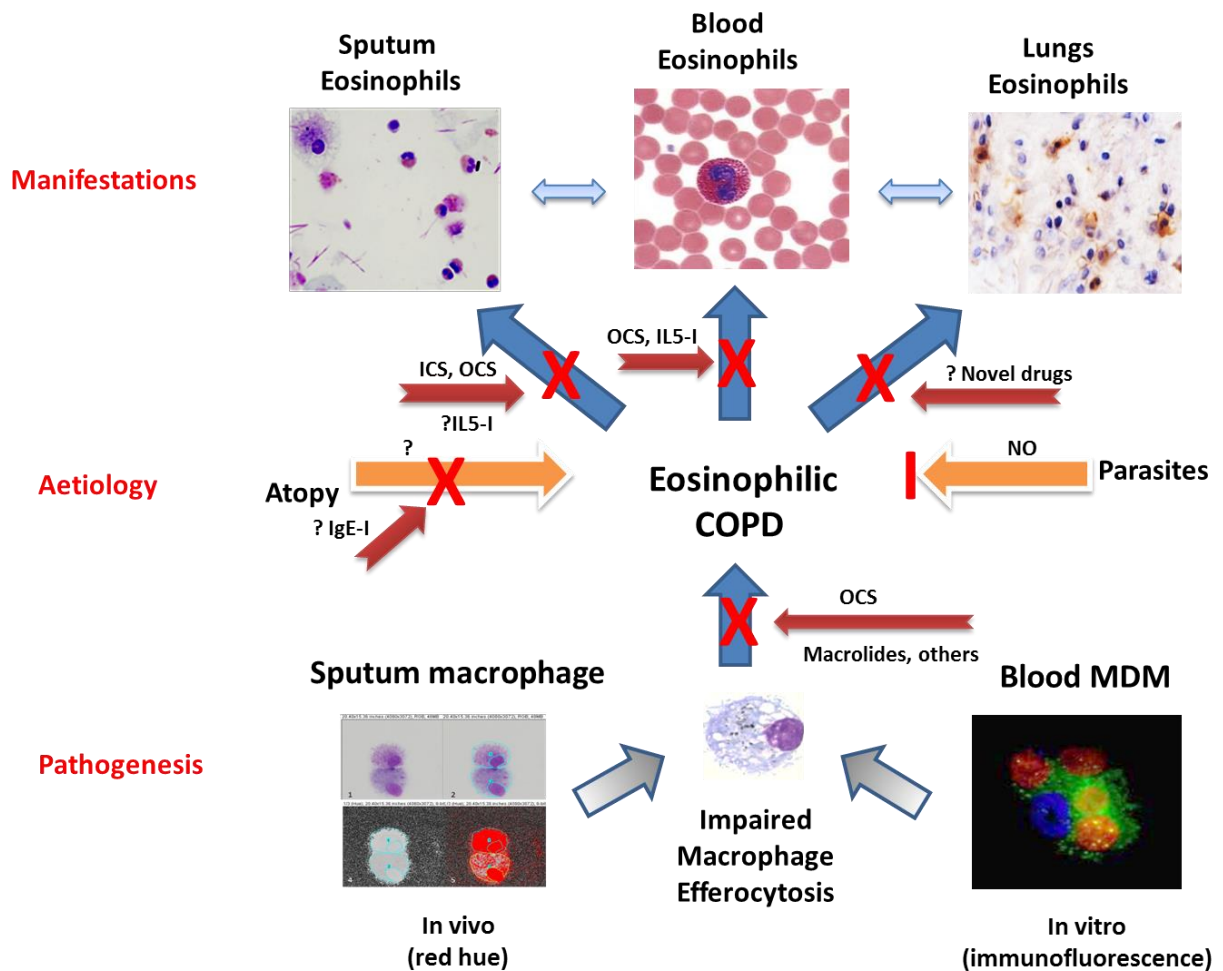
Finding aetiology is always a challenge that motivates many researchers. Despite this thesis did not provide a definitive cause of the eosinophilia in COPD, but at least it was able to evaluate some possible etiologies such as atopy and exclude some others including parasites exposure, albeit not completely. The role of atopy if proved in the future is likely to be a

potential therapeutic target using probably Omalizumab a humanized anti-Ig-E monoclonal antibody that has been extensively tried in atopic asthma (Humbert, Busse *et al.* 2014), urticaria (McCormack 2014) and other allergic diseases.

Although the concept of macrophages red hue was previously validated and utilised clinically in asthma to predict risk of the emergence of a sputum eosinophilia in the future and poor asthma control after weaning from corticosteroid (Kulkarni, Hollins *et al.* 2010), this is the first time that this index is verified in COPD. Application of this tool clinically revealed at least two novel and important findings. Firstly, eosinophilic contribution to the total inflammatory burden in COPD might be under-estimated as great number of COPD subjects (group D) had high red hue despite the lack of sputum eosinophilia, which suggests that this might be a consequence of the presence of eosinophilic reservoir in the bronchial mucosa. Secondly, COPD exacerbation severity and frequency are associated with impaired macrophage efferocytosis of eosinophils, which again may be used clinically to target this pathway (Figure 9-1). These observations signify the importance that use of sputum eosinophil count in clinical practice should be complemented by measuring red hue too as has been previously highlighted (Kulkarni, Hollins *et al.* 2010). Additionally, as stated previously in chapter 7, this thesis was not able to confirm or exclude impaired efferocytosis as a potential contributing factor in the pathogenesis of eosinophilic inflammation in COPD.

The work in this thesis concludes that the mechanism and aetiologies of eosinophilic inflammation in COPD are likely to be multifactorial. Altogether, these finding may help us to understand the complexity of disease further and may have important implications for developing new therapies that might improve the outcome in patients with COPD.





**Figure 9-1 Summary diagram illustrating the eosinophilic airway inflammation and potential therapeutic target points.**

IgE-I: immunoglobulin-E inhibitors; IL5-I: Interleukin-5 inhibitors; ICS: inhaled corticosteroids; OCS: oral corticosteroids.

## 9.2 Limitations

The limitations for each individual study have been discussed in the relevant chapters. Only the general limitations will be discussed here.

Compliance to treatment has not been evaluated although some efforts were made to measure it, but the available data was limited and insufficient for analysis and compliance in general is expected to be good.

A potential criticism of the thesis is the use of indirect methods such as blood eosinophils instead of sputum, red hue as indirect method of the eosinophil efferocytosis. However, these might be considered strengths as well, since this thesis provided potential alternative simpler and quicker ways to measure more complex processes. Additionally, blood eosinophil has been already proved previously to be clinically useful (Bafadhel, McKenna *et al.* 2012) and the findings from red hue were confirmed *in vitro* in a separate experiment in this thesis (chapter 7) and also in previous a published work (Kulkarni, Hollins *et al.* 2010), although further larger studies are required to validate these methods and measure their repeatability.

A further limitation of this work is that using indirect immunofluorescence staining instead of flow cytometry in the *in vitro* experiment for eosinophils efferocytosis described in chapter 7. However, this technically was not possible as the flow cytometry requires larger number of cells than was available in the experiment. Moreover, this method is also well established and was extensively used previously, although it is more subjective, therefore blinding was used to eliminate any observer bias. Another obstacle in this context is the use of alveolar macrophages to study the efferocytosis of eosinophils instead of MDM. Again, the lack of

sufficient amount of sputum and cells in the sputum plug hinders this and also a lot of subjects were unable to produce sputum even after induction and some others had a very poor lung function to perform induction of sputum. In addition, I have already proved indirectly that alveolar macrophages have impaired ability to efferocytose eosinophils and as I mentioned in chapter 7, the demonstration of this impairment experimentally in macrophages derived from blood monocytes suggests that the problem found is intrinsic abnormality in the blood rather than a result to the exposure to lungs environment.

In this thesis, corrections for multiple comparisons were not made. For this reason I have been cautious in conclusions that have been drawn, not to over interpret any findings. Another potential limitation is that techniques such as the principal component analysis (PCA) have not been used in this thesis to understand complex datasets without underlying hypothesis and therefore avoiding multiplicity. However, factor analysis was already used for the complex dataset from the previous studies (Bafadhel, McKenna *et al.* 2011, Bafadhel, McKenna *et al.* 2012) and the results have already been mentioned in this thesis.

One of the other important technical limitations in my PhD study is the complexity of the experiments I performed and lack of time. Optimising the conditions of the procedure of *in vitro* experiment took about six months and involved doing 4 to 5 preliminary trials. Each experiment, which includes four stages, takes two weeks to finish. The first step is to take blood from COPD or healthy control and if a patients do not turn up or change their mind for any reason in the last moment, I have to find alternative, which is not always possible. Secondly, withdrawing blood from eosinophil donors requires also to have a spare and feasible donor always available to avoid wasting time, wasting the first patients' blood and resources in case the second donor does not turn up. The next challenge is the number of

retrieved eosinophils, especially from mild asthmatic, is usually small; a lot of these cells adhere to the flasks and it is difficult to retrieve eosinophils without killing them. The blood eosinophils count prior to the experiment might be helpful, but not always accurate, especially if the result was long time before the experiment and also some donors have variable cell counts. The timing of the experiment is also challenging. Although, the eosinophil isolation experiment is mostly started in the early morning, but the process takes about 5-6 hours to complete. The incubation period of eosinophils for 48 hours to induce apoptosis commences from the time the eosinophils are kept in the incubator, which is usually at 2 or 3 in the afternoon. This means that phagocytosis assay cannot be performed before this time following 2 days, which usually finishes late evening or sometimes midnight. In one occasion, I had to cancel the assay and repeat all the experiment again as the incubated monocytes found all dead after 6 days for unknown reason. The next step is the immunofluorescence staining, which also takes about 8 hours to complete and the final stage is visualising the stained cells and counting them under the microscope, which is sometimes challenging and time consuming. Measuring the red hue is also in itself time consuming as it takes 2-3 hours in average to look for and image the macrophages and then about 2 hours to measure the red hue. So, in average 2 samples require one full day to finish them completely.

Another small technical issue was the small sizes of several bronchial biopsies obtained by bronchoscope with very little representative area of lamina propria which made it impossible to be used for cell counting as they are below the required area specified in the inclusion criteria. Therefore, these subjects were excluded which resulted in a relatively smaller study sample size than was expected.

### 9.3 Plans for future work and future view

#### 9.3.1 Plans for future work

Information about the relationship between sputum eosinophilia and mortality is very scarce and only few epidemiological studies based on old data from asthma patients exist in the literature (Hospers, Schouten *et al.* 2000, Hospers, Schouten *et al.* 1999). It would be interesting to perform a mortality study to investigate this further in COPD.

The relationship between eosinophils and bacteria is not known and evidences from this thesis and previous work suggest that bacteria rarely coexist with sputum eosinophilia and that human eosinophils possess bactericidal properties (Persson, Andersson *et al.* 2001). This can be investigated indirectly by measuring the bacterial load and culture in those subjects who participated in clinical trial pre and post-depletion of the eosinophils in the blood (Brightling, Bleecker *et al.* 2014) and directly *invitro* using lung tissue model that can be exposed to bacterial before feeding with eosinophils. In fact, only few *invitro* studies, performed in mice (Linch, Kelly *et al.* 2009) and human (Yazdanbakhsh, Eckmann *et al.* 1986) exists in the literature and these studies suggest that eosinophils may have antibacterial and phagocytic activities against bacteria.

It would be vital to study the eosinophilic inflammatory profile extensively in multiple levels prospectively and simultaneously. e.g. genome, blood, sputum using sputum induction non-invasively and invasively using bronchoscopy in addition to CT imaging, in large population of COPD and appropriately matched normal subjects. This might be also possible if data from the ongoing large studies such as EvA (Ziegler-Heitbrock, Frankenberger *et al.* 2012) and COPDMAP (<http://www.copdmap.org>) can be used for this purpose.

There are no data in the literature about occupational exposure and risk of development of sputum eosinophilia in COPD. It would be interesting to investigate any relationship between the two in the future using large number of COPD and healthy control subjects.

The red hue is a promising biomarker that needs further exploration and validation. It would be interesting to study the dynamics of red hue over time at stable visits and measure its reproducibility during stable and exacerbation events. Further work is also required to explore the relationship between the eosinophils efferocytosis and lung function decline and frequency of exacerbations and find out the mechanism behind it as well. It would be also worth studying the eosinophils efferocytosis impairment in the alveolar macrophages to validate the results from peripheral blood. These future investigations may help gaining additional information about this tool and if the red hue is well validated then it might be used as a potential target in clinical trials in the future.

### 9.3.2 Future view

The accumulation of evidence over the last 20 years has demonstrated that measuring eosinophilic inflammation in lung disease has clinical utility in supporting a diagnosis and directing corticosteroid therapy. Within the next five years there will be an increase in the application of measuring eosinophilic inflammation particularly in airways disease. This will be supported by several international guidelines on asthma and chronic cough. There remains a need for a simple peripheral blood biomarker of eosinophilic inflammation, although none has yet to demonstrate greater clinical utility than a sputum eosinophil count. The peripheral blood eosinophil count may provide sufficient information to direct therapy and recent evidence does suggest this may have immediate clinical utility (Bafadhel, McKenna *et al.* 2012). Serum periostin has emerged as a potential biomarker of a Th2 high phenotype and in a study of anti-IL13, those patients with high periostin had a greater clinical response in terms of lung function than those with low periostin (Corren, Lemanske *et al.* 2011). Periostin is reported to be a good biomarker of composite eosinophilic inflammation in the airway wall and sputum (Jia, Erickson *et al.* 2012). Whether this biomarker has a more generalisable role in directing other therapies in asthma and COPD will need to be tested and such studies are planned. The need for a wider application of biomarkers of eosinophilic inflammation to aid diagnosis and stratify patients for therapeutic interventions will become more acute as current biological therapies in late phase clinical trials enter the clinic. This will bring exciting opportunities for clinicians and their patients, but will mean that there is a greater challenge to identify the right drug to the right patient and the right time. Measuring eosinophilic inflammation in COPD will be pivotal in making these critical decisions.

## **Chapter 10 References**



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## **Chapter 11 Appendix**

The following pages show copies of the volunteer information sheets and consents forms used in the thesis.

*Caring at its best*

**Glenfield Hospital**  
Groby Road  
Leicester  
LE3 9QP

## Subject Information Leaflet and Consent Form

**Study Title:** Use of Tissue from Lung Resections to Investigate the Molecular and Functional Mechanisms of Human Lung Disease  
Tel: 0300 303 1573  
Switchboard Fax: 0116 258 3950  
Minicom: 0116 287 9852

**Chief Investigator:** Professor Andrew Wardlaw  
**Principle Investigator:** Professor Andrew Wardlaw

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

### Introduction

Lung disease causes pain, discomfort and can prevent sufferers from carrying out everyday activities. Whilst available treatments including steroids and other drugs may relieve symptoms, none provide a cure. Operations may help some people. More research is needed to find new treatments that can cure lung disease.

### What is the purpose of the study?

In order to find the causes of lung disease such as COPD and lung cancer and to find new ways of treating these diseases we have to do more research. It is ideal to do the research on tissue from human lungs because we are investigating a human disease. The lung research teams at Glenfield Hospital Leicester, Birmingham Heartlands Hospital, and Walsgrave Hospital, Coventry have joined together in order to collaborate on studies into lung disease using human lung tissue. A numbers of diseases will be studied and the lung tissue will be used in a number of different laboratory studies. The lung tissue will be used to improve our understanding of lung disease which may then lead to the development of new treatments for lung disease. These experiments will be done on lung tissue that has been removed from patients as part of their medical treatment, which would otherwise be destroyed. In some cases we may also wish to take a blood sample to compare the findings in the lung tissue and blood.

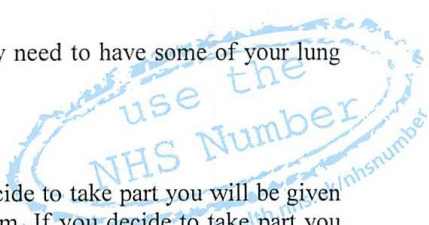
### Why have I been chosen?

You have been chosen because your doctor has said that you may need to have some of your lung removed to treat your disease.

### Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

Chairman Mr Martin Hindle Chief Executive Mr John Adler



**What will happen to me if I take part?**

You are about to undergo an operation for your current condition. We would like to retain some of the spare lung tissue that will be removed as part of your operation, which would otherwise be destroyed. If you are willing to take part in this research, we will pass the surplus lung tissue to the collaborating hospitals. The surgeon will not remove any extra lung tissue for this research. We will also record some information about your recent medical history, medicines taken and reason for the operation from your medical records. In terms of your operation, stay in hospital and subsequent follow up there will be no difference to what will happen to you whether you take part in the study or not except that in a few cases we may wish to take an extra blood sample of approximately 15mL (about three tablespoons full) before your operation.

**What happens if I don't want to take part?**

Nothing, you simply don't sign this form. This will not affect your medical care or your legal rights in any way.

**What rights do I have to the results of the research?**

You are being asked to donate your tissue as a gift to the researchers in the hospitals involved. Any information derived directly or indirectly from this research by the collaborating hospitals, as well as any patents, diagnostic tests, drugs, or biological products developed directly or indirectly as a result, are the sole property of the hospitals (or their successors, licensees, and assigns) and may be used for commercial purposes. You have no right to this property or to any share of the profits that may be earned directly or indirectly as a result of this research. However, in signing this form and donating a blood sample, you do not give up any rights that you would otherwise have as a participant in research.

**What do I have to do?**

There is nothing extra to do as a result of being part of this study

**What are the possible disadvantages and risks of taking part?**

There are no disadvantages or risks to taking part in the study over and above the normal risks associated with this surgery, which you require as part of your care. If you are asked to donate an additional blood sample there may be some discomfort of the needle being inserted into a vein in your arm and the possibility of bruising developing afterwards around the area that the needle was inserted. This should disappear in a few days

**What are the possible benefits of taking part in the study?**

There are no direct benefits. Taking part in this study means that you may possibly help suffers of lung disease in the future, as information about the changes that occur in the lung may be used to develop new treatments.

### **What if something goes wrong?**

We do not think there is any significant risk of any harm occurring as a result of participating in this study. However if you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms would be available to you.

### **Will my taking part in this study be kept confidential?**

All information resulting from you taking part in the study will be stored both in paper and computerised form, and will be treated confidentially. You will be identified in the computer by a number and only your doctor will be able to identify the number as belonging to you. The study records and your medical records will not be made available in any form to anyone other than authorised representatives of all three health authorities (Leicester, Birmingham, and Coventry & Warwickshire). In all instances, your confidentiality will be maintained, in accordance with the Data Protection Act or as local laws permit.

Regulatory authorities may wish to check that this research has been done properly, they may have access to your files and know your identity, but they are under a duty of confidentiality not to disclose details to others.

### **What will happen to the samples that I have donated?**

The samples will be processed by the research team and used in a range of experiments into the causes of lung disease. Samples may be transported to other hospitals in the collaborating group to do further experiments including tests to develop new drugs. Those samples that are not fully used up in experiments may be stored by the research team, for use in future experiments, for up to 20 years. With your consent your samples and data may also be used in other ethically approved research and by other academic partners or industrial collaborators. In all instances your data will be anonymised.

### **Who is organising and funding the research?**

The research is collaboration between the lung research teams at the hospitals in Leicester, Coventry and Birmingham. The study is organised and operated by the individual hospitals involved.

### **Can I withdraw my consent?**

You may withdraw your consent to the use of your data and samples at any time. If you withdraw your permission consent before your donated tissue and data are used, we will not use the data and the samples will be destroyed. If you withdraw your consent after your tissue sample has been sent for analysis we will ensure that your sample(s) are destroyed. However, if analysis has already been performed by ourselves we are not obliged to destroy results of this research.

**Who has reviewed the study?**

The study has been reviewed by the research teams within the consortium and by the members of the Department of Respiratory Medicine, Allergy and Thoracic Surgery. Individual research projects where we use the lung tissue have been reviewed by a variety of charities and funding organisations

If you have any further questions about this study please do discuss them with:

**Professor Wardlaw or Mr Waller (0116 2563841)**

This document must be kept in the investigator's study file and retained for a minimum period of 20 years after completion of the study.



*Caring at its best*

**Consent Form**

**Glenfield Hospital**

**Study Title:** Use of Tissue from Lung Resections to Investigate the Molecular and Functional Mechanisms of Human Lung Disease

Groby Road  
Leicester  
LE3 9QP

Tel: 0300 303 1573  
Fax: 0116 258 3950  
Minicom: 0116 287 9852

1. I confirm that I have read and understood the patient information form on the above project and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand why the research is being done and any foreseeable risks involved.

Initials

☐

2. I agree to donate as a gift a sample of tissue for research in the above project which together with my data may also be used in other ethically approved research and by academic partners or industrial collaborators. I understand how the sample will be collected and that giving the sample is voluntary. I am free to withdraw my approval for use of the sample at any time without giving any reason and without my medical treatment or legal rights being affected. In all instances my data will be anonymised.

☐

3. I understand that relevant sections of my medical notes and/or study data may be looked at by responsible individuals from the study team, the sponsor, NHS Trust or from regulatory authorities where it is relevant to my taking part in the research. I give permission for these individuals to access my records. I also give permission for my medical records to be looked at and information taken from them to be treated in strict confidence by responsible people from Glenfield Leicester, Heartlands Birmingham, and Walsgrave Coventry.

☐

4. I understand that my doctor will be informed if any of the results of the tests done as part of the research are important for my health.

☐

5. I understand that I will not benefit financially if this research leads to a new treatment or medical test.

☐

6. I do know where to contact Professor Wardlaw, if I need further information.

☐

7. Do you agree to take part in this study?

YES	NO
-----	----

Signed: ..... Date: .....

Name (Block capitals) .....

I, (Name of investigator, block letters) .....  
have explained the nature and purpose of the study to .....  
and believe that he/she understands what the study involves.

Signed: ..... Trust Headquarters, Level 3, Balmoral Building, Leicester Royal Infirmary, Leicester, LE1 5WW.  
Website: www.leicestershospitals.nhs.uk  
Chairman Mr Martin Hindle Chief Executive Mr John Adler



Glenfield Hospital  
Groby Road  
Leicester  
LE3 9QP

**PARTICIPANT INFORMATION SHEET  
& CONSENT FORM**

Tel: 0300 303 1573  
Fax: 0116 258 3950  
Minicom: 0116 287 9852

**Healthy Volunteer**

**TITLE: An open study to measure imaging biomarkers and inflammatory cells, mediators and biomarkers from blood, urine and airway samples from healthy volunteers, asthma patients and COPD patients in stable disease and during acute exacerbation.**

**Principal Investigator: Professor Christopher Brightling**

You may contact the Research Nurses on 0116 258 3119 or the study doctor Professor Brightling on 0116 2583998 or via airpager through switchboard.

**Invitation**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it involves. Please take time to read the following information carefully and discuss it with others if you wish.

**What is the purpose of the study?**

Asthma and COPD (Chronic Obstructive Pulmonary Disease) means there is damage to the lungs, which prevents them from working as well as people without asthma or COPD. We do not fully understand the cause but if we can find out, it will help to devise the best treatment for these diseases. To do this we would like to collect and compare sputum and blood to further understand asthma, COPD and other diseases from healthy people.

**Do I have to take part?**

It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw or not take part at any time will not affect the standard of care you receive.

Information Sheet and Consent Form (HV)  
Version 11 16-May-12

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1 for patient; 1 for researcher; 1 to be kept with hospital notes  
REC Ref: 08/H0406/189

**What will happen to me if I take part?**

The study involves a preliminary information visit (Visit 1) followed by a screening visit (Visit 2) followed by routine visits (Visit 3 onwards). There will be a maximum of 12 visits per year.

At Visit 1 the study doctor will explain the details of the study. If you agree to take part, you will be asked to sign the consent form.

The tests and visits are outlined here:

	Screening Visit	Routine Visit
Medical History and Review	✓	✓
Physical Exam	✓	
Breathing Tests	✓	✓
DEXA scan and Chest X Ray (if needed)	✓	
CT scan (optional)	✓	
MRI scan (optional)	✓	
Bronchoscopy (optional)	✓	
Allergy Skin Prick Tests	✓	✓
Blood and sputum collection	✓	✓
Nasal sampling	✓	✓
Urine Sample	✓	✓

**Sputum Collection:** To help you to produce sputum you may need to inhale a salty solution for three 5-minute periods. This can cause some chest tightness, wheezing and/or cough. These can all readily be reversed by inhaling a bronchodilator (ventolin)

**Blood Collection:** You will be asked to provide no more than 100mL of blood every 3 months (100ml is equivalent to 10 tablespoons). This may cause some mild discomfort and occasionally some bruising.

**Breathing Tests:** This will involve breathing out as hard as you can several times, repeated once more after inhaling Ventolin (Spirometry). Other tests involve breathing in safe gas mixtures and blowing out as long as you can to measure the irritation in your lungs. One breathing test is carried sitting in a transparent glass booth. The breathing test may cause some temporary light headedness, and coughing.

**Nasal sampling:** A small brush is inserted and gently rubbed inside the nostril. This takes a few seconds to do and may cause sneezing and or coughing. Occasionally there may be a little discomfort and rarely minor bleeding. A small piece of paper will be inserted into the nostril to collect nasal fluid.

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**Skin Prick Tests:** This test is to determine if you are allergic to any common allergens found in the air. A series of test droplets are placed on your forearm and then introduced into the skin using a small lancet. If you are allergic to the substance the surrounding skin will redden and may itch.

**Urine sample:** A portion of urine will be collected.

**DEXA scan:** This is like a chest x-ray, but requires you to lie down. It measures the proportion of muscle, fat and bone density in your body. The radiation dose from each DEXA scan is equivalent to radiation from less than 1/10<sup>th</sup> of a chest x-ray. Chest x-rays expose you to a minimum amount of radiation.

**CT scan (optional):** A CT scan is a type of X-ray examination that gives much more information than a normal X-ray. It produces detailed images of the lungs, breathing passages and blood vessels. The scanner itself is shaped like a large donut and is not enclosed. You will have two scans, one with your lungs empty and one with your lungs full. This is so that we can tell if air is getting trapped in your lungs when you breathe out.

The CT scan will expose you to radiation. The dose of radiation will be higher than you would normally be exposed to, and this may cause a very small increase in the risk of developing cancer.

Low radiation dose CT scans will be performed for the purpose of this study. Each CT scan will be equivalent to approximately eight months' natural radiation exposure in the UK. We will closely monitor the radiation dose due to CT scans during the course of the study and the upper limit of radiation exposure will be equivalent to two years' natural radiation exposure in the UK.

The natural risk of fatal cancer in the UK population is about 1 in 4. We estimate that the amount of additional radiation exposure in this study will increase this risk slightly to 1 in 3.994.

**MRI scan (optional):** An MRI scan uses a powerful magnet and a computer to produce detailed images of any part of the body. In this case we will be looking particularly at the lungs. An MRI scan does not involve X-rays. You will be asked to lie on the scan table and breathe a small quantity of helium gas – This is completely harmless. You will then be moved slowly into the scanner, which is shaped like a tube. The scan itself will take about 15 minutes.

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You **cannot** have an MRI scan if:

- you have a pacemaker or have ever had heart surgery
- you have ever had any metal fragments in your eyes
- you have any implants or surgical clips in your body or head
- you have had an operation in the last six weeks
- you are pregnant or breast-feeding
- you suffer with severe claustrophobia.

**Bronchoscopy (optional):** Bronchoscopy is a procedure that allows doctors to see inside the breathing passages and take samples. It is performed using a thin flexible tube that has a camera on the end, called a bronchoscope. The bronchoscope has ports that allow instruments to be introduced for taking samples.

The procedure should take about 30 minutes from start to finish. First an anaesthetic spray will be applied to the back of your throat and an anaesthetic gel to your nostrils. You will be given an injection to make you feel sleepy (sedation) unless you would prefer not to receive this. The bronchoscope is inserted through the nose or mouth and slowly advanced into the breathing passages. The tube is much thinner than the breathing passages and does not restrict your ability to breathe. Once in the correct position, the doctor performing the procedure will pass local anaesthetic into the breathing passages and take the samples that are needed.

To remove secretions from the breathing passages, sterile water is inserted through the bronchoscope into the breathing tubes and then removed again via the bronchoscope. It may cause you to cough but is not painful. Cells from the lining of the breathing passages are collected by gently rubbing a soft brush against the side of the breathing passages. Small samples of the walls of the breathing passages are collected using forceps. A small sample of lung tissue will be taken using forceps. This may be performed under X-ray guidance.

Common side effects include a small amount of blood from the nostril or on coughing, a sore throat or a hoarse voice for up to a few days following the test.

Less common side effects may be due to the effect of sedation, which can affect the breathing or cause excessive sleepiness. Occasionally (in about 1 in 20 cases), people having a biopsy taken from the lung can have a small amount of air leak from the lung (pneumothorax). This usually just requires admission to hospital overnight for observation. In rare cases the leaked air needs to be removed using a special tube (chest drain).

After the test, you will be monitored for 2-3 hours in the Endoscopy Department and if we have taken a sample of lung tissue we will perform a chest X-ray to make sure there is no air leak. If your pulse, blood pressure and oxygen levels are fine, as well as your X-ray, then you will be able to go home. Your throat may feel a little numb from the anaesthetic spray but this

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will wear off after a few hours. You should not eat for about three hours after the test, until your swallowing feels normal.

You may provide any single sample or combination of samples of blood and sputum, on these visits.

### **Expenses and payments**

Travel and car park charges will be reimbursed to you throughout the study. For the inconvenience of the bronchoscopy test you will be additionally compensated £200.

### **What if new information becomes available?**

As part of this study we may uncover medical conditions not previously recognised (e.g. diabetes, abnormal liver function), if this happens we will assess your condition and manage you accordingly which may result in referral to other specialist teams or back to your GP for further care.

### **What if something goes wrong?**

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Professor Brightling 0116 258 3998). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

### **Will any genetic tests be done?**

Yes. Your blood sample will be used to look at the patterns in the inherited material that may be linked to an increased risk of developing airway disease. We will not be looking at your personal genetics. This is preliminary work which would not affect insurance status or be used to give information for other family members and therefore the results will not be made available to you.

### **Will my taking part in this study be kept confidential?**

By signing this form you consent to the Study Doctor and his or her staff collecting and using personal data about you for the study ("Study Data"). This includes: your date of birth, your sex, your ethnic origin and personal data on your physical or mental health or condition. Your consent to the use of Study Data does not have a specific expiration date, but you may withdraw your consent at any time by notifying the Study Doctor.

Any Study Data shared with our research collaborators in other academic institutions and pharmaceutical research companies is protected by the use of a code (the "Code"), which is a number specific to you. The Study Doctor is in control of the Code key, which is needed to connect Study Data to you. A person appointed by our research collaborators in other

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academic institutions and pharmaceutical research companies, regulatory authorities or other supervisory bodies may review any Study Data held by the Study Doctor.

The Study Doctor will use Study Data to conduct the Study. Our research collaborators in other academic institutions and pharmaceutical research companies may use Study Data for research related to the development of pharmaceutical products, diagnostics or medical aids. The Study Doctor's institution and our research collaborators in other academic institutions and pharmaceutical research companies are each responsible for their handling of Study Data in accordance with applicable Data Protection law(s).

The Leicester Glenfield Hospital and our research collaborators in other academic institutions and pharmaceutical research companies may share Study Data with other companies within their group (where applicable), with service providers, contractors and with research institutions and research based commercial organisations, who will use Study Data only for the purposes described above.

The Leicester Glenfield Hospital and our research collaborators in other academic institutions and pharmaceutical research companies may transfer Study Data to countries outside of the UK and the European Economic Area (EEA) for the purposes described in this document. Please be aware that the laws in such countries may not provide the same level of data protection as in the UK and may not stop Study Data from being shared with others. However, rest assured that all Study Data that is transferred would be coded, so your identity is masked. Please note, the results of the Study may be published in medical literature, but you will not be identified.

You have the right to request information about Study Data held by the Study Doctor and our research collaborators in other academic institutions and pharmaceutical research companies. You also have the right to request that any inaccuracies in such data be corrected. If you wish to make a request, then please contact the Study Doctor.

If you withdraw your consent, the Study Doctor will no longer use Study Data or share it with others. Our research collaborators in other academic institutions and pharmaceutical research companies may still use Study Data that was shared with it before you withdrew your consent for the legitimate purposes detailed in this document. By signing this form you consent to the use of Study Data as described in this form

How will my samples be used?

All the testing of your samples, now and in the future, will be performed for research and development purposes only. Any information derived directly or indirectly from this research or any optional future research, as well as any patents, diagnostic tests, drugs, or biological products developed, are the property of the researchers. The results from this research and any future research may be used for commercial purposes. You will have no right to this

Information Sheet and Consent Form (HV)

Version 11 16-May-12

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1 for patient; 1 for researcher; 1 to be kept with hospital notes

REC Ref: 08/H0406/189

property or to any share of the profits that may be earned directly or indirectly as a result of this or any future research. However, in signing this form and donating sample(s) for this and any future research, you do not give up any rights that you would otherwise have as a participant in such research.

Your samples will be kept for up to 15 years after the study is completed and at that time any remaining samples will be destroyed or anonymised i.e. the link between you and your samples will be broken so that the samples can never be traced back to you.

You may withdraw your consent to the use of your biological sample(s) at any time by contacting your Study Doctor/nurse. If a link between you and your samples exists and the samples have not been anonymised then we will ensure that your biological sample(s) will be destroyed. However, if any analysis has already been performed then neither we nor our research collaborators in other academic institutions and pharmaceutical research companies are obliged to destroy the results of this research.

**Who has reviewed the study?**

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

**Thank you for reading this**

**Please keep a copy of this information sheet and the enclosed consent form.**



Patient name, address, DOB (*or*  
*ID label*)

Groby Road  
Leicester  
LE3 9QP

**Study Number: UHL 10613**

**Patient Identification Number for this study:**

Tel: 0300 303 1573  
Fax: 0116 258 3950  
Minicom: 0116 287 9852

**CONSENT FORM: Healthy volunteer**

**TITLE: An open study to measure imaging biomarkers and inflammatory cells, mediators and biomarkers from blood, urine and airway samples from healthy volunteers, asthma patients and COPD patients in stable disease and during acute exacerbation.**

Name of Investigator: **Professor Brightling**

**Please initial box**

I confirm that I have read and understand the information sheet dated 16/05/2012, version 11 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐

I understand that relevant sections of my medical notes and/or study data may be looked at by responsible individuals from the study team, the sponsor, NHS Trust or from regulatory authorities where it is relevant to my taking part in the research. I give permission for these individuals to access my records

☐

I understand that I am consenting for samples of blood, sputum, urine and nasal samples to be used in research along with coded study data by Leicester Glenfield Hospital, academic & industry partners at routine visits and whilst in hospital. I am aware that the specific nature of the research will vary and is not known fully at this time.

☐

I give permission for my samples to be used for genetic tests. I understand that my blood sample will be used to look at the patterns in the inherited material that may be linked to an increased risk of developing airway disease. I understand that this is preliminary work and would not affect insurance status or be used to give information for other family members and therefore the results will not be made available to me.

☐

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REC Ref: 08/H0406/189

I agree to take part in the above study

☐

I agree to undertake bronchoscopy as part of this study (optional).

☐ ☐

Yes No

I agree to have CT scans as part of this study (optional).

☐ ☐

Yes No

I agree to have one or more MRI scans as part of this study (optional).

☐ ☐

Yes No

\_\_\_\_\_  
Name of Patient

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Name of Person taking consent  
(if different from researcher)

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Researcher

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature

Information Sheet and Consent Form (HV)  
Version 11 16-May-12

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1 for patient; 1 for researcher; 1 to be kept with hospital notes  
REC Ref: 08/H0406/189

Glenfield Hospital  
Groby Road  
Leicester  
LE3 9QP

**PARTICIPANT INFORMATION SHEET  
& CONSENT FORM**

Tel: 0300 303 1573  
Fax: 0116 258 3950  
Minicom: 0116 287 9852

**COPD**

**TITLE: An open study to measure imaging biomarkers and inflammatory cells, mediators and biomarkers from blood, urine and airway samples from healthy volunteers, asthma patients and COPD patients in stable disease and during acute exacerbation.**

**Principal Investigator: Professor C Brightling**

You may contact the Research Nurses on 0116 258 3119 or the study doctor Professor Brightling on 0116 258 3998 or via airpager through switchboard.

**Invitation**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it involves. Please take time to read this information sheet and discuss it with others if you wish.

**What is the purpose of the study?**

You have been invited to take part in this study because you have COPD (Chronic Obstructive Pulmonary Disease). We do not fully understand the cause of COPD, but if we can find out, it will help to design the best treatment for the disease. To do this we would like to collect and analyse samples of blood, sputum and urine, to further understand COPD and other diseases. All the tissue samples you have donated will be used in research by Leicester Glenfield Hospital and our research collaborators in other academic institutions and pharmaceutical research companies.

**Do I have to take part?**

It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw or not take part at any time will not affect the standard of care you receive.

**What will happen to me if I take part?**

Patient Information Sheet and Consent Form (COPD)

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1 for patient; 1 for researcher; 1 to be kept with hospital notes

REC Ref: 08/H0406/189

The study involves a preliminary information visit (Visit 1) followed by a screening visit (Visit 2) followed by routine visits (Visit 3 onwards). There will be a maximum of 12 visits per year to provide samples.

At Visit 1 the study doctor will explain the details of the study. If you agree to take part, you will be asked to sign the consent form.

*The tests and visits are outlined overleaf:*

	Screening Visit	Routine Visits	Exacerbations
Detailed review of medical history	✓		
Physical Exam & screening blood test	✓		
Breathing tests	✓	✓	✓
DEXA scan (& CXR if needed)	✓		✓
CT scan (optional)	✓		
MRI scan (optional)	✓		
Bronchoscopy (camera test)	✓		
Questionnaires	✓	✓	✓
Study blood and urine collection	✓	✓	✓
Sputum collection	✓	✓	✓

**Sputum Collection:** To help you to produce sputum you may need to inhale a salty solution for three 5-minute periods. This can cause some chest tightness, wheezing and/or cough. These can all readily be reversed by inhaling a bronchodilator (ventolin)

**Blood Collection:** You will be asked to provide no more than 100mL of blood every 3 months (100ml is equivalent to 10 tablespoons). This may cause some mild discomfort and occasionally some bruising.

**Breathing Tests:** This will involve breathing out as hard as you can several times, repeated once more after inhaling Ventolin (Spirometry). The breathing test may cause some temporary light headedness, and coughing.

**Questionnaires:** You will be asked to complete questionnaires regarding your illness.

**DEXA scan:** This is like a chest x-ray, but requires you to lie down. It measures the proportion of muscle, fat and bone density in your body. The radiation dose from each DEXA scan is equivalent to radiation from less than 1/10<sup>th</sup> of a chest x-ray. Chest x-rays expose you to a minimum amount of radiation.

Patient Information Sheet and Consent Form (COPD)

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**Urine test:** A portion of urine will be collected

You may provide any single sample or combination of samples of blood and sputum, on these visits. We would also like to collect samples from you if you have a flare up of COPD. This additional part of the study is voluntary. You can still participate in the scheduled visits described above without having to consent to providing samples if you should have an exacerbation.

**CT scan (optional):** A CT scan is a type of X-ray examination that gives much more information than a normal X-ray. It produces detailed images of the lungs, breathing passages and blood vessels. The scanner itself is shaped like a large donut and is not enclosed. You will have two scans, one with your lungs empty and one with your lungs full. This is so that we can tell if air is getting trapped in your lungs when you breathe out.

The CT scan will expose you to radiation. The dose of radiation will be higher than you would normally be exposed to, and this may cause a very small increase in the risk of developing cancer.

Low radiation dose CT scans will be performed for the purpose of this study. Each CT scan will be equivalent to approximately eight months' natural radiation exposure in the UK. We will closely monitor the radiation dose due to CT scans during the course of the study and the upper limit of radiation exposure will be equivalent to two years' natural radiation exposure in the UK.

The natural risk of fatal cancer in the UK population is about 1 in 4. We estimate that the amount of additional radiation exposure in this study will increase this risk slightly to 1 in 3.994.

**MRI scan (optional):** An MRI scan uses a powerful magnet and a computer to produce detailed images of any part of the body. In this case we will be looking particularly at the lungs. An MRI scan does not involve X-rays. You will be asked to lie on the scan table and breathe a small quantity of helium gas – This is completely harmless. You will then be moved slowly into the scanner, which is shaped like a tube. The scan itself will take about 15 minutes.

You **cannot** have an MRI scan if:

- a) you have a pacemaker or have ever had heart surgery
- b) you have ever had any metal fragments in your eyes
- c) you have any implants or surgical clips in your body or head
- d) you have had an operation in the last six weeks
- e) you are pregnant or breast-feeding.

**Bronchoscopy (optional):** Bronchoscopy is a procedure that allows doctors to see inside the breathing passages and take samples. It is performed using a thin flexible tube that has a camera on the end, called a bronchoscope. The bronchoscope has ports that allow instruments to be introduced for taking samples.

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The procedure should take about 30 minutes from start to finish. First an anaesthetic spray will be applied to the back of your throat and an anaesthetic gel to your nostrils. You will be given an injection to make you feel sleepy (sedation) unless you would prefer not to receive this. The bronchoscope is inserted through the nose or mouth and slowly advanced into the breathing passages. The tube is much thinner than the breathing passages and does not restrict your ability to breathe. Once in the correct position, the doctor performing the procedure will pass local anaesthetic into the breathing passages and take the samples that are needed.

To remove secretions from the breathing passages, sterile water is inserted through the bronchoscope into the breathing tubes and then removed again via the bronchoscope. It may cause you to cough but is not painful. Cells from the lining of the breathing passages are collected by gently rubbing a soft brush against the side of the breathing passages. Small samples of the walls of the breathing passages are collected using forceps. A small sample of lung tissue will be taken using forceps. This may be performed under X-ray guidance.

Common side effects include a small amount of blood from the nostril or on coughing, a sore throat or a hoarse voice for up to a few days following the test.

Less common side effects may be due to the effect of sedation, which can affect the breathing or cause excessive sleepiness. Occasionally (in about 1 in 20 cases), people having a biopsy taken from the lung can have a small amount of air leak from the lung. This usually just requires admission to hospital overnight for observation. In rare cases the leaked air needs to be removed using a special tube (chest drain).

After the test, you will be monitored for 1-2 hours in the Endoscopy Department and if we have taken a sample of lung tissue we will perform a chest X-ray to make sure there is no air leak. If your pulse, blood pressure and oxygen levels are fine, as well as your X-ray, then you will be able to go home. Your throat may feel a little numb from the anaesthetic spray but this will wear off after a few hours. You should not eat for about three hours after the test, until your swallowing feels normal.

### **Expenses and payments**

Travel and car park charges will be reimbursed to you throughout the study. For the inconvenience of the bronchoscopy test you will be additionally compensated £200.

### **What do I have to do?**

During the study you will be encouraged to use your regular medication. Before the visits you will need to withhold certain inhalers and tablets, as these will affect the breathing tests. This is routine and you will be given written information about this. It is important that you restart your regular medication after each visit.

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REC Ref: 08/H0406/189

**What if new information becomes available?**

As part of this study we may uncover medical conditions not previously recognised (e.g. diabetes, abnormal liver function), if this happens we will assess your condition and manage you accordingly which may result in referral to other specialist teams or back to your GP for further care.

**What if something goes wrong?**

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Professor Brightling 0116 258 3998). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

**Will any genetic tests be done?**

Yes. Your blood sample will be used to look at the patterns in the inherited material that may be linked to an increased risk of developing airway disease. We will not be looking at your personal genetics. This is preliminary work which would not affect insurance status or be used to give information for other family members and therefore the results will not be made available to you.

**Will my taking part in this study be kept confidential?**

By signing this form you consent to the Study Doctor and his or her staff collecting and using personal data about you for the study ("Study Data"). This includes: your date of birth, your sex, your ethnic origin and personal data on your physical or mental health or condition. Your consent to the use of Study Data does not have a specific expiration date, but you may withdraw your consent at any time by notifying the Study Doctor.

Any Study Data shared with our research collaborators in other academic institutions and pharmaceutical research companies is protected by the use of a code (the "Code"), which is a number specific to you. The Study Doctor is in control of the Code key, which is needed to connect Study Data to you. A person appointed by our research collaborators in other academic institutions and pharmaceutical research companies, regulatory authorities or other supervisory bodies may review any Study Data held by the Study Doctor.

The Study Doctor will use Study Data to conduct the Study. Our research collaborators in other academic institutions and pharmaceutical research companies may use Study Data for research related to the development of pharmaceutical products, diagnostics or medical aids. The Study Doctor's institution and our research collaborators in other academic institutions and pharmaceutical research companies are each responsible for their handling of Study Data in accordance with applicable Data Protection law(s).

The Leicester Glenfield Hospital and our research collaborators in other academic institutions and pharmaceutical research companies may share Study Data with other companies within their group (where applicable), with service providers, contractors and with research

Patient Information Sheet and Consent Form (COPD)

Version 10 dated 22 Jun 2012

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1 for patient; 1 for researcher; 1 to be kept with hospital notes

REC Ref: 08/H0406/189

institutions and research based commercial organisations, who will use Study Data only for the purposes described above.

The Leicester Glenfield Hospital and our research collaborators in other academic institutions and pharmaceutical research companies may transfer Study Data to countries outside of the UK and the European Economic Area (EEA) for the purposes described in this document. Please be aware that the laws in such countries may not provide the same level of data protection as in the UK and may not stop Study Data from being shared with others. However, rest assured that all Study Data that is transferred would be coded, so your identity is masked. Please note, the results of the Study may be published in medical literature, but you will not be identified.

You have the right to request information about Study Data held by the Study Doctor and our research collaborators in other academic institutions and pharmaceutical research companies. You also have the right to request that any inaccuracies in such data be corrected. If you wish to make a request, then please contact the Study Doctor.

If you withdraw your consent, the Study Doctor will no longer use Study Data or share it with others. Our research collaborators in other academic institutions and pharmaceutical research companies may still use Study Data that was shared with it before you withdrew your consent for the legitimate purposes detailed in this document. By signing this form you consent to the use of Study Data as described in this form

### **How will my samples be used?**

All the testing of your samples, now and in the future, will be performed for research and development purposes only. Any information derived directly or indirectly from this research or any optional future research, as well as any patents, diagnostic tests, drugs, or biological products developed, are the property of the researchers. The results from this research and any future research may be used for commercial purposes. You will have no right to this property or to any share of the profits that may be earned directly or indirectly as a result of this or any future research. However, in signing this form and donating sample(s) for this and any future research, you do not give up any rights that you would otherwise have as a participant in such research.

Your samples will be kept for up to 15 years after the study is completed and at that time any remaining samples will be destroyed or anonymised i.e. the link between you and your samples will be broken so that the samples can never be traced back to you.

You may withdraw your consent to the use of your biological sample(s) at any time by contacting your Study Doctor/nurse. If a link between you and your samples exists and the samples have not been anonymised then we will ensure that your biological sample(s) will be destroyed. However, if any analysis has already been performed then neither we nor our research collaborators in other academic institutions and pharmaceutical research companies are obliged to destroy the results of this research.

Patient Information Sheet and Consent Form (COPD)

Version 10 dated 22 Jun 2012

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1 for patient; 1 for researcher; 1 to be kept with hospital notes

REC Ref: 08/H0406/189



**Who has reviewed the study?**

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

**Thank you for reading this**

**Please keep a copy of this information sheet and the enclosed consent form.**

Patient Information Sheet and Consent Form (COPD)

Version 10 dated 22 Jun 2012

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1 for patient; 1 for researcher; 1 to be kept with hospital notes

REC Ref: 08/H0406/189

Patient name, address, DOB  
(or ID label)

Glenfield Hospital  
Groby Road  
Leicester  
LE3 9QP

**Study Number: UHL 10613**

**Patient Identification Number for this study:**

Tel: 0300 303 1573  
Fax: 0116 258 3950  
Minicom: 0116 287 9852

**CONSENT FORM: COPD**

**TITLE: An open study to measure imaging biomarkers and inflammatory cells, mediators and biomarkers from blood, urine and airway samples from healthy volunteers, asthma patients and COPD patients in stable disease and during acute exacerbation.**

Name of Investigator: **Professor Brightling**

**Please initial box**

I confirm that I have read and understand the information sheet dated 16/05/2012, version 11 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐

I understand that relevant sections of my medical notes and/or study data may be looked at by responsible individuals from the study team, the sponsor, NHS Trust or from regulatory authorities where it is relevant to my taking part in the research. I give permission for these individuals to access my records

☐

I understand that I am consenting for samples of blood, sputum, urine and nasal samples to be used in research along with coded study data by Leicester Glenfield Hospital, academic & industry partners at routine visits and whilst in hospital. I am aware that the specific nature of the research will vary and is not known fully at this time.

☐

I give permission for my samples to be used for genetic tests. I understand that my blood sample will be used to look at the patterns in the inherited material that may be linked to an increased risk of developing airway disease. I understand that this is preliminary work and would not affect insurance status

☐

Patient Information Sheet and Consent Form (COPD)  
Version 10 dated 22 Jun 2012

---

1 for patient; 1 for researcher; 1 to be kept with hospital notes  
REC Ref: 08/H0406/189

or be used to give information for other family members and therefore the results will not be made available to me.

I agree to take part in the above study

☐

I agree to undertake bronchoscopy as part of this study (optional).

☐☐

Yes

No

I agree to have CT scans as part of this study (optional).

☐☐

Yes

No

I agree to have one or more MRI scans as part of this study (optional).

☐☐

Yes

No

\_\_\_\_\_  
Name of Patient

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Name of Person taking consent  
(if different from researcher)

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Researcher

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature

Patient Information Sheet and Consent Form (COPD)

Version 10 dated 22 Jun 2012

1 for patient; 1 for researcher; 1 to be kept with hospital notes

REC Ref: 08/H0406/189

Glenfield Hospital  
Groby Road  
Leicester  
LE3 9QP

Tel: 0300 303 1573  
Fax: 0116 258 3950  
Minicom: 0116 287 9852

## **PARTICIPANT INFORMATION SHEET & CONSENT FORM**

### **Asthma**

**TITLE: An open study to measure imaging biomarkers and inflammatory cells, mediators and biomarkers from blood, urine and airway samples from healthy volunteers, asthma patients and COPD patients in stable disease and during acute exacerbation.**

**Principal Investigator: Professor C Brightling**

You may contact the Research nurses on 0116 2583119 or the study doctor, Prof C Brightling on 0116 2583998 or via airpager through switchboard.

### **Invitation**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it involves. Please take time to read this information sheet and discuss it with others if you wish.

### **What is the purpose of the study?**

You have been invited to take part because you have asthma. We do not fully understand the cause of asthma but if we can find out, it will help to design the best treatment for the disease. To do this we would like to collect and analyse samples of blood and sputum, to further understand asthma and other diseases. All the tissue samples you have donated will be used in research by Leicester Glenfield Hospital and our research collaborators in other academic institutions and pharmaceutical research companies.

### **Do I have to take part?**

It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw or not take part at any time will not affect the standard of care you receive.

Patient Information Sheet and Consent Form (Asthma)  
Version 10 dated 26/08/2011

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1 for patient; 1 for researcher; 1 to be kept with hospital notes  
REC Ref: 08/H0406/189

### What will happen to me if I take part?

The study involves a preliminary information visit (Visit 1) followed by a screening visit (Visit 2) followed by routine visits (Visit 3 onwards). There will be a maximum of 12 visits per year to provide samples.

At Visit 1 the study doctor will explain the details of the study. If you agree to take part, you will be asked to sign the consent form.

*The tests and visits are outlined overleaf:*

	Screening Visit	Routine Visit	Exacerbations
Medical History and Review	✓	✓	✓
Physical Exam	✓		✓
Breathing Tests	✓	✓	✓
DEXA scan and (Chest X Ray if needed)	✓		✓
CT scan (optional)	✓		
MRI scan (optional)	✓		
Bronchoscopy (optional)	✓		
Questionnaires	✓	✓	✓
Blood and sputum collection	✓	✓	✓
Allergy Skin Prick Tests	✓	✓	
Nasal sampling	✓	✓	✓
Urine sample	✓	✓	✓

**Sputum Collection:** To help you to produce sputum you may need to inhale a salty solution for three 5-minute periods. This can cause some chest tightness, wheezing and/or cough. These can all readily be reversed by inhaling a bronchodilator (ventolin)

**Blood Collection:** You will be asked to provide no more than 100 ml of blood every 3 months (100ml is equivalent to 10 tablespoons), however the amount usually required is half this (50mls or 5 tablespoons). This may cause some mild discomfort and occasionally some bruising.

**Breathing Tests:** This will involve breathing out as hard as you can several times, repeated once more after inhaling ventolin (Spirometry). Another test to measure the irritation in your lungs, involves breathing in a safe gas mixture and blowing out as long as you can. One breathing test is carried out sitting in a transparent glass booth. The breathing test may cause some temporary light headedness, and coughing. You may be asked to keep a record of your peak flow in either a diary or electronic format.

**Nasal sampling:** A small brush is inserted and gently rubbed inside the nostril. This takes a few seconds to do and may cause sneezing and or coughing. Occasionally there may be a little discomfort and rarely minor bleeding. A small piece of paper will be inserted into the nostril to collect nasal fluid.

**Skin Prick Tests:** This test is to determine if you are allergic to any common allergens found in the air. A series of test droplets are placed on your forearm and then introduced into the skin using a small lancet. If you are allergic to the substance the surrounding skin will redden and may itch.

**Urine sample:** A portion of urine will be collected.

Patient Information Sheet and Consent Form (Asthma)

Version 10 dated 26/08/2011

1 for patient; 1 for researcher; 1 to be kept with hospital notes

REC Ref: 08/H0406/189

**DEXA scan:** This is like a chest x-ray, but requires you to lie down. It measures the proportion of muscle, fat and bone density in your body. The radiation dose from each DEXA scan is equivalent to radiation from less than 1/10<sup>th</sup> of a chest x-ray. Chest x-rays expose you to a minimum amount of radiation.

You may provide any single sample or combination of samples of blood and sputum, on these visits. We would also like to collect samples from you if you have a flare up of asthma. This additional part of the study is voluntary. You can still participate in the scheduled visits described above without having to consent to providing samples if you should have an exacerbation.

**CT scan (optional):** A CT scan is a type of X-ray examination that gives much more information than a normal X-ray. It produces detailed images of the lungs, breathing passages and blood vessels. The scanner itself is shaped like a large donut and is not enclosed. You will have two scans, one with your lungs empty and one with your lungs full. This is so that we can tell if air is getting trapped in your lungs when you breathe out.

The CT scan will expose you to radiation. The dose of radiation will be higher than you would normally be exposed to, and this may cause a very small increase in the risk of developing cancer.

Low radiation dose CT scans will be performed for the purpose of this study. Each CT scan will be equivalent to approximately eight months' natural radiation exposure in the UK. We will closely monitor the radiation dose due to CT scans during the course of the study and the upper limit of radiation exposure will be equivalent to two years' natural radiation exposure in the UK.

The natural risk of fatal cancer in the UK population is about 1 in 4. We estimate that the amount of additional radiation exposure in this study will increase this risk slightly to 1 in 3.994.

**MRI scan (optional):** An MRI scan uses a powerful magnet and a computer to produce detailed images of any part of the body. In this case we will be looking particularly at the lungs. An MRI scan does not involve X-rays. You will be asked to lie on the scan table and breathe a small quantity of helium gas – This is completely harmless. You will then be moved slowly into the scanner, which is shaped like a tube. The scan itself will take about 15 minutes.

You **cannot** have an MRI scan if:

- f) you have a pacemaker or have ever had heart surgery
- g) you have ever had any metal fragments in your eyes
- h) you have any implants or surgical clips in your body or head
- i) you have had an operation in the last six weeks
- j) you are pregnant or breast-feeding.

**Bronchoscopy (optional):** Bronchoscopy is a procedure that allows doctors to see inside the breathing passages and take samples. It is performed using a thin flexible tube that has a camera on the end, called a bronchoscope. The bronchoscope has ports that allow instruments to be introduced for taking samples.

Patient Information Sheet and Consent Form (Asthma)  
Version 10 dated 26/08/2011

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1 for patient; 1 for researcher; 1 to be kept with hospital notes  
REC Ref: 08/H0406/189

The procedure should take about 30 minutes from start to finish. First an anaesthetic spray will be applied to the back of your throat and an anaesthetic gel to your nostrils. You will be given an injection to make you feel sleepy (sedation) unless you would prefer not to receive this. The bronchoscope is inserted through the nose or mouth and slowly advanced into the breathing passages. The tube is much thinner than the breathing passages and does not restrict your ability to breathe. Once in the correct position, the doctor performing the procedure will pass local anaesthetic into the breathing passages and take the samples that are needed.

To remove secretions from the breathing passages, sterile water is inserted through the bronchoscope into the breathing tubes and then removed again via the bronchoscope. It may cause you to cough but is not painful. Cells from the lining of the breathing passages are collected by gently rubbing a soft brush against the side of the breathing passages. Small samples of the walls of the breathing passages are collected using forceps. A small sample of lung tissue will be taken using forceps. This may be performed under X-ray guidance.

Common side effects include a small amount of blood from the nostril or on coughing, a sore throat or a hoarse voice for up to a few days following the test.

Less common side effects may be due to the effect of sedation, which can affect the breathing or cause excessive sleepiness. Occasionally (in about 1 in 20 cases), people having a biopsy taken from the lung can have a small amount of air leak from the lung. This usually just requires admission to hospital overnight for observation. In rare cases the leaked air needs to be removed using a special tube (chest drain).

After the test, you will be monitored for 1-2 hours in the Endoscopy Department and if we have taken a sample of lung tissue we will perform a chest X-ray to make sure there is no air leak. If your pulse, blood pressure and oxygen levels are fine, as well as your X-ray, then you will be able to go home. Your throat may feel a little numb from the anaesthetic spray but this will wear off after a few hours. You should not eat for about three hours after the test, until your swallowing feels normal.

### **Expenses and payments**

Travel and car park charges will be reimbursed to you throughout the study. For the inconvenience of the bronchoscopy test you will be additionally compensated £200.

### **What do I have to do?**

During the study you will be encouraged to use your regular medication. Before the visits you will need to withhold certain inhalers and tablets, as these will affect the breathing tests. This is routine and you will be given written information about this. It is important that you restart your regular medication after each visit.

### **What if new information becomes available?**

As part of this study we may uncover medical conditions not previously recognised (e.g. diabetes, abnormal liver function), if this happens we will assess your condition and manage you accordingly which may result in referral to other specialist teams or back to your GP for further care.

### **What if something goes wrong?**

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Professor Brightling 0116 258 3998). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

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**Will any genetic tests be done?**

Yes. Your blood sample will be used to look at the patterns in the inherited material that may be linked to an increased risk of developing airway disease. We will not be looking at your personal genetics. This is preliminary work which would not affect insurance status or be used to give information for other family members and therefore the results will not be made available to you.

**Will my taking part in this study be kept confidential?**

By signing this form you consent to the Study Doctor and his or her staff collecting and using personal data about you for the study ("Study Data"). This includes: your date of birth, your sex, your ethnic origin and personal data on your physical or mental health or condition. Your consent to the use of Study Data does not have a specific expiration date, but you may withdraw your consent at any time by notifying the Study Doctor.

Any Study Data shared with our research collaborators in other academic institutions and pharmaceutical research companies is protected by the use of a code (the "Code"), which is a number specific to you. The Study Doctor is in control of the Code key, which is needed to connect Study Data to you. A person appointed by our research collaborators in other academic institutions and pharmaceutical research companies, regulatory authorities or other supervisory bodies may review any Study Data held by the Study Doctor.

The Study Doctor will use Study Data to conduct the Study. Our research collaborators in other academic institutions and pharmaceutical research companies may use Study Data for research related to the development of pharmaceutical products, diagnostics or medical aids. The Study Doctor's institution and our research collaborators in other academic institutions and pharmaceutical research companies are each responsible for their handling of Study Data in accordance with applicable Data Protection law(s).

The Leicester Glenfield Hospital and our research collaborators in other academic institutions and pharmaceutical research companies may share Study Data with other companies within their group (where applicable), with service providers, contractors and with research institutions and research based commercial organisations, who will use Study Data only for the purposes described above.

The Leicester Glenfield Hospital and our research collaborators in other academic institutions and pharmaceutical research companies may transfer Study Data to countries outside of the UK and the European Economic Area (EEA) for the purposes described in this document. Please be aware that the laws in such countries may not provide the same level of data protection as in the UK and may not stop Study Data from being shared with others. However, rest assured that all Study Data that is transferred would be coded, so your identity is masked. Please note, the results of the Study may be published in medical literature, but you will not be identified.

You have the right to request information about Study Data held by the Study Doctor and our research collaborators in other academic institutions and pharmaceutical research companies. You also have the right to request that any inaccuracies in such data be corrected. If you wish to make a request, then please contact the Study Doctor.

If you withdraw your consent, the Study Doctor will no longer use Study Data or share it with others. Our research collaborators in other academic institutions and pharmaceutical research companies may still use Study Data that was shared with it before you withdrew your consent for the legitimate purposes detailed in this document. By signing this form you consent to the use of Study Data as described in this form.

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**How will my samples be used?**

All the testing of your samples, now and in the future, will be performed for research and development purposes only. Any information derived directly or indirectly from this research or any optional future research, as well as any patents, diagnostic tests, drugs, or biological products developed, are the property of the researchers. The results from this research and any future research may be used for commercial purposes. You will have no right to this property or to any share of the profits that may be earned directly or indirectly as a result of this or any future research. However, in signing this form and donating sample(s) for this and any future research, you do not give up any rights that you would otherwise have as a participant in such research.

Your samples will be kept for up to 15 years after the study is completed and at that time any remaining samples will be destroyed or anonymised i.e. the link between you and your samples will be broken so that the samples can never be traced back to you.

You may withdraw your consent to the use of your biological sample(s) at any time by contacting your Study Doctor/nurse. If a link between you and your samples exists and the samples have not been anonymised then we will ensure that your biological sample(s) will be destroyed. However, if any analysis has already been performed then neither we nor our research collaborators in other academic institutions and pharmaceutical research companies are obliged to destroy the results of this research.

**Who has reviewed the study?**

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

**Thank you for reading this**

**Please keep a copy of this information sheet and the enclosed consent form.**

Patient name, address, DOB  
(or ID label)

Glenfield Hospital  
Groby Road  
Leicester  
LE3 9QP

**Study Number: UHL 10613**

**Patient Identification Number for this study:**

Tel: 0300 303 1573  
Fax: 0116 258 3950  
Minicom: 0116 287 9852

**CONSENT FORM: Asthma**

**TITLE: An open study to measure imaging biomarkers and inflammatory cells, mediators and biomarkers from blood, urine and airway samples from healthy volunteers, asthma patients and COPD patients in stable disease and during acute exacerbation.**

Name of Investigator: **Professor Brightling**

**Please initial box**

I confirm that I have read and understand the information sheet dated 16/05/2012, version 11 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐

I understand that relevant sections of my medical notes and/or study data may be looked at by responsible individuals from the study team, the sponsor, NHS Trust or from regulatory authorities where it is relevant to my taking part in the research. I give permission for these individuals to access my records

☐

I understand that I am consenting for samples of blood, sputum, urine and nasal samples to be used in research along with coded study data by Leicester Glenfield Hospital, academic & industry partners at routine visits and whilst in hospital. I am aware that the specific nature of the research will vary and is not known fully at this time.

☐

I give permission for my samples to be used for genetic tests. I understand that my blood sample will be used to look at the patterns in the inherited material that may be linked to an increased risk of developing airway disease.

☐

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I understand that this is preliminary work and would not affect insurance status or be used to give information for other family members and therefore the results will not be made available to me.

I agree to take part in the above study

☐

I agree to undertake bronchoscopy as part of this study (optional).

☐☐

Yes

No

I agree to have CT scans as part of this study (optional).

☐☐

Yes

No

I agree to have one or more MRI scans as part of this study (optional).

☐☐

Yes

No

\_\_\_\_\_  
Name of Patient

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Name of Person taking consent  
(if different from researcher)

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Researcher

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature

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