

**Characterization of COPD with biomarkers of
inflammation and quantitative CT**

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Declaration

This MD thesis has been completed by Joy J Miller.

The work is my own and contributions from members of the ELEGI-COLT research group at the University of Edinburgh are gratefully acknowledged.

This work has not been submitted for any other degree or professional qualification except for this MD.

Signed:

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Abstract

Background

The aim of this thesis was to investigate the role of inflammation in the airways and in the systemic compartment in COPD compared to healthy subjects, and to investigate the relationship between inflammation and the clinical and radiological features of COPD.

Methods

182 COPD patients and 96 healthy control subjects were recruited. Post-bronchodilator spirometry, smoking history, body mass index (BMI), exacerbation frequency, St George's Respiratory Questionnaire (SGRQ) scores, MRC dyspnoea and chronic bronchitis scores, oxygen saturations, 6 minute walking distance and BODE index scores were recorded. Highly sensitive C-reactive protein (CRP), total white cell count and neutrophils were measured in blood. Percentage (%) neutrophils, IL-1 β , IL-6 and IL-8 were measured in induced sputum. COPD patients had a quantitative CT scan on inspiration and expiration. Lung volume and density (pixel index -910 HU, pixel index -950 HU, and 15th percentile) were determined using in-house software.

Results

Systemic inflammation was increased in COPD subjects compared to healthy controls: CRP ($p<0.001$), blood neutrophils ($p<0.001$). Pulmonary inflammation was also increased in COPD subjects compared to healthy controls: induced sputum % neutrophils ($p=0.001$), IL-6 ($p=0.02$) and IL-8 ($p=0.01$). Induced sputum IL-1 β was not increased in COPD compared to controls. Blood neutrophils, CRP, sputum IL-6 and IL-8 were higher in COPD patients compared to healthy controls after adjusting for age, gender and smoking status. Induced sputum was not reliably attainable particularly in healthy control subjects (sample available for 65% of COPD subjects and 26% of healthy controls). In COPD subjects, blood neutrophils ($p=0.03$) and sputum % neutrophils ($p=0.004$) were independently related to FEV₁ after adjusting for age, gender, smoking status and inhaled corticosteroid use. Blood neutrophils and CRP correlated significantly with each other ($r=0.408$, $p<0.001$), but were not associated with any of the sputum markers of inflammation. Systemic inflammation was associated with SGRQ total score (neutrophils $p=0.02$, CRP $p<0.001$) and MRC dyspnoea score (neutrophils $p=0.005$, CRP $p=0.003$) after adjusting for FEV₁ and smoking status. Blood neutrophils ($p=0.02$) were associated with oxygen saturations $\leq 93\%$. BODE index scores correlated with blood neutrophils, ($p=0.005$), CRP ($p=0.03$) and sputum IL-8 ($p=0.02$). After adjusting for potential confounding factors; exacerbation frequency and BMI were not associated with markers of blood or sputum inflammation. Quantitative CT lung volume and density (pixel index -910, pixel index -950 and 15th percentile) on inspiration and expiration were associated with post-bronchodilator FEV₁ % predicted and FEV₁/FVC ratio ($p<0.001$) but not with any of the markers of pulmonary or systemic inflammation. Expiratory CT parameters correlated better with lung function than inspiratory parameters. Lung volumes were inversely associated with BMI on inspiration ($r=-0.225$, $p=0.015$) and expiration ($r=-0.296$, $p=0.001$). BMI was associated with 15th percentile on inspiration ($r=0.518$, $p<0.001$) and expiration ($r=0.534$, $p<0.001$) and inversely with pixel index -910 and -950 ($p<0.001$). BMI was associated with lung density independent of airflow limitation ($p=0.03$). Lung volume and density were not associated with age, gender, current smoking status, smoking pack years, SGRQ scores, MRC dyspnoea or chronic bronchitis score, exacerbation frequency, oxygen saturations or BODE index.

Conclusion

Markers of inflammation in blood and sputum were increased in COPD compared to healthy controls. Blood and sputum % neutrophils were associated with airflow limitation, but not with lung density. Pulmonary and systemic inflammatory markers had different profiles according to different clinical features of COPD. Inflammatory markers and lung density may be useful in characterizing COPD patients in addition to airflow limitation alone.

List of Abbreviations

2D, 3D	2 dimensional, 3 dimensional
6MWD	6 minute walking distance (metres)
%LAA	Percentage of low attenuation areas
AECOPD	Acute exacerbations of COPD
ANOVA	Analysis of variance
B	Regression co-efficient
BMI	Body mass index
BODE	Body mass index, airflow Limitation, Dyspnoea and Exercise capacity index
BOLD	Burden of Lung Disease initiative, Iceland
CD4 ⁺	T helper cells
CD8 ⁺	Cytotoxic T cells
CO	Carbon monoxide
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-reactive protein
CT	Computed tomography
Df	Degrees of freedom
DOSE	Dyspnoea, airflow Obstruction, Smoking status, and Exacerbation frequency index
DTT	Dithiotreitol
ELISA	Human enzyme linked immunosorbent assay
Exp (B)	Relative odds
FEV ₁	Forced expiratory volume in 1 second
FFM	Fat free mass
FVC	Forced vital capacity
GOLD	Global initiative for chronic Obstructive Lung Disease
HATS	Histone acetyl transferase
HBSS	Hank's balanced salt solution
HDAC	Histone deacetylase
HIF-1	Hypoxia inducible factor 1
HRCT	High resolution computed tomography
hsCRP	C-reactive protein measured by a highly sensitive assay
HU	Hounsfield unit
IHD	Ischaemic heart disease
IL-1 β , 6 and 8	Interleukin-1 beta, 6 and 8
IQR	Interquartile range
kV	Kilovolt
LOD	Limit of detection
LTB ₄	Leukotriene B ₄
LTOT	Long term oxygen therapy
mA	Milliampere
MDA	Malonyldialdehyde
MCID	Minimal clinically important difference
MLD	Mean lung density
MMP	Matrix metalloproteinases
MMRC	Modified MRC dyspnoea score (MRC score – 1)
MRC	Medical Research Council
MRI	Magnetic resonance imaging

mRNA	Messenger ribonucleic acid
NETT	National Emphysema Treatment Trial
NF- κ B	Nuclear factor kappa B
NHANES	National Health and Nutrition Examination Survey
NOTT	Nocturnal Oxygen Treatment Trial
PaO ₂	Arterial partial pressure of oxygen
PD 15	15 th percentile point
PI	Pixel index
PMN	Polymorphonuclear neutrophils
ROS	Reactive oxygen species
SaO ₂	Haemoglobin oxygen saturation
SD	Standard deviation
SE	Standard error
SGRQ	St George's respiratory questionnaire
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox-equivalent antioxidant capacity
TGF- β	Transforming growth factor beta
TNF- α	Tumour necrosis factor alpha
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

List of Associated Publications

Abstracts presented

Miller JJ, Frazer GA, Anderson D, McLaughlin M, Poland P, McGuinness C, Barr JE, Deans A, Donaldson K, MacNee W. Factors influencing induced sputum cytokines and VEGF in a population of stable COPD patients. European Respiratory Society, Munich 2006. (Poster discussion)

Miller JJ, Connell M, Barr JE, Deans A, Frazer GA, Murchison JT, MacNee W. Quantitative CT characterisation of stable COPD patients relates to COPD severity and exacerbation frequency. European Respiratory Society, Munich 2006. (Oral presentation)

Miller JJ, Mills NL, Baird RA, Frazer GA, Anderson D, Barr JE, Deans A, Newby DE, MacNee W. Airways limitation is associated with increased cardiac injury and systemic inflammation in patients with stable COPD. European Respiratory Society, Munich 2006. (Oral presentation)

Miller JJ, Anand A, Robinson SD, Wilkinson IB, McEniery CM, Frazer GA, Donaldson K, Newby DE, MacNee W, Mills NL. Systemic inflammation and increased arterial stiffness in patients with chronic obstructive pulmonary disease. European Respiratory Society, Munich 2006. (Oral presentation)

Miller JJ, Mills N, Anand A, Frazer G, Robinson S, Wilkinson I, McEniery C, Donaldson K, Newby D, MacNee W. Increased arterial stiffness and blood pressure in patients with chronic obstructive airways disease. Proc Am Thorac Soc. 2006;3:130. American Thoracic Society, San Diego 2006. (Thematic Poster)

Miller JJ, Macleod F, Connell M, Anderson D, Barr JE, Deans A, MacNee W, Murchison JT. Airway wall dimensions on CT in COPD patients. American Thoracic Society, San Francisco 2007. (Thematic poster)

Miller JJ, Anderson S, Mills NL, McAllister DA, Maclay JD, Donaldson K, Newby DE, MacNee W. Chest pain is associated with increased platelet activation in AECOPD. American Thoracic Society, San Francisco 2007. (Poster discussion)

Miller JJ, Mair G, MacLeod F, Connell M, Anderson DG, Donaldson K, Murchison J, MacNee W. CT assessment of airway dimensions and clinical parameters in COPD. European Respiratory Society, Stockholm 2007. (Electronic poster)

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Frazer GA, **Miller JJ**, Barr J, Deans A, Poland C, McGuinness C, Donaldson K, MacNee W. Predictors of exacerbation frequency in a cohort of subjects with chronic obstructive pulmonary disease. Proc Am Thorac Soc. 2006;3:A603. American Thoracic Society, San Diego 2006. (Thematic poster)

Lee J, **Miller J**, Barr J, Deans A, Thompson C, Poland C, MacDougall M, MacNee W. Characterisation of frequent exacerbators of chronic obstructive pulmonary disease (COPD) using the body mass index, airflow limitation, dyspnoea and exercise capacity (BODE) index. British Thoracic Society, London 2006. (Oral presentation)

McAllister DA, Maclay JD, Mills NL, Mair G, **Miller JJ**, Anderson D, Newby DE, Murchison JT, MacNee W. Emphysema severity is associated with arterial stiffness, a marker of cardiovascular risk. S139; A55. British Thoracic Society, London 2007. (Thematic poster)

Journal Publications

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Miller JJ, MacNee W. What's new in COPD? Scottish Medical Journal. May 2007 52(2):36-41.

Mair G, **Miller JJ**, McAllister D, Maclay J, Connell M, Murchison J, MacNee W. CT emphysema distribution: relationship to clinical features in a cohort of smokers. Eur Respir J. 2009 33:536-542.

Mair G, Maclay J, **Miller JJ**, McAllister D, Connell M, Murchison JT, MacNee W. Airway dimensions in COPD: relationships with clinical variables. Resp Med. 2010 (in press)

McAllister DA, Maclay JD, Mills NL, Mair G, **Miller JJ**, Anderson D, Newby DE, Murchison JT, MacNee W. Arterial stiffness is independently associated with emphysema severity in patients with COPD. Am J Respir Crit Care Med. 2007 176:1208-14.

Chapter 1: Introduction

Chronic Obstructive Pulmonary Disease (COPD) is characterized by chronic airflow limitation and is currently defined in terms of the forced expiratory volume in 1 second (FEV_1) for the purpose of diagnosis and treatment. (1) The FEV_1 is also used to define disease severity in COPD, (2) and has long been associated with prognosis. (3, 4) The FEV_1 is an important predictor of outcome in COPD. Epidemiological studies in the general population have shown that a reduction in FEV_1 is an independent predictor of all cause mortality. (5) However, although FEV_1 is useful as a defining variable for COPD, which is an otherwise heterogeneous disease, it is limited as an endpoint for COPD in several ways.

An ideal diagnostic marker of disease would be dichotomous in order to differentiate disease and non-disease, but FEV_1 is a continuous variable with an arbitrary cut-off that has been refined over time. (6) However, the use of defined cut-offs has come under criticism recently due to the propensity for under- and over-diagnosis depending on age and gender. (7-9) This may, for example, lead to an over-diagnosis of COPD in older age groups as the FEV_1/FVC ratio reduces with age. (10-12) FEV_1 is also limited as a marker for monitoring disease progression. It is estimated that 1000 people need to be followed up for 3 years to detect a variation of only 20mls per year in FEV_1 . (13) This has implications for clinical trials which require large sample sizes and a longer period of follow up. Mortality in COPD patients is often due to factors other than

the severity of airflow limitation. At least one third to one half of deaths in COPD patients are due to a non-respiratory cause. (5, 14) Although FEV₁ is still the strongest predictor of outcome in COPD, other clinical parameters, for example body mass index and C-reactive protein (CRP), predict mortality in COPD patients independently of FEV₁ and smoking status. (14-16) In addition, FEV₁ does not account for the variation in clinical presentation, disease severity, and the rate of disease progression in COPD. (17) The degree of airflow limitation correlates poorly with symptoms (18) and is not associated with the extra-pulmonary features seen in COPD. (19) In fact, the regulatory requirement that FEV₁ be used as the main endpoint in clinical trials in COPD may actually inhibit the development of treatments that might impact on other aspects of the disease such as inflammation and cachexia. (6) There is therefore a need for markers to better characterize COPD to augment the current definitions of disease severity and prognosis that are based on lung function.

A biomarker can be defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic interventions.” (20) In clinical practice, biomarkers can be used to establish a diagnosis, to monitor disease severity or to predict prognosis. They may also be useful in defining disease phenotypes and response to a therapeutic intervention. (21) A COPD “phenotype” has been defined as one of “the outward physical manifestations of patients with COPD; anything that is part of their observable structure, function or behaviour.” (22) An ideal biomarker should be able to “indicate a patho-

physiological process relevant to the disease of interest, distinguish a disease phenotype, respond to treatment in a time frame that precedes changes in clinical status, be easily measured, minimally invasive, reproducible, and properly validated." (21, 23) Many potential biomarkers have been investigated as a means of further characterizing COPD. The following text will focus on some of the markers that have potential for defining COPD phenotypes. These include biological markers, symptomatic markers, physiological markers, and markers of structure and anatomy.

Biological markers

There are three established mechanisms for the pathogenesis of COPD. These are inflammation, oxidative stress and protease/anti-protease imbalance. (24) Inflammation is a central feature of COPD and is included in the working definition of the disease. (2, 25) Each of these pathogenic mechanisms can be directly linked by inflammation and are not mutually exclusive. For example, oxidative stress induced by cigarette smoke up-regulates the inflammatory process by activating the transcription factor nuclear factor κ B (NF- κ B). This in turn induces the transcription of pro-inflammatory cytokines. (26, 27) The main inflammatory effectors cells in COPD are neutrophils and macrophages. They are key sources of proteases in the lungs and therefore directly impact on the balance of between proteases and anti-proteases. Inflammatory cells and mediators may therefore be useful biomarkers in COPD.

Pulmonary inflammation

Pulmonary inflammation is a key pathological process in COPD. (28) Airway inflammation, parenchymal destruction and tissue remodeling occur in COPD as a result of the complex interaction of cells and inflammatory mediators. All smokers have some inflammation in the lungs particularly in their small airways. (29) However, in 15-20% of smokers (30) and more commonly in heavy smokers, (31) the “inhalation of noxious particles or gases” results in a persistent chronic abnormal inflammatory response in the lungs that persists even after cessation of cigarette smoking. (1) This chronic inflammation, when established, is self-perpetuating. (24) The mechanism behind the transformation from the ‘normal’ to the abnormal inflammatory response in the development of COPD, and the reason it occurs in only a proportion of smokers is unknown, but genetic factors may be implicated. (32) Expression of pro-inflammatory genes is determined in part by the modification of histones, for example, by acetylation and deacetylation. The inhibition of histone deacetylase, which is involved in the control of gene transcription, may have a role in enhancing the inflammatory response. Histone acetyl transferases (HATS) activate, whereas histone deacetylase (HDAC) represses gene expression. Cigarette smoke exposure inhibits histone deacetylation leading to the increased transcription of pro-inflammatory genes. (33)

Many methods for assessing pulmonary inflammation have been explored. Exhaled breath condensate, sputum and induced sputum, bronchoalveolar lavage, endobronchial biopsy and resected lung specimens have all been

analyzed. (34) In these samples, the cellular composition of the airways has been examined and many inflammatory mediators have been identified and quantified. There is also some evidence that biomarkers of inflammation and oxidative stress may predict therapeutic responses. For example, there is some evidence that the presence of airway eosinophilia may predict response to steroid therapy in patients with airways disease. (35, 36)

Neutrophils

The neutrophil is a key cell in the pathogenesis of COPD. (37) Neutrophilic inflammation occurs in healthy lungs and constitutes an important part of the innate immune response. The neutrophil is characteristically the predominant cell type in induced sputum and bronchoalveolar lavage fluid in subjects with COPD. (38) Neutrophils are activated to release reactive oxygen species (ROS) and proteases. Once released, these result in damage to lung cells. (39)

Neutrophil proteases such as neutrophil elastase, cathepsins and matrix metalloproteinases (MMPs) are thought to induce lung damage through degradation of the extracellular matrix. (40) The serine proteases secreted by neutrophils are also potent stimulators of mucus secretion in the airways. (41, 42) The function and activity of neutrophils is altered in COPD. Neutrophils in the airways in COPD patients are activated such that there is an increased release of reactive oxygen species and proteases. (34) The rate of neutrophil apoptosis is also increased in COPD patients compared to controls, and is increased further in COPD patients with more severe airflow limitation ($FEV_1 < 50\%$ predicted). (43) Neutrophil numbers in the airways increase as

airflow limitation progresses. (44) Increased numbers of induced sputum neutrophils have been associated with a more rapid decline in FEV₁. (45) A meta-analysis of cross-sectional studies in COPD found that the sputum neutrophil count was one of the few biomarkers showing a trend towards a separation between stages of disease in COPD. (46)

Macrophages

Macrophages are also key cells which mediate and perpetuate the chronic inflammatory process in COPD. (47, 48) Macrophages are increased in all regions of the lungs in COPD patients, whereas neutrophils are increased predominantly in the airways. (34) As with neutrophils, the number of alveolar macrophages in the airways increases with the degree of airflow limitation in COPD. (49) Activated macrophages in COPD patients release increased numbers of pro-inflammatory cytokines and matrix-metalloproteinases. Alveolar macrophages from COPD patients have a greater release of MMP-9 than those from healthy smokers. (50, 51) Interestingly, corticosteroids are less effective in inhibiting release of these mediators from alveolar macrophages in COPD patients compared to controls, (52) possibly as a result of reduced HDAC.

Lymphocytes

T lymphocytes are involved in both the innate and adaptive immune responses but predominantly in the latter. Originating from thymocytes, they differentiate into CD8⁺ (cytotoxic) and CD4⁺ (T helper) cells. These lineages further differentiate into type 1 or type 2 cells which differ in their cytokine profiles. Type 1 cells secrete interferon- γ , which activates macrophages in response to

bacterial and viral infections. Type 2 cells secrete cytokines such as IL-4, IL-5 and IL-13, which are involved in the IgE mediated response usually associated with asthma and allergy. (53) CD8⁺ T lymphocytes cause cell destruction both by inducing lysis of cell membranes by granzymes and perforins, and by inducing apoptosis. (54) There is some evidence that CD8⁺ and CD4⁺ T cells proliferate in regional lymph nodes in a sustained immune response to chronic infection or to other antigenic stimuli. (55)

T lymphocytes are increased in the lung parenchyma and airways of COPD patients and the ratio of CD8⁺ to CD4⁺ T cells is increased. (47) Finkelstein and co-workers found that the number of T lymphocytes in the alveolar wall was increased in smokers and correlated with the extent of emphysema. (56) CD8⁺ T lymphocytes have been associated with the degree of airflow limitation in COPD. (57) An association has been found between smoking pack years and both the number of T cells in the alveolar wall and the degree of apoptosis. (58) CD8⁺ cells are increased in the circulation of COPD patients and the number of abnormally activated T cells is increased. (59, 60) The function of these cells may be affected by cigarette smoking and inhaled corticosteroid use. (59) It has even been proposed that COPD may be an autoimmune disease that is mediated by auto-reactive T cells. These may respond to auto-antigens on lung cells structurally altered by neutrophil and macrophage mediated acute inflammation. (58, 61)

Other cells and inflammatory mediators in the lungs

A number of other inflammatory cells and mediators are active in the inflammatory process in COPD. Eosinophils are more typically associated with asthma, but are increased in the airways of some COPD patients. When identified in COPD patients, sputum eosinophilia was associated with increased corticosteroid responsiveness. (36) However, a meta-analysis found that macrophage and neutrophil counts distinguished between COPD patients and healthy subjects, whereas eosinophil counts did not. (46) Interestingly, patients with asthma who develop chronic airflow limitation have a higher number of neutrophils in the airways. (62)

Dendritic cells have a vital role in the detection of microorganisms and in linking the innate and adaptive immune responses. (63) They are found in increased numbers in the airways of smokers. These antigen-presenting cells are instrumental in the activation of both the innate and adaptive immune responses and in the activation of other inflammatory cells. They are present in abundance in all regions of the lungs and may initiate an immune response to inhaled particles in cigarette smoke. (34)

Epithelial cells are important in the defense of the airways through secretion of mucus, anti-oxidants, anti-proteases and IgA. (34) These cells also have a role in aiding neutrophil migration from the circulation into the airways by the release

of inflammatory mediators such as IL-1 β and IL-8 in response to cigarette smoke. (64, 65)

Cytokines are central to the inflammatory process in COPD although their role and interactions in the pathogenesis of COPD is not fully understood. They have a role in the recruitment of inflammatory cells and the activation and suppression of inflammatory and immune responses. More than 50 cytokines have been identified in asthma and COPD. These can be classified into lymphokines, pro-inflammatory cytokines, chemokines, anti-inflammatory cytokines and growth factors. (66)

Tumour necrosis factor alpha (TNF α), interleukin-1 beta (IL-1 β) and interleukin-6 (IL-6) are pro-inflammatory cytokines and amplify the inflammatory cascade through the activation of NF- κ B. Increased TNF α is associated with the weight loss seen in COPD. Blocking the action of TNF α is efficacious in chronic inflammatory conditions like rheumatoid arthritis and inflammatory bowel disease. TNF α blockade has some benefits in asthma, (67, 68) but trials of anti-TNF α therapy have not been effective in COPD. (69, 70) In fact, an increased incidence of infection and neoplasia was observed in COPD patients treated with TNF antibody. (70) However TNF α levels may be affected by factors not considered in these studies, such as disease severity and exacerbation frequency. IL-6 is a pro-inflammatory cytokine. Increased IL-6 levels in induced sputum have been described in stable COPD patients and further increases are seen during exacerbations. (71) Sputum IL-6 levels

increase over time in COPD and higher levels are associated with a more rapid decline in FEV₁. (72) IL-8 (or CXCL-8) is a chemokine. IL-8, leukotriene B₄ (LTB₄) and TNF α act as chemotactic factors and facilitate neutrophil and monocyte migration into the airway lumen. (73) Other facilitators of neutrophil migration such as the intra-cellular adhesion molecule (ICAM) -1 (74) are also up regulated in COPD. E-selectin enables adhesion of circulating neutrophils, and is up-regulated on vascular endothelial cells in bronchial biopsy samples in COPD. (74) IL-8 levels have been increased in sputum in COPD compared with smoking and non-smoking controls in small studies. (75) IL-8 was increased in bronchoalveolar lavage fluid (76) in stable COPD and IL-8 levels correlated with neutrophil numbers. (71)

IL-1 β is a potent activator of macrophages in COPD and promotes the release of inflammatory cytokines, chemokines and MMP-9. (66) Matrix-metalloproteases are proteolytic enzymes that degrade the extra-cellular matrix and this action is opposed by specific anti-proteases (Tissue inhibitors of MMPs – TIMPs). They are involved in pulmonary remodeling and have been implicated in the pathogenesis of COPD and asthma. (77) Levels of MMP-1, -8 and -9 were increased in sputum in COPD patients compared to healthy smokers and asthmatics. (78)

Growth factors such as TGF- β stimulate the proliferation of fibroblasts, resulting in fibrosis of the small airways. (66) Vascular endothelial growth factor (VEGF) is associated with increased vascularity and vascular leakage in asthma. But

where VEGF levels were increased in asthma, (79) levels of VEGF and its receptors were reduced in the lungs and sputum from COPD patients. Blockage of the VEGF receptor led to apoptosis and the development of emphysema in rat models. (80)

Systemic inflammation in COPD

In addition to the effects on the lungs, COPD is now considered to have important systemic effects. (2) A large body of evidence indicates that a systemic inflammatory response is present in a proportion of patients with COPD. (81, 82) Airflow limitation is associated with increased systemic inflammation in population studies. In the Burden of Lung Disease (BOLD) initiative in Iceland, investigators found that increased circulating IL-6 and CRP were associated with reduced FEV₁ and FVC (n=758). (83) A French population study of 531 subjects followed over 8.5 years found that increases in CRP over time were associated with a greater decline in FEV₁. (84) Data from The Third National Health and Nutrition Examination Survey (NHANES) in the United States demonstrated a relationship between systemic inflammation (neutrophils, fibrinogen and CRP) and reduced FEV₁ that was independent of current smoking status. (85)

Co-morbidity and systemic features of COPD

Several co-morbidities are considered to be systemic sequelae of COPD, such as atherosclerosis, (86) weight loss, (87, 88) skeletal muscle dysfunction, (89) osteoporosis, (90) depression and anxiety. (91) These associations are particularly important as they have a major influence on morbidity and

mortality in COPD. (92) In fact, the presence of co-morbid conditions, and in particular cardiovascular disease, is an independent predictor of mortality in COPD patients. (90, 93) And yet the majority of therapies for COPD currently focus on the airways. While these treatments improve symptoms, they do not convincingly impact on mortality. Systemic inflammation could explain some of the systemic consequences of COPD and its associated co-morbid conditions. Treating co-morbidities may reduce mortality in COPD, (92) but understanding the mechanisms behind the systemic inflammatory state and the association between local airway and systemic inflammation is likely to further expand the therapeutic possibilities.

Markers of systemic inflammation in COPD

Markers of systemic inflammation may be useful in predicting prognosis in COPD. A meta-analysis of systemic inflammatory markers found that blood leukocytes, C-reactive protein, fibrinogen and TNF α levels were increased in COPD patients compared to healthy controls. These markers of systemic inflammation were increased in COPD patients irrespective of smoking status. (82) Increased circulating leukocytes are associated with the symptoms traditionally associated with COPD such as chronic cough, sputum production and dyspnoea. (94, 95) Increased peripheral blood white cell count is associated with airflow limitation, rate of decline in FEV₁ and smoking status. (96, 97) It is also associated with increased all cause mortality and with mortality associated with cardiovascular disease, independent of smoking history and the degree of airflow limitation. (95) Fibrinogen is an acute phase reactant, synthesized by

the liver under the control of IL-6. Raised levels of circulating fibrinogen and blood neutrophils are also associated with a faster rate of decline in FEV₁. (72)

C-reactive protein is a strong independent predictor of hospitalization and death in COPD, independently of FEV₁ and smoking history. (16, 98) C-reactive protein is widely used in clinical practice to detect and monitor systemic inflammation. Like fibrinogen, it is produced by hepatocytes in response to stimulation by IL-6. In addition to being a marker of inflammation, it has a role in the regulation of the inflammatory cascade by activating complement, augmenting leukocyte adhesion and recruiting pro-inflammatory cytokines. (85, 88, 99) What is less clear is the extent to which CRP is itself pro-inflammatory. This has been the subject of debate in the cardiovascular literature. (100, 101) There is evidence that CRP is pro-inflammatory in the setting of acute ischaemia where it has been found to increase tissue damage through a complement dependent mechanism. (102) It has also been associated with the progression of atherosclerosis. (103) An elevated resting level of CRP in COPD has been confirmed in population studies, (104) and levels increase with the severity of airflow limitation. (105) Raised CRP levels are associated with a more rapid decline in FEV₁. (106) It is now established as a marker of chronic inflammation with prognostic implications, not only in COPD. (16, 107)

Theories linking pulmonary and systemic inflammation in COPD

The role of systemic inflammation in the pathogenesis of COPD and its relationship with pulmonary inflammation is not well understood. (82) The

most common theory is that pulmonary inflammation “spills over” into the systemic compartment, resulting in chronic low-grade inflammation. It is postulated that cytokines such as IL-6 reach the systemic circulation and increase the production of inflammatory mediators like CRP in hepatocytes. (108) These then perpetuate the low-grade systemic inflammation. This theory is supported by evidence that pulmonary anti-inflammatory therapies such as inhaled corticosteroids may improve outcome in COPD. (109) Inhaled corticosteroids may reduce systemic markers of inflammation in COPD and resulted in a 40% reduction in circulating CRP in one study, (110) but other studies showed conflicting results. (111, 112) There is a lack of evidence of an association between pulmonary and systemic markers of inflammation in COPD. Diesel exhaust inhalation causes a rise in markers of acute inflammation in the pulmonary and systemic compartments in healthy volunteers. (113, 114) But a number of studies have failed to show a relationship between markers of pulmonary and systemic inflammation in COPD subjects. (72, 115, 116)

The alternative theory of reverse causation (that systemic inflammation drives pulmonary inflammation) must also be considered. There is evidence that inflammatory cytokines increase adhesion of inflammatory cells to the pulmonary capillary endothelium, augmenting neutrophil sequestration in the pulmonary vasculature with a resultant increase in the influx of inflammatory mediators from the circulation to the lungs. (84) Cardiovascular disease is strongly associated with increased markers of systemic inflammation such as CRP and IL-6. (104) In chronic inflammatory conditions like diabetes, obesity

and ischaemic heart disease (IHD), surface adhesion molecules such as ICAM-1 and VCAM-1 are up-regulated on the endothelium, causing leukocyte adhesion and enhanced chemotaxis.

Smoking is an obvious link between COPD and systemic inflammation. But hypertension, ischaemic heart disease, osteoporosis, muscle weakness and diabetes mellitus are more common in COPD, independently of the effects of smoking. (89) It is possible that a core susceptibility to inflammation is represented by each of these co-morbidities. Inactivity is also a factor common to many of these conditions and has been proposed as a possible cause of systemic inflammation. (91) Hypoxic, genetic and autoimmune mechanisms may provide additional links between systemic and pulmonary inflammation. Skeletal muscles and pulmonary hyperinflation are other potential causative factors associated with systemic inflammation in COPD. (88, 117)

Symptomatic and physiological markers

Although FEV₁ is established as an outcome measure in COPD, it is not the best indicator of a patient's functional abilities. Many clinical markers of symptoms and function have been investigated in COPD with a view to identifying additional disease phenotypes, predicting outcome and monitoring response to therapeutic interventions. This thesis will focus on the symptom scores St George's Respiratory Questionnaire (SGRQ) and MRC dyspnoea score, exacerbation frequency, body mass index (BMI), oxygen saturations and the Body mass index, airflow Obstruction, Dyspnoea and Exercise capacity (BODE)

index. (118) These features were chosen as non-invasive, well validated measures associated with prognostic importance in COPD. They also each depict manifestations of COPD that potentially link pulmonary and systemic inflammation.

Health status

FEV₁ is not closely associated with health-related quality of life in COPD patients. (119) Tools measuring health status and quality of life take into account symptoms, physical function, psychosocial and emotional factors, activity levels and the degree of adaptation to disease. (120) Therapeutic impact on symptoms and quality of life are more important to patients than improvement in spirometry. (121) Symptoms like dyspnoea – a key determinant of quality of life in COPD - are poorly correlated with FEV₁ (122) FEV₁ takes no account of physiological factors such as dynamic hyperinflation, which is associated with an increased perception of breathlessness. (123) Health status tools like the St Georges Respiratory Questionnaire (SGRQ) and the MRC dyspnoea score are linked to prognosis in COPD. (118, 124) These tools are increasingly used as outcome measures in clinical trials. (125) Health status can be improved by use of inhaled bronchodilators, inhaled corticosteroids and pulmonary rehabilitation. (126-128)

The SGRQ is a well-validated, self-administered questionnaire with 76 weighted components. It is specific to patients with respiratory disease. It gives an overall score (total score) that can be broken down into three domains: symptoms,

activity and the impact of disease on daily life. (129) A 4-point change in the SGRQ total score is established as the minimal clinically important difference (MCID) when it used as an outcome measure in clinical trials. (125) In the National Emphysema Treatment Trial (NETT), a sustained improvement in SGRQ total score was seen at 2 years post lung volume reduction surgery. (130) The SGRQ may capture features of COPD not encompassed in lung function. For example, SGRQ total score was associated with multi-dimensional tools for the assessment of COPD severity such as the BODE index, though not with GOLD stage which is based on spirometry. (131, 132) SGRQ predicts mortality after adjusting for age, FEV₁ and BMI (133) and is a more consistent predictor of mortality than other quality of life scores. (89) In a study of 102 subjects with COPD, SGRQ was associated with increased sputum macrophages, although there was no association between SGRQ and FEV₁. (117) Low SGRQ scores were associated with raised systemic CRP. (105) Combinations of inhaled corticosteroid and long acting beta agonists improve health status in COPD. (127) In a study of 289 COPD patients, inhaled fluticasone and salmeterol led to an improvement in health status. But while specific lung markers of inflammation improved (surfactant protein D), there was no difference in general systemic inflammatory markers (CRP or IL-6). (110) This suggests that the improvement in health status may be independent of systemic inflammation.

Exacerbation frequency

Exacerbations have been extensively studied in COPD. They are associated with reduced quality of life, (134) increased hospitalization, morbidity and mortality. (135-137) Increased frequency of exacerbations may be associated with a faster decline in lung function. (138, 139) The definition of an exacerbation of COPD is based on respiratory symptoms. An exacerbation is defined as an acute increase in cough, dyspnoea and/or sputum production that may require a change to regular medication. (1, 140) Exacerbation frequency may be used to define clinical phenotypes of COPD. The exacerbation rate in COPD is variable.

Donaldson and co-workers measured exacerbation frequency in their cohort using diary cards. They reported a mean annual exacerbation frequency of 2.5 and they reported that the exacerbation frequency in the first year was highly correlated with exacerbation frequency in subsequent years. (139) In contrast, a significant proportion of COPD patients experience no exacerbations in an average year. This group may therefore constitute a distinct clinical phenotype. (6) Frequency of exacerbations is increasingly used as an endpoint in clinical trials. (6, 141) Treatment with combined inhaled corticosteroid/long acting beta agonist or with long acting anti-cholinergic pharmacotherapy have both been associated with a significant reduction in exacerbations frequency and a decline in FEV₁. (142, 143)

The aetiology of exacerbations in COPD is not fully understood. They are not explained by infection alone. While around 50% of exacerbations are associated

with a bacterial infection (usually with *Haemophilus influenzae*, *Moraxella catarrhalis*, or *Streptococcus pneumoniae*), (137) the causative role of bacteria in exacerbations has been questioned. (144) A longitudinal cohort study of 104 patients over 7 years found insignificant differences in bacterial load during exacerbations, though there was a small but significant change in the concentration of new strains of *Haemophilus influenzae* and *Moraxella catarrhalis*. (145) Viruses are implicated in exacerbations in asthma and COPD, (146) and air pollution has been proposed to have a role in inducing exacerbations of COPD. (147) Genetic (148) and immunological factors (149) in response to tobacco smoke are associated with susceptibility to exacerbations. Exacerbations are associated with increased pulmonary inflammation. Neutrophils, eosinophils and other inflammatory mediators are increased in the airways during exacerbations compared to the stable state. (150) Exacerbation frequency is also linked with increases in many markers of systemic inflammation. (151, 152) For example, in microarray analysis, 24 serum biomarkers were associated with exacerbation frequency (153) Systemic inflammation and inactivity are key mechanisms relating COPD to its associated co-morbidities. Exacerbations are associated with both of these factors and may be a key link between pulmonary and systemic inflammation and the systemic consequences of COPD. (91)

Body composition (Body Mass Index) and the BODE index

Body mass index is an established measure of nutritional status. (154) A proportion of COPD patients develop significant loss of body mass with

progression of disease. In the GOLD stage IV group from the Copenhagen City Heart Study, 30% of women and 15% of men were underweight (BMI <18.5%). (15) The clinical features associated with this phenotype are low body mass index, reduced fat free mass (a surrogate for skeletal muscle mass), skeletal muscle dysfunction, exercise intolerance and leg weakness. (90) The causes of reduced body mass index and muscle dysfunction in COPD are likely to be multifactorial. These include inactivity, steroid use, poor nutrition and the effects of systemic inflammation. (19, 155, 156) Systemic markers of oxidative stress and the pro-inflammatory cytokine TNF α have been linked with the 'cachectic' phenotype of COPD. (157) CRP predicted body mass index and fat free mass independently of FEV₁, age and sex in COPD patients. (105) Body mass index is also associated with systemic consequences of COPD such as osteoporosis. (158) Low body mass index and fat free mass are independent predictors of mortality in COPD and this association was stronger in more severe COPD. (15, 159, 160) Body mass index and fat free mass correlate significantly with exercise capacity (6 minute walking distance). (154) The recognition that body mass index and exercise capacity have prognostic significance over and above airflow limitation has led to interest in the development of composite clinical endpoints. The most well known of these is the BODE index. The BODE index is a composite 10-point scoring system based on the clinical indices of Body mass index (BMI), airways Obstruction, Dyspnoea and Exercise capacity (6 minute walking distance). The composition and scoring system used in the BODE index is outlined in detail in Chapter 2. This composite score is based on non-invasive clinical outcomes and has been validated such that a one point increase in the

BODE index has been associated with a 34% increase in mortality in COPD in one study population. It has been suggested that the BODE index is a more effective predictor of mortality than FEV₁ alone. (118)

Hypoxia

Hypoxaemia can be measured non-invasively and simply using an oxygen saturation monitor or more invasively by arterial blood gas. The arterial partial pressure of oxygen has been associated with outcome in COPD (46) and oxygen supplementation is one of the few therapeutic measures known to improve survival in COPD. (161, 162) In addition to improving pulmonary vascular resistance and polycythaemia, (161) exercise capacity, dyspnoea and sleep consolidation may also be improved with oxygen therapy. (163) Although hypoxia is more common in severe COPD, it does not develop in all patients with severe airflow limitation. Hypoxia in COPD may be in part genetically mediated. The transcription factors NF- κ B and hypoxia inducible factor-1 (HIF-1) induce gene transcription in response to alveolar oxygen levels. HIF-1 consists of two subunits (α and β). HIF-1 α expression in the lungs is regulated by the concentration of inspired oxygen. (164) This factor may also induce anorexia through induction of the promoter for the leptin gene. (165)

Hypoxia provides a possible causative link between pulmonary and systemic inflammation. Hypoxia was associated with increased systemic inflammation with raised circulating IL-6 and CRP, (166) increased platelet activation and the up-regulation of intracellular adhesion molecules. (167) These factors, in

addition to hypoxia-induced haemodynamic stress, which results in tachycardia and peripheral vasoconstriction, may be one reason for the increased incidence of cardiovascular disease in COPD. (168) Hypoxia may increase inflammation through mechanisms that alter vascular responses. (169, 170) Remy-Jardin and colleagues reported interesting findings regarding emphysema and inflammation using imaging techniques. (171) They found that emphysematous regions often developed at a site that had previously shown nodules or ground glass and suggested that these areas may have represented a region of inflammation or possibly fibrosis. In the presence of inflammation, the usual pulmonary response of diverting blood flow from hypoxic alveolar regions is reversed. Some smokers may exhibit blunting of this process, and in these individuals, inflammatory regions associated with hypoxia may experience a further limitation in blood flow, resulting in tissue destruction. (172)

Anatomical markers

COPD is a pathologically heterogeneous disease and results in characteristic anatomical alterations in the lungs. The main features contributing to airflow limitation in COPD are parenchymal destruction (emphysema) and remodeling of the small airways. (173) Chronic bronchiolitis and emphysema have long been recognized as key components of COPD. (174) Emphysema causes loss of lung elasticity and an increase in total lung volume, whereas inflammation and fibrosis of the membranous bronchioles, accompanied by mucus plugging contribute to small airway narrowing. (175-177) There is evidence that airway remodeling and emphysema develop through different pathogenic mechanisms.

(177-179) As future treatments are likely to target these processes separately, (180) it is likely to become important to characterize patients into emphysema and airway phenotypes for the purposes of treatment and prognosis. CT provides a means to differentiate these pathological subtypes. (181-183) This section will illustrate the technological considerations necessary for the measurement of lung density and airway caliber using computed tomography. Potential anatomical phenotypes of COPD identifiable on CT will then be discussed with respect to their utility as markers of disease.

CT measurement of lung density

CT has been used to provide an objective measure of lung density in Hounsfield Units (HU) since the 1980's. (184, 185) The most widely used method for objectively quantifying emphysema using CT is the "density mask" technique. (186) In the acquisition of scan data for density analysis, software is used to separate lung regions from the more dense structures (e.g. chest wall, mediastinum and hila) by a process known as segmentation. (187) The development of density mask software is based on the allocation of 3D regions (voxels) of signal intensity, which are reconstructed using a mathematical algorithm into 2D regions (pixels). The reconstruction algorithm allocates a value based on the signal intensity of each voxel, taking account that of neighbouring voxels. This filter or 'kernel' can be altered depending on the purpose of the scan. For example a soft filter is more representative for lung density analysis, whereas a hard filter will give more image definition for assessing, for example, airway wall dimensions. Regions of signal density are

then allocated a density value in Hounsfield Units based on their intensity index on a gray scale. For example, zero HU represents the density of water and -1000 HU represents the density of air. A threshold is then applied close to the HU value for air to dichotomize regions to be denoted as 'normal' lung parenchyma or as emphysematous. Normal lung at full inspiration has a mean density between -750 and -850 HU on 10mm slices. Emphysema was initially defined as a lung density < 2 standard deviations below average density. This is approximately a density of < 900 HU but can vary depending on the reconstruction algorithm, slice thickness, the use of contrast, and differences in collimation. No fixed value has yet been established for this threshold, but commonly used cut-offs are -910 and -950 HU. Emphysema is considered to be present when $> 10\%$ of pixels fall below this threshold. (188) The low-density threshold to quantify emphysema has been validated against pathology. (184, 186, 189)

Alternatives to the density mask technique such as the volume fraction of emphysema, mean lung density (MLD) and a CT air-trapping index are also in common use. (190) Emphysematous regions are often described as the percentage of low attenuation areas (%LAA) below a given threshold in HU, e.g. %LAA-950. (191) European studies of alpha-1 antitrypsin deficiency developed a measurement known as the "percentile point" which was initially utilized in Edinburgh. (192) Dirksen and coworkers analyzed lung density using 5-15% of the lowest attenuation voxels. The "percentile point" (e.g. 5th or 15th percentile point) is advantageous in having less variability than the density mask

technique. It is also significantly less variable than measurements of pulmonary function. (193)

Practical considerations

CT protocols for calculating lung density are different from the usual clinical protocols used primarily for image analysis. The radiation dose from a normal helical CT scan of the thorax is estimated to be approximately 8.9-10.9 milliSieverts (mSv). Clinical scans use a current of 180-240 milli-Ampere seconds (mAs) in order to achieve greater image clarity. Quantitative CT scans for the assessment of emphysema can be undertaken using a lower current (20-40mAs). This reduces the total radiation dose by approximately 10-fold, (194) making CT a more attractive option for phenotyping COPD and for assessing the progression of disease over time. In order to standardize lung density measurement, the effects of slice thickness, collimation and reconstruction algorithm must be taken into account. (195) With respect to computed tomography, collimation refers to the beam width, with thinner collimation (e.g. 1mm compared to 2.5mm) resulting in increased sensitivity to smaller regions of emphysema. Edge enhancement filters (e.g. "bone" or "lung") are used for image enhancement in clinical scans. This alters the density measurement and should not be used in scans for emphysema quantification. (188)

Scanners are usually calibrated at water density (zero HU). For accuracy in densitometry, calibration at air density (-1000 HU), which is closer to the density of emphysematous regions, is also required. Calibration with both air

and water phantoms is recommended. (196) This is particularly important for multi-centre and longitudinal studies, as have been pioneered in alpha-1 antitrypsin deficiency. (197)

In addition to technical variables, patient factors such as changes in lung volume affect the density measurement. (198, 199) Although some studies support the use of spirometric gating to help standardize the degree of inspiration, it is generally accepted that verbal coaching of subjects - to sustain first a breath hold in inspiration, then again in expiration - yields acceptable results. (200) Whether scans for lung density should be taken in inspiration or expiration is a source of debate. Expiratory scans may relate better to physiology as they represent emphysema and gas trapping even when the inspiratory scan is normal. (201, 202)

Emphysema phenotypes

CT lung density measurements have been investigated in relation to physiological measures of pulmonary function. (181, 182, 192, 203, 204)

Despite the same spirometric stage of disease in COPD, the quantity of emphysema can vary widely. (205) The extent of emphysema does not therefore fully account for the expiratory airflow limitation. (206, 207) Patients can have severe airflow limitation with limited or no emphysema. (206, 208) Macroscopic emphysema has been demonstrated in never-smokers (209) and in post-mortem studies significant emphysema can be present in patients with normal lung function. (210) In a cohort undergoing CT scans for lung cancer

screening, 78% of smokers had emphysema in the presence of normal spirometry. (211) Although an inverse relationship between the extent of emphysema and FEV₁ exists, (211) quantitative analysis of lung density correlates better with carbon monoxide diffusion capacity and the degree of microscopic emphysema (212) than with FEV₁. (207) Some studies found that lung density parameters on expiratory scans correlated more closely with pulmonary function tests, with correlation co-efficients between lung density and FEV₁ improving from 0.5-0.7 between inspiration and expiration. (213, 214)

In addition to whole lung density measurements, the zone distribution of emphysema may be important in phenotyping COPD. Pathologists describe three major different patterns of emphysema. Centrilobular emphysema is a "breakdown in alveolar walls in the central portion of the acini, initially sparing the peripheral parts of the acinus and lobule." Panlobular emphysema is the term for "destruction of all portions of the lobule out to the periphery," Paraseptal emphysema is "tissue destruction exclusively in the periphery of the lobule." (188) Classically, centrilobular and paraseptal emphysema were thought to have upper lobe predominance and be more common in smokers, while panlobular emphysema has been shown to predominate in the lower lobes in patients with alpha-1 antitrypsin deficiency. (215, 216) These subtypes may be important for COPD phenotyping as different patterns of emphysema type and distribution may develop through different pathological mechanisms. For example, one study found that narrower peripheral airways were found in

centrilobular emphysema suggesting that an airborne mechanism might induce centrilobular emphysema, whereas a blood-borne mechanism might better explain the panlobular phenotype. (217) There is evidence to suggest that regional blood flow abnormalities may be one of the primary abnormalities in the pathogenesis of emphysema. (171, 218, 219)

Some progress has been made in using CT to assess the pathological types of COPD (centrilobular, paraseptal and panlobular), (220) by identifying different sizes and distributions of emphysematous regions, although visual assessment is still needed to differentiate emphysema types. Software is used to divide the lungs for example, into left and right lungs, upper and lower halves, and inner and outer (or core and rind) regions based on 50% of the lung volume.

Separation of the lungs into different lobes, though more complex, has also been achieved. (172) But for clinical purposes, separation by zones may be adequate as was demonstrated in patient selection for the NETT trial. (130) Software used to identify 'hole size distribution' or the 'Alpha' technique was developed to determine proportions of coalescent areas of emphysematous lung. (221)

This method is based on a fractal geometry technique to make a log-log plot of the frequency distribution of 'holes' of a pre-determined area below a given density in HU. 'Alpha' is the gradient of the slope of the log-log relationship of hole-size versus the percentage of holes of that size. This gradient lessens in the presence of larger emphysematous regions. (172, 221)

The relationship between the zone distribution of emphysema and lung function has been investigated. Lower zone emphysema is more closely correlated with spirometry than upper zone emphysema. (203, 222, 223 2004, p07330)} Core predominant emphysema is also more closely correlated with pulmonary function. (203, 224) While lower zone and core emphysema correlate more closely with airflow limitation, upper zone and core predominant emphysema are more closely related to carbon monoxide transfer factor. (191, 225) Several studies have found gender differences in the distribution of emphysema. (226-228) For example, core predominant emphysema was more often found in women in the NETT trial. (226)

Emphysema severity and distribution on CT has been utilized in clinical practice. The distribution of emphysema on CT has been used to assess patients before and after lung volume reduction surgery. (224, 229, 230) Clinical trials in alpha-1 antitrypsin deficiency have pioneered the development of protocols for CT measurement of emphysema in a longitudinal setting for use in clinical trials. (193, 231)

There are few studies investigating emphysema quantity and distribution phenotypes with clinical parameters in COPD other than lung function. (191, 203) Gas trapping on expiratory scans has been found to correlate with dyspnoea. (213) Makita and co-workers found that symptoms of bronchitis were equally distributed irrespective of the degree of emphysema, but that patients with most emphysema had lower health related quality of life scores.

(205) Low body mass index was associated with low lung density but not with airway dimensions on CT. (205, 232) The extent of emphysema on CT was associated with fat-free mass but not with C- reactive protein in a cohort of smokers. (233) Emphysema has also been associated with an increase in sputum MMP-9 (associated with lung parenchymal destruction) and with worsening BODE index score. (234)

CT measurement of airways

CT protocols for measuring airway dimensions are less well developed than those for lung densitometry. Three-dimensional reconstruction of multidetector-row scans enables the accurate measurement of airway dimensions to a diameter of approximately 2mm (corresponding to 6th generation airways) (235, 236) and a lumen as small as 1 mm in diameter. (237) There is as yet, no consensus for the best method of assessing airways with CT. (238) Some studies focus on airways cut in cross-section on standard CT images, such as the apical segmental bronchus of the right upper lobe. Manual measurements (using electronic calipers) have also been used, though this method is open to significant inter-operator variation. Nakano and coworkers (181) measured the dimensions in a single airway that was most often cut in cross section on CT (right apical segmental bronchus), while Parr and coworkers used a visual scoring system to estimate airway dimensions using a technique initially utilized by Bhalla and co-workers. (239, 240)

Semi-quantitative techniques have been developed to help reduce sources of error. Techniques for accurately determining the wall edge have been explored. Of these, the full-width-half maximum technique is the most commonly used. (241-243) X-ray attenuation is measured along a ray projected from a central seed point in the airway. Wall thickness is determined as the point at which attenuation is half way between the minimum attenuation in the lumen or parenchyma and the maximum attenuation in the middle of the airway wall. This measure overestimates wall area and underestimates lumen area, with error increasing in the smaller airways. (244) Alternatives have been explored such as the “maximum likelihood method” and ellipse fitting algorithms. (241, 245) Montaudon and coworkers demonstrated that removing the effect of obliquity improved the accuracy of airway measurement. They used software to map the pathway of an airway, producing wall measurements orthogonal to the airway centre. This technique reduced the potential for over or underestimation of wall dimensions due to obliquity. (235)

Multi-detector row CT now enables image acquisition at $\leq 1\text{mm}$ slice thickness. This allows for segmentation of the airways using a seed-point in the trachea out to the 5th or 6th airway generation. Airways can then be labeled anatomically to determine branch points and airway generation, although this can be problematic due to anatomical variation. Different nomenclatures have been utilized to aid anatomical identification, but there is increasing difficulty in discerning branch points in the smaller airway divisions. (172, 246, 247) Fully automated software is currently being developed using volumetric techniques,

which may reduce several of these sources of error. However, problems with branch identification, combined with difficulties in the accuracy of airway wall identification (particularly in the small airways), and patient factors like the degree of inspiration and anatomical variation are yet to be overcome. (238)

Airway phenotypes

Remodeling of the airways is common to many chronic lung diseases. In asthma wall thickness in the conducting airways can be increased between 50 and 300%. (248) The pathological features of airway remodeling in COPD include squamous metaplasia, loss of cilia, submucosal gland hypertrophy, neovascularisation, increased smooth muscle mass and fibrosis. (249) Hogg and co-workers first described the pivotal role of the small airways in COPD in 1968, when they showed that the major site of airway limitation in patients with COPD is in airways <2mm in internal diameter. (177) More recently, it was demonstrated that airway dimensions in COPD parallel the stage of disease as defined by the degree of airflow limitation. (250) Animal (251) and human studies (177) have shown that airway remodeling contributes to approximately 25% of the airflow limitation in COPD. Airway dimensions of the large and small airways are attenuated in COPD such that there is a reduction in lumen area and increased wall thickness. (252) Bronchial wall thickening is an independent predictor of FEV₁. (236, 253)

Airway dimensions have been related to morphometric measures and lung function. (181, 254) Nakano and colleagues were first to report an association

between airway wall thickness and FEV₁ in patients with COPD. They measured the lumen area, radius, and airway wall thickness of the apical segmental bronchus of the right upper lobe. A subsequent study analyzing airways in 114 male smokers showed that wall thickness and percentage wall area correlated independently with spirometric measurements of airflow limitation, but not with lung diffusing capacity (a physiological surrogate of emphysema severity). (181) Hasegawa and co-workers studied 52 patients with stable COPD and found that airway luminal area and wall area were significantly correlated with FEV₁ (percent predicted) and that the correlation coefficients improved as the airways became smaller in size from the 3rd to the 6th generation, concluding that airflow limitation in COPD is more closely related to the dimensions of the smaller airways than the more proximal large airways. (236) Nakano and co-workers (254) assessed airway dimensions with CT scans and lung morphometry in 22 resected lungs (mean FEV₁ 83% predicted). They found that airway dimensions on CT correlated with morphometric measurements of airways dimensions in airways down to an internal diameter of approximately 1.27mm. They also concluded that CT consistently overestimated airway dimensions particularly in the smaller airways and that the larger cartilaginous airways provided a reasonable surrogate measure of small airways ($r^2=0.57$, $p<0.01$). Few studies have examined the relationship between CT measurements of airway dimensions and clinical features other than lung function in COPD. (213, 255-257)

Summary

Many potential markers that might augment COPD phenotyping in addition to FEV₁ have therefore been explored. Numerous markers of inflammation and oxidative stress in various body fluid compartments have been analyzed, but many of the studies have been small and often report conflicting results. Clinical features have been utilized with some success to help predict prognosis in COPD. In addition, CT scanning now provides a method for investigating anatomical profiles as outcome measures in COPD.

There is a need to investigate potential COPD phenotypes in a single well-characterized cohort of COPD patients and controls in order to establish their inter-relationship with one another. Most studies in the literature investigate inflammatory markers in only one body fluid compartment and often do not take account the potential confounding effect of smoking history. It is also difficult to compare reports due to differences in study methods. Clinical features such as body mass index have been investigated with spirometry and prognosis but little work has been done on the relationship between some of these clinical features of COPD and markers of inflammation. Lung density measured by CT has been investigated extensively with lung function parameters but again, there is little work assessing relationships between lung density, clinical features and markers of inflammation in COPD.

Aims

The aim of this thesis was to explore phenotypes of COPD using inflammatory markers and computed tomography. A cohort of COPD patients and control subjects was characterized with respect to post-bronchodilator spirometry, quality of life, and physiological features (dyspnoea, exacerbations, body mass index, oxygenation and the BODE index). Quantitative computed tomography was used to measure lung volume and density in the COPD cohort. Biomarkers of pulmonary and systemic inflammation were related to the clinical phenotypes defined by spirometry, symptoms, and clinical and anatomical features. The specific aims were to investigate the role of inflammation in the airways and in the systemic compartment in COPD patients compared to controls, and to investigate the relationship of inflammation to clinical and anatomical phenotypes of COPD.

We hypothesize that:

1. Inflammation in the pulmonary and systemic compartments will be increased in COPD patients compared to controls.
2. Airflow limitation will be associated with increased inflammation in the pulmonary and systemic compartments in COPD patients.
3. Inflammation will be associated with clinical features known to be associated with outcome in COPD.
4. Increased airflow limitation and more inflammation will be associated with the extent of emphysema on CT.

Chapter 2: Methods

Study subjects

COPD patients were recruited from general practices and respiratory medical outpatient clinics at the Royal Infirmary of Edinburgh. Subjects had a clinical history consistent with COPD, previous or current exposure to tobacco smoke with at least a 10 pack year smoking history, and spirometric evidence of chronic irreversible airflow limitation (defined as a post bronchodilator FEV_1/FVC ratio $<70\%$ with $<15\%$ or 200ml reversibility following 2.5 mg of nebulized salbutamol). (2) Patients were studied when clinically stable, at least 6 weeks post-exacerbation. Subjects with other respiratory conditions, systemic inflammatory diseases or prescribed regular oral corticosteroids were excluded. Healthy control subjects were recruited through advertisements placed in the Edinburgh area and by word of mouth. The Lothian Regional Ethics Committee granted ethical approval. Written informed consent was obtained from all subjects.

Study design

This was a prospective cross-sectional case-control study. Subjects were asked to refrain from smoking for 12 hours preceding assessment. Exhaled CO measurements (Bedfont piCO smokerlyzer, Bedfont scientific Ltd., UK) were used to confirm abstinence indicated by a value of $<10\text{ppm}$. All medications were withheld for at least 12 hours, with long acting anti-cholinergic treatment being withheld for 24 hours. A structured questionnaire was administered to

record past medical history, smoking status, current medication, MRC dyspnoea score, MRC chronic bronchitis score, total St George's Respiratory Questionnaire (SGRQ) score and SGRQ subcategory scores. The SGRQ is composed of three subcategories – symptoms, activity and impact. These combine as a total score, which is the mean of the three subcategories. Subcategory scores range from 0-100, with higher scores representing greater impairment. A difference of 4 points is considered a clinically significant difference. (258) Annual exacerbation frequency (as defined by Rodriguez-Roisin and co-workers) (140) was assessed by patient recall. Spirometry with reversibility to 2.5mg nebulized salbutamol was performed in accordance with ATS/ERS standards (2) (Vitalograph Alpha Spirometer, Ennis, Ireland). Disease severity was classified according to GOLD stage. (25) Height and weight were recorded and Body Mass Index (BMI) was calculated as weight in kilograms divided by height in metres squared (kg/m^2). Venous blood was collected and stored at -80°C . Six minute walking distance (6MWD) was measured following the recognition that exercise tolerance was an important predictor of mortality. (118) 6MWD was performed according to ATS guidelines, including ATS guidance on statements used to “encourage” patients to continue. (259) The Body mass index, degree of airflow Obstruction and Dyspnoea, and Exercise capacity (BODE) Index score was calculated for the COPD subjects. (118) Components of the BODE index are displayed in Table 2.1.

Table 2.1. Variables and point values used for the computation of the Body mass index, degree of airflow Obstruction and Dyspnoea, and Exercise capacity (BODE) Index. The total possible values range from 0-10. (118)

Variable	Points on BODE Index			
	0	1	2	3
FEV ₁ , % predicted	≥65	50-64	36-49	≤35
Distance walked in 6 minutes, m	≥350	250-349	150-249	≤149
MMRC dyspnoea scale	0-1	2	3	4
Body mass index, kg/m ²	>21	≤21		

MMRC = modified MRC dyspnoea score. Scores range from 0-4, with a score of 4 indicating that the patient is too breathless to leave the house or becomes breathless when dressing or undressing.

Sputum induction

Sputum induction was performed according to a validated procedure. (45) In brief, subjects were asked to rinse their mouth with water and gargle prior to attempting sputum induction. COPD patients were asked to expectorate sputum, which was then discarded. Nebulized hypertonic saline was inhaled for 5 minutes via an ultrasonic nebulizer (Devilbiss Healthcare, Heston, UK) during normal tidal breathing first with 3, then 4 and 5% saline. FEV₁ was measured between each inhalation. Sputum induction was not undertaken in subjects with FEV₁ ≤ 0.5 litres. The procedure was abandoned in subjects whose FEV₁ fell to < 80% of baseline.

Subject recruitment, baseline history, examination, height and weight, CO measurement, administration of symptom questionnaires, spirometry, collection of blood samples, sputum induction and 6 minute walking distance measurements were made by me with the help of two experienced research nurses.

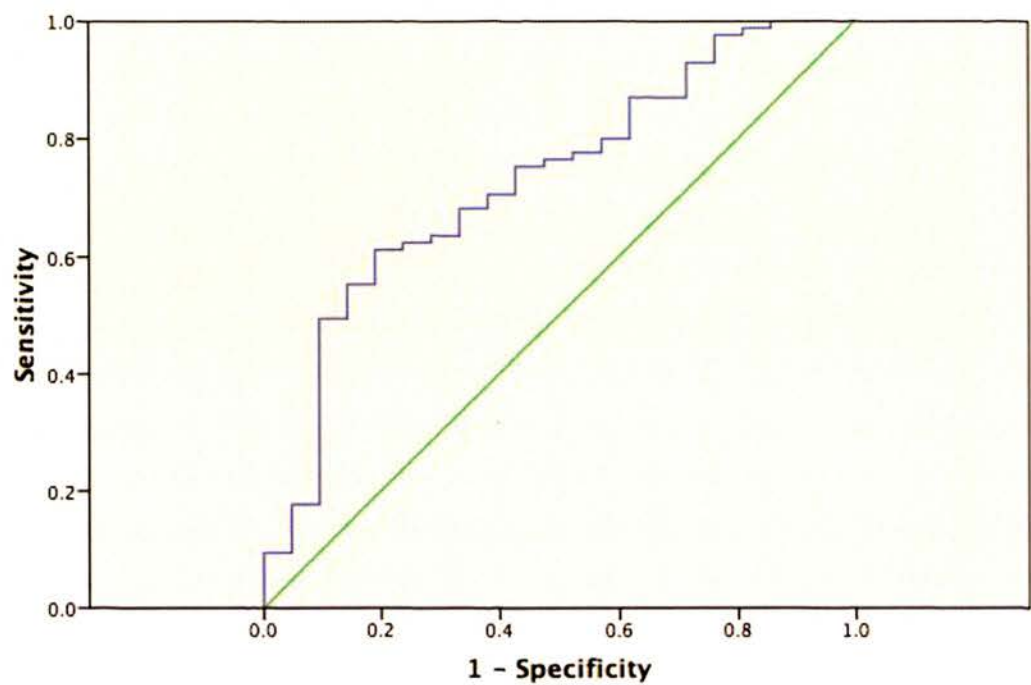
Laboratory analysis

Sputum samples were examined as soon as possible and within 2 hours of production. The weight of the sample was recorded. Sputum was separated from contaminating saliva macroscopically using forceps and was placed in a pre-weighed falcon tube and the weight of sputum was recorded. Each sputum sample was processed with 4 times its weight of dithiotreitol (DTT, 0.1% in distilled water), vortexed for 15 seconds and rocked for 15 minutes. The suspension was filtered through 50 μ m nylon gauze and centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted and stored at -80°C for further analysis. The cell pellet was re-suspended with approximately 1ml of Hank's balanced salt solution (HBSS) according to macroscopic estimation of the pellet size. The total cell count was determined with a haemocytometer. Trypan blue was used to determine cell viability (blue cells being non-viable). The percentage of viable and non-viable cells per gram of original sputum was determined and the cell suspension mixed with HBSS to obtain a cell count of $0.6-1.0 \times 10^6$ cells/ml of suspension. Cytospins were made using a Cytotek cytocentrifuge. Cytospin slides were stained with DiffQuik to obtain differential cell counts made by counting 400 cells per slide. (260, 261)

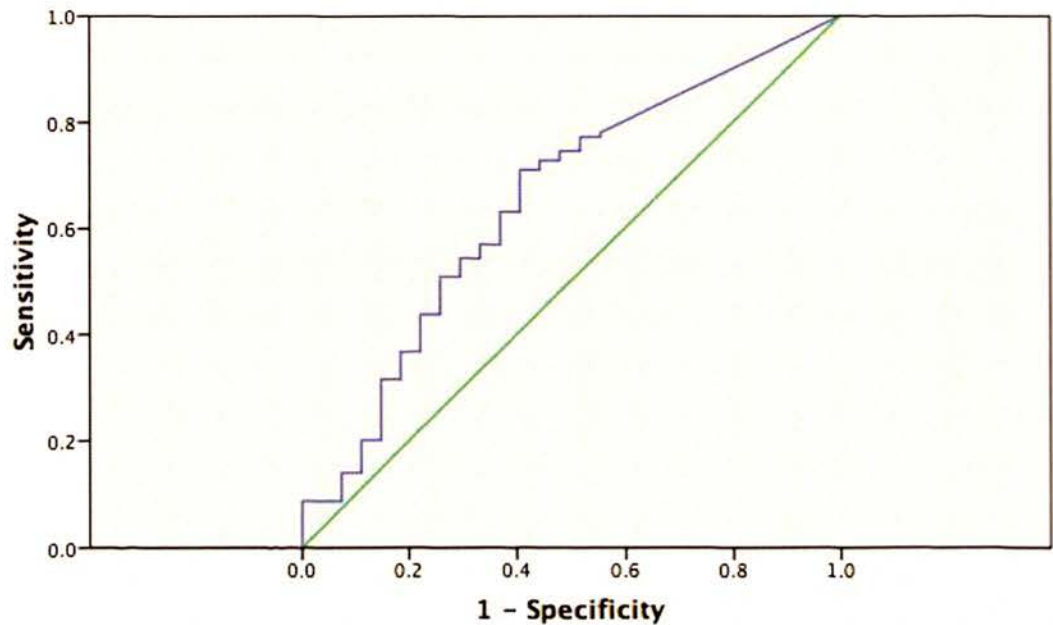
IL-8, IL-6, and IL-1 β concentrations in sputum supernatant were assessed by a human enzyme linked immunosorbent assay. A monoclonal antibody specific for the cytokine was pre-coated onto a microtitre plate and standards and samples added. After washing, an enzyme linked polyclonal antibody was added followed by a substrate solution for colour development and intensity reading (Duoset ELISA - R&D Systems, Oxon, UK). The limit of detection for each of the assays was 1.95 pg/ml for IL-1 β , 4.69 pg/ml for IL-6, and 15.63 pg/ml for IL-8 (based on the lowest standard of an eight point standard curve). The co-efficient of variation for each of the assays was 19% for IL-1 β , 14% for IL-6, and 8% for IL-8. Receiver-operator curves (ROC) for COPD patients and healthy subjects are shown in Figure 2.1 for induced sputum % neutrophils, IL-6 and IL-8 with the area under the curve (AUC) and confidence intervals (CI). IL-1 β was not significantly different between COPD patients and controls.

Figure 2.1 ROC curves for induced sputum inflammatory markers

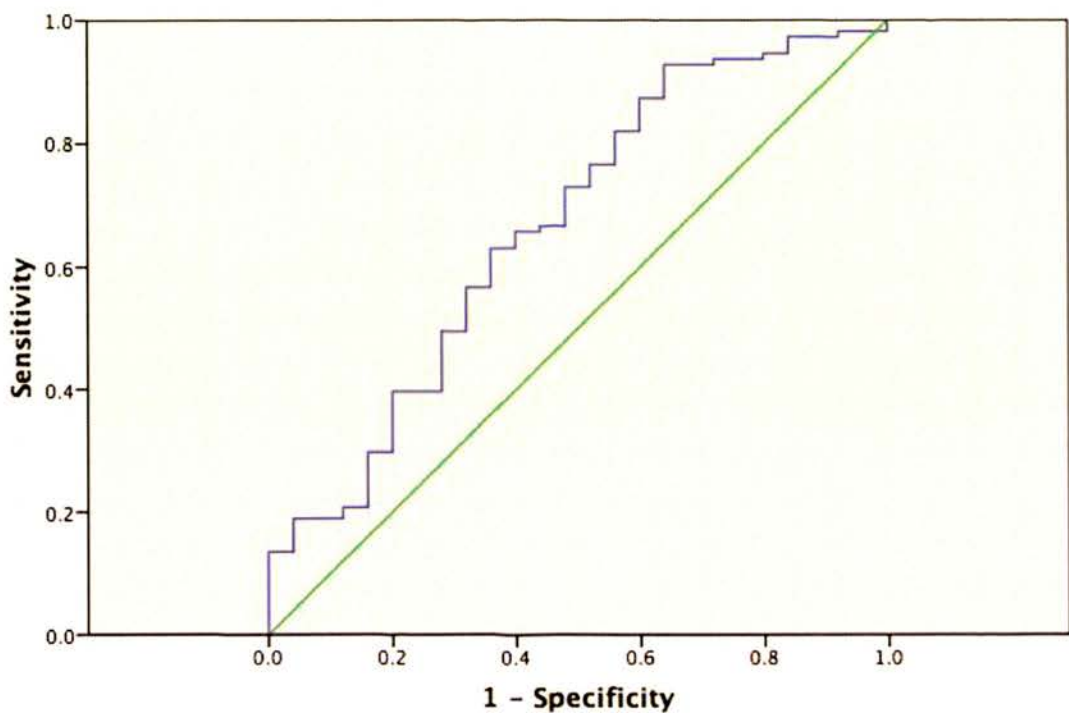
2.11 Induced sputum % neutrophils: AUC 0.73, (CI 0.64-0.85)



2.12 Induced sputum IL-6: AUC 0.65, CI (0.53-0.77)



2.13 Induced sputum IL-8: AUC 0.66, (CI 0.54-0.79)



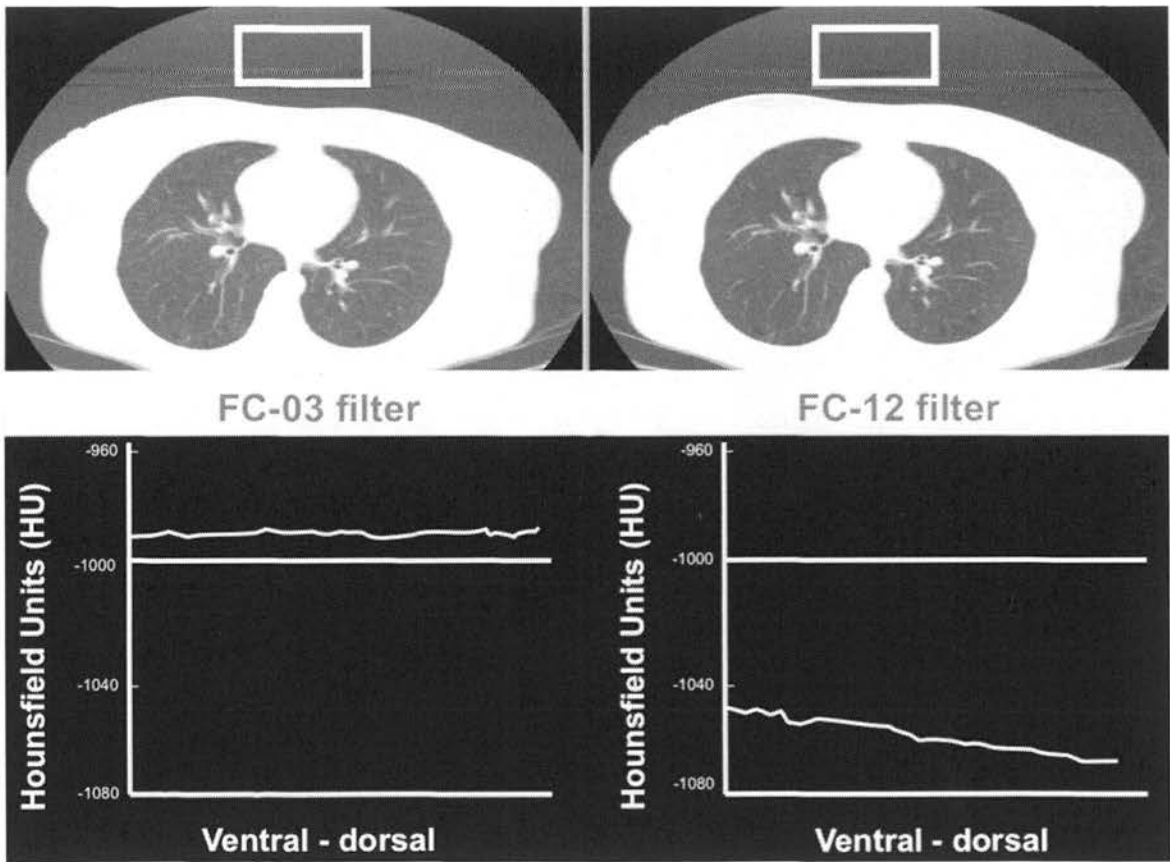
Full blood count was measured (Sysmex XE 2100, Norderstedt, Germany) and serum C-reactive protein (CRP) concentration was measured using a highly sensitive immunonephelometric assay (Behring BN II nephelometer, Dade Behring, Marburg, Germany). I took part in the laboratory analysis of induced sputum and blood samples with the help of two experienced laboratory technicians. Highly sensitive CRP assays were conducted by laboratory technicians in a nearby laboratory.

CT protocol

Subjects underwent low dose thoracic CT scanning at full inspiration and on expiration. I attended CT scans with the study subjects and undertook standardized coaching to achieve as close to total lung capacity and functional residual capacity as possible. No intravenous contrast was administered. Scans were acquired in the caudo-cranial direction to reduce artefact. All scans were acquired using a 16-slice Toshiba Aquilion (Toshiba Medical Systems, Tokyo, Japan) CT scanner (135kV, 40mA, rotation time 0.5 sec, 16 x 1mm collimation, pitch 1.45, FC-03 filter, reconstructed at 2.5mm intervals with 5mm thick slices for lung density analysis. Radiation dose was low at around 1 mSv per scan.

Scanner calibration was undertaken according to the manufacturer's instructions within 3 hours of the study scan being conducted. The Hounsfield Unit (HU) for air was recalibrated using a method similar to that described by Stoel and Stolk (195) using the FC-03 filter to correct for the air offset in Toshiba CT scanners (about -985 HU instead of the nominal -1000 HU with this filter). A more detailed explanation of the air calibration method used can be found in the Appendix. The difference between the FC-03 and FC-12 filters is demonstrated in Figure 2.2. Water calibration was also undertaken every 3 months using a phantom. Calibration procedures were undertaken by the CT radiographers and supervised by the medical physicist.

Figure 2.2. Air calibration using the FC-03 filter compared to the FC-12 filter. A “region of interest” for air calibration was designated ventrally (indicated by the white rectangle above each CT image). The graph corresponding to each filter shows the difference in air calibration measurement for each. The FC-03 filter gives a constant measurement for air in Hounsfield Units, compared to the significant drift seen with the FC-12 filter.



Software was developed in-house by Martin Connell (medical physicist) to measure lung volume and whole lung density. Data collected using this software has been published in a number of papers. (222, 262, 263) Following segmentation of the lungs from the chest wall, mediastinum and trachea, lung volume was calculated on inspiration and expiration. Densitometric indices were extracted from the frequency distribution histogram of lung voxels. While “voxel” is the most accurate term representing 3 dimensional units of volume, the terms voxels and pixels (2-D) are used interchangeably in the CT literature. For simplicity the term “pixels” will be used throughout the thesis. Lung density was measured with the 15th percentile point (PD15), and with the pixel index at the -950 threshold (PI-950) and the -910 threshold (PI-910). Pixel indices indicate the percentage of pixels with density values below -950 or below -910 Hounsfield Units (Figure 2.2). (194) The 15th percentile is defined as the value in Hounsfield Units below which lie 15% of the lowest density pixels. (254) Figures 2.3 and 2.4 show the pixel frequency distribution histogram with the derivation of density indices used in the analysis. Airway dimensions including lumen area, wall thickness and percentage wall area were measured as part of the study but the airway data required further validation that was outwith the scope of this thesis.

Figure 2.3. The graph shows the frequency distribution histogram of CT pixels for a subject with emphysema compared to a subject with normal lung density (adapted from Parr et al, 2009). (264) The figure shows the position of the -910 and -950 HU thresholds (not to scale). The number of pixels at each threshold is higher in patients with emphysema than with normal lung density. -1000 HU is equivalent to the density of air.

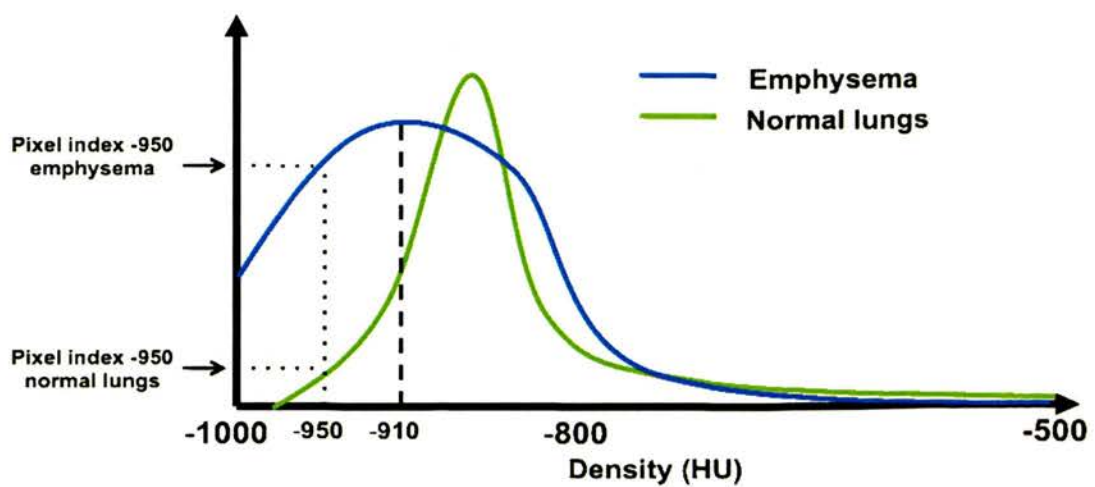
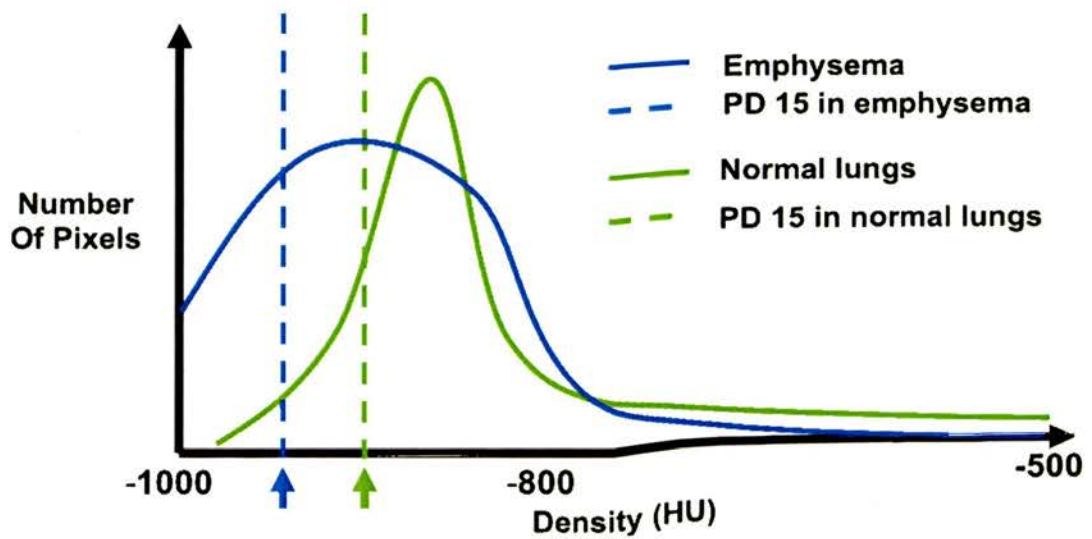


Figure 2.4. The graph shows the 15th percentile (PD 15) on the frequency distribution histogram of CT pixels for a subject with emphysema compared to a subject with normal lung density (adapted from Parr et al, 2009). (264) The figure demonstrates that the 15th percentile is lower in patients with emphysema than in patients with normal lung density (not to scale). -1000 HU is equivalent to the density of air.



Data analysis and statistics

The method of data presentation and statistical analysis is provided in detail with each chapter. I did the data analysis with guidance from the statistician. A sample size calculation was not undertaken due to a lack of prior data. Analyses were performed using SPSS version 16 (for Mac). A p value of < 0.05 was considered significant except where multiple comparisons were made where a significance level was taken as 0.05 divided by the number of comparison tests e.g. if 6 comparisons were made then the significance level was $p < 0.008$. (265)

Logistic regression results were displayed in tabular form as B, standard error, degrees of freedom (df), Wald, significance and Exp (B). With each table the -2 log likelihood, Cox & Snell R square and Nagelkerke R square were displayed in addition to the overall significance. B is the regression coefficient. The Wald test indicates the statistical significance of each co-efficient (B) in the model. When the sample size is small, the -2 log likelihood is more reliable than the Wald test. (266) Exp (B) is the relative odds (or odds ratio) for the factor in question e.g. in COPD patients compared to healthy controls. Therefore for each unit in variation in that factor (e.g. each year change in age) a subject is Exp (B) times more likely to be a COPD patient than a healthy subject. The -2 log likelihood is a measure of deviance or variation in the model. It has a chi-squared distribution and is a measure of how well the model explains variations in the outcome of interest. A lower value indicates a closer representation of this outcome e.g. in COPD patients compared to healthy subjects. The Cox & Snell R square and

Nagelkerke R square values represent the proportion of variation explained by the model including all the factors in the table. For example, when Cox & Snell R square is 0.354 and Nagelkerke R square is 0.494 (as in Table 3.7), the model explains 35.4 to 49.4% of the variation between healthy controls and COPD subjects. (267)

Multiple linear regression data were presented in tabular form where: B is the regression co-efficient (the slope of the regression line for a given variable); SE is the standard error (of the slope); t is the test statistic (B/SE) and p is the statistical significance.

CHAPTER 3: Systemic and pulmonary markers of inflammation in COPD subjects and healthy controls

Introduction

Inflammation in the pulmonary and systemic compartments is central to the pathogenesis of COPD. Inflammation is part of the working definition of the disease (2) and has implications for prognosis. (28, 81, 82) Markers of inflammation may therefore be useful in defining clinical phenotypes of COPD. An ideal diagnostic marker should be able to differentiate COPD subjects from healthy controls, should relate to the severity of disease or a disease phenotype and should be responsive to therapeutic interventions. However, in clinical practice few biological markers achieve this. (21) In the case of FEV₁, it is achieved using an arbitrary cut-off that has been refined over time. (6) The FEV₁ is still the strongest predictor of outcome in COPD, but additional clinical parameters have been found to predict prognosis in COPD patients independently of FEV₁ and smoking status. (14-16) In order to characterize phenotypes of COPD using markers of inflammation, it is important to know if these markers are significantly different in COPD patients compared to healthy subjects and if they are affected by smoking status.

The association between COPD and cigarette smoking has long been recognized. (268) There is an established direct causal link between tobacco smoke and COPD. (269) However, only 10-15% of smokers develop COPD, (270, 271) and the cumulative smoking history in smoking pack years is not strongly associated

with the degree of airflow limitation. (46) Cigarette smoking is associated with pulmonary inflammation. Leukocytes, and in particular neutrophils in the lungs, are increased in cigarette smokers. (272) Smoking cessation is associated with a reduction in lung inflammation in asymptomatic smokers (273) and slows the rate of decline in lung function in early COPD. (274) But a prospective study of smoking cessation in COPD patients found that airway inflammation increased even after smoking cessation. (275)

Systemic inflammation is increased in COPD. (276) Cigarette smoking has a long recognized association with blood leukocytosis. (277) However, an association has been reported between systemic inflammation and airflow limitation that is independent of smoking status. (82, 85) The NHANES III study reported that current smoking added to the systemic inflammation present in subjects with airflow limitation. (85) An increase in systemic inflammatory markers (peripheral white cell count and CRP) is associated with poor outcome in COPD, independent of smoking status and the degree of airflow limitation. (16, 95)

Sputum induction provides a relatively non-invasive means of assessing the cellular and inflammatory composition of the airways. Sputum induction procedures have been standardized. (278) But obtaining a reliable sputum sample is not always possible. (175) However, when compared to analysis of pulmonary inflammation by alternative means, induced sputum remains an attractive alternative for assessing inflammatory biomarkers in COPD. (23) For example, the accurate measurement of inflammatory markers in exhaled breath

condensate remains controversial (279) and bronchoalveolar lavage and lung biopsy are more invasive. Induced sputum has a higher yield of viable cells than spontaneous sputum. (71) It has been shown to be repeatable but with significant inter- and intra-patient variability. (280) Induced sputum can be used to obtain samples from the airways of subjects who do not usually produce sputum.

This chapter investigates the utility of markers of inflammation in blood and induced sputum as potential biomarkers in COPD, taking into account current smoking status. The hypothesis is that markers of inflammation in blood and induced sputum will be increased in COPD patients compared to controls after controlling for the effect of smoking.

Aims

In a cohort of stable COPD subjects and healthy controls:

1. To investigate levels of blood and sputum markers of inflammation in COPD patients compared to controls.
2. To investigate the effect of smoking status on markers of blood and sputum inflammation.

Methods and data analysis

Subjects were recruited as described in Chapter 2. Demographic data were recorded for COPD subjects and healthy controls according to smoking status. COPD and control subjects were compared with the unpaired t-test (parametric) or Mann-Whitney U tests (non-parametric) for continuous variables and with the Chi-squared test for categorical data. COPD subjects and control groups were then analyzed according to smoking status. Groups were compared using analysis of variance (ANOVA) or the Kruskal Wallis test. This was followed by the Bonferroni post-hoc test or the Mann Whitney U test for comparison between groups.

Significant variables in the univariate analysis were analyzed using binary logistic regression. For the purpose of inclusion in the regression analysis, significance was taken as $p < 0.1$. Subjects were divided by smoking status into two categories: current smokers and ex- or non-smokers. CRP and sputum IL-8 were log-transformed to achieve a normal distribution for analysis. In the absence of pre-defined cut-off values, low and high categories (50th percentiles) were defined for sputum % neutrophils ($\leq 87\%$ and $> 87\%$) and sputum IL-6 (≤ 22 pg/ml and > 22 pg/ml). COPD patients, male sex, current smokers and the “high” category for sputum % neutrophils and sputum IL-6 were the reference categories.

Results

182 COPD subjects and 96 healthy controls were recruited. The baseline characteristics of the COPD patients and healthy controls are shown in Table 3.1. The COPD patients were older than the controls and had a higher proportion of men. In the COPD group, disease severity ranged from GOLD stage I to IV: GOLD I n=13 (7%), GOLD II n=70 (39%), GOLD III n=73 (40%), GOLD IV n=26 (14%). As expected, the COPD groups had significantly more airflow limitation and lower oxygen saturations than controls. 65% of COPD patients and 26% of control subjects were able to produce an induced sputum sample for analysis. Sputum induction was not attempted in COPD subjects whose baseline FEV₁ was \leq 0.5 litres (n=8).

Table 3.1. Characteristics of COPD patients and healthy controls. Data are presented as number (%) or mean \pm SD. Groups were compared with the unpaired t-test (continuous variables) or Chi-square test* (categorical variables).

	COPD	Controls
	n=182	n=96
Age	68 \pm 8	58 \pm 11*
Gender, (% male) *	112 (62%)	41 (43%)*
Smoking, pack years	48 \pm 24	18 \pm 16*
Body mass index, kg/m²	26 \pm 6	27 \pm 4*
Oxygen saturation, %	95 \pm 2	98 \pm 2*
FEV₁, post bronchodilator % predicted	50 \pm 19	106 \pm 14*
FVC, post bronchodilator % predicted	79 \pm 21	104 \pm 49*
FEV₁/FVC ratio, % post bronchodilator	48 \pm 13	79 \pm 7*
Induced sputum sample*	119 (65%)	25 (26%)*

* = p<0.05

The subjects were divided according to smoking status for further analysis (Table 3.2). Age was significantly different between groups ($p < 0.001$) except between COPD smokers and healthy ex-smokers ($p = 0.9$) and between healthy smokers and healthy ex- and non-smokers ($p = 0.3$ and 1.0 respectively). Cumulative smoking history in pack years was significantly higher in COPD patients compared to controls ($p < 0.001$) except for healthy smokers ($p = 0.03$). Oxygen saturation, FEV_1 and FEV_1/FVC ratio were significantly lower in COPD patients compared to the control groups ($p < 0.001$). BMI was significantly lower in COPD current smokers ($p < 0.05$). When analyzed according to smoking status, current smokers in both groups produced a higher yield of induced sputum: 78% COPD smokers compared to 50% of COPD ex-smokers, 52% healthy smokers compared to 17% of healthy ex-smokers and 19% of healthy non-smokers.

Table 3.2. Clinical characteristics of COPD patients and healthy controls are presented according to smoking status. Data are presented as number (%) or mean \pm SD. Groups are compared with ANOVA or Chi squared* tests.

	COPD smokers	COPD ex- smokers	Healthy smokers	Healthy ex- smokers	Healthy non- smokers
	n=76	n=106	n=23	n=41	n=32
Age, years	65 \pm 8	71 \pm 7	∇ 55 \pm 11	\S 63 \pm 10	∇ 55 \pm 9
Gender, % male*	\S 52%	72%	∇ 30%	\S 51%	∇ 40%
Smoking, pack years	51 \pm 25	44 \pm 24	∇ 30 \pm 13	∇ 26 \pm 11	\P 0 \pm 1
Body mass index, kg/m²	\P 24 \pm 5	27 \pm 5	27 \pm 4	28 \pm 5	27 \pm 5
Oxygen saturations, %	95 \pm 2	96 \pm 2	∇ 97 \pm 1	∇ 97 \pm 2	∇ 98 \pm 1
FEV₁, post bronchodilator % predicted	51 \pm 19	52 \pm 19	∇ 100 \pm 12	∇ 105 \pm 16	∇ 111 \pm 12
FVC, post bronchodilator % predicted	77 \pm 21	79 \pm 23	∇ 101 \pm 25	∇ 109 \pm 16	∇ 113 \pm 18
FEV₁/FVC ratio % post bronchodilator	52 \pm 14	49 \pm 14	∇ 78 \pm 8	∇ 79 \pm 7	∇ 81 \pm 7

∇ = significantly different from both COPD groups (p<0.05).

\S = significantly different from COPD ex-smokers (p<0.05).

\P = significantly different from all other groups (p<0.05).

\P = significantly different from COPD ex-smokers and healthy ex-smokers (p<0.05).

Systemic inflammation

Markers of systemic inflammation were compared between COPD patients and controls (Table 3.3). White cell count, neutrophils and C-reactive protein were each significantly higher in COPD patients compared to healthy subjects. These markers were analyzed in COPD patients and healthy controls according to smoking status (Table 3.4).

Table 3.3. Markers of systemic inflammation in COPD patients and healthy controls are displayed. Data are presented as mean \pm SD (parametric) or median (IQR) (non-parametric). Data were analyzed with the unpaired t-test. As CRP was not normally distributed, it was log-transformed for analysis.

	COPD	CONTROLS	p
Blood white cell count, $\times 10^9$	7.6 \pm 2	6.4 \pm 2	<0.001
Blood neutrophil count, $\times 10^9$	4.9 \pm 2	3.8 \pm 1	<0.001
hsCRP, mg/L	3.8 (2-7)	1.4 (1-3)	<0.001

Table 3.4. Markers of systemic inflammation are displayed for COPD patients and healthy controls according to smoking status. Data are presented as mean \pm SD. Differences between groups were analyzed with ANOVA and the Bonferonni post-hoc test. CRP was not normally distributed. It is presented as median (IQR) and was log-transformed for analysis.

	COPD smokers	COPD ex-smokers	Healthy smokers	Healthy ex-smokers	Healthy non-smokers	p
	n=76	n=106	n=23	n=41	n=32	
Blood white cell count, $\times 10^{-9}$	7.9 \pm 2	7.4 \pm 2	7.1 \pm 2	*6.3 \pm 2	∇ 6.0 \pm 1	<0.001
Blood neutrophil count, $\times 10^{-9}$	5.1 \pm 2	4.7 \pm 2	4.3 \pm 1	∇ 3.6 \pm 1	∇ 3.6 \pm 1	<0.001
hsCRP, mg/L	3.7 (2-8)	4.4 (2-7)	∇ 1.6 (1-2)	∇ 1.9 (1-3)	∇ 1.3 (1-2)	<0.001

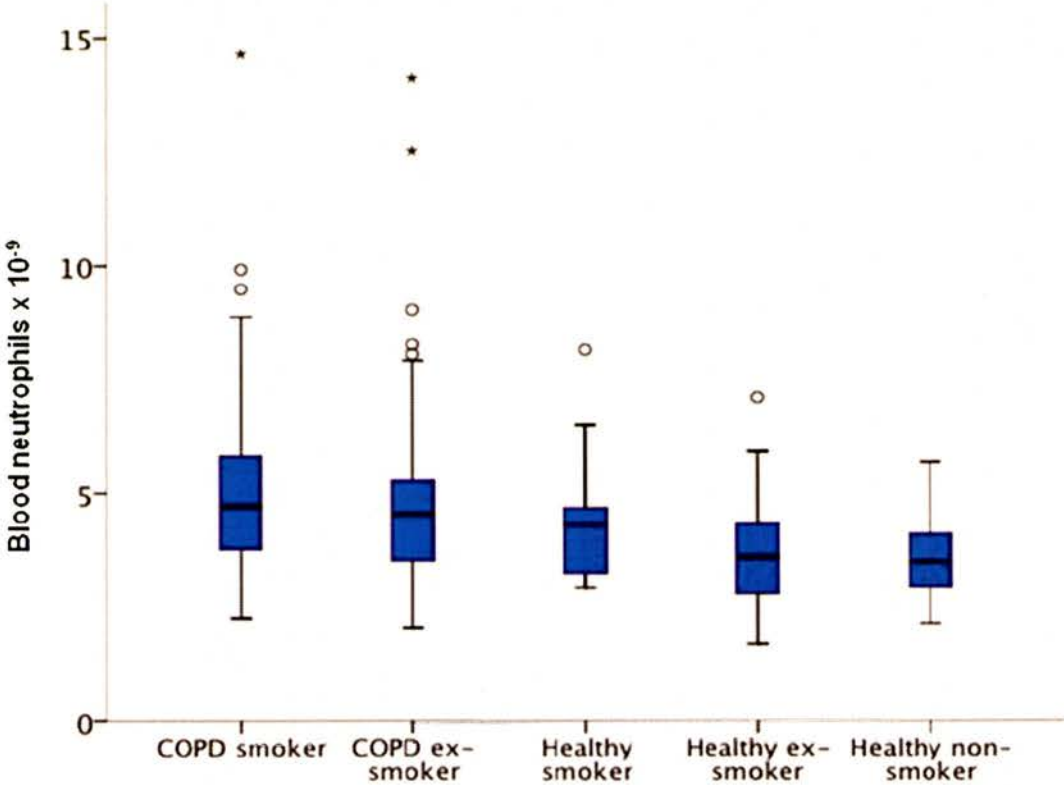
∇ = significant difference between this group and COPD smokers and COPD ex-smokers ($p < 0.05$);

* = significant difference between this group and COPD smokers only ($p < 0.05$)

When analyzed according to current smoking status, blood leukocytes were significantly higher in COPD current and ex-smokers compared to healthy ex- and non-smokers ($p<0.001$). There was no significant difference between current or ex-smoking COPD patients and healthy current smokers (Figure 3.1).

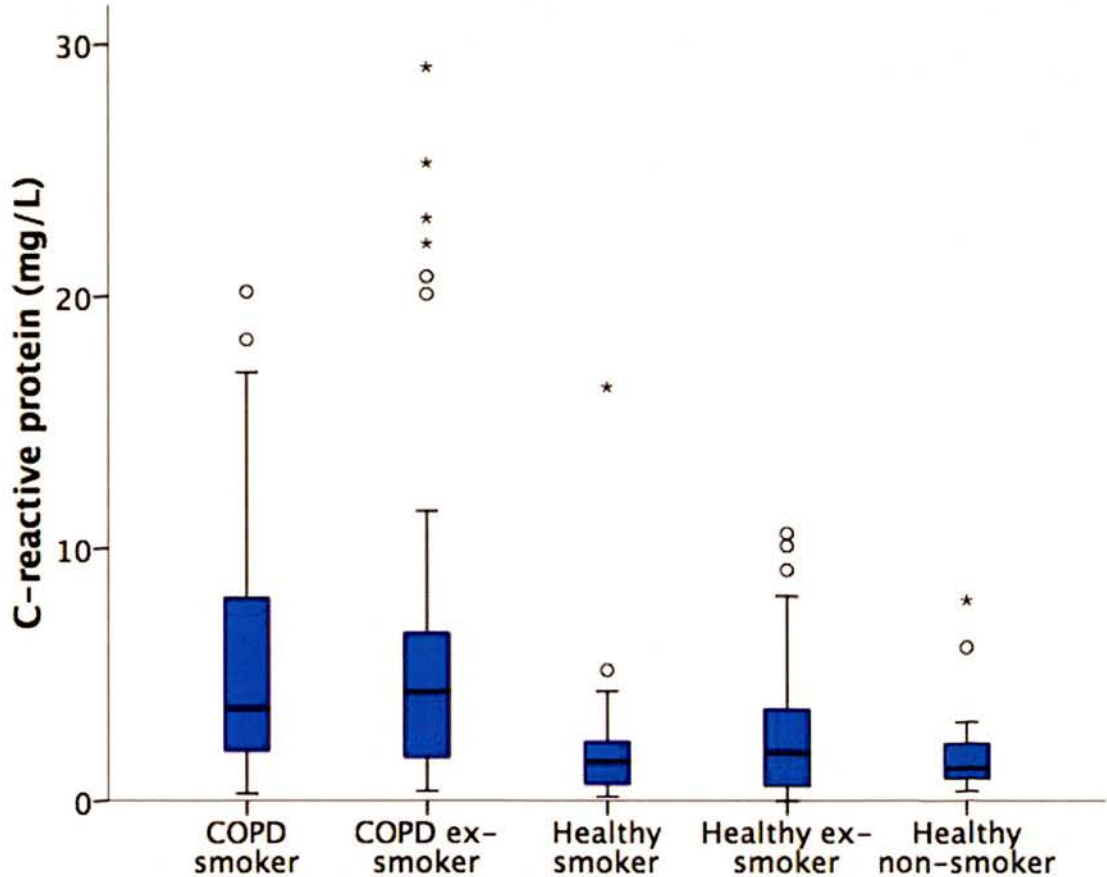
C-reactive protein was compared between groups according to smoking status. There was no difference between CRP levels in COPD smokers compared to COPD ex-smokers ($p=0.96$). CRP was significantly higher in COPD smokers compared to healthy smokers ($p<0.001$), healthy ex-smokers ($p=0.002$), and healthy non-smokers ($p<0.001$). CRP was also significantly higher in COPD ex-smokers compared to healthy smokers ($p<0.001$), healthy ex-smokers ($p=0.004$), and healthy non-smokers ($p<0.001$). CRP was therefore significantly increased in COPD patients compared to controls irrespective of current smoking activity (Figure 3.2).

Figure 3.1. Box and whisker plot showing blood neutrophil counts in COPD patients and healthy control subjects according to smoking status ($p<0.001$, ANOVA). Blood neutrophils were higher in COPD current and ex-smokers compared to healthy ex- and non-smokers ($p<0.001$). There was no significant difference in blood neutrophils between COPD smokers or ex-smokers and healthy smokers ($p=0.2$, $p=0.8$).



Box plot values:
The top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and central line represents the 50th percentile. The whiskers represent the highest and lowest values that are not outliers or extreme values. Outliers (between 1.5 and 3 times the interquartile range) are represented by circles (o). Extreme values (more than 3 times the interquartile range) are represented by stars (*).

Figure 3.2. Box and whisker plots showing blood C-reactive protein levels in COPD and control subjects according to smoking status. COPD smokers and ex-smokers had significantly higher CRP compared to healthy smokers ($p<0.001$), healthy ex-smokers ($p=0.002$ and $p=0.004$) and healthy non-smokers ($p<0.001$).



CRP values for outliers not seen due to scale of x axis: COPD smoker 136, COPD ex-smokers 71 and 75, healthy ex-smoker 32, healthy non-smoker 327.

Pulmonary inflammation

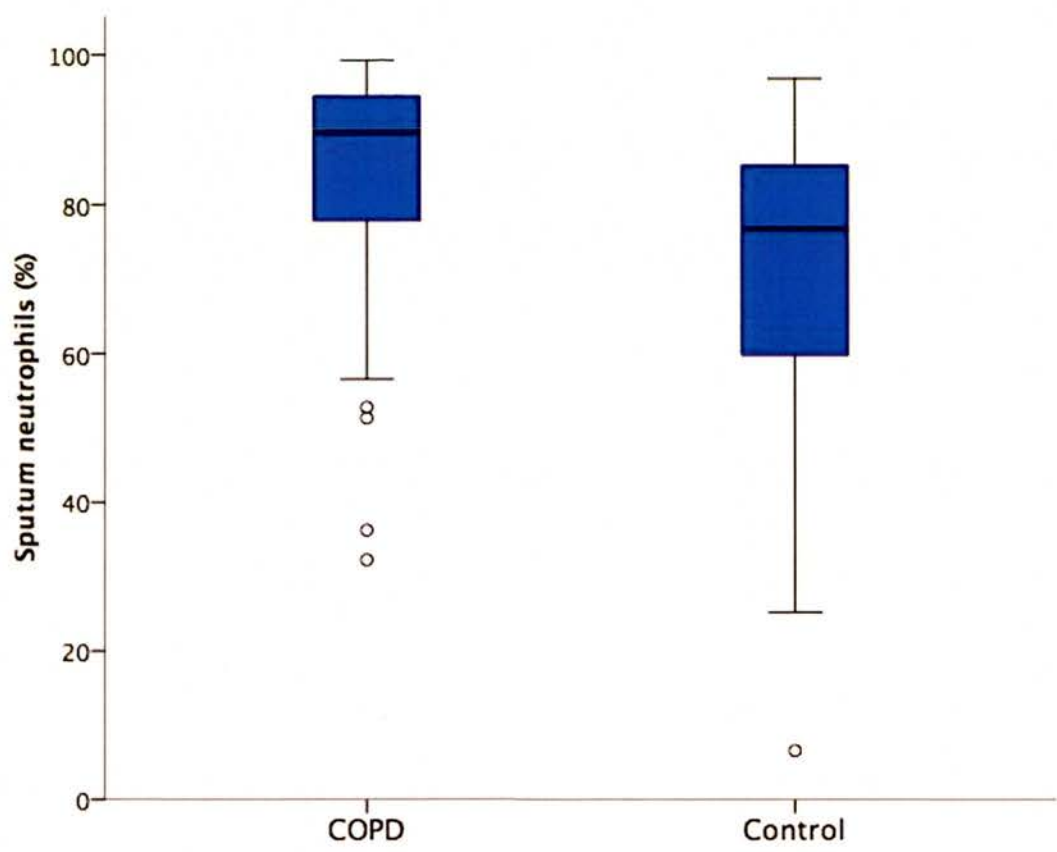
Percentage of neutrophils in induced sputum and levels of IL-1 β , IL-6 and IL-8 were measured in COPD patients and healthy controls (Table 3.5). Sputum IL-1 β was below the limit of detection of the assay in 33% of COPD patients and 32% of healthy controls. There was no significant difference either in the number of patients with detectable sputum IL-1 β ($p=0.5$) or of the level of IL-1 β ($p=0.3$) between COPD patients and controls. Sputum IL-6 was below the limit of detection of the assay in 22% of COPD patients and 40% of healthy controls ($p=0.03$). Induced sputum neutrophils, IL-6 and IL-8 were significantly higher in COPD patients compared to healthy controls (Figures 3.3, 3.4 and 3.5).

Table 3.5. Inflammatory markers in induced sputum are displayed in COPD patients and healthy controls. Data are presented as median (IQR) or *number (%). Groups were compared using the Mann Whitney U test or *Chi-square test.

	COPD n=112	Controls n=25	p
Neutrophils, %	89 (78-95)	77 (60-85)	0.001
IL-1β, pg/ml	9 (0-35)	5 (0-15)	0.326
IL-1β < LOD*	37 (33%)	8 (32%)	0.536
IL-6, pg/ml	28 (6-62)	8 (0-36)	0.018
IL-6 < LOD*	25 (22%)	10 (40%)	0.027
IL-8, pg/ml	458 (266-797)	279 (158-584)	0.010

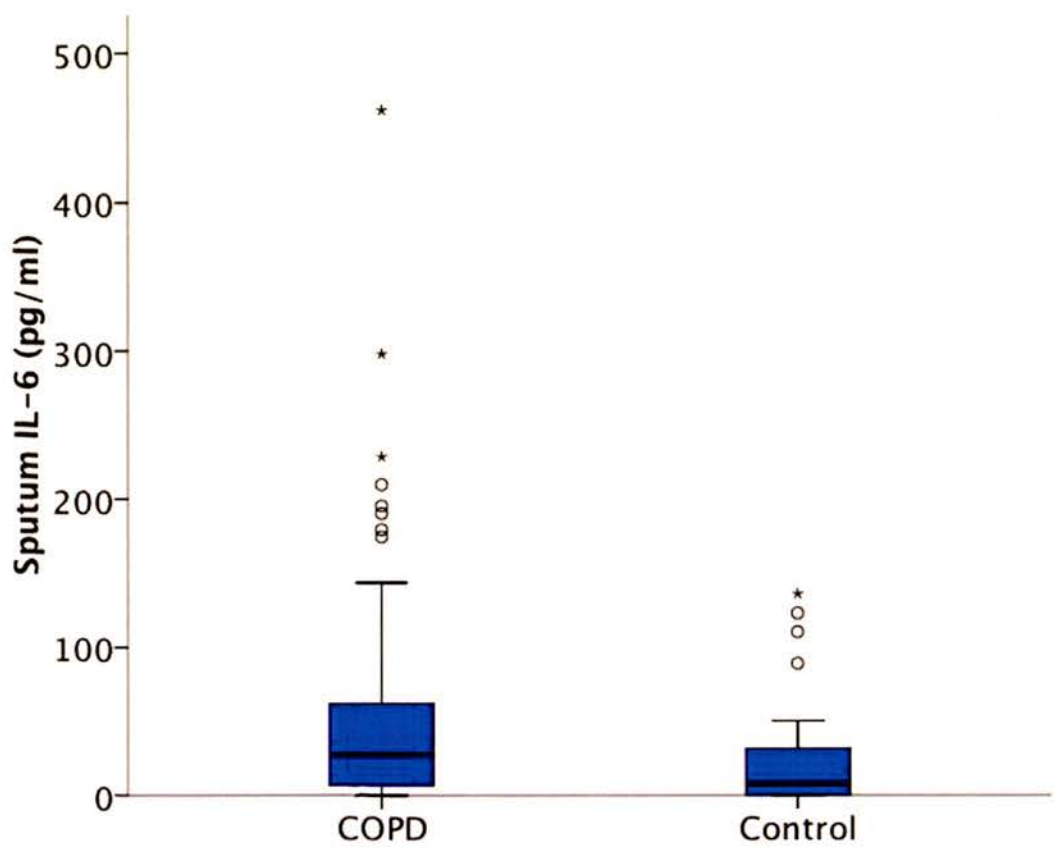
LOD = limit of detection of assay

Figure 3.3. Box and whisker plot showing the percentage of neutrophils in induced sputum in COPD patients and healthy controls (p=0.001).



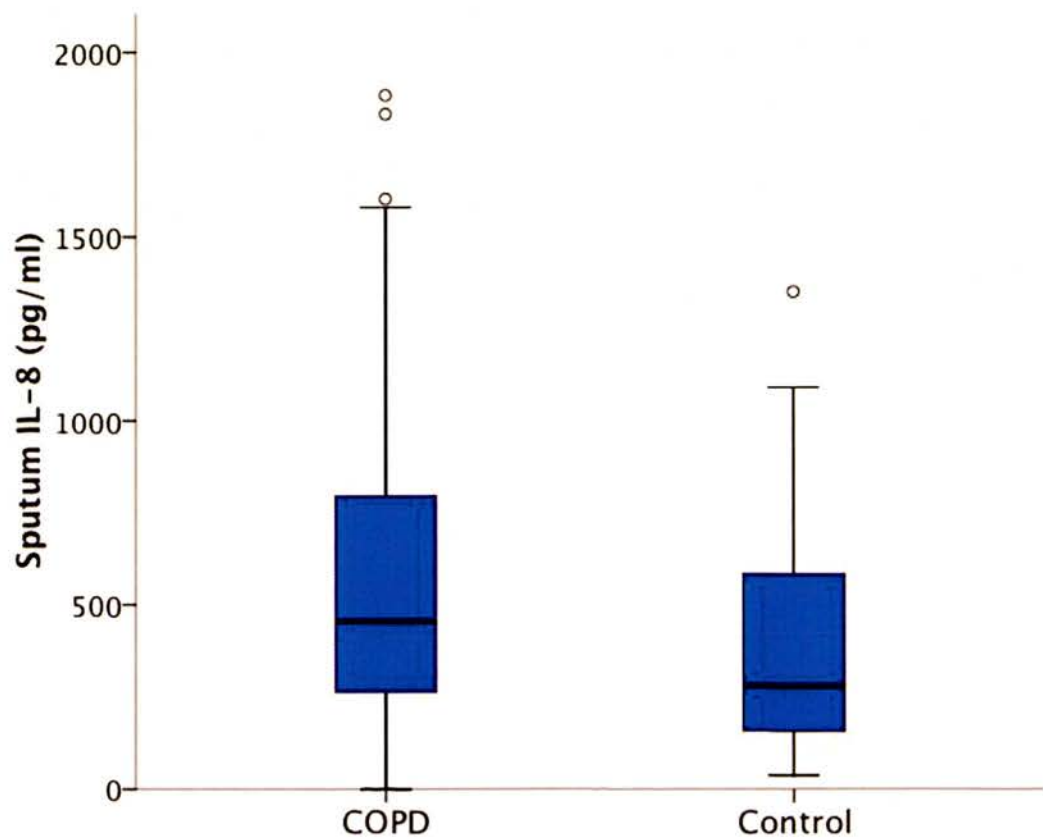
Box plot values:
The top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and central line represents the 50th percentile. The whiskers represent the highest and lowest values that are not outliers or extreme values. Outliers (between 1.5 and 3 times the interquartile range) are represented by circles (o). Extreme values (more than 3 times the interquartile range) are represented by stars (*).

Figure 3.4. Box and whisker plot showing induced sputum IL-6 levels in COPD patients and healthy controls (p=0.02).



Box plot values:
The top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and central line represents the 50th percentile. The whiskers represent the highest and lowest values that are not outliers or extreme values. Outliers (between 1.5 and 3 times the interquartile range) are represented by circles (o). Extreme values (more than 3 times the interquartile range) are represented by stars (*).

Figure 3.5. Box and whisker plot showing levels of induced sputum IL-8 levels in COPD patients compared to healthy controls (p=0.01).



Box plot values:
The top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and central line represents the 50th percentile. The whiskers represent the highest and lowest values that are not outliers or extreme values. Outliers (between 1.5 and 3 times the interquartile range) are represented by circles (o). Extreme values (more than 3 times the interquartile range) are represented by stars (*).

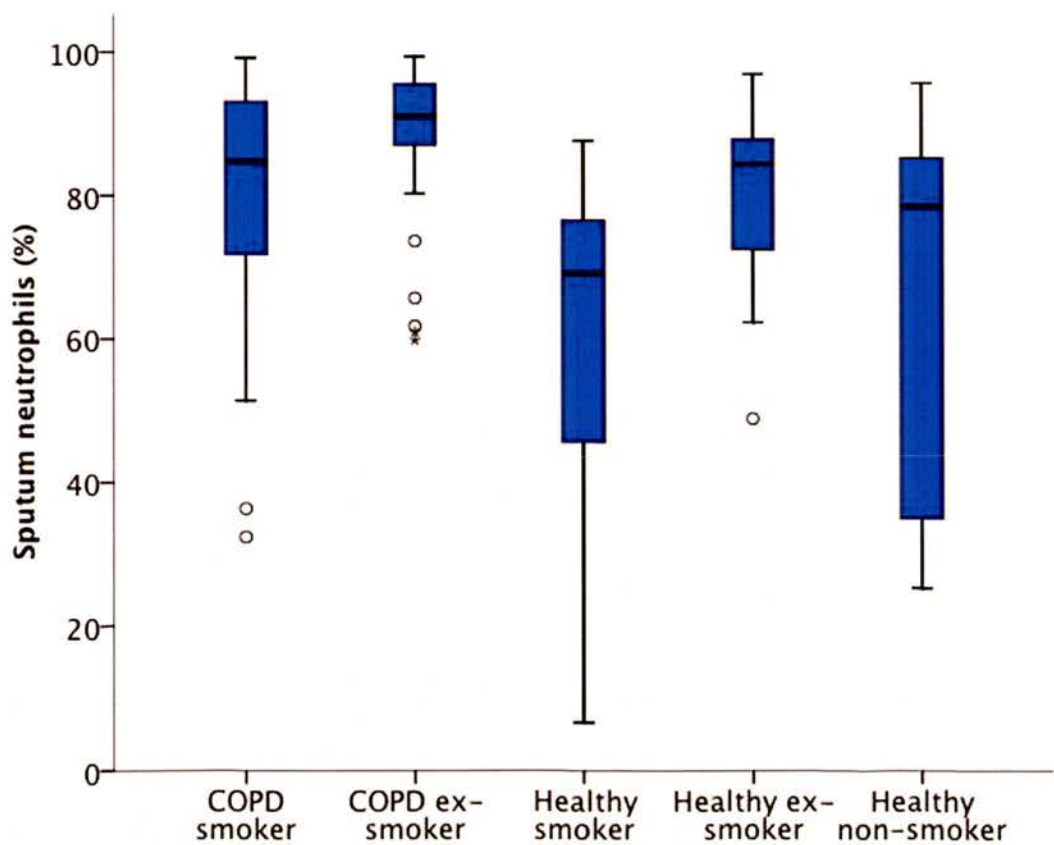
Sputum markers of inflammation were analyzed according to smoking status (Table 3.6). As sputum IL-6 and IL-1 β levels were often below the limit of detection of the assay, the number of subjects whose results were below the limit of detection were tabulated for these cytokines. Sputum neutrophils were significantly different between groups but there were no significant differences in % neutrophils in sputum between the control groups (Figure 3.6). There were no significant differences in sputum cytokines.

Table 3.6. Sputum inflammatory markers in COPD and control subjects are displayed according to smoking status. Data are presented as median (IQR) or *number (%). Groups were compared using the Kruskal Wallis test with the Mann Whitney U for post-hoc analysis or the *Chi-square test.

	COPD smokers	COPD ex-smokers	Healthy smokers	Healthy ex-smokers	Healthy non-smokers	p
	n=59	n=53	n=12	n=7	n=6	
Neutrophils %	85 (72-93)	91 (87-96)	69 (46-76) [∇]	84 (62-90)	78 (35-85)	<0.001
IL-1β, pg/ml	2 (0-26)	12 (4-39)	8 (0-17)	7 (1-12)	5 (0-15)	0.101
IL- 1β < LOD*	27 (46%) [∇]	10 (19%)	5 (42%)	2 (29%)	1 (17%)	0.049
IL-6, pg/ml	22 (7-51)	34 (0-88)	16 (0-45)	0 (0-19)	2 (0-46)	0.127
IL- 6 < LOD*	11 (19%)	14 (26%)	4 (33%)	4 (57%)	2 (33%)	0.062
IL-8, pg/ml	455 (307-769)	462 (230-937)	267 (160-594)	230 (150-365)	755 (310-1200)	0.052

[∇] = significant difference between this group and COPD ex-smokers (p<0.005)
LOD = limit of detection of assay

Figure 3.6. Box and whisker plot showing the percentage of induced sputum neutrophils in COPD patients and healthy controls according to smoking status ($p<0.001$). Sputum % neutrophils were increased in COPD ex-smokers compared to COPD current smokers ($p=0.006$), COPD smokers compared to healthy smokers ($p=0.02$), COPD ex-smokers compared to healthy smokers ($p<0.001$) and COPD ex-smokers compared to healthy non-smokers ($p=0.02$). There was no significant difference between COPD smokers and healthy non-smokers ($p=0.3$). The only significant difference was in COPD ex-smokers compared to healthy smokers after correcting for multiple comparisons ($p<0.005$).



Multiple regression models

In the univariate analysis age, gender and current smoking status, blood neutrophils, CRP, sputum % neutrophils, sputum IL-6 and IL-8 were significantly different between COPD patients and healthy controls. These factors were entered into a binary logistic regression model. Age, gender and current smoking status were investigated with blood and sputum inflammatory markers separately (Tables 3.7 – 3.10) and then together (Tables 3.11-3.12). Blood neutrophils and CRP were significantly and independently increased in COPD patients compared to healthy controls after adjusting for age, gender and current smoking status (Table 3.7). COPD patients had significantly higher sputum neutrophils after adjusting for age and smoking status ($p=0.034$) but not after adjusting for gender ($p=0.065$) (Table 3.8). IL-6 ($p=0.005$) and IL-8 ($p=0.002$) were significantly higher in COPD patients compared to healthy controls after adjusting for age, gender and current smoking status (Tables 3.9 and 3.10). When included in the model together, as expected, they were not independent of each other. When analyzed together with markers of systemic inflammation, sputum IL-6 and IL-8 were not independent of blood neutrophils. But sputum IL-6 ($p=0.008$) and sputum IL-8 ($p=0.003$) and CRP were significantly and independently higher in COPD patients compared to healthy controls (Table 3.11 and 3.12).

When cumulative smoking history (smoking pack years) was included in the model instead of current smoking status, blood neutrophils ($p=0.009$), CRP

($p=0.03$), sputum IL-6 ($p=0.043$) and sputum IL-8 ($p=0.006$) were significantly higher in COPD patients. Sputum % neutrophils ($p=0.3$) were not significantly higher in COPD patients after adjusting for smoking pack years. CRP and neutrophils were not independent of each other when included in the model together (CRP $p=0.01$, blood neutrophils $p=0.12$). Sputum IL-6 and IL-8 were not independent of each other (IL-6 $p=0.043$, IL-8 $p=0.023$). CRP and sputum IL-8 were independent of each other after adjusting for smoking pack years (Table 3.13) but sputum IL-6 was not independent of CRP ($p=0.053$).

Table 3.7. Binary logistic regression of blood neutrophils and CRP in COPD patients compared to healthy controls, adjusting for age, gender and current smoking status.

	B	SE	Wald	df	p	Exp (B)
Intercept	-10.1	1.549	42.7	1	<0.001	<0.001
Age	0.129	0.022	33.7	1	<0.001	1.9
Current smoker	1.730	0.414	17.5	1	<0.001	5.6
Blood neutrophils	0.328	0.153	4.6	1	0.032	1.4
Log CRP	0.353	0.175	4.1	1	0.044	1.4
Male sex	-0.618	0.368	2.8	1	0.093	0.5

-2 Log likelihood 205.7 (Cox % Snell R Square 0.354, Nagelkerke R square 0.494), $p<0.001$

Table 3.8. Binary logistic regression of sputum % neutrophils in COPD patients compared to healthy controls, adjusting for age, gender and current smoking status.

	B	SE	Wald	df	p	Exp (B)
Intercept	10.3	3.081	11.1	1	0.001	<0.001
Age	0.174	0.045	14.7	1	<0.001	2.1
Current smoker	2.627	0.846	9.6	1	0.002	13.8
Sputum % neutrophils	-1.4	0.779	3.4	1	0.065	0.2
Male sex	0.731	0.680	1.2	1	0.283	2.1

-2 Log likelihood 64.3 (Cox % Snell R Square 0.322, Nagelkerke R square 0.511), $p<0.001$

Table 3.9. Binary logistic regression of sputum IL-6 in COPD patients compared to healthy controls, adjusting for age, gender and current smoking status.

	B	SE	Wald	df	p	Exp (B)
Intercept	-8.07	2.112	14.6	1	<0.001	<0.001
Age	0.142	0.032	19.8	1	<0.001	1.2
Sputum IL-6	1.636	0.585	7.8	1	0.005	0.2
Current smoker	1.649	0.650	6.4	1	0.011	5.2
Male sex	1.319	0.597	4.9	1	0.027	3.7

-2 Log likelihood 94.2 (Cox % Snell R Square 0.266, Nagelkerke R square 0.426), $p<0.001$

Table 3.10. Binary logistic regression of sputum IL-8 in COPD patients compared to healthy controls, adjusting for age, gender and current smoking status.

	B	SE	Wald	df	p	Exp (B)
Intercept	-15.3	3.6	17.8	1	<0.001	<0.001
Age	0.129	0.032	16.4	1	<0.001	1.1
Log sputum IL-8	1.205	0.386	9.7	1	0.002	3.3
Current smoker	1.542	0.667	5.3	1	0.021	4.7
Male sex	1.434	0.629	5.2	1	0.023	4.2

-2 Log likelihood 87.5 (Cox % Snell R Square 0.266, Nagelkerke R square 0.430), $p<0.001$

Table 3.11. Binary logistic regression of CRP and sputum IL-6 in COPD patients compared to healthy controls, adjusting for age, gender and current smoking status.

	B	SE	Wald	df	p	Exp (B)
Intercept	-11.2	2.846	15.6	1	<0.001	<0.001
Age	0.124	0.034	13.1	1	<0.001	1.1
Male sex	1.900	0.698	7.4	1	0.006	6.7
Sputum IL-6	1.772	0.663	7.1	1	0.008	5.8
Current smoker	1.617	0.702	5.3	1	0.021	5.0
Log CRP	0.724	0.325	4.9	1	0.026	2.1

-2 Log likelihood 80.4 (Cox % Snell R Square 0.304, Nagelkerke R square 0.492), $p<0.001$

Table 3.12. Binary logistic regression of CRP and sputum IL-8 in COPD patients compared to healthy controls, adjusting for age, gender and current smoking status.

	B	SE	Wald	df	p	Exp (B)
Intercept	-15.8	4.016	15.4	1	<0.001	<0.001
Age	0.116	0.034	11.5	1	0.001	1.1
Sputum IL-8	1.298	0.433	9.0	1	0.003	3.7
Male sex	1.802	0.699	6.6	1	0.010	6.1
Current smoker	1.539	0.722	4.5	1	0.033	4.7
Log CRP	0.661	0.329	4.0	1	0.045	1.9

-2 Log likelihood 74.3 (Cox % Snell R Square 0.303, Nagelkerke R square 0.497), $p<0.001$

Table 3.13. Binary logistic regression of CRP and sputum IL-8 in COPD patients compared to healthy controls, adjusting for age, gender and smoking pack years.

	B	SE	Wald	df	p	Exp (B)
Intercept	-13.8	4.0	12.2	1	<0.001	0.001
Smoking pack years	0.086	0.026	11.1	1	0.001	1.1
Log sputum IL-8	1.2	0.471	6.7	1	0.010	3.4
Log CRP	0.751	0.374	4.0	1	0.044	2.1
Age	0.063	0.035	3.2	1	0.073	1.1
Male sex	1.4	0.79	3.2	1	0.073	4.1

-2 Log likelihood 62.1 (Cox % Snell R Square 0.366, Nagelkerke R square 0.601), $p < 0.001$

Discussion

The aim of this chapter was to establish whether biomarkers of pulmonary and systemic inflammation differentiated COPD from healthy controls. Each of the biomarkers analyzed was increased in COPD compared to controls with the exception of induced sputum IL-1 β . This finding remained consistent after adjusting for age, gender and smoking history (both current smoking status and smoking pack years), except for sputum neutrophils, which were increased in COPD patients after adjusting for current smoking status but not after adjusting for smoking pack years. After adjusting for potential confounding factors including smoking status and smoking pack years, CRP and sputum IL-8 were independently increased in COPD patients. The yield of induced sputum was low, particularly in healthy subjects.

Pulmonary and systemic inflammation in COPD

Markers of systemic inflammation were significantly higher in COPD subjects than in healthy controls in our study. This is in keeping with previously published work. (82, 104) Induced sputum markers of inflammation (percentage neutrophils, IL-6 and IL-8) were also significantly increased in COPD subjects compared to healthy controls. This confirms previous published work in the case of IL-8 in studies investigating between 13 and 33 COPD patients. (75, 281, 282) But while IL-6 in induced sputum has been associated with COPD disease severity, (283) there were no comparable studies showing results of induced sputum IL-6 between COPD patients and healthy controls. This may be partly due to our finding that in a large number of healthy subjects,

IL-6 in induced sputum was below the limit of detection of the assay. The level of induced sputum IL-6 in our cohort was lower than that found in COPD patients in the study by Hacievliyagil and co-workers. (283) But this may be due to the use of a chemiluminescence method instead of an ELISA. They also had a smaller number of COPD patients (n=24). Bhowmik and co-workers also found higher levels of IL-6 (median 64 pg/ml) and IL-8 (median 3953 pg/ml) than seen in our COPD patients, and used the same R&D ELISA assay used in our cohort. (71) However, they sometimes took several attempts to obtain an induced sputum sample, and some patients were sampled only 3 weeks post-exacerbation. In a meta-analysis of potential biomarkers in COPD, Franciosi and co-workers reported that there was insufficient data on IL-6 in BAL and induced sputum to draw conclusions. (46) And Comandini and co-workers do not list IL-6 in an otherwise comprehensive review of induced sputum biomarkers in COPD. (284) No studies investigating IL-1 β in induced sputum from healthy smokers has yet been published. (284) Our results therefore add significantly to the literature by evaluating IL-1 β , IL-6 and IL-8 in the same cohort of COPD patients compared to healthy controls.

IL-1 β was the only marker measured in our cohort that was not increased in COPD patients compared to controls. Reported levels of IL-1 β in COPD have been contradictory. Studies in BAL suggest that IL-1 β may be up regulated in smokers and COPD subjects. (285, 286) Whereas one EBC study and two ex-vivo studies found that cigarette smoking either reduced IL-1 β or did not affect the levels. (287-289) It may be that the importance of IL-1 β in the pathogenesis of

COPD is in its role as a mediator of airway remodeling and fibrosis, rather than as an inflammatory mediator, (290, 291) as IL-1 β had a role in the induction of emphysema and small airway remodeling in mice, with an effect that is similar to TNF α . (292) Or perhaps the increased pro-inflammatory action of IL-1 β in COPD is best determined not by measuring overall levels of IL-1 β , but by measuring the difference between IL-1 and its antagonists (IL-1sRII and IL-1RA), which are found in reduced numbers in COPD. (293) One study found reduced levels of both IL-1 antagonists in plasma from COPD patients compared to controls. (293) However, it is important to remember that our assessment of IL-1 β in induced sputum uses a technique to sample airway secretions and not the distal airspaces, which may be the main site of inflammation in COPD. (294) Our results suggest that further measurement of IL-1 β in induced sputum is not likely to be helpful in differentiating COPD patients from healthy subjects.

Effects of smoking on pulmonary and systemic inflammation

Acute smoking, current smoking and cumulative smoking history may all impact on inflammation. We controlled for the effect of acute smoking by asking patients to abstain from smoking for 12 hours prior to testing, and confirmed abstinence with an exhaled carbon monoxide measurement. The effects of current smoking and cumulative smoking history in pack years were controlled for in the logistic regression analysis.

Blood leukocytes and CRP were both increased in COPD independently of smoking status. This has been previously reported, (82, 85, 276) but

importantly, we found these systemic inflammatory markers were increased in COPD independently of each other. No other studies were found investigating both of these systemic inflammatory markers in the same cohort of patients. Different profiles of these markers have been reported in relation to smoking habit. A UK population study (n=6902) found that current smoking had a more pronounced effect on circulating white blood cells than the cumulative effect of smoking in pack years. (295) In addition to increased numbers of circulating neutrophils, current cigarette smoking was associated with an increase in neutrophil myeloperoxidase activity (296) and increased activation of epithelial cells. (297) Peripheral blood white count was associated with annual decline in FEV₁ in current smokers in a longitudinal study, but not in ex- or never-smokers. (96) In contrast, CRP may be elevated in ex-smokers. In the Framingham cohort, evaluation of a panel of inflammatory markers showed that CRP was significantly higher in ex-smokers compared to never-smokers. (298) While most smoking related inflammatory changes reversed with smoking cessation, (299) CRP levels remained elevated in "healthy" ex-smokers for 10-20 years after quitting. (300) There was a trend towards an increased CRP in both ex-smoking groups in our cohort, which may in part be explained by the higher body mass index in these groups. But mechanisms for the more chronic elevation of CRP seen in ex-smokers are not fully understood. (299)

When assessed according to smoking status and smoking pack years, sputum IL-6 and IL-8 were significantly higher in COPD patients compared to healthy subjects, but sputum % neutrophils were not. However, the results should be

interpreted with caution due to small sample numbers from healthy control subjects. In addition, levels of sputum IL-6 and IL-1 β were below the limit of detection of the assays in a large proportion of subjects. Airway inflammation has previously been associated with cumulative smoking history, but while the induced sputum markers IL-6, IL-8 and TNF α have been associated with smoking pack years in COPD exacerbations, (283) there is little in the literature regarding induced sputum markers and cumulative smoking history in stable COPD subjects. This may be partly due to our finding that induced sputum markers such as IL-6 and IL-1 β are difficult to detect in stable subjects. IL-8 has been associated with smoking pack years in asthma. (301) Interestingly, Ito and co-workers found that genetic polymorphisms associated with a high production of IL-8 were more likely to be found in never- and ex-smokers. They therefore proposed that the inflammatory phenotype associated with increased IL-8 levels might act as a deterrent to cigarette smoking. (302) Previous studies have investigated markers of airway inflammation in other body fluid compartments in response to cumulative smoking history and current smoking status. Studies in BAL found that neutrophils were highly correlated both with smoking pack years ($r^2=0.65$) and with the number of cigarettes smoked per day ($r^2=0.41$), (303) which is consistent with our findings in induced sputum. A dose-response relationship was found between smoking pack years and neutrophils, macrophages, IL-1 β and IL-8 in BAL. (286) One study of 9 healthy young smokers reported lower IL-6 levels in BAL and EBC in these subjects compared to never-smokers. Two of these smokers with the highest BAL cell counts also had higher IL-8 levels than non-smoking controls. (304) While

assessment of induced sputum inflammatory profiles are known not to be representative of the more distal airspaces, it is interesting to speculate that IL-8 may be an important factor in smokers who subsequently develop COPD. We found that while smoking history significantly influenced sputum % neutrophils, sputum IL-6 and IL-8 remained significantly elevated in COPD patients after adjusting for both current smoking status and cumulative smoking history in pack years. These markers may therefore be useful in further characterizing COPD patients. In addition, we found that blood CRP and sputum IL-8 were independently increased in COPD patients after adjusting for smoking history, suggesting that pulmonary and systemic inflammation are independent of each other in COPD.

There was a trend towards higher levels of sputum % neutrophils in ex-smoking COPD subjects and healthy ex-smokers compared to COPD current smokers and healthy current smokers. Increased airway inflammation has been reported after smoking cessation in COPD patients, but not in healthy smokers. (275, 305) This suggests that in a proportion of subjects, cigarette smoking induces an abnormal inflammatory response, which persists even after smoking cessation. It was proposed that this was partly due to ongoing repair of smoking related tissue damage. (275) But it raises the possibility of an autoimmune (306) or infective (307) mechanism resulting in persistent inflammation in susceptible smokers. Smoking cessation has nevertheless been associated with a reduced rate of decline in FEV₁ in early COPD. (308) From our results it is not possible to make conclusions about the effect of smoking status on markers of

sputum inflammation due to small numbers in the healthy control groups. But it is interesting that there was a trend towards higher sputum % neutrophils in ex-smoking groups suggestive of ongoing airway inflammation in these patients, even in the “healthy” ex-smoking group.

Additional factors influencing inflammation

Several factors may have influenced levels of inflammatory markers in addition to COPD and smoking history. We found no difference in systemic inflammation in men compared to women in the COPD group or the healthy controls, but sputum neutrophils were higher in men in the cohort as a whole, which could be explained by cumulative smoking history. However, each of the inflammatory markers measured (except sputum % neutrophils and sputum IL-1 β) remained significantly elevated in COPD patients after controlling for gender and smoking status. Results from the MONICA survey found significant gender differences in systemic inflammation in response to smoking. (309) In this study peripheral WCC was significantly associated with current smoking, number of cigarettes smoked per day, smoking pack years and the duration of smoking in years in men and women. CRP was also associated with these parameters in men. While CRP was associated with smoking pack years in women, there was no relationship between CRP and smoking status, number of cigarettes smoked per day or the duration of smoking (years) in women. This group reported a reduction in WCC and CRP after smoking cessation in men but not in women. (309) This gender difference in levels of CRP and white blood cells adds to our finding that levels of these markers were also differently affected by smoking

history. This suggests that CRP and blood neutrophils may reflect different systemic inflammatory pathways.

Inflammatory markers other than those measured may play an important role in the inflammatory response in COPD. Circulating polymorphonuclear neutrophils (PMNs) are increased in the circulation of smokers as a result of chronic stimulation of the bone marrow. (310) Circulating IL-6 stimulates bone marrow to release leukocytes (311) and is associated with leukocyte sequestration in the lung in animal models. (312, 313) In the MONICA III North Glasgow study, IL-6 was increased in the circulation of current smokers, whereas ex-smokers had similar levels to never smokers. This group also found that IL-6 correlated significantly with blood leukocytes and fibrinogen. (314) Pathways involving IL-6 may therefore be associated with neutrophilia and are affected by smoking status. While we cannot substantiate these results, as IL-6 was not measured in blood in our subjects, it is interesting that induced sputum levels of IL-6 and circulating CRP were independently increased in COPD after adjusting for current smoking status.

Oxidative stress is another factor potentially linking smoking and inflammation. One puff of cigarette smoke contains 10^{17} oxidant molecules. (315) However, data linking oxidative stress to systemic inflammation show mixed results. Systemic oxidative stress was increased in current smokers who had an increased plasma oxidative burden and reduced levels of plasma antioxidants. Thiobarbituric acid reactive substances (TBARS) were increased in smokers

compared to non-smokers (316) and were associated with increased airflow limitation. (317) However, although the Trolox-equivalent anti-oxidant capacity (TEAC) of plasma was significantly lower in smokers than non-smokers, (318) no relationship was found between TEAC and airflow limitation. (319) Therefore, while current smoking is associated with an increased systemic oxidative burden, the relationship between systemic oxidative stress and airflow limitation remains unclear.

Bacterial load can influence airway inflammation in subjects with COPD. *Haemophilus influenza* colonisation was associated with increased sputum neutrophils, IL-1 β and IL-12, but not with IL-6 or IL-8 in patients with moderate COPD. (320) A study of 30 COPD patients found that bacterial load and induced sputum IL-8 were associated with the rate of decline in FEV₁ over a 12 month period. (321) Bacterial load was not investigated in our cohort.

Inhaled corticosteroids may have influenced levels of sputum inflammation in our COPD subjects, but several studies report no effect on sputum IL-8 in response to inhaled corticosteroid use. (322-324) This is investigated further in our COPD cohort in Chapter 4, but the use of inhaled corticosteroids was not associated with the ability to produce sputum in our patients.

Induced sputum yield

Production of induced sputum in this cohort was low compared to other studies (325-327) particularly in the control groups. Current smoking was associated

with a higher yield of induced sputum samples as was a history of symptoms of chronic bronchitis. The low yield of analyzed samples of induced sputum may in part be due to a low threshold for discarding unsuitable samples. However, other important factors may also be important. The low yield in COPD subjects is partly because sputum induction was not attempted in those with a very low post-bronchodilator FEV₁ (≤ 0.5 litre) (n=8). Most studies publishing data from induced sputum samples are in small numbers of subjects. Many studies simply do not comment on the yield of acceptable induced sputum samples (e.g. O'Donnell and co-workers) (328) and therefore may include only those subjects producing sputum. Some groups have overcome this problem by pre-selecting patients for study based on their known ability to produce adequate samples of induced sputum. (329) A study of similar design to ours had an even lower sputum yield, reporting induced sputum IL-8 results for 43 of 148 stable COPD subjects. In this study, spontaneous and induced sputum were both used for analysis. (72)

A wide range of levels of inflammatory cytokines was seen in our "healthy" controls. This may indicate that production of adequate induced sputum is not "normal," but may be associated with underlying asymptomatic airway inflammation. Snell and Newbold (330) question the ability of 'healthy' and therefore usually non-spontaneous sputum producers to produce a sample of induced sputum. However, they quote an 80-90% sample yield from sputum induction in their 'healthy subjects' overall. The two largest studies of induced sputum in healthy controls, both of which were designed to establish the normal

variation in differential cell counts in healthy subjects, reported a yield of 81% (n=118) and 84% (n=114) respectively. (326, 331) The first of these studies used the same hypertonic saline nebulization protocol used in our cohort – 3%, 4% and 5% saline nebulized for 5 minutes each. (326) In this study, subjects had a median induced sputum % neutrophils of 37%. This is much lower than in our healthy subjects (median 77%) but the age of the healthy subjects in this study was not mentioned and it was likely that subjects were much younger than in our study. Spanevello and co-workers also reported a low median percentage of induced sputum neutrophils (29%). They used a sputum induction protocol comprising 4.5% nebulized saline for increasing time periods – for 1, 2 4, 8 and 16 minutes. Again, the age of the cohort is not reported, but that it had a “normal age distribution,” and subjects are likely therefore to be substantially younger than in our study. (331)

Numbers of induced sputum neutrophils increase with age. (332) While our COPD patients were not age matched with healthy controls, this was taken into account as a potential confounding factor in the logistic regression analysis. The percentage of sputum neutrophils in our healthy subjects was consistent with the 11 patients over 60 in the study by Thomas and co-workers, in which the neutrophil differential was 68.5%, compared to 26.9% in those under 30. This study also found that sputum neutrophil differential count does not continue to increase after 50 years of age. (332) Another potential confounding factor is inhaled corticosteroid use. The effect of inhaled corticosteroid use in our cohort was further analyzed in Chapter 4, where we found that COPD patients taking

inhaled corticosteroids did not have increased markers of blood or sputum inflammation. Induced sputum neutrophil differential count in a previous study was not affected by inhaled or oral corticosteroid use. (333)

Limitations

We had a low yield of induced sputum in this cohort. This has potential to introduce bias. In addition, the stability and reproducibility of induced sputum cytokine data is not clearly established. This would be particularly pertinent in longitudinal analysis. A significant number of induced sputum cytokines were below the limit of detection of the assay for IL-1 β and IL-6. For this reason, work to define a valid cut-off point for positive values for these cytokines in COPD might be useful. However, as a proportion of values were below the limit of detection it may simply mean that these markers are not useful in differentiating between COPD patients and healthy controls. Caution should be exercised with cut-off values used for sputum % neutrophils and IL-6 used in the regression analysis as the 50th percentiles are different for COPD patients and controls. An exclusion bias was implemented for COPD subjects (avoidance of sputum induction in those with a low FEV₁) for safety reasons. (334-336) Had these patients produced sputum, it is likely that it would have served to strengthen our finding that inflammation is increased in COPD, as published work suggests that many inflammatory markers increase with the severity of airflow limitation. (45, 46, 325) In addition, the detection of soluble mediators may have been affected by the use of the mucolytic agent dithiothreitol (DTT). While this agent improves cell yield by improving the homogenisation of

induced sputum, the yield of soluble factors like cytokines may be reduced.

(337)

It would have been interesting to assess a larger selection of markers of systemic inflammation. Blood levels of IL-1 β , IL-6 and IL-10 were analyzed in a subset of 39 COPD patients and controls from this cohort. Of these only IL-6 was significantly higher in COPD subjects ($p=0.049$). In the full cohort analysis, blood leukocytes and highly sensitive C-reactive protein were chosen to measure systemic inflammation due to their important association with prognosis in COPD. (16, 95)

Conclusion

Systemic and pulmonary inflammation was increased in COPD patients compared to healthy controls after adjusting for age, gender, current smoking status and smoking pack years. Induced sputum IL-1 β was not increased in COPD compared to controls. Induced sputum was not reliably attainable in healthy control subjects. After adjusting for current smoking status and smoking pack years, CRP and sputum IL-8 were significantly and independently increased in COPD patients and may therefore be useful in the characterization of subjects with COPD.

CHAPTER 4: Pulmonary and systemic inflammation and airflow limitation in COPD

Introduction

COPD is characterized by excessive inflammation in the lungs, (1, 2) but it is accepted that in addition to abnormal inflammation in the lung parenchyma, COPD is associated with a systemic inflammatory response. (19, 338) We have shown an increase in both lung and systemic inflammation in COPD patients compared to healthy subjects in Chapter 3. Several studies confirm that markers of systemic inflammation such as numbers of blood neutrophils, blood white cell count and CRP are increased in stable COPD compared to healthy subjects, (46, 85, 339) but the cause of systemic inflammation in COPD is not known. One hypothesis is that lung inflammation causes systemic inflammation by a “spill-over” effect. (338) Relatively few studies have investigated the relationship between inflammatory markers in the systemic and pulmonary compartments in the same cohort of COPD patients, and these studies are in small patient numbers. (115, 116, 340, 341) None of them found a direct association between markers of pulmonary and systemic inflammation. It was therefore suggested that pulmonary and systemic inflammation might be regulated independently. (116) Indirect mechanisms linking pulmonary and systemic inflammation in COPD have therefore been considered, including alterations in mechanisms controlling degradation and repair and the hypothesis that COPD is an autoimmune disease. (155, 342)

Markers of systemic inflammation have been associated with disease severity and prognosis in COPD independent of airflow limitation and smoking history. (95) CRP and blood neutrophils are two of the most frequently assessed markers of systemic inflammation in COPD populations. They have both been consistently associated with all cause mortality and airflow limitation in population studies. (5, 83-85) Both lung and blood neutrophil numbers have been linked to airflow limitation and the rate of decline in FEV₁. (44, 45, 96, 97) Assays measuring low levels of (highly sensitive) CRP enable the investigation of "resting" levels in chronic disease (343) and it is now established as a marker of chronic inflammation with prognostic implications, not only in COPD. (16, 107)

Pulmonary markers of inflammation have been associated with airflow limitation in COPD. Not only are neutrophils the predominant cells in the airways in the early stages of COPD, they are also increasingly prevalent with increasing severity of the disease. (46) In a meta-analysis of cross-sectional studies in COPD, sputum neutrophils and IL-8 were the only sputum biomarkers showing a trend to separation between stages of disease in COPD, with inadequate data for sputum IL-6. (46)

This chapter investigates the relationship between markers of inflammation in induced sputum and blood in a cohort of stable COPD patients. The hypotheses are that pulmonary and systemic inflammatory markers will correlate significantly with each other and with airflow limitation in stable COPD patients.

Aims

In a cohort of stable COPD subjects:

1. To investigate the relationship between markers of inflammation in the pulmonary and systemic compartments.
2. To investigate the relationship between pulmonary and systemic inflammation and airflow limitation.

Methods and data analysis

One hundred and eighty two COPD patients were recruited as described in Chapter 2. Demographic data were compared for those with and without an induced sputum sample. Continuous variables were compared using the unpaired t-test (parametric) or Mann-Whitney U test (non-parametric). Categorical variables were compared with the Chi-square test. Blood and sputum inflammatory markers were correlated with each other and with post-bronchodilator spirometry using Pearson's correlation (parametric) and Spearman's correlation (non-parametric) co-efficients. The severity of airflow limitation by GOLD stage was analyzed with the blood and sputum inflammatory markers using ANOVA (parametric variables) or the Kruskal-Wallis (non-parametric variables) test. Dunnett's T3 post-hoc test was used for comparison between groups. Variables significantly associated with post-bronchodilator FEV₁ (% predicted) in univariate analysis ($p < 0.1$) were entered into a multiple linear regression model. Sputum neutrophils were cubed for analysis in the linear regression model in order to obtain a normal distribution but were also analyzed in 2 categories: highest ($> 89.6\%$) and lowest ($\leq 89.6\%$) 50th percentiles. CRP and sputum IL-8 were log-transformed to achieve normal distribution.

Results

In the cohort of 182 patients, mean post-bronchodilator FEV₁ was $50 \pm 19\%$ predicted, post-bronchodilator FVC $79 \pm 21\%$ predicted, and FEV₁/FVC ratio $48 \pm 13\%$ predicted. GOLD stage ranged from I to IV (GOLD I n=13 (7%), GOLD II n=70 (39%), GOLD III n=73 (40%), GOLD IV n=26 (14%). Baseline data for these patients is displayed in Table 4.1.

One hundred and nineteen subjects (65%) produced a sample of induced sputum. Sputum induction was not attempted in 8 COPD subjects (4%) whose baseline FEV₁ was ≤ 0.5 litres. Subjects producing induced sputum had the same degree of airflow limitation as those without induced sputum. Subjects able to produce a sample of induced sputum reported a higher number of exacerbations, poorer quality of life (SGRQ total score) and a higher percentage had chronic bronchitic symptoms. There were significantly more current smokers in this group although they had a lower cumulative smoking history in pack years than subjects not producing an induced sputum sample. Systemic markers of inflammation were the same for both groups (Table 4.2).

Table 4.1. Clinical data for the COPD cohort is displayed. Data are presented as number (%) for categorical variables, mean \pm SD for parametric data, and *median (IQR) for non-parametric data.

COPD subjects	n=182
Age	68 \pm 8
Gender (% male)	112 (62%)
Smoking pack years	48 \pm 24
Taking inhaled corticosteroids (%)	121 (66%)
Taking statins (%)	23 (13%)
FEV₁, post bronchodilator % predicted	50 \pm 19
FVC, post bronchodilator % predicted	79 \pm 21
FEV₁/FVC ratio, post bronchodilator	0.48 \pm 0.1
St Georges Respiratory Questionnaire (SGRQ) total score	51 \pm 20
Body mass index, kg/m²	26 \pm 6
Oxygen saturation, %	95 \pm 2
Annual exacerbation frequency	2 (1-3)*
MRC chronic bronchitis symptoms	83 (46%)
MRC dyspnoea score	3 (2-4)*
Blood white cell count, x10⁹/L	7.6 \pm 2
Blood neutrophil count, x10⁹/L	4.9 \pm 2
Blood hsCRP, mg/L	3.8 (2-7)*

Table 4.2. Clinical data are presented for subjects with and without induced sputum samples. Results are presented as number (%) for categorical variables, mean \pm SD for parametric data and *median (IQR) for non-parametric data. Groups are compared with the un-paired t-test, Mann-Whitney U test* or Chi-square test.[†]

	Induced sputum produced (n=119)	Induced sputum not produced (n=63)
Age	67 \pm 9	70 \pm 7*
Gender (% male) [†]	77 (65%)	35 (56%)
Current smokers [†]	58 (49%)	18 (29%)*
Smoking pack years	43 \pm 19	51 \pm 26*
Taking inhaled corticosteroids (%) [†]	75 (63%)	46 (73%)
Taking statins (%) [†]	14 (12%)	9 (14%)
Post bronchodilator FEV ₁ , % predicted	50 \pm 20	50 \pm 18
Post bronchodilator FVC, % predicted	78 \pm 21	79 \pm 20
Post bronchodilator FEV ₁ /FVC ratio	0.49 \pm 0.1	0.48 \pm 0.1
St Georges Respiratory Questionnaire (SGRQ) total score	53 \pm 19	44 \pm 20*
Body mass index, kg/m ²	26 \pm 5	25 \pm 6
Oxygen saturation, %	96 \pm 2	95 \pm 2
Annual exacerbation frequency*	2 (1-3)	1 (0-2)*
MRC chronic bronchitis symptoms [†]	61 (55%)	22 (39%)*
MRC dyspnoea score*	3 (2-4)	3 (2-4)
Blood white cell count, x10 ⁹ /L	7.8 \pm 2	7.3 \pm 2
Blood neutrophil count, x10 ⁹ /L	5.0 \pm 2	4.6 \pm 2
Blood hsCRP, mg/L*	3.9 (2-8)	3.3 (1-7)

* = p<0.05

Systemic inflammatory markers were analyzed with airflow limitation, measured by post-bronchodilator FEV₁, FVC and FEV₁/FVC ratio (Table 4.3). Blood total white cell count and neutrophil count correlated significantly with FEV₁ and FVC but not FEV₁/FVC ratio. There was no correlation between CRP and spirometric measures. The relationships between markers of lung inflammation and post-bronchodilator spirometry are shown in Table 4.4. Percentage neutrophils in sputum correlated inversely with FEV₁ and FEV₁/FVC ratio. There was also a negative association between sputum % neutrophils and FVC ($r=-0.198$, $p=0.051$). IL-1 β , IL-6 and IL-8 in induced sputum did not show any significant correlations with spirometric values. Total blood white cell count ($p=0.044$), neutrophil counts ($p=0.012$) and induced sputum % neutrophils ($p=0.002$) increased with severity of COPD as assessed by the GOLD stage (Figures 4.1 and 4.2).

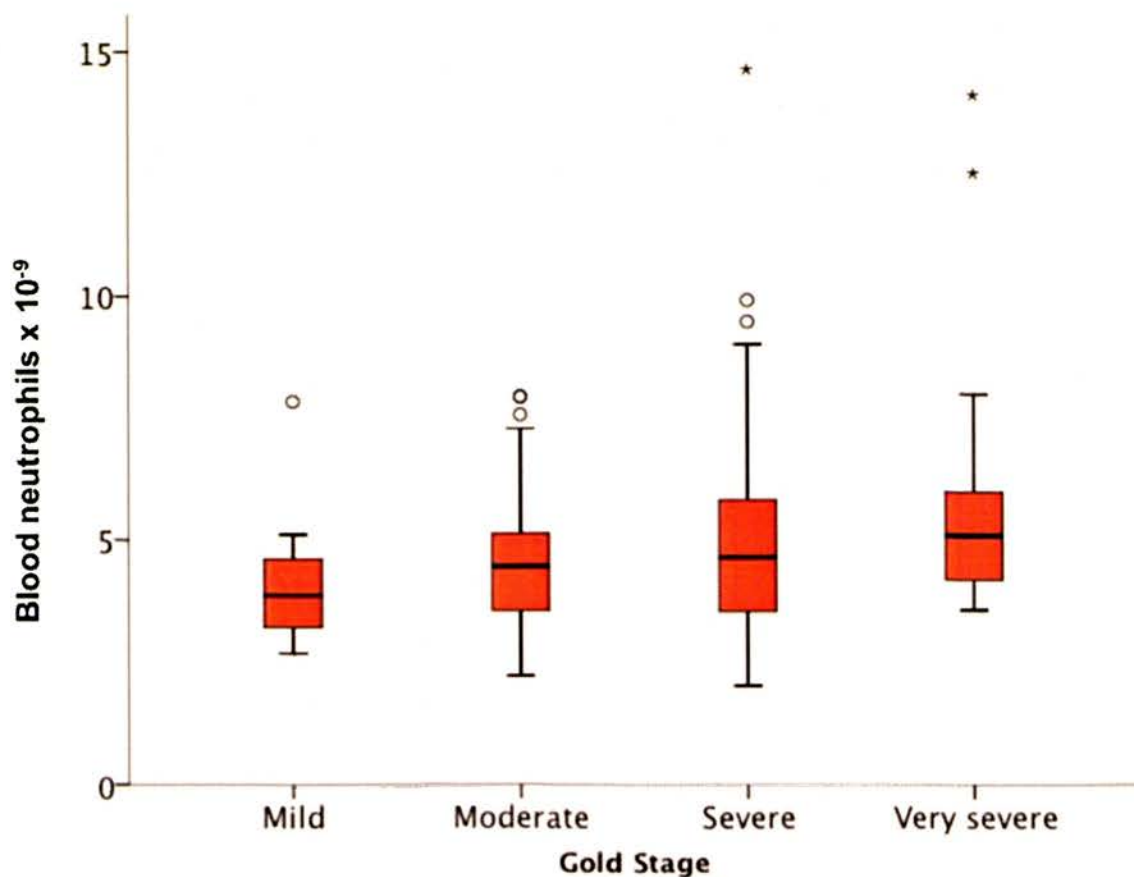
Table 4.3. Correlations between markers of systemic inflammation and post-bronchodilator spirometry are displayed. Data are presented as Pearson's correlation co-efficient (parametric data) or *Spearman's correlation co-efficient (non-parametric data) with statistical significance.

		Correlation	p
Post bronchodilator FEV₁, % predicted	White cell count, x10 ⁹ /L	-0.245	0.001
	Neutrophils, x10 ⁹ /L	-0.267	<0.001
	C-reactive protein, mg/L	-0.117*	0.119
Post bronchodilator FVC, % predicted	White cell count, x10 ⁹ /L	-0.248	0.001
	Neutrophils, x10 ⁹ /L	-0.260	0.001
	C-reactive protein, mg/L	-0.110*	0.144
Post bronchodilator FEV₁/FVC ratio	White cell count, x10 ⁹ /L	-0.067	0.382
	Neutrophils, x10 ⁹ /L	-0.082	0.283
	C-reactive protein, mg/L	-0.011*	0.884

Table 4.4. Correlations between markers of inflammation in induced sputum and post-bronchodilator spirometry are displayed. Data are presented as Spearman's correlation co-efficient with statistical significance.

		Correlation	p
Post bronchodilator FEV₁, % predicted	Neutrophils, %	-0.312	0.002
	IL-1 β , pg/ml	-0.163	0.083
	IL-6, pg/ml	-0.019	0.840
	IL-8, pg/ml	-0.148	0.119
Post bronchodilator FVC, % predicted	Neutrophils, %	-0.198	0.051
	IL-1 β , pg/ml	-0.067	0.483
	IL-6, pg/ml	-0.123	0.193
	IL-8, pg/ml	0.031	0.745
FEV₁/FVC ratio, post bronchodilator	Neutrophils, %	-0.255	0.011
	IL-1 β , pg/ml	-0.096	0.309
	IL-6, pg/ml	-0.038	0.685
	IL-8, pg/ml	-0.100	0.294

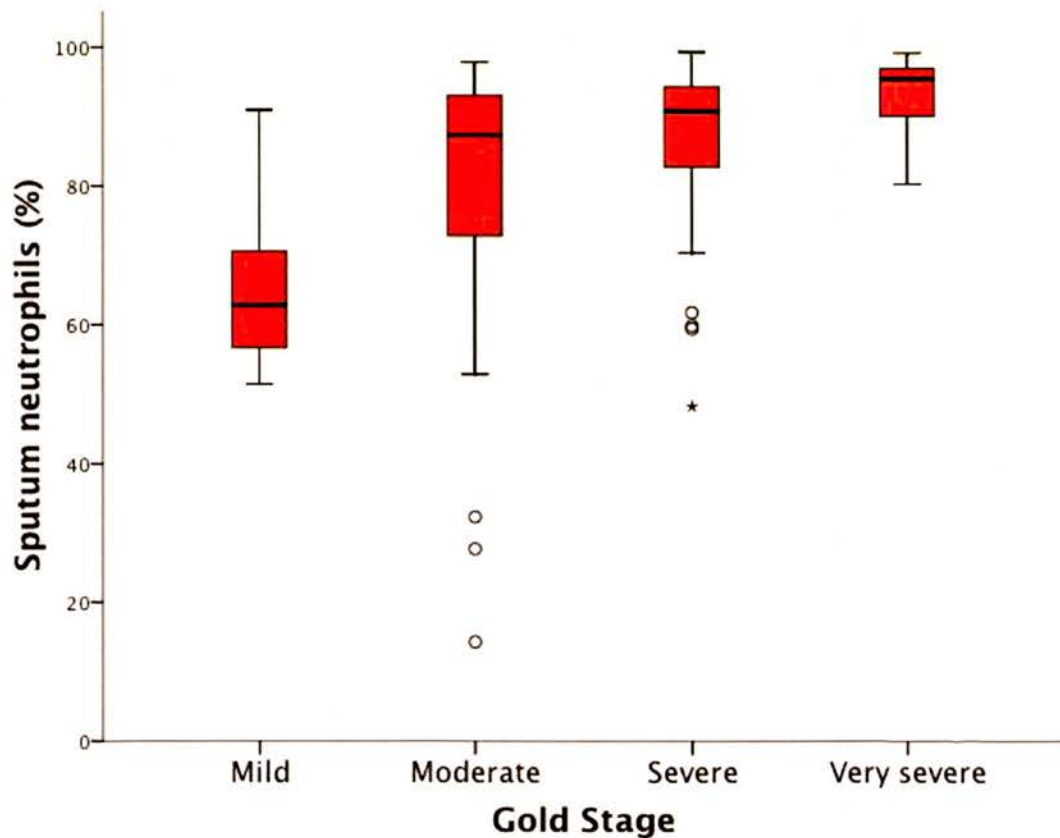
Figure 4.1. Box and whisker plot showing the relationship between blood neutrophils and the severity of airflow limitation by GOLD stage in the COPD cohort ($p=0.012$, ANOVA). There were no significant differences between groups on post-hoc analysis.



GOLD stages: GOLD stage I (Mild): FEV₁/FVC ratio < 70% predicted, FEV₁% predicted > 80% (n=13); GOLD stage II (Moderate): FEV₁/FVC ratio < 70% predicted, FEV₁% predicted 50–80% (n=68); GOLD stage III (Severe): FEV₁/FVC ratio < 70% predicted, FEV₁% predicted 30–50% (n=72); GOLD stage IV (Very Severe): FEV₁/FVC ratio < 70% predicted, FEV₁% predicted < 30% (n=25)

Box plot values: The top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and central line represents the 50th percentile. The whiskers represent the highest and lowest values that are not outliers or extreme values. Outliers (between 1.5 and 3 times the interquartile range) are represented by circles (o) beyond the whiskers. Extreme values (more than 3 times the interquartile range) are represented by stars (*) beyond the whiskers.

Figure 4.2. Box and whisker plot showing the relationship between induced sputum % neutrophils and airflow limitation by GOLD stage in the COPD cohort ($p=0.002$, Kruskal-Wallis). There were significant differences between GOLD stages I and III ($p=0.003$) and I and IV ($p=0.002$).



GOLD stages: GOLD stage I (Mild): FEV_1/FVC ratio < 70% predicted, $FEV_1\%$ predicted > 80% ($n=13$); GOLD stage II (Moderate): FEV_1/FVC ratio < 70% predicted, $FEV_1\%$ predicted 50–80% ($n=68$); GOLD stage III (Severe): FEV_1/FVC ratio < 70% predicted, $FEV_1\%$ predicted 30–50% ($n=72$); GOLD stage IV (Very Severe): FEV_1/FVC ratio < 70% predicted, $FEV_1\%$ predicted < 30% ($n=25$)

Box plot values: The top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and central line represents the 50th percentile. The whiskers represent the highest and lowest values that are not outliers or extreme values. Outliers (between 1.5 and 3 times the interquartile range) are represented by circles (o) beyond the whiskers. Extreme values (more than 3 times the interquartile range) are represented by stars (*) beyond the whiskers.

The relationship between induced sputum and blood inflammatory markers was assessed (Table 4.5). Blood neutrophils and CRP correlated significantly with each other ($r=0.41$, $p<0.001$). Sputum neutrophils were not significantly associated with blood neutrophils ($r=0.12$, $p=0.23$) or CRP ($r=0.15$, $p=0.15$). The sputum cytokines correlated significantly with each other ($p<0.001$) but only IL- 1β correlated with sputum % neutrophils ($p<0.001$). Blood inflammatory markers showed no significant correlations with any of the induced sputum inflammatory markers.

Table 4.5. Correlations between markers of inflammation in blood and induced sputum are displayed. Data are presented as Spearman's correlation co-efficient and statistical significance ($p<0.001$) * is indicated.

	Blood Neutrophils	Blood hsCRP	Sputum % Neutrophils	Sputum IL- 1β	Sputum IL-6
Blood hsCRP	0.408*				
Sputum % Neutrophils	0.124	0.148			
Sputum IL-1β	0.145	0.088	0.403*		
Sputum IL-6	0.091	-0.005	0.134	0.440*	
Sputum IL-8	0.058	-0.078	0.173	0.438*	0.347*

The cohort was analyzed according to inhaled corticosteroid (ICS) use (Table 4.6). 66% of patients were using ICS at the time of data collection. When divided by severity of airflow limitation, 6 (46%) of GOLD stage I, 40 (57%) GOLD stage II, 55 (75%) GOLD stage III, and 20 (77%) GOLD stage IV patients were on ICS treatment. ICS use did not significantly affect the number of patients producing an induced sputum sample and there was no significant difference in any of the pulmonary inflammatory markers measured in patients using ICS compared to those not using ICS, but CRP was significantly higher in patients using ICS in the mild-moderate GOLD stage group ($p=0.009$).

Table 4.6. Markers of inflammation in blood and sputum according to GOLD stage and inhaled corticosteroid use (ICS). GOLD stages are divided into I-II (mild-moderate) and III-IV (severe-very severe). Results are displayed as mean \pm SD or *median (IQR). CRP was significantly higher in subjects taking ICS therapy in the mild-moderate GOLD group ($p=0.009$).[∇]

GOLD STAGE	I and II		III and IV	
	Yes n=46	No n=37	Yes n=75	No n=24
<i>BLOOD</i>				
WCC, $\times 10^9/L$	7.4 \pm 2	6.9 \pm 2	7.9 \pm 2	8.3 \pm 3
Neutrophils, $\times 10^9/L$	4.5 \pm 1	4.3 \pm 2	5.1 \pm 2	5.6 \pm 3
CRP, mg/L*	4.8 (2-8) [∇]	2.9 (1-5) [∇]	3.3 (2-8)	4.7 (3-8)
<i>SPUTUM</i>				
Neutrophils, %*	86 (73-92)	79 (62-93)	91 (84-95)	91 (80-95)
IL-1 β , pg/ml*	4.9 (0-22)	5.5 (0-26)	14 (0-53)	7 (0-106)
IL-6, pg/ml*	25 (0-47)	19 (8-44)	46 (6-88)	28 (12-44)
IL-8, pg/ml*	392 (230-677)	394 (257-876)	460 (230-867)	579 (436-1164)

Statin therapy was investigated in the cohort (Table 4.7). 23 patients (13%) were taking statin therapy at the time of data collection. One patient (8%) in GOLD stage I, 13 (23%) GOLD stage II, 5 (7%) GOLD stage III, and 4 (18%) patients in GOLD stage IV were taking a statin. In the whole COPD cohort, there was no significant difference in statin use when analyzed by COPD severity according to GOLD stage ($p=0.18$, Pearson's Chi sq). There was no difference in statin use between patients producing and not producing an induced sputum sample, and there was no difference in any of the pulmonary or systemic inflammatory markers measured in patients taking statins compared to those not on statins. Statin use was therefore not included in the multiple regression analysis. When divided according to COPD severity by GOLD stage, sputum % neutrophils ($p=0.023$) were significantly higher in patients with mild-moderate COPD (GOLD stages I and II) receiving statin therapy, but only 9 subjects produced an induced sputum sample in this group.

Table 4.7. Markers of inflammation in blood and sputum according to GOLD stage and statin use. GOLD stages are divided into I-II (mild-moderate) and III-IV (severe-very severe). Results are displayed as mean \pm SD or *median (IQR). Sputum neutrophils were significantly higher in subjects taking statin therapy in the mild-moderate GOLD groups ($p=0.023$).[∇]

GOLD STAGE	I and II		III and IV	
STATIN	Yes n=14	No n=69	Yes n=9	No n=90
<i>BLOOD</i>				
WCC, $\times 10^9/L$	7.3 \pm 2	7.1 \pm 2	8.9 \pm 4	7.9 \pm 2
Neutrophils, $\times 10^9/L$	4.8 \pm 1	4.4 \pm 1	6.1 \pm 3	5.2 \pm 2
CRP, mg/L*	4.8 (2-8)	2.9 (1-5)	3.3 (2-8)	4.7 (3-8)
<i>SPUTUM</i>				
Neutrophils, %*	93 (88-95) [∇]	79 (62-92) [∇]	91 (90-94)	91 (83-95)
IL-1 β , pg/ml*	19 (2-37)	4 (0-20)	16 (10-25)	11 (0-60)
IL-6, pg/ml*	39 (8-47)	19 (0-40)	15 (0-72)	36 (10-88)
IL-8, pg/ml*	596 (207-737)	383 (253-743)	476 (229-1173)	479 (297-849)

Multiple linear regression

The linear regression model (Table 4.8) shows that sputum % neutrophils and blood neutrophils were independently associated with post-bronchodilator % predicted FEV₁ after adjusting for age, gender, current smoking status and inhaled corticosteroid use. Post-bronchodilator % predicted FEV₁ was significantly lower in female subjects, those using inhaled corticosteroids, and in subjects with higher blood neutrophils counts and higher sputum % neutrophils.

Blood neutrophils ($p=0.01$) and sputum % neutrophils ($p=0.001$) remained significantly and independently associated with post-bronchodilator % predicted FEV₁ when the model was repeated with smoking pack years instead of current smoking status (adjusted R square 0.183) and when sputum % neutrophils were analyzed as a categorical variable in high and low 50th percentiles. IL-1 β was not associated with post-bronchodilator % predicted FEV₁ after adjusting for potential confounding factors ($p=0.1$).

Table 4.8. Multiple linear regression of sputum % neutrophils and blood neutrophils with FEV₁ (post-bronchodilator % predicted) as the dependent variable, adjusting for age, gender, current smoking status and inhaled corticosteroid use.

	B	SE	t	p
Intercept	58.8	15.1	3.884	<0.001
Age	-0.031	0.209	-0.146	0.884
Male sex	10.342	3.411	3.032	0.003
Current smoker	3.825	3.515	1.088	0.279
ICS	-10.281	3.267	-3.147	0.002
Blood neutrophils	-1.721	0.757	-2.275	0.025
Sputum neutrophils % (cubed)	-1.83 x 10 ⁻⁵	<0.001	-2.917	0.004

R squared = 0.299 (Adjusted R Squared = 0.251), p<0.001

B = regression co-efficient; SE = standard error; t = B/SE; p = significance. ICS = inhaled corticosteroid use.

Discussion

The aim of this chapter was to investigate the relationship between pulmonary and systemic inflammation and the relationship of inflammatory markers to airflow limitation in COPD. The markers of systemic inflammation (CRP and neutrophils) correlated significantly with each other, but neither was associated with markers of pulmonary inflammation. Blood and sputum neutrophils were independently associated with airflow limitation but did not correlate with each other. Blood CRP and sputum cytokines were not related to airflow limitation in this cohort. The strength of this study is in the investigation of both systemic and pulmonary inflammation in the same COPD cohort, with adjustment for potential confounding factors. The results show that neutrophilic inflammation in the pulmonary and systemic compartments is associated with airflow limitation, but suggest that pulmonary and systemic inflammation are not directly related to each other.

This is the first study to investigate these inflammatory markers in the lung and circulation in the same cohort of COPD patients. Neutrophils increased with the severity of airflow limitation in both the pulmonary and systemic compartments. Blood and sputum neutrophils both correlated with post-bronchodilator FEV₁ ($r=0.261$ and $r=0.311$ respectively). This is consistent with the existing literature, which shows that numbers of neutrophils in the small airways (250) and induced sputum (45, 325) increase with the severity of airflow limitation in COPD. However, contrary to expectation, levels of blood

and sputum neutrophils did not correlate significantly with each other.

Therefore, although they were both associated with the severity of airflow limitation, they were independent of each other, suggesting that they are not causally related. In fact, although they correlated significantly with each other, none of the sputum markers correlated significantly with either of the systemic inflammatory markers that were measured.

The ability of pulmonary inflammation to induce a systemic inflammatory response has been reported. For example, a systemic response has been elicited in response to the inhalation of noxious particles like diesel exhaust fumes.

(108, 114) However, a lack of correlation between lung and systemic markers has previously been reported in COPD. Vernooij and co-workers found no relationship between blood and sputum levels of soluble TNF α , although the cohort that was studied was small (n=18). (116) Bizeto and co-workers also investigated blood and sputum markers of inflammation in a small cohort (n=20) and found that sputum neutrophils increased with the severity of airflow limitation but CRP showed no relationship with COPD severity. (115)

Another UK study found no relationship between blood and sputum markers – circulating fibrinogen and sputum IL-6 – in stable COPD patients. (72) Our data concur with these results, which suggest that there is not a direct relationship between pulmonary and systemic inflammation in COPD. The hypothesis that there is an overflow of inflammatory mediators from the lung into the systemic compartment (82) in COPD is not supported by these results. Other mechanisms linking systemic and pulmonary inflammation in COPD have been considered.

Possible candidates include systemic hypoxia, (344) genetic factors and autoimmune mechanisms. (306) However, there is a lot of variability in the data and the sample sizes in our study are small.

It is interesting that CRP, unlike blood leukocytes, was not related to the severity of airflow limitation in this cohort, and yet CRP was significantly increased in COPD patients compared to healthy controls (see Chapter 3). Blood neutrophils and CRP were only moderately correlated with each other, suggesting that the increase in these markers in COPD may derive from different mechanisms. While this may be partly due to the effects of cigarette smoking (which was significantly associated with increased levels of blood and sputum neutrophils), neutrophils in blood and sputum were significantly associated with the severity of airflow limitation independently of current smoking status or smoking pack years. The cause of systemic inflammation in COPD has not been determined. But the presence of systemic inflammation in COPD has been associated with poor prognosis and has been linked to clinical phenotypes such as cachexia, as well as cardiovascular disease and osteoporosis. (82) Our results suggest that CRP and blood neutrophils may each have a role in characterizing COPD with regard to disease stage and prognosis, but this would need further investigation in longitudinal studies. Further investigation of clinical features such as exacerbation frequency and oxygenation may further elucidate factors influencing the presence of systemic inflammation in COPD.

Sputum cytokines were not related to airflow limitation in this cohort. More current smokers produced an induced sputum sample, which may have influenced the results but was not significantly associated with airflow limitation in multiple regression analysis. We found no difference in sputum markers between COPD current and ex-smokers. In fact contrary to expectation current smokers had a trend towards lower levels of induced sputum % neutrophils. The lack of association seen between sputum cytokines and lung function in our cohort is in contrast to results from smaller studies. Keatings and co-workers found that sputum IL-8 and neutrophils were increased in COPD patients compared to healthy smokers (n=14), and Yamamoto and co-workers found that IL-8 levels increased with the severity of airflow limitation (n=33). (75, 281) It is likely that the variability in detectable levels of cytokines contributed to the disparity of results found between our results and those from smaller studies. And yet, despite a low yield of induced sputum, we analyzed sputum from 119 COPD patients, which is a substantially greater sample size. Airflow limitation represents only one method of measuring disease severity in COPD. This is recognized in the combined European Respiratory Society and American Thoracic Society COPD guidelines. (2) It may be that sputum cytokines may reflect a different aspect of disease severity in COPD and may have a role in differentiating clinical phenotypes such as frequent exacerbators and those with cachexia in the COPD population.

Limitations

Our data suggested that pulmonary and systemic inflammation were independent of each other in COPD. However, induced sputum is thought to mainly be representative of inflammation in the larger airways and may not closely represent inflammation in the distal airspaces or lung parenchyma.

(345) A percentage of patients were unable to produce an acceptable induced sputum sample. Successful sputum induction is not always possible, particularly in patients with more severe COPD. (175) It is likely that patients with more severe airflow limitation would have increased levels of sputum inflammation, which would serve to strengthen our results.

Conclusion

Neutrophils in blood and induced sputum were independently related to the severity of airflow limitation in COPD. Systemic inflammatory markers (CRP and neutrophils) were associated with each other but not with airflow limitation.

Blood markers did not correlate with sputum markers of inflammation. These findings suggest that lung and systemic inflammation may be regulated independently in COPD.

CHAPTER 5: Inflammation and clinical phenotypes associated with prognosis in COPD

Introduction

COPD is defined in terms of FEV₁ for the purpose of diagnosis and treatment. But FEV₁ does not capture all aspects of COPD and in particular, does not reflect the systemic features of the disease. (2) Additional variables have been used to better describe COPD and its functional and phenotypic heterogeneity. Many of these factors have been used as outcome measures, or are associated with outcome measures in COPD. (346) Examples include measurement of health related quality of life using the St George's Respiratory Questionnaire (SGRQ), MRC dyspnoea score, body mass index (BMI), exacerbation frequency and measurement of oxygenation (using either the partial arterial pressure of oxygen or haemoglobin oxygen saturation). In addition to providing useful endpoints in COPD studies, some of these measures used to describe COPD may relate to different sub-types or phenotypes within the COPD population. Some of these features relate to outcomes better than FEV₁ alone. For example, low BMI has been associated with prognosis in COPD (347) and frequent exacerbations in COPD subjects have been associated with a faster rate of decline in FEV₁. (139)

Inflammation is central to the definition of COPD (2) and is postulated to provide a mechanistic link between COPD and some of its systemic effects and co-morbidities. (276) If this is the case, it is likely that markers of inflammation

will be associated with some of the features of COPD associated with outcome measures other than FEV₁. To investigate this hypothesis, the following outcome measures were selected for investigation of their association with markers of pulmonary and systemic inflammation in a stable COPD population.

St George's Respiratory Questionnaire

Quality of life measures have been linked to prognosis in COPD and are often utilized as outcome measures in clinical trials. (6, 124) Numerous questionnaires exist to measure quality of life in patients with respiratory disease. One of the best known is the St George's Respiratory Questionnaire (SGRQ). SGRQ is a validated tool for assessing the impact of COPD on health related quality of life (129) and has been identified as an important predictor of mortality in COPD. (124) The SGRQ is composed of three domains: symptoms, activity and the impact of COPD on quality of life. These are combined as a total score, which is the mean of the three subcategories. Subcategory scores range from 0-100, with higher scores representing greater impairment. A difference of 4 points is considered a clinically significant difference. (258)

MRC dyspnoea score

Dyspnoea is a complex subjective sensation, which is relevant to the assessment of cardiac and respiratory disease. (348) The MRC dyspnoea score was developed in the 1940's when questions were devised to quantify breathlessness in Welsh coalminers with pneumoconiosis. By the 1950's it was established in clinical practice and for research purposes. (349) It comprises 5 simple categories which ask a patient to define the presence of dyspnoea

according to the activity level at which it is experienced. The MRC dyspnoea score is now regarded as an important clinical endpoint, which is independently associated with all cause mortality, not only in COPD patients. (95) In the assessment of COPD, it has been incorporated in the BODE index due to its importance as an independent prognostic indicator. (118) The MRC dyspnoea score correlates poorly with FEV₁ and as such may provide important additional information on functional status in COPD. (122)

Body mass index

A number of COPD patients experience significant loss of body mass in association with progression of disease. Loss of body mass has a number of associated clinical features, including low fat free mass (a surrogate for skeletal muscle mass), skeletal muscle dysfunction, exercise intolerance and lower limb muscle weakness. (90) Low body mass index and fat free mass are independent predictors of mortality in COPD and this association is stronger in more severe COPD. (15, 159, 160) Multi-dimensional staging systems using clinical features like BMI and dyspnoea have been shown to improve the prediction of prognosis in COPD. (347)

Exacerbation frequency

Frequent exacerbations of COPD are associated with reduced quality of life, (134) increased hospitalization, morbidity and mortality and a faster decline in lung function. (135-139, 350, 351) Reducing exacerbation frequency is a therapeutic target in COPD and frequency of exacerbations is increasingly used as an endpoint in clinical trials. (6, 141) The annual number of exacerbations

varies between COPD patients. A median annual exacerbation frequency of 2.5 per year is typical in COPD populations. (71, 139) Exacerbation frequency recorded in the first year is highly correlated with exacerbation frequency in subsequent years in individual subjects. (139) The “frequent exacerbator” constitutes a recognized clinical phenotype in COPD, meanwhile a significant proportion of COPD patients with the opposite “phenotype” experience no exacerbations in an average year. (6) Due to its association with prognosis in COPD, exacerbation frequency has been incorporated in the DOSE multidimensional scoring system and is associated with hospital admissions and respiratory failure. (352)

Hypoxia

Hypoxia is associated with prognosis in COPD (46) and can be measured reliably and non-invasively by pulse oximetry. (353) Oxygen supplementation is one of the few therapeutic measures known to improve survival in COPD. (161, 162) In addition to improving pulmonary vascular resistance and polycythaemia; (161) exercise capacity, dyspnoea and sleep consolidation may also be improved with oxygen therapy. (163) Hypoxia is more common in severe COPD, and yet it does not develop in all patients with severe airflow limitation. Chronic hypoxaemia increases mortality at any stage of the disease. (354)

BODE index

A number of multi-dimensional scoring indices have been developed in order to better reflect the multi-system effects of COPD. The best known of these is the

Body mass index, airflow Obstruction, Dyspnoea and Exercise capacity Index (BODE). (122) The BODE index is a better predictor of mortality in COPD than FEV₁ alone. (122)

In this chapter, the validated quality of life measure SGRQ, the MRC dyspnoea score and the pulmonary and systemic features of COPD measured by body mass index, exacerbation frequency, oxygenation and the BODE index will be explored in relation to markers of systemic and pulmonary inflammation in a cohort of COPD patients. These features have been chosen due to their known association with prognosis in COPD. The hypothesis is that these features will be significantly associated with pulmonary and systemic inflammation in COPD patients.

Aims

To investigate the associations between pulmonary and systemic inflammation in a cohort of stable COPD subjects and the following clinical parameters:

1. Quality of life and dyspnoea
2. Body mass index
3. Exacerbation frequency
4. Oxygenation
5. BODE index

Methods and data analysis

182 COPD patients were recruited as described in Chapter 2. Inflammatory markers in blood and induced sputum were analyzed with clinical features in the recruited COPD cohort. SGRQ total scores were analyzed with inflammatory markers using the Pearson's or Spearman's correlation co-efficients. SGRQ total score was then categorized in quartiles for further analysis. Groups were compared with the Kruskal Wallis test with Mann-Whitney post hoc analysis (abnormally distributed variables) or ANOVA with the Bonferroni post hoc test (normally distributed variables). The MRC dyspnoea score was categorized into 3 groups for analysis. Dyspnoea scores were grouped together as 1-2, 3 and 4-5 to obtain equal numbers between groups for comparison. Body mass index, exacerbation frequency and oxygen saturations were categorized for analysis. BMI was categorized into 2 groups ($\leq 21\text{kg/m}^2$ and $>21\text{kg/m}^2$). This division is utilized in the BODE index as an inflection in survival has been shown to occur in COPD patients with a BMI of $\leq 21\text{kg/m}^2$. (118) Oxygen saturation was categorized using a cut-off of $\leq 93\%$. Pulse oximetry $\text{SaO}_2 \leq 92\%$ effectively represents systemic hypoxia (sensitivity 100%, specificity 86%) compared to measurement by arterial blood gas analysis. (355) A cut-off of $\leq 93\%$ was chosen in this study to give large enough numbers for comparison in each group. 25 patients had $\text{SaO}_2 \leq 93\%$. In patients with induced sputum for analysis, 14 subjects had $\text{SaO}_2 \leq 93\%$ whereas only 7 subjects with $\text{SaO}_2 \leq 92\%$ had induced sputum for analysis. Exacerbation frequency was dichotomized into ≤ 2 exacerbations per year and > 2 exacerbations per year. Other groups

have utilized this cut-off. (71) BODE index total scores, which followed a normal distribution, were analyzed with inflammatory markers using Pearson's and Spearman's correlation co-efficients.

Statistically significant factors in the univariate analysis were analyzed using multiple regression models. SGRQ total score was investigated with multiple linear regression. Exacerbation frequency, BMI and oxygen saturations were analyzed in binary logistic regression models using the above categories. MRC dyspnoea score was also analyzed using binary logistic regression, with subjects categorized into scores of ≤ 2 or > 2 in order to obtain approximately equal numbers between groups. Age, gender and height were not included separately in models as these variables were accounted for as part of the FEV₁ (post-bronchodilator % predicted).

Results

Baseline data for the cohort of 182 COPD patients is shown in Table 5.1.

Table 5.1. Clinical data for the COPD cohort. Data are presented as number (%) for categorical variables, mean \pm SD for parametric data, and *median (IQR) for non-parametric data.

COPD subjects	n=182
Age	68 \pm 8
Gender (% male)	112 (62%)
Smoking pack years	48 \pm 24
Inhaled corticosteroids (% taking inhaled steroids)	121 (66%)
FEV₁, post bronchodilator % predicted	50 \pm 19
FVC, post bronchodilator % predicted	79 \pm 21
FEV₁/FVC ratio, post bronchodilator	0.48 \pm 0.1
St Georges Respiratory Questionnaire (SGRQ) total score	51 \pm 20
SGRQ impact	38 \pm 22
SGRQ symptoms	62 \pm 21
SGRQ activity	66 \pm 24
Body mass index, kg/m²	26 \pm 6
Oxygen saturation, %	95 \pm 2
Annual exacerbation frequency	2 (1-3)*
MRC chronic bronchitis symptoms	83 (46%)
MRC dyspnoea score	3 (2-4)*
6 minute walking distance, m	63 \pm 38
BODE index	6.4 \pm 2

Health related quality of life: SGRQ

The SGRQ total score was available for 179 patients. Baseline data are shown in Table 5.2. There was no significant difference in age, gender, inhaled corticosteroid use or smoking pack year history when analyzed according to SGRQ total score in quartiles. Inhaled corticosteroid treatment was significantly associated with a poorer SGRQ symptoms score ($p=0.048$) but not with the other domains, but there was a trend towards a greater ICS use with worsening SGRQ symptoms, activity and impact domains ($p<0.1$). Patients with poorer quality of life were significantly more likely to be current smokers, had significantly increased airflow limitation and were more likely to be able to produce a sample of induced sputum. Correlations between SGRQ total score and markers of blood and sputum inflammation are shown in Table 5.3. SGRQ symptoms, activity and impact domains were also analyzed with the sputum and blood markers. All domains of SGRQ were associated with blood leukocytes and CRP, but not with sputum neutrophils or cytokines. The association between SGRQ in quartiles and blood neutrophils and CRP are shown in Figures 5.1 and 5.2.

Blood neutrophils and CRP were investigated with SGRQ total score using linear regression to adjust for confounding factors (Tables 5.4 and 5.5). Blood neutrophils were significantly associated with SGRQ total score independent of smoking status, induced sputum production and post-bronchodilator % predicted FEV₁. This significance remained when the model was repeated with

smoking pack years instead of current smoking status (blood neutrophils $p=0.019$). Log CRP was significantly associated with SGRQ total score independent of smoking status, induced sputum production and post-bronchodilator % predicted FEV₁. This significance remained when the model was repeated with smoking pack years instead of current smoking status (log CRP $p<0.001$). There was a stronger association between SGRQ total score and log CRP (adjusted R sq 0.356) than with blood neutrophils (adjusted R sq 0.271). But when included in the model together, CRP and blood neutrophils were not independently associated with SGRQ.

Table 5.2. Baseline characteristics of the COPD cohort categorized by St George's Respiratory Questionnaire total score (quartiles I-IV from lowest to highest) are displayed. Cut-points for each quartile are 0-33.6 (I), 33.7-51.5 (II), 51.6-65.6 (III), 65.7-100 (IV). Data are presented as number (%) for categorical variables and mean \pm SD for continuous variables. Significance between groups taken at $p < 0.008$ using ANOVA and Dunnett's T3 post hoc test for continuous variables and Pearson's Chi squared test for categorical variables.

SGRQ total score (quartiles)	I	II	III	IV	p
	n=45	n=45	n=45	n=44	
Age	68 \pm 10	68 \pm 7	68 \pm 8	68 \pm 8	0.976
Gender, % male	30 (67%)	26 (58%)	28 (62%)	27 (61%)	0.858
Current smokers	10 (22%)	15 (33%)	22 (49%)	20 (45%)	0.002
Smoking pack years	40 \pm 18	48 \pm 26	51 \pm 26	54 \pm 24*	0.027
Inhaled corticosteroids	24 (53%)	30 (67%)	35 (78%)	30 (68%)	0.106
Post bronchodilator predicted FEV₁, %	61 \pm 19	54 \pm 18	45 \pm 16*	38 \pm 15* [∇]	<0.001
Post bronchodilator predicted FVC, %	89 \pm 18	82 \pm 22	76 \pm 18*	66 \pm 18* [∇]	<0.001
Post bronchodilator FEV₁/FVC	0.53 \pm 0.1	0.51 \pm 0.1	0.46 \pm 0.1	0.43 \pm 0.1*	0.001
Induced sputum produced	21 (47%)	31 (69%)	33 (73%)	33 (75%)	0.016

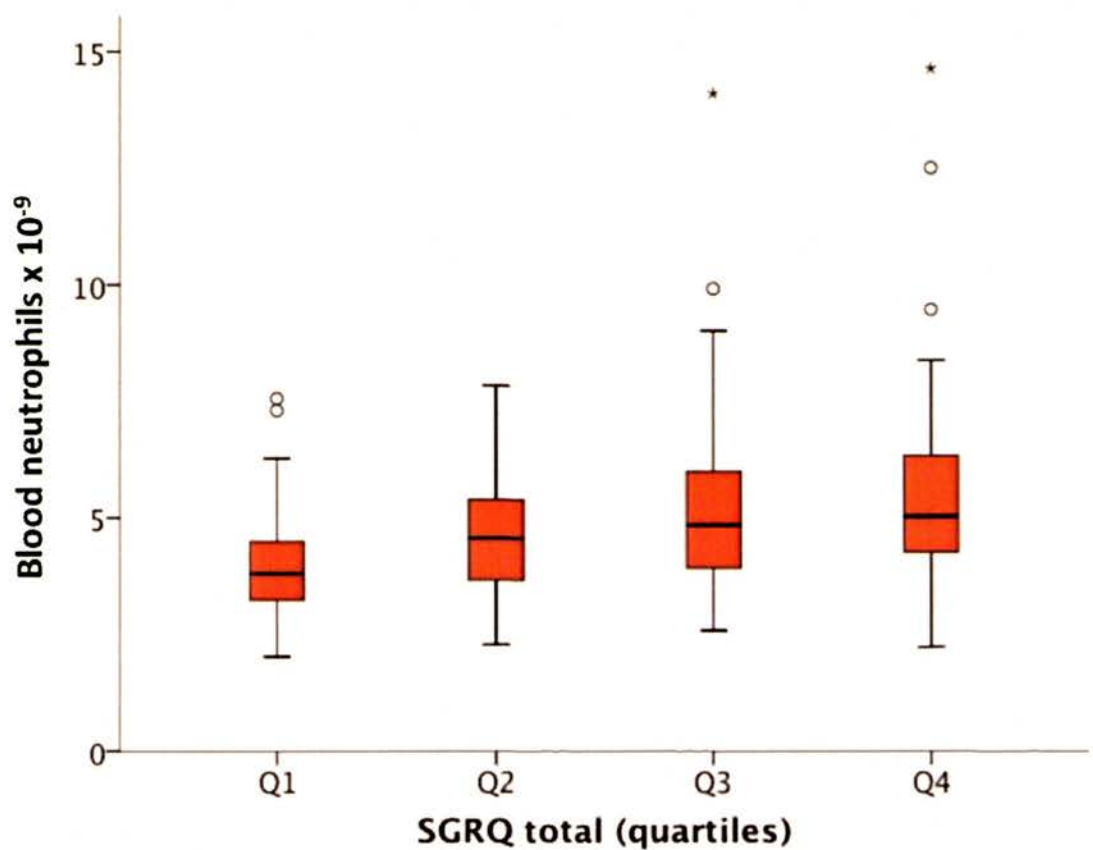
* = significantly different from quartile I.

[∇] = significantly different from quartile II.

Table 5.3. Correlations between St George's Respiratory Questionnaire total scores and markers of pulmonary and systemic inflammation in the COPD cohort. Data are presented as Pearson's* (parametric data) or Spearman's correlation (non-parametric data) co-efficients.

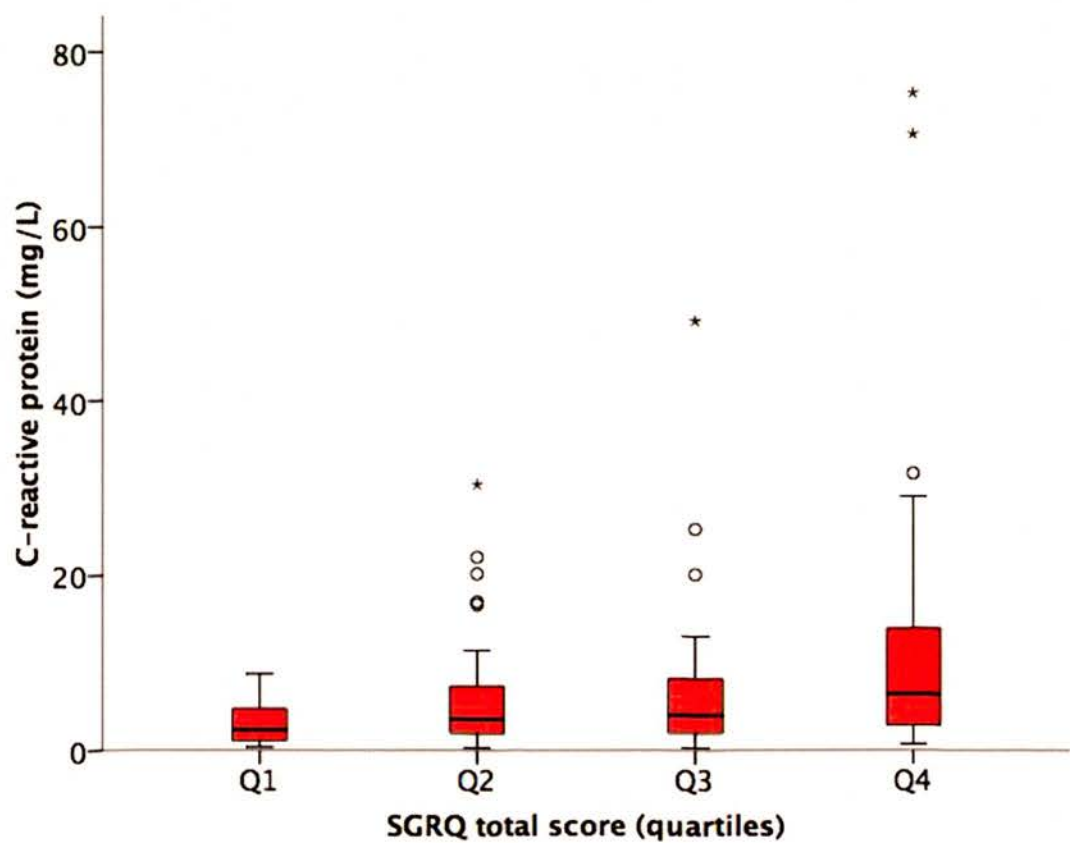
SGRQ total score	Correlation	p
White cell count, x10⁹/L*	0.339*	<0.001
Blood neutrophils, x10⁹/L*	0.306*	<0.001
C-reactive protein, mg/L	0.323	<0.001
Sputum neutrophils, %	-0.032	0.753
Sputum IL-1β, pg/ml	-0.023	0.810
Sputum IL-6, pg/ml	0.000	0.996
Sputum IL-8, pg/ml	-0.018	0.848

Figure 5.1. Box and whisker plot showing the relationship between blood neutrophils and St George’s Respiratory Questionnaire total score in quartiles in the COPD cohort ($p<0.001$). Patients in the highest quartile report the poorest quality of life. In the post-hoc analysis, significant differences existed between quartiles 1 and 3 ($p=0.003$), and 1 and 4 ($p=0.002$).



Box plot values: The top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and central line represents the 50th percentile. The whiskers represent the highest and lowest values that are not outliers or extreme values. Outliers (between 1.5 and 3 times the interquartile range) are represented by circles (o) beyond the whiskers. Extreme values (more than 3 times the interquartile range) are represented by stars (*) beyond the whiskers.

Figure 5.2. Box and whisker plot showing the relationship between highly sensitive C-reactive protein and St George’s Respiratory Questionnaire total score in quartiles in COPD patients ($p<0.001$). Patients in the highest quartile report the poorest quality of life. In the post-hoc analysis significant differences were seen between quartiles 1 and 3 ($p=0.009$) and quartiles 1 and 4 ($p<0.001$).



Outlier in quartile 4: CRP value=136.

Table 5.4. Multiple linear regression of blood neutrophils with SGRQ total score as the dependent variable, adjusting for current smoking status, induced sputum production and post-bronchodilator % predicted FEV₁.

	B	SE	t	p
Intercept	59.7	8.454	7.056	<0.001
FEV₁ post-bronchodilator % predicted	-0.396	0.072	-5.509	<0.001
Induced sputum produced	7.481	2.774	2.697	0.008
Current smoker	6.209	2.666	2.329	0.021
Blood neutrophils	1.723	0.700	2.461	0.015

R squared = 0.288 (Adjusted R Squared = 0.271), p<0.001

Table 5.5. Multiple linear regression of CRP with SGRQ total score as the dependent variable, adjusting for current smoking status, induced sputum production and post-bronchodilator % predicted FEV₁.

	B	SE	t	p
FEV₁ (post-bronchodilator % predicted)	-0.478	0.067	-7.105	<0.001
Log CRP	4.397	1.135	3.874	<0.001
Induced sputum produced	7.734	2.685	2.880	0.004
Intercept	37.4	13.5	2.777	0.006
Current smoker	7.535	2.753	2.737	0.007

R squared = 0.378 (Adjusted R Squared = 0.356), p<0.001

Dyspnoea

MRC dyspnoea scores were available for 180 COPD patients. Subjects were categorized into groups according to the MRC dyspnoea score: 1-2 (n=71), 3 (n=46), and 4-5 (n=63). Baseline data are shown in Table 5.6. There was no significant difference in age, gender, smoking status, smoking pack years, inhaled corticosteroid use or in the number of patients producing a sample of induced sputum when analyzed according to MRC dyspnoea score. There was a significant association between increased dyspnoea and increased airflow limitation measured by post bronchodilator FEV₁ and FVC % predicted and FEV₁/FVC ratio.

Increased MRC dyspnoea score was associated with increased systemic inflammation measured by blood leukocytes and CRP. There was no relationship between dyspnoea and induced sputum markers of inflammation (Table 5.7). Box and whisker plots showing the relationship between MRC dyspnoea score and blood neutrophils and CRP are shown in Figures 5.3 and 5.4.

Binary logistic regression models of blood neutrophils and CRP with MRC dyspnoea score are shown in Table 5.8. Blood neutrophils (p=0.005) and CRP (p=0.003) were significantly associated with MRC dyspnoea score independent of smoking pack years and FEV₁ but blood neutrophils were not associated with MRC dyspnoea score independently of CRP.

Table 5.6. Baseline characteristics of the COPD cohort are shown when categorized by MRC dyspnoea score. Data are presented as number (%) for categorical variables and mean \pm SD for continuous variables. Significance between groups taken at $p < 0.017$ using ANOVA and Dunnett's T3 post hoc test for continuous variables and Pearson's Chi squared test for categorical variables.

MRC dyspnoea score	1-2	3	4-5	p
	n=71	n=46	n =63	
Age	67 \pm 9	68 \pm 6	69 \pm 8	0.128
Gender, % male	43 (61%)	26 (57%)	41 (65%)	0.659
Current smokers	24 (34%)	18 (39%)	26 (41%)	0.826
Smoking pack years	43 \pm 19	48 \pm 24	54 \pm 28	0.030
Inhaled corticosteroids	42 (59%)	34 (74%)	45 (71%)	0.171
Post bronchodilator predicted FEV₁, %	60 \pm 18	45 \pm 15*	41 \pm 16*	<0.001
Post bronchodilator predicted FVC, %	89 \pm 20	75 \pm 18*	70 \pm 20*	<0.001
Post bronchodilator FEV₁/FVC	0.53 \pm 0.1	0.47 \pm 0.1	0.45 \pm 0.1*	0.001
Induced sputum produced	48 (68%)	52 (52%)	73 (73%)	0.069

* = significantly different from MRC dyspnoea score 1-2.

Table 5.7. Pulmonary and systemic inflammatory markers are displayed according to the different categories of MRC dyspnoea score in the COPD cohort. Results are presented as mean \pm SD* or median (IQR). Higher MRC dyspnoea scores indicate more breathlessness.

MRC dypnoea score	1-2 n=71 sputum n=47	3 n=46 sputum n=23	4-5 n=63 sputum n=43	p
White cell count, $\times 10^9/L^*$	6.9 \pm 2	7.7 \pm 2	8.4 \pm 3	0.001
Blood neutrophils, $\times 10^9/L^*$	4.2 \pm 1	5.0 \pm 2	5.6 \pm 3	<0.001
C-reactive protein, mg/L	2.8 (1-5)	4.5 (2-7)	6.0 (2-9)	0.002
Sputum neutrophils, %	88 (76-92)	90 (80-96)	92 (74-95)	0.163
Sputum IL-1 β , pg/ml	4 (0-16)	15 (0-42)	14 (0-66)	0.103
Sputum IL-6, pg/ml	28 (10-60)	20 (0-47)	27 (0-94)	0.611
Sputum IL-8, pg/ml	451 (316-781)	526 (271-850)	451 (213-885)	0.805

Figure 5.3. Box and whisker plot showing the relationship between blood neutrophils and MRC dyspnoea score in the COPD cohort ($p<0.001$). In the post-hoc analysis, there was a significant difference between groups 1-2 and 3 ($p=0.013$) and groups 1-2 and 4-5 ($p<0.001$).

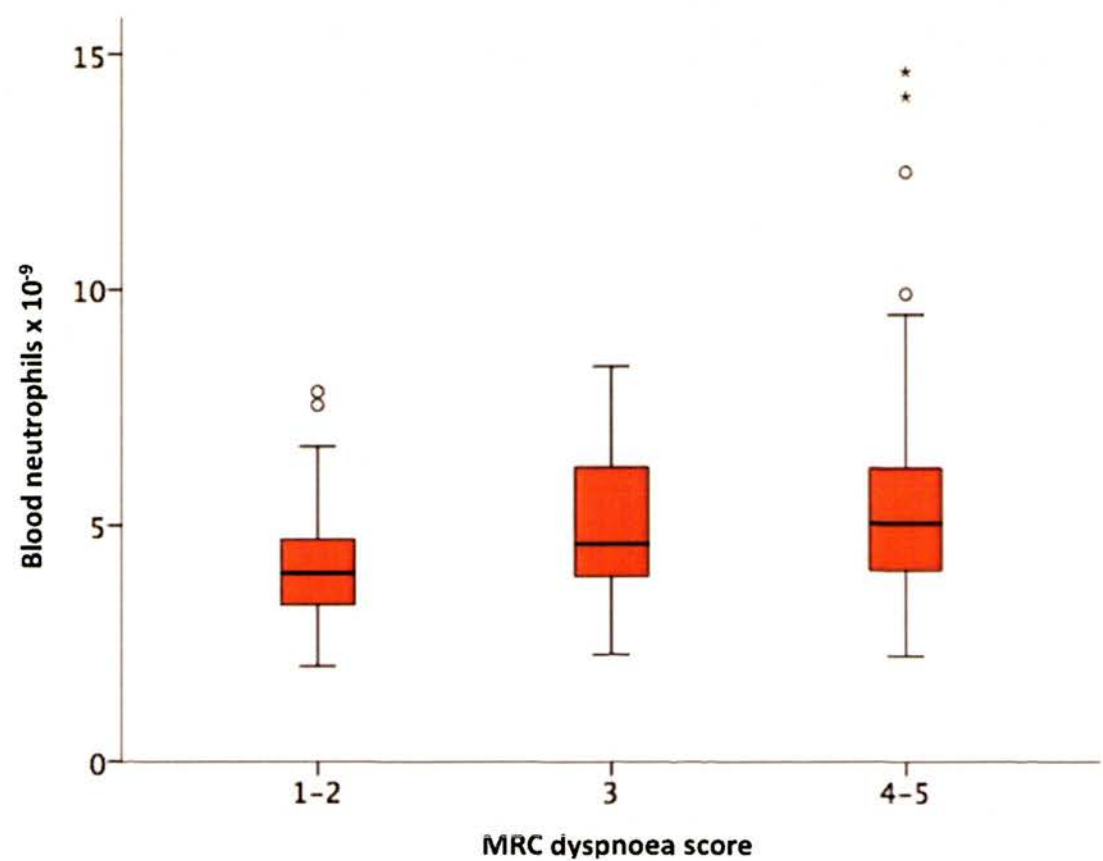
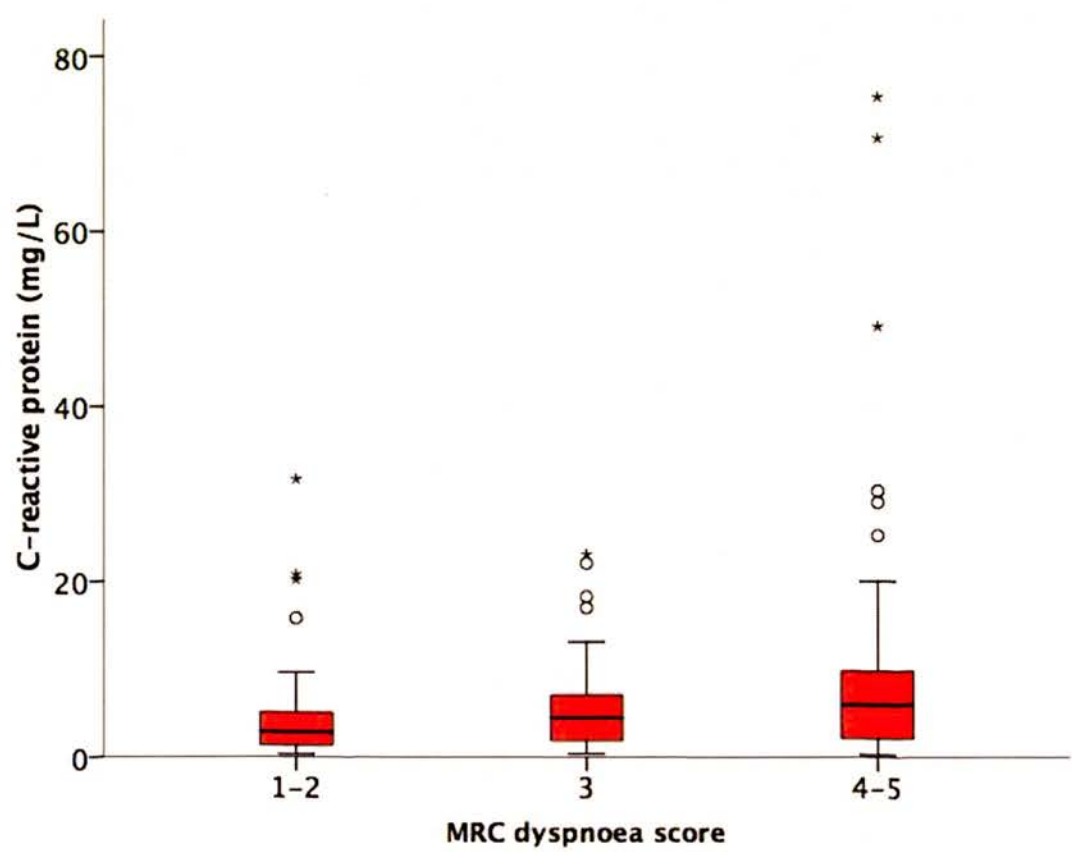


Figure 5.4. Box and whisker plot showing the relationship between highly sensitive C-reactive protein and MRC dyspnoea score in the COPD cohort ($p=0.002$). Statistically significant differences existed between groups in the post-hoc analysis for groups 1-2 and 4-5 ($p<0.001$).



Outlier for subject with MRC dyspnoea score level 5, CRP value = 136.

Table 5.8. Binary logistic regression of blood neutrophils with MRC dyspnoea score (≤ 2 or > 2) as the outcome variable, adjusting for smoking pack years and post-bronchodilator % predicted FEV₁.

	B	SE	df	Wald	p	Exp (B)
FEV₁ post-bronchodilator % predicted	-0.060	0.012	1	23.40	<0.001	0.942
Log CRP	0.452	0.198	1	5.184	0.023	1.571
Blood neutrophils	0.257	0.140	1	3.365	0.067	1.293
Smoking pack years	0.013	0.009	1	2.041	0.153	1.013
Intercept	1.199	1.006	1	1.422	0.233	3.317

-2 Log likelihood 169.0 (Cox % Snell R Square 0.280, Nagelkerke R square 0.380), $p < 0.001$

Body mass index

Baseline characteristics and inflammatory markers for the COPD cohort according to body mass index are shown in Table 5.9. BMI was available for 181 patients. There was no significant difference in age, gender, inhaled corticosteroid use, statin use or in the frequency of induced sputum samples produced between COPD patients with a BMI $\leq 21\text{kg/m}^2$ compared to subjects with a BMI $> 21\text{kg/m}^2$. Although there was no difference in smoking pack years between the groups, there were significantly more current smokers in the low BMI group ($p<0.001$). These patients also had a significantly lower post-bronchodilator FEV₁ % predicted and FEV₁/FVC ratio. BMI $\leq 21\text{kg/m}^2$ was associated with significantly higher sputum neutrophils and IL-6 (Figures 5 and 6). There was no significant association between BMI and sputum IL-1 β ($p=0.07$) or IL-8 ($p=0.08$).

A binary regression model was used to investigate sputum % neutrophils and sputum IL-6 with BMI adjusting for potential confounding factors (Table 5.10). Sputum % neutrophils ($p=0.042$) but not IL-6 ($p=0.058$) were significantly associated with BMI after adjusting for current smoking status, but neither were significantly associated with BMI after adjusting for FEV₁.

Table 5.9. Baseline characteristics and markers of inflammation for the COPD cohort are shown according to body mass index (low $\leq 21\text{kg/m}^2$, normal/high $> 21\text{kg/m}^2$). Data are presented as number (%) for categorical variables, mean \pm SD for parametric variables and median (IQR)* for non-parametric variables. Groups were compared using the Chi-squared test (categorical), t-test (parametric) and Mann-Whitney U test (non-parametric). * Significance is taken at the $p < 0.05$ level.

Body Mass index	$\leq 21\text{kg/m}^2$	$> 21\text{kg/m}^2$	p
	n=46 sputum n=21	n=135 sputum n=94	
Age	67 \pm 7	68 \pm 8	0.370
Gender, % male	24 (52%)	87 (64%)	0.162
Current smokers	28 (61%)	39 (29%)	<0.001
Smoking pack years	46 \pm 2	49 \pm 3	0.467
Inhaled corticosteroids	29 (63%)	91 (67%)	0.593
Post bronchodilator predicted FEV ₁ , %	42 \pm 2	53 \pm 2	0.001
Post bronchodilator predicted FVC, %	76 \pm 2	80 \pm 2	0.315
Post bronchodilator FEV ₁ /FVC	0.43 \pm 0.1	0.5 \pm 0.1	0.001
Induced sputum produced	29 (63%)	90 (67%)	0.720
White cell count, $\times 10^9/\text{L}$	7.9 \pm 3	7.5 \pm 2	0.410
Blood neutrophils, $\times 10^9/\text{L}$	5.3 \pm 3	4.8 \pm 2	0.154
C-reactive protein, mg/L*	3.6 (2-6)	4.0 (2-7)	0.375
Sputum neutrophils, %*	95 (80-97)	88 (76-93)	0.020
Sputum IL-1 β , pg/ml*	29 (0-81)	7 (0-28)	0.211
Sputum IL-6, pg/ml*	48 (19-93)	22 (0-53)	0.023
Sputum IL-8, pg/ml*	481 (416-756)	431 (241-843)	0.453

Figure 5.5. Box and whisker plot showing the relationship between induced sputum neutrophils (%) and BMI in the COPD cohort. BMI $\leq 21\text{kg/m}^2$ (n=21), BMI $> 21\text{kg/m}^2$ (n=94), p=0.02.

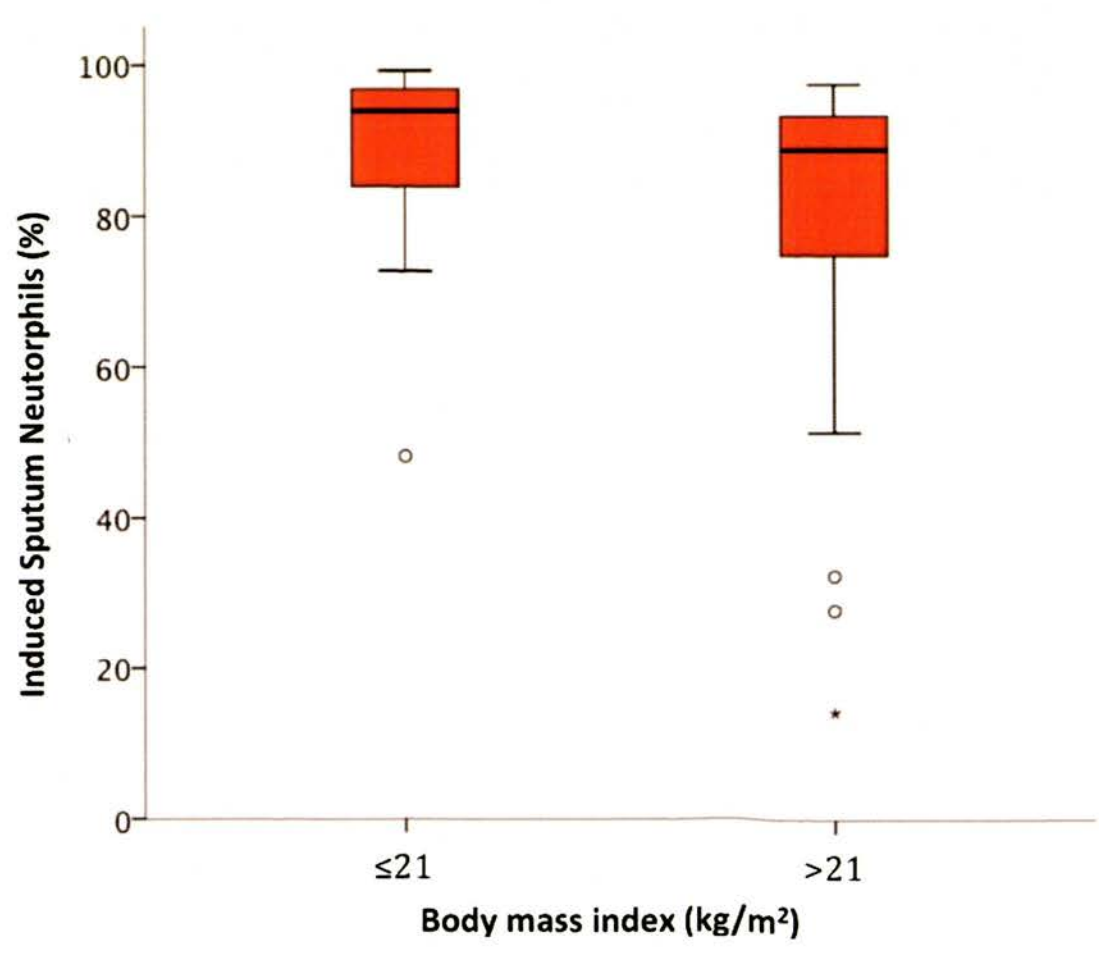


Figure 5.6. Box and whisker plot showing the relationship between BMI and induced sputum IL-6 in COPD patients. BMI $\leq 21\text{kg/m}^2$ (n=21), BMI $>21\text{kg/m}^2$ (n=94), p=0.02.

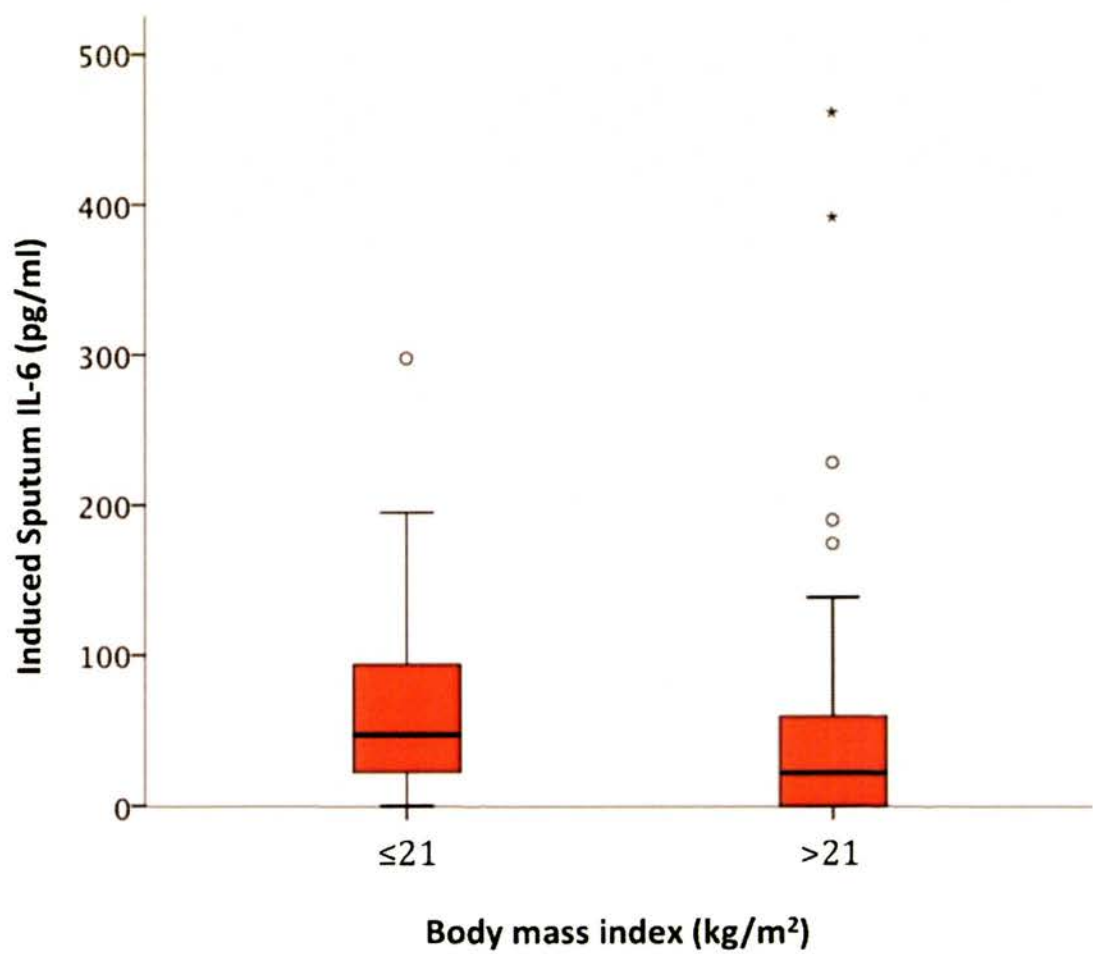


Table 5.10. Binary logistic regression of sputum % neutrophils and sputum IL-6 with BMI (> 21 kg/m²) as the outcome variable, adjusting for current smoking status and post-bronchodilator % predicted FEV₁.

	B	SE	df	Wald	p	Exp (B)
Current smoker	-1.683	0.673	1	6.3	0.012	0.186
Intercept	4.854	1.95	1	6.2	0.013	128.2
Sputum % neutrophils, x10³	0.0001	0.0001	1	2.5	0.116	1.000
Sputum IL-6	-1.010	0.647	1	2.4	0.118	0.364
FEV₁ post-bronchodilator % predicted	0.022	0.021	1	1.1	0.285	1.000

-2 Log likelihood 71.7 (Cox % Snell R Square 0.139, Nagelkerke R square 0.232), *p*<0.001

Exacerbations

Baseline data and markers of inflammation according to exacerbation frequency are shown in Table 5.11. 20% of patients in this cohort reported no exacerbations in the previous 12 months (n=35). There was no difference in gender, smoking status or smoking pack years when analyzed according to exacerbation frequency. COPD patients with > 2 exacerbations per year were significantly younger, were more likely to be taking inhaled corticosteroids, and were more likely to have produced a sample of induced sputum. 114 patients produced induced sputum samples. 70 of these had ≤ 2 exacerbations per year (comprising 40% of the full cohort and 62% of sputum producers). 44 patients producing induced sputum had > 2 exacerbations per year (comprising 25% of the full cohort and 38% of sputum producers). Patients experiencing > 2 exacerbations per year had significantly lower post-bronchodilator FEV₁ and FVC % predicted and a lower FEV₁/FVC ratio.

Increased blood white cell count was significantly associated with > 2 exacerbations per year (p=0.01) and there was a trend towards an association between blood neutrophils and exacerbation frequency, which did not achieve statistical significance (p=0.06). Induced sputum IL-6 was significantly increased in COPD patients with > 2 exacerbations per year (Figure 5.7). In binary logistic regression analysis total WCC, blood neutrophils and sputum IL-6 were not associated with exacerbation frequency after adjusting for FEV₁ (Tables 5.12 and 5.13).

Table 5.11. Baseline characteristics and inflammatory markers in the COPD cohort categorized according to exacerbation frequency (≤ 2 and > 2 per year). Data are presented as number (%) for categorical variables, mean \pm SD for parametric variables and median (IQR)* for non-parametric variables. Groups were compared using the Chi-squared test (categorical), t-test (parametric) and Mann-Whitney U test (non-parametric). * Significance is taken at the $p < 0.05$ level.

Exacerbation frequency	≤ 2	> 2	p
	n=122 sputum n=73	n=60 sputum n=46	
Age	69 \pm 8	66 \pm 8	0.012
Gender, % male	75 (61%)	37 (62%)	1.00
Current smokers	43 (35%)	25 (42%)	0.424
Smoking pack years	48 \pm 24	48 \pm 24	0.886
Inhaled corticosteroids	75 (61%)	46 (77%)	0.046
Post bronchodilator predicted FEV ₁ , %	53 \pm 19	43 \pm 18	0.001
Post bronchodilator predicted FVC, %	82 \pm 20	71 \pm 20	<0.001
Post bronchodilator FEV ₁ /FVC	0.50 \pm 0.1	0.46 \pm 0.1	0.084
Induced sputum produced	73 (60%)	46 (77%)	0.031
White cell count, $\times 10^9/L$	7.3 \pm 2	8.3 \pm 3	0.011
Blood neutrophils, $\times 10^9/L$	4.7 \pm 2	5.4 \pm 2	0.055
C-reactive protein, mg/L*	3.7 (2-6)	4.9 (2-10)	0.107
Sputum neutrophils, %*	90 (76-94)	89 (77-95)	0.986
Sputum IL-1 β , pg/ml*	7 (0-31)	11 (0-91)	0.262
Sputum IL-6, pg/ml*	16 (0-47)	40 (8-109)	0.018
Sputum IL-8, pg/ml*	436 (268-783)	478 (242-971)	0.648

Figure 5.7. Box and whisker plot showing the relationship between induced sputum IL-6 levels and COPD exacerbations. Annual exacerbations ≤ 2 (n=73), annual exacerbations > 2 (n=46), p=0.018.

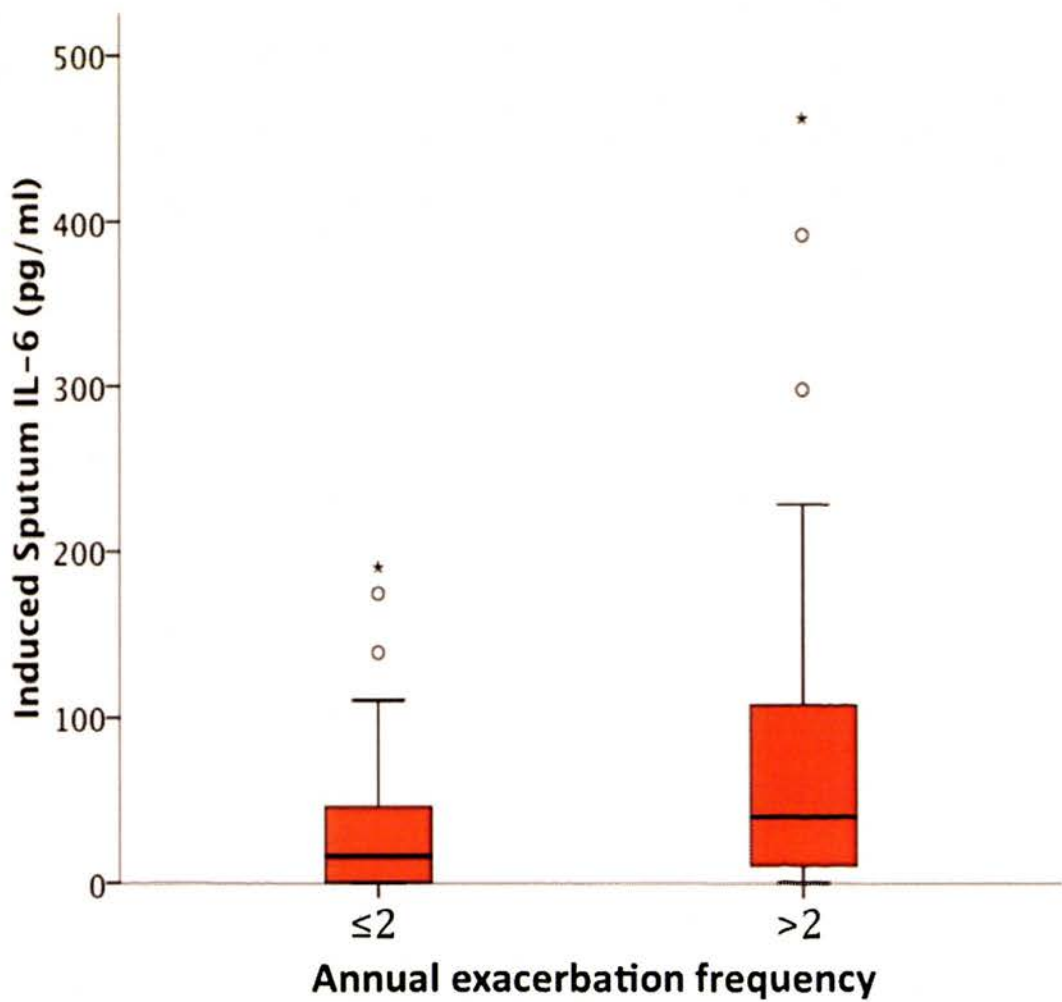


Table 5.12. Binary logistic regression of blood neutrophils with exacerbation frequency (> 2 per year) as the outcome variable, adjusting for post-bronchodilator % predicted FEV₁.

	B	SE	Wald	df	p	Exp (B)
FEV₁ post bronchodilator % predicted	-0.028	0.010	7.501	1	0.006	0.973
Blood neutrophils	0.117	0.089	1.739	1	0.187	1.124
Intercept	-0.003	0.730	0.001	1	0.997	0.997

-2 Log likelihood 205.8 (Cox % Snell R Square 0.071, Nagelkerke R square 0.099), p=0.002

Table 5.13. Binary logistic regression of sputum IL-6 with exacerbation frequency (> 2 per year) as the outcome variable, adjusting for post-bronchodilator % predicted FEV₁.

	B	SE	Wald	df	p	Exp (B)
FEV₁ post bronchodilator % predicted	-0.033	0.013	6.963	1	0.008	0.967
Intercept	0.092	0.897	0.011	1	0.918	1.096
Sputum IL-6	0.092	0.897	0.011	1	0.918	1.096

-2 Log likelihood 141.2 (Cox % Snell R Square 0.098, Nagelkerke R square 0.133), p=0.002

Oxygen saturations

Oxygen saturations were recorded in 172 subjects. Baseline data and inflammatory markers according to oxygenation are shown in Table 5.14. There was no difference in age, gender, smoking status, smoking pack years, inhaled corticosteroid use or production of induced sputum according to oxygen saturation. Subjects with oxygen saturations below 93% had more severe airflow limitation (mean post-bronchodilator FEV₁ 37% predicted) compared to the normoxic group (mean post-bronchodilator FEV₁ 52% predicted, $p < 0.001$). Blood and sputum neutrophils were significantly increased in COPD patients with lower oxygen saturations (Figures 5.8 and 5.9) but only blood neutrophils remained significantly associated with oxygen saturations after adjusting for potential confounding factors (Table 5.15).

Table 5.14. Baseline characteristics of the COPD cohort categorized according to oxygen saturations measured by pulse oximetry ($\leq 93\%$ and $> 93\%$). Data are presented as number (%) for categorical variables, mean \pm SD for parametric variables and median (IQR)* for non-parametric variables. Groups were compared using the Chi-squared test (categorical), t-test (parametric) and Mann-Whitney U test (non-parametric).* Significance is taken at the $p < 0.05$ level.

Oxygen saturation	$\leq 93\%$ n=26 n=17 sputum	$> 93\%$ n=146 n=89 sputum	p
Age	69 \pm 7	68 \pm 8	0.668
Gender, % male	16 (62%)	86 (61%)	1.00
Current smokers	10 (38%)	53 (37%)	0.397
Smoking pack years	53 \pm 23	47 \pm 24	0.217
Inhaled corticosteroids	17 (65%)	97 (68%)	0.821
Post bronchodilator predicted FEV ₁ , %	37 \pm 18	52 \pm 18	<0.001
Post bronchodilator predicted FVC, %	69 \pm 23	81 \pm 20	0.013
Post bronchodilator FEV ₁ /FVC	0.42 \pm 0.1	0.49 \pm 0.1	0.015
Induced sputum produced	17 (65%)	94 (66%)	1.00
White cell count, $\times 10^9/L$	8.8 \pm 4	7.4 \pm 2	0.059
Blood neutrophils, $\times 10^9/L$	6.2 \pm 3	4.7 \pm 2	0.031
C-reactive protein, mg/L*	5.5 (4-9)	3.5 (2-7)	0.071
Sputum neutrophils, %*	95 (88-97)	88 (74-93)	0.004
Sputum IL-1 β , pg/ml*	15 (0-54)	8 (0-30)	0.441
Sputum IL-6, pg/ml*	21 (0-78)	27 (8-62)	0.587
Sputum IL-8, pg/ml*	455 (310-748)	451 (266-867)	0.888

Figure 5.8. Box and whisker plot showing the relationship between oxygen saturation and blood neutrophils. Oxygen saturations $\leq 93\%$ (n=26), oxygen saturations $> 93\%$ (n=146), p=0.03.

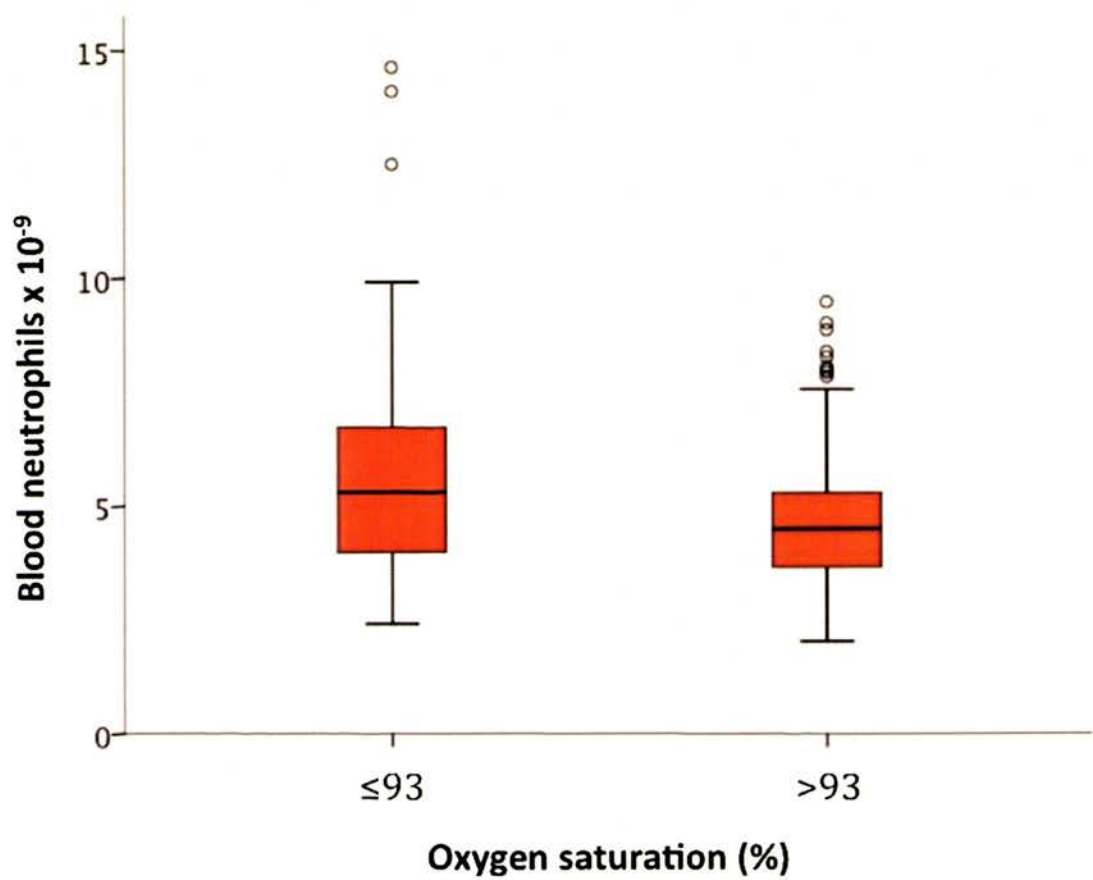


Figure 5.9. Box and whisker plot showing the relationship between induced sputum neutrophils and oxygen saturations in COPD patients. Oxygen saturations $\leq 93\%$ (n=17), oxygen saturations $> 93\%$ (n=89), p=0.004.

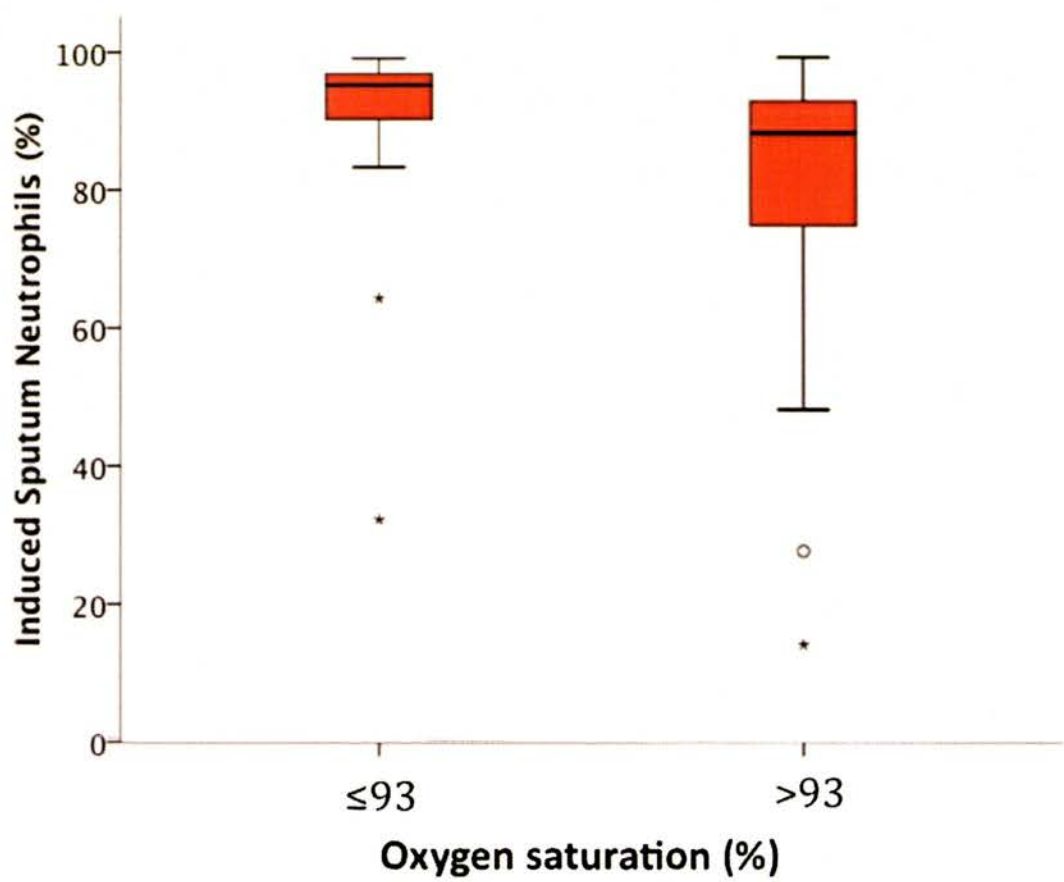


Table 5.15. Binary logistic regression of blood neutrophils with oxygen saturations (> 93%) as the outcome variable, adjusting for FEV₁ (post-bronchodilator % predicted). The model was repeated with log CRP and sputum neutrophils but neither was significantly associated with oxygen saturations after adjusting for FEV₁.

	B	SE	df	Wald	p	Exp (B)
FEV₁ post-bronchodilator % predicted	0.042	0.16	1	7.288	0.007	1.043
Blood neutrophils	-0.253	0.109	1	5.422	0.020	0.777
Intercept	1.186	0.962	1	1.519	0.218	3.27

-2 Log likelihood 120.7 (Cox % Snell R Square 0.111, Nagelkerke R square 0.194), $p<0.001$

BODE index

Six minute walking distance was recorded in 112 patients and the BODE index was calculated for these subjects. Induced sputum data were available for 62 of these patients. Age, gender, smoking status and smoking pack year history were not associated with the total BODE index score. BODE index score correlated significantly with SGRQ total score (0.660, $p<0.001$), exacerbation frequency (0.411 $p<0.001$) and oxygen saturations (-0.265, $p=0.006$). Blood white cell count, blood neutrophils, log CRP and log sputum IL-8 correlated significantly with total BODE index score (Table 5.16). SGRQ total score, exacerbation frequency, oxygen saturations, blood neutrophils, log CRP and log IL-8 were analyzed with BODE index score using linear regression. SGRQ remained significantly associated with BODE index after including the other variables in the model (Table 5.17).

Table 5.16. BODE index correlations with inflammatory markers. Data are presented as Pearson's correlation co-efficient (parametric data) and *Spearman's correlation co-efficient (non-parametric data) with statistical significance.

	Correlation	p
White cell count, x10⁹/L	0.256	0.008
Blood neutrophils, x10⁹/L	0.271	0.005
Log C-reactive protein, mg/L	0.213	0.025
Sputum neutrophils, %*	0.020	0.882
Sputum IL-1β, pg/ml*	-0.120	0.354
Sputum IL-6, pg/ml*	-0.070	0.586
Log sputum IL-8, pg/ml	-0.302	0.022

Table 5.17 Multiple linear regression model of BODE index score with log induced sputum IL-8 after controlling for SGRQ total score, exacerbation frequency and oxygen saturations. The model was repeated with blood neutrophils and log CRP but neither were significantly associated with BODE index after controlling for the other variables.

	B	SE	t	p
Oxygen saturation %	-0.055	0.089	-0.626	0.534
Intercept	10.852	8.385	1.294	0.202
Exacerbation frequency	0.147	0.091	1.612	0.113
Log sputum IL-8	-0.350	0.209	-1.674	0.100
SGRQ total score	0.056	0.009	6.520	<0.001

R squared = 0.578 (Adjusted R Squared = 0.545), p<0.001

Discussion

The aim of this chapter was to investigate the relationship between inflammation and clinical parameters that have been associated with prognosis in COPD. Quality of life and dyspnoea were associated with markers of systemic inflammation after adjusting for potential confounding factors, but were not associated with markers of airway inflammation. Hypoxia was associated with increased blood neutrophils after controlling for potential confounding factors. Markers of systemic inflammation and sputum IL-8 correlated with total BODE index score. Exacerbation frequency and BMI were not associated with blood or sputum inflammation after controlling for airflow limitation. Sputum IL-6 and IL-1 β were not associated with any of the clinical parameters investigated.

Inflammation and quality of life in COPD

Systemic inflammation was associated with health related quality of life in this cohort but pulmonary inflammation was not. Blood neutrophils and CRP were significantly associated with all domains of SGRQ independent of smoking status, induced sputum production and post-bronchodilator % predicted FEV₁. This is consistent with previous reports with respect to CRP. Garrod and co-workers found that CRP and IL-6 were associated with SGRQ and MRC dyspnoea scores in COPD subjects (n=41), whereas TNF α and neopterin were not. (356) Broekhuizen and co-workers found that COPD patients with CRP levels >4.21mg/L had higher SGRQ total scores compared to those with low systemic levels of CRP. (105) No previous reports of an association between leukocytosis and quality of life in COPD were found. Only one study reported an association

between increased numbers of sputum inflammatory cells and SGRQ in COPD (n=102), but this study did not investigate markers of systemic inflammation and found no association between SGRQ and FEV₁. (117) Our findings therefore add to the existing literature by investigating blood and sputum inflammation with respect to quality of life and dyspnoea in a single COPD cohort.

The association we found between systemic inflammation and poor quality of life may have implications for symptom management in COPD. An improvement in health-related quality of life in COPD has been seen in response to therapeutic interventions, even when there was no improvement in lung function. (110, 127) This may be because treatments impact on systemic and pulmonary inflammation separately. For example, while treatment with oral theophylline reduced sputum neutrophils and the sputum total inflammatory cell count in one study, other sputum markers (IL-8 and MPO), CRP and QOL scores did not improve. (357) Therefore, while there was some evidence that lung inflammation was reduced in response to theophylline, there was not the same effect on systemic inflammation. Combination inhaled corticosteroids and bronchodilators may also reduce pulmonary inflammation but do not seem to reduce systemic inflammation. (110, 358) Perng and co-workers reported a reduction in sputum IL-8 and MMP-9 following treatment with inhaled fluticasone and salmeterol. But there was no change in induced sputum cell counts, serum CRP, lung function or quality of life. (358) Inhaled fluticasone and salmeterol led to an improvement in health status and lung inflammation (surfactant protein D) in a study of 289 patients, but there was no difference in

systemic inflammatory markers (CRP or IL-6). (110) Treatment with ICS was a potential confounding factor in our study but was controlled for in the analysis. Our data showed a consistent relationship between health related quality of life and systemic inflammation, so perhaps therapies targeting systemic inflammation may result in improved quality of life in COPD.

Inflammation and low body mass index

BMI was not associated with systemic leukocytosis in our COPD cohort after controlling for airflow limitation. This is in keeping with an otherwise healthy population where obesity has been identified as a cause of leukocytosis. (359) But there is some evidence to support increased systemic inflammation in COPD patients with low BMI. Circulating TNF α and IL-1 β have been associated with cachexia in COPD. (157, 293, 360) Casadevall and co-workers found that IL-6 and TNF α were significantly increased in the intercostal muscles of COPD patients with a normal BMI compared to healthy subjects. (361) There is a relationship between systemic inflammation and cachexia in other diseases. IL-1, IL-6, interferon- γ and TNF α are the "classic cachectic cytokines". (362) Systemic inflammation with increased pro-inflammatory cytokines contributes significantly to cancer cachexia. (363, 364) Circulating IL-6 and blood neutrophils were associated with cachexia in gastro-oesophageal cancer patients, whereas IL-1, IL-8 and TNF α were not. (364) There are little data on systemic neutrophilia and BMI in COPD. This may be due to difficulty in extrapolating confounding factors, but we found no association after adjusting for disease severity and smoking habit.

We found no relationship between low BMI and CRP. This is also in concordance with published data suggesting that CRP is more strongly associated with obesity. A recent study of 628 COPD patients found, after adjusting for confounding factors, that CRP was increased in obese and overweight COPD patients, but not in those with a low BMI. (365) Broekhuizen and co-workers found a positive association between CRP and BMI (n=102), after adjusting for FEV₁, age, sex, TL_{CO} and FFM. (105) A smaller study found that CRP and TNF α were raised in COPD patients with a low BMI, n=35. (366) A Norwegian population study reported that elevated BMI was a strong predictor of raised CRP, independent of airflow limitation. (367) CRP levels may be influenced by statin use and possibly also by inhaled corticosteroids. In the Norwegian population cohort (n=3877), statin use was strongly associated with low CRP, whereas inhaled corticosteroids were not. (367) In a COPD cohort however, inhaled corticosteroid use was associated with lower circulating CRP. (111) Treatment with pravastatin reduced systemic inflammation (CRP) and improved exercise capacity in a randomized, double-blind, placebo controlled trial in COPD (n=125). (368) We found no association between markers of systemic inflammation and BMI after adjusting for potential confounding factors including the effects of statins and inhaled corticosteroids.

There is a complex interaction between nutrition, exercise, dyspnoea and cachexia in COPD. (88, 154) Systemic inflammation has been proposed as a factor in this interaction and there is some data to support this. (88, 369) Eid

and co-workers found increased levels of systemic inflammation (IL-6, TNF α and their soluble receptors) in COPD patients with a low creatinine-height index even when BMI was normal. (88) Systemic levels of TNF α and CRP were lower in more active COPD patients (n=341). (370) Whereas reduced physical activity in COPD patients was associated with higher CRP and fibrinogen and with left ventricular dysfunction. (371) Adipose tissue may influence levels of systemic inflammation. It has been proposed that inappropriate levels of leptin and TNF α may lead to altered nutritional parameters and BMI in COPD. (372) Circulating IL-6 and BMI were predictive of increased insulin resistance in a COPD population (n=56). (158) TNF α and oxidative stress burden have been proposed to link weight loss and muscle wasting in COPD. But BMI was not associated with either circulating TNF α or oxidative stress burden measured by malonyldialdehyde (MDA) in a study of male COPD patients, n=52 (373) or with serum or sputum TNF α levels in another study, n=26. (374)

Body mass index was associated with increased sputum leukocytes and sputum IL-6 after adjusting for current smoking status but not after adjusting for airflow limitation. BMI has not previously been associated with increases in induced sputum cells or cytokines. There was no association between BMI and sputum cell counts in a normal adult population or in those with asthma, n=727. (375) In 100 COPD patients, Ischaki and co-workers found that FFM was more closely associated with disease severity (GOLD stage) than was BMI (n=100), but neither parameter was associated with induced sputum neutrophils or LTB-4. (154) No other studies investigating the relationship between sputum IL-6 and

BMI in COPD could be found. Our finding that BMI was not associated with blood or sputum inflammation is in concordance with existing literature. But the role of blood neutrophils may merit further investigation in the sub-phenotype of COPD patients who lose body mass progressively over time.

Inflammation and exacerbation frequency

Frequent exacerbators had increased blood neutrophils and increased sputum IL-6 in this cohort, but neither was associated with exacerbation frequency after adjusting for FEV₁. COPD exacerbations have been extensively investigated with respect to inflammation. Our data confirm the findings of others that sputum IL-6 was increased in COPD patients with > 2 exacerbations per year. (71, 72, 150, 376) But many of these studies did not control for confounding factors such as the severity of airflow limitation. Bhowmik and coworkers described increased IL-6 in sputum in stable COPD patients with > 2 exacerbations per year (n=44), with a further increase in IL-6 during an exacerbation (n=37). (71) Aaron and coworkers (377) found an increase in IL-8 and TNF α in induced sputum during COPD exacerbations (n=14) with levels returning to baseline after the exacerbation. Microbial colonization in COPD was associated with sputum neutrophilia. (320) In the study by Marin and co-workers, 71% of moderate COPD patients in the stable state had evidence of bacterial colonization and this was associated with sputum neutrophilia and a faster decline in FEV₁. This study measured an array of sputum cytokines (TNF α , IL-1 β , IL-6, IL-8, IL-10 and IL-12) but only IL-1 β and IL-12 were increased in patients with bronchial colonization, and this was only the case for patients with *Haemophilus*

influenzae. (320) Aaron and co-workers (377) found no difference in induced sputum cytokine levels in patients with and without a documented bacterial or viral infection, suggesting that bacterial colonization is not likely to be solely responsible for the increased level of sputum IL-6 found in our cohort. There is therefore substantial evidence that markers of blood and sputum inflammation increase during COPD exacerbations, but after controlling for confounding factors, there was no baseline increase in these markers in stable COPD patients.

Exacerbation frequency is associated with increased systemic inflammation in COPD. (72, 151, 152, 378) Hurst and co-workers from the ECLIPSE study have recently shown that WCC was associated with exacerbation frequency after controlling for potential confounding factors including FEV₁. (378) This suggests that our study may have been under-powered to show this. Other studies have shown associations between markers of systemic inflammation and exacerbations frequency. For example, in microarray analysis, 24 serum biomarkers (a variety of chemoattractants, and markers of inflammation, destruction and repair) were associated with exacerbation frequency in severe COPD, although markers such as C-reactive protein were not included in the analysis. (153) CRP has been found to predict exacerbations in COPD in one study, (379) and levels take longer to return to baseline following an exacerbation. (376) But in our cohort, CRP was not significantly associated with exacerbation frequency in stable COPD patients. In the study by Perera and co-workers, CRP was elevated at day 14 post-exacerbation, (376) whereas our

COPD patients were sampled at least 6 weeks post-exacerbation. Donaldson and co-workers found that sputum IL-6 and plasma fibrinogen increased during exacerbations, and yet there was no relationship between these 2 markers. They therefore suggested that mechanisms other than “overflow” of pulmonary inflammation into the systemic compartment might be responsible for the systemic inflammation found during exacerbations. (72) We found a similar inflammatory profile in blood and sputum from COPD patients with a low BMI and from frequent exacerbators. While this may be due to confounding from disease severity and smoking history, systemic inflammation and inactivity are key mechanisms relating COPD to its associated co-morbidities. Exacerbations are associated with both of these factors and may be a key link between pulmonary and systemic inflammation and the systemic consequences of COPD. (72, 91)

Inflammation and hypoxia

Blood leukocytes were associated with hypoxaemia in this cohort after adjusting for potential confounding factors. Few studies have reported a relationship between neutrophilia and hypoxaemia. An influx of neutrophils and macrophages to the alveolar space occurred in response to moderate hypoxaemia in rats, (164) and neutrophil numbers are increased in muscle fibres from hypoxic COPD patients. (380) Hypoxia provides a potential causative link between pulmonary and systemic inflammation. Hypoxia induces expression of inflammatory cytokines *in vitro* (381-383) through the activation of NFκB. (384) Yet human and animal studies report mixed associations

between hypoxaemia and levels of inflammatory cytokines. Savransky and co-workers and Mikko and co-workers found no increase in inflammatory cytokines in response to hypoxaemia. (385, 386) Whereas Jammes and co-workers found an inverse relationship between resting levels of inflammatory cytokines (IL-6 and TNF α), oxidative stress (TBARS) and pO₂ in COPD patients, with a further increase in IL-6 in response to exercise induced hypoxaemia. They suggested therefore that intermittent hypoxia might have a role in the induction of inflammation in COPD. (387) Studies at altitude describe an increase in systemic inflammation (IL-1 receptor antagonist, IL-6 and CRP) that may be hypoxia induced. (166) We did not find a relationship between CRP and hypoxaemia, but tissue hypoxia was independently associated with CRP in one study of 50 patients. (388)

Hypoxaemia is associated with increased platelet activation, the up-regulation of intracellular adhesion molecules (167) and the development of atherosclerosis. (385) Some of these factors, in addition to hypoxia induced haemodynamic stress may be associated with the increased incidence of cardiovascular disease in COPD. (168) Hypoxaemia increases inflammation through mechanisms that alter vascular responses. (169, 170) Activated T cells from COPD patients secreted significantly more VEGF in response to hypoxia than cells from healthy subjects. (386) Hypoxic COPD patients with pulmonary hypertension confirmed on right heart catheterization had increased levels of circulating IL-6 and an increase in IL-6 mRNA in response to hypoxia. (389) Hypoxia inducible factor (HIF-1 α) has a key role in the regulation of pulmonary

vascular remodeling in response to hypoxaemia. (Wouters PATS 2005) It was proposed that the primary defect causing emphysema might result from disordered vascular flow mediated by hypoxic pulmonary vasoconstriction. (172) Remy-Jardin and colleagues (171) found that emphysematous regions often developed at a site that had previously shown signs of inflammation (nodules or ground glass). In the presence of inflammation, the usual pulmonary response of diverting blood flow from hypoxic alveolar regions is reversed. Neutrophils may be implicated in this process. In some cases, smokers exhibit blunting of this process. In these individuals, inflammatory regions associated with hypoxaemia experience a further limitation in blood flow, resulting in tissue destruction. (172) Further work is required to investigate the role of neutrophils in response to hypoxia in COPD.

The BODE index

Blood neutrophils, CRP and sputum IL-8 were significantly associated with the BODE index. This is interesting as the BODE index was designed as a composite score to represent both the pulmonary and systemic features of COPD. The BODE index has previously been associated with markers of systemic inflammation. (153, 371) But this is the first study to find an association between BODE score and sputum inflammation. IL-8 was not associated with any of the other clinical features of COPD assessed in this chapter. Further work will be required to establish whether measurement of these inflammatory markers can add to the important prognostic information provided by the BODE index.

Limitations

We investigated the association between BMI and inflammation. While BMI is associated with prognosis in COPD, it has been proposed that fat free mass might be a more pertinent measurement of weight loss and muscle dysfunction in COPD. (154) However, both BMI and FFM are independent predictors of mortality in COPD, (15) and BMI instead of FFM is incorporated in the BODE index. (118)

Exacerbation frequency was recalled subjectively by study subjects and may therefore be prone to inaccuracy. This was not primarily designed as a study of COPD exacerbations. Exacerbations have been the focus of study by other research groups who used more accurate recording of exacerbation frequency and symptoms, for example with diary cards and telephone follow up. (71, 72) However, our results are concordant with the findings of others. Microbiology assessment in sputum was not made in this study, and bacterial colonization is a potential cause of an increased frequency of exacerbations in COPD. Studies investigating sputum inflammation in response to bacterial colonization in COPD show conflicting results. (320, 377)

Hypoxia was measured using oxygen saturations instead of the more accurate arterial blood gas measurement. Pulse oximetry is particularly useful as a non-invasive measure of relative hypoxia (355) in clinical practice and is now the main tool utilized in the British Thoracic Society oxygen prescribing guidelines. (390) A large meta-analysis reported that pulse oximetry was accurate to within

2% (± 1 SD) or 5% (± 2 SD) compared to PaO_2 over the SaO_2 range 70%-100%.

(391)

Conclusions

Different profiles of pulmonary and systemic inflammation were expressed with different clinical features of COPD. Increased systemic inflammation was associated with reduced quality of life and more dyspnoea. Blood neutrophilia was associated with hypoxia. Inflammatory markers in blood and sputum were not associated with BMI or exacerbation frequency after controlling for potential confounding factors. BODE index was associated with both pulmonary and systemic inflammation. Pulmonary and systemic markers of inflammation may be useful in further defining clinical phenotypes for the purpose of prognosis and treatment in COPD.

CHAPTER 6. Quantitative computed tomography evaluation of COPD with clinical and inflammatory features

Introduction

COPD is a pathologically heterogeneous disease. With the increasing use of radiological imaging in clinical practice, CT scanning may be useful in further characterizing COPD. COPD results in characteristic anatomical changes in the lungs, particularly resulting in the development of emphysema. Despite the same stage of COPD according to lung function, the quantity of emphysema can vary widely. (205) Patients can have severe airflow limitation with minimal or no emphysema. (206, 208) Yet macroscopic emphysema has been demonstrated in never-smokers (209) and post-mortem studies have found significant emphysema in patients with normal lung function. (210) The extent of emphysema does not fully account for the expiratory airflow limitation in COPD, as small airway disease is the predominant factor. (206, 207)

CT can be used to provide an objective measure of lung density in Hounsfield Units (HU). (184, 185, 204) The most widely used method for objectively quantifying emphysema using CT is the “density mask” technique, (186) in which regions of signal density are allocated a value in Hounsfield Units (HU) based on their intensity index on a grey scale. Zero HU represents the density of water and -1000 HU represents the density of air. A threshold can be applied (close to the HU value for air) to dichotomize regions to be denoted as ‘normal’ lung parenchyma or as emphysematous. No fixed value has yet been established

for this threshold, but commonly used cut-offs are -910 and -950 HU. (188) The low-density threshold that quantifies emphysema has been validated against pathology (184, 186, 212, 392)

An alternative measurement of lung density was pioneered in COPD patients in Edinburgh in the 1980's. (204) This measurement, known as the "percentile point," was used to characterize the low-density region of the frequency distribution histogram. This measurement was then developed further in European studies of alpha-1 antitrypsin deficiency. Dirksen and coworkers analyzed lung density using 5-15% of the lowest attenuation voxels (e.g. 5th or 15th percentile point). (193) The 15th percentile is defined as the value in Hounsfield Units below which lie 15% of the lowest density lung voxels. (264) This method is less variable than the density mask technique and is significantly less variable than measurements of pulmonary function. (193) In the EXACTLE study of 77 patients with alpha-1 antitrypsin deficiency, Parr and co-workers found that CT densitometry was able to detect progressive loss of lung tissue over a 2.5-year follow-up period and was more sensitive than lung function or quality of life measures. (393)

In this study, a CT protocol and software were developed to measure lung volume and density in a cohort of stable COPD patients. Density was measured using both a density mask (pixel index threshold -910 and -950 HU) and the 15th percentile point. These parameters were then related to clinical and inflammatory parameters in a COPD cohort. The hypothesis was that both

techniques for measurement of density would be similarly associated with lung function; and that both high lung volumes and low lung density would be associated with reduced lung function. The secondary objective was to determine the association between lung density and markers of systemic and airway inflammation and clinical features of the disease.

Aims

In a cohort of stable COPD patients to investigate the relationship between CT lung volume and density parameters with:

1. Lung function
2. Markers of pulmonary and systemic inflammation
3. Clinical features

Methods and data analysis

COPD patients were recruited and underwent low dose thoracic CT scanning at full inspiration and on expiration as described in Chapter 2. The details of patient recruitment and the CT protocol are described in detail in Chapter 2. Demographic data for the COPD cohort was tabulated using mean (standard deviation) for normally distributed variables, number (%) for categorical data, and median (inter-quartile range) for abnormally distributed data. CT data on lung volumes and density on inspiration and expiration were recorded. Density was measured using the density mask (pixel index -950 HU and pixel index -910 HU) and the 15th percentile point. Pixel index -950 (inspiration only) and pixel index -950 and -910 (expiration) had a skewed distribution and were log-transformed for analysis. CT measurements were then analyzed with spirometry, markers of inflammation and clinical features using Pearson's correlation for normally distributed variables, Spearman's correlation for abnormally distributed variables and the Chi-squared test for categorical variables. Clinical features and inflammatory markers were categorized or log-transformed for analysis as described in Chapter 5. Factors associated with lung density in univariate analysis were analyzed in a multiple linear regression model.

Results

CT scans were obtained on inspiration and expiration in 117 COPD patients.

Clinical baseline data for these patients compared to patients in the same cohort who did not undergo a CT scan are displayed in Table 6.1. More male (64%) than female subjects (36%), and more ex-smokers (70%) than current smokers (30%) attended for a CT scan. COPD severity by GOLD stage ranged from I to IV (GOLD I n=9 (8%), GOLD II n=49 (42%), GOLD III n=45 (38%), GOLD IV n=14 (12%). 67 of 117 patients (57%) produced an adequate sample of induced sputum for analysis. Compared to these patients, COPD patients from the same cohort who did not have a CT scan were more likely to be current smokers ($p=0.008$), to have a lower post bronchodilator FVC ($p=0.02$), to have higher SGRQ scores in all domains and a higher MRC dyspnoea score. Subjects who had not had a CT scan did not have 6 minute walking distance recorded and therefore did not have a BODE index calculated.

Table 6.1. Clinical data for the COPD cohort who had a CT scan compared to COPD patients for whom a CT scan was not obtained. Data are presented as mean \pm SD for parametric variables, *median (IQR) for non-parametric variables and † number (%) for categorical variables.

COPD subjects		CT	No CT	p
		n=117	n=65	
Age		68 \pm 8	69 \pm 8	0.395
Gender, % male †		74 (64%)	38 (58%)	0.530
Current smokers, % †		35 (30%)	36 (55%)	0.008
Smoking pack years		45 \pm 21	52 \pm 29	0.087
FEV ₁ , post bronchodilator % predicted		51 \pm 19	47 \pm 19	0.113
FVC, post bronchodilator % predicted		81 \pm 21	74 \pm 20	0.013
FEV ₁ /FVC ratio, post bronchodilator		0.49 \pm 0.1	0.48 \pm 0.1	0.727
Body Mass Index, kg/m ²		26 \pm 6	25 \pm 5	0.151
Oxygen saturation, %		96 \pm 2	95 \pm 3	0.225
Annual exacerbation frequency*		2 (1-3)	2 (1-3)	0.639
St George's Respiratory Questionnaire	Symptoms	60 \pm 22	67 \pm 18	0.022
	Activity	62 \pm 25	74 \pm 20	<0.001
	Impact	34 \pm 21	44 \pm 22	0.002
	Total score	47 \pm 20	57 \pm 181	0.001
MRC dyspnoea score †	1-2	56 (49%)	15 (23%)	0.001
	3	28 (24%)	18 (28%)	
	4-5	31 (27%)	31 (48%)	
MRC chronic bronchitis score †	Yes	52 (49%)	31 (48%)	1.00
	No	55 (51%)	32 (49%)	
6 minute walking distance, m		73 \pm 53	-	
BODE index		6.7 \pm 3	-	

Examples of the CT images obtained are shown in Figure 6.1. CT measurements of lung volume and density were made on inspiration and expiration. These results are displayed in Table 2. Values for air calibration are also shown in inspiration and expiration. As described more fully in the main methods section, CT scanner calibration was undertaken according to the manufacturer's instructions. The Hounsfield Unit (HU) value for air was recalibrated using a method similar to that described by Stoel and Stolk. (195) The FC-03 filter was used to correct for the air offset in the Toshiba CT scanner. CT values for lung density shown in Table 6.2 were corrected according to the value for air calibration.

Figure 6.1. CT images of COPD subjects with (a) mild emphysema and (b) severe emphysema. The subject in image (b) also has upper lobe predominant emphysema. The images were acquired at full inspiration and full expiration (see Chapter 2). Using density mask software, the lungs have been segmented from other anatomical structures (e.g. mediastinum and chest wall). Air in the trachea has also been segmented out of the analysis. The highlighted regions represent emphysema (regions of lung density below -950 Hounsfield Units). Each colour represents a confluent region of emphysema of a different size: red representing the largest confluent regions, followed by yellow and green, with blue representing the smallest confluent regions of low lung density.

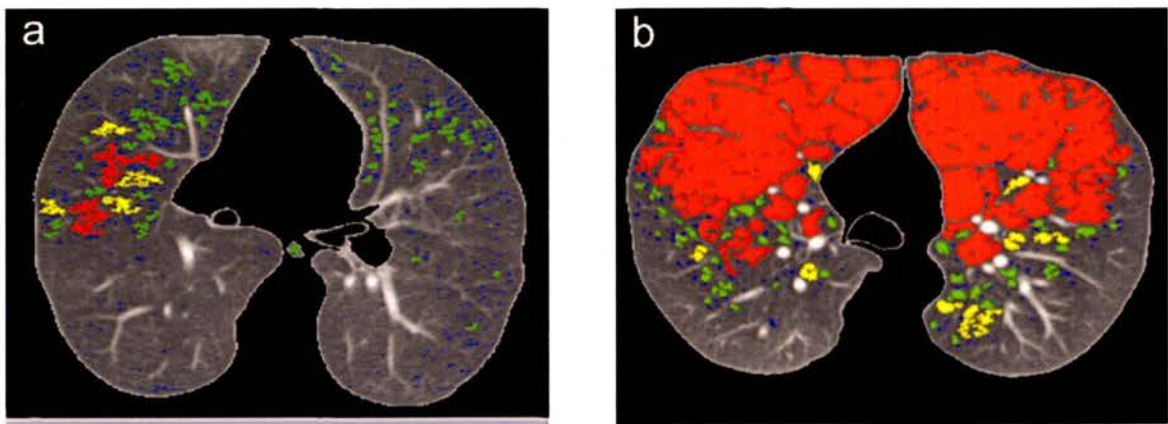


Table 6.2. CT parameters are shown for the COPD cohort (n=117) on inspiration and expiration. Data are presented as mean \pm SD for parametric variables and median (IQR)* for non-parametric variables. Pixel index and 15th percentile values are corrected for air calibration. Pixel index -950 (inspiration and expiration) and pixel index -910 (expiration only) were log-transformed to achieve normal distribution. Pixel index -910 (inspiration) was normally distributed.

CT parameters	Inspiration	Expiration
Volume, L	6.4 \pm 2	4.9 \pm 1
Pixel index -950, %	7% (2-17%)*	3% (1-11%)*
Log pixel index -950	-2.95 \pm 1.5	-3.73 \pm 1.8
Pixel index -910, %	33% \pm 18%	14% (6%-32%)*
Log pixel index -910	-1.3 (0.8)	-2.2 (1.3)
15th percentile point, HU	-934 \pm 29	-910 \pm 41
Air calibration, HU	-987 \pm 1	-987 \pm 2

CT parameters and lung function

Correlations between CT parameters of lung volume, density in inspiration and expiration and lung function parameters are shown in Tables 6.3 and 6.4. Lung volume and density (pixel index -950, pixel index -910 and 15th percentile point) were significantly associated with airflow limitation measured by post-bronchodilator % predicted FEV₁ and by FEV₁/FVC ratio on inspiration and expiration ($p < 0.001$). The strongest correlations were seen for FEV₁/FVC ratio and the expiratory values. Of the lung density parameters measured, pixel index -910 was most strongly correlated with FEV₁ and FEV₁/FVC ratio. Post-bronchodilator % predicted FVC was not associated with CT lung volume or density measurements on inspiration, but there was a significant relationship with lung volume on expiration ($p = 0.003$). This “post-bronchodilator percent predicted FVC” measurement has intrinsic correction for height and gender. Post-bronchodilator FVC (not taken as % predicted) however, was significantly associated with lung volume on inspiration ($r = 0.450$, $p < 0.001$), and expiration ($r = 0.212$, $p = 0.022$) but not with any of the measurements of CT lung density.

Table 6.3. Correlation of lung function with CT parameters on inspiration.

Pearson's correlation coefficients are displayed for each parameter, together with statistical significance. Pixel index -950 was log-transformed for analysis to achieve normal distribution. Pixel index -910 on inspiration was normally distributed.

Lung function	CT parameters, inspiration	Correlation	p value
FEV₁, post bronchodilator % predicted			
	Lung volume, L	-0.337	<0.001
	Log pixel index, -950 HU	-0.472	<0.001
	Pixel index, -910 HU	-0.504	<0.001
	15 th percentile	0.415	<0.001
FVC, post bronchodilator % predicted			
	Lung volume, L	-0.141	0.134
	Log pixel index, -950 HU	-0.055	0.560
	Pixel index, -910 HU	-0.077	0.411
	15 th percentile	-0.042	0.657
FEV₁/FVC ratio, post bronchodilator			
	Lung volume, L	-0.423	<0.001
	Log pixel index, -950 HU	-0.688	<0.001
	Pixel index, -910 HU	-0.731	<0.001
	15 th percentile	0.588	<0.001

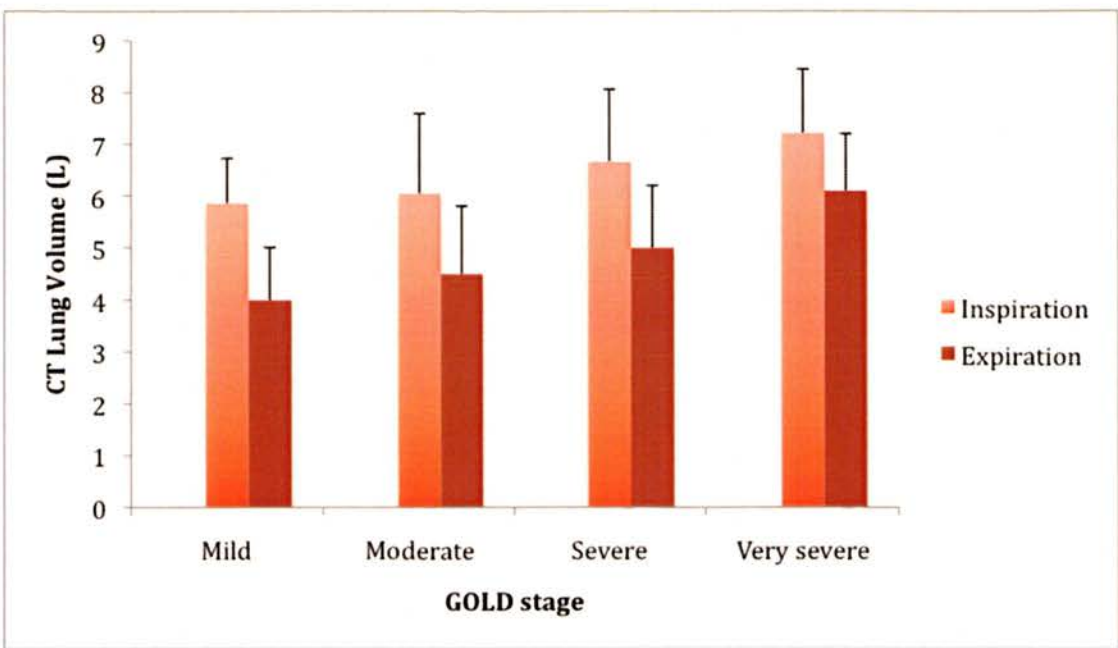
Table 6.4. Correlation of lung function with CT parameters on expiration.

Pearson's correlation coefficient is displayed for each parameter, with statistical significance. Pixel index -950 and pixel index -910 were log-transformed for analysis to achieve normal distribution.

Lung function	CT parameters, expiration	Correlation	p value
FEV₁, post bronchodilator % predicted			
	Lung volume, L	-0.481	<0.001
	Log pixel index, -950 HU	-0.512	<0.001
	Log pixel index, -910 HU	-0.545	<0.001
	15 th percentile	0.498	<0.001
FVC, post bronchodilator % predicted			
	Lung volume, L	-0.271	0.003
	Log pixel index, -950 HU	-0.085	0.366
	Log pixel index, -910 HU	-0.123	0.188
	15 th percentile	0.103	0.276
FEV₁/FVC ratio, post bronchodilator			
	Lung volume, L	-0.504	<0.001
	Log pixel index, -950 HU	-0.701	<0.001
	Log pixel index, -910 HU	-0.702	<0.001
	15 th percentile	0.636	<0.001

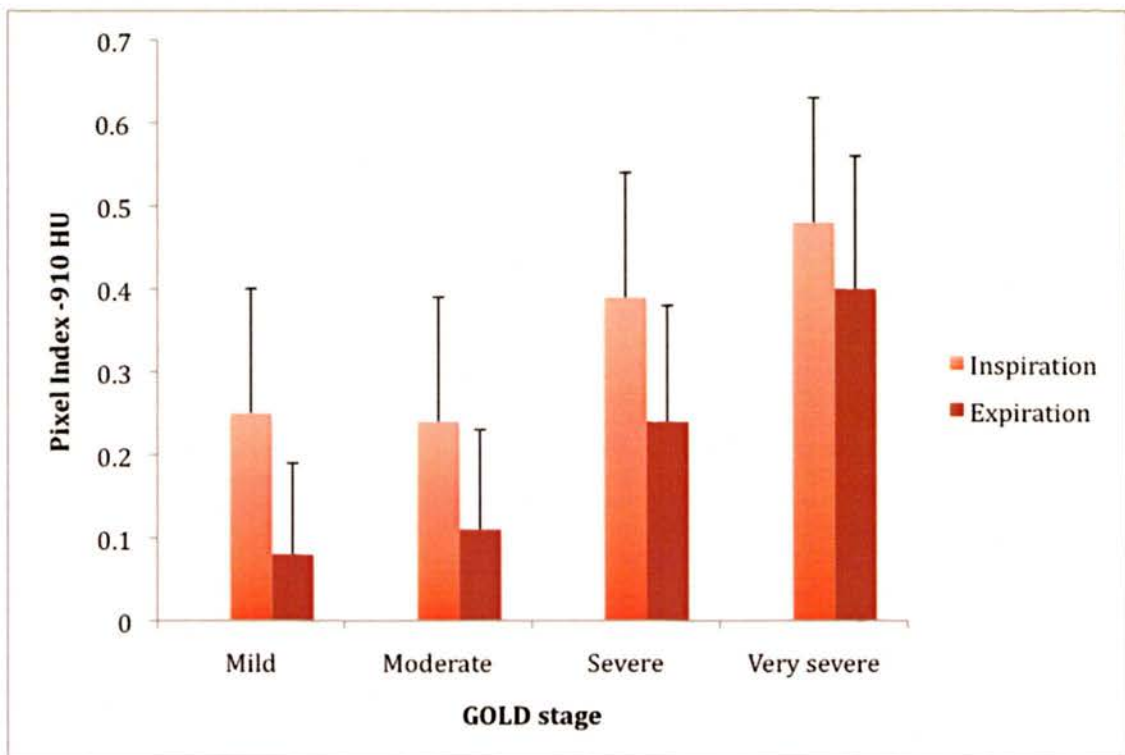
CT lung volumes on inspiration and expiration increased significantly with COPD severity according to GOLD stage, $p < 0.001$ (Figure 6.2). Lung density on inspiration and expiration was significantly lower with more severe COPD according to GOLD stage, $p < 0.001$ (Figures 6.3 and 6.4), irrespective of the density parameter that was measured. The difference in lung volumes and lung density between inspiration and expiration is smaller with worsening airflow limitation.

Figure 6.2. The relationship between the severity of airflow limitation (GOLD stage) and CT lung volumes on inspiration and expiration for the COPD cohort. More severe airflow limitation was associated with higher lung volumes on inspiration ($p=0.017$) and expiration ($p<0.001$, ANOVA) and a smaller inspiratory/expiratory volume ratio. The histograms represent the mean and the error bars represent the standard deviation.



Number of subjects for each GOLD stage: I (mild) $n=13$, II (moderate) $n=70$, III (severe) $n=73$, IV (very severe) $n=26$.
 Bonferroni post-hoc analysis on inspiration: GOLD II: GOLD IV $p=0.04$. Bonferroni post-hoc analysis on expiration: GOLD I:III $p=0.02$, GOLD I:IV $p<0.001$, GOLD II:III $p=0.045$, GOLD II:IV $p<0.001$, GOLD III: GOLD IV $p=0.02$.

Figure 6.3. The relationship between CT lung density (Pixel Index -910) and airflow limitation (GOLD stage) on inspiration and expiration in the COPD cohort. More severe airflow limitation is associated with lower lung density (higher pixel index at the -910 threshold) on inspiration ($p<0.001$) and expiration ($p<0.001$, ANOVA). The histograms represent the mean and the error bars represent the standard deviation.



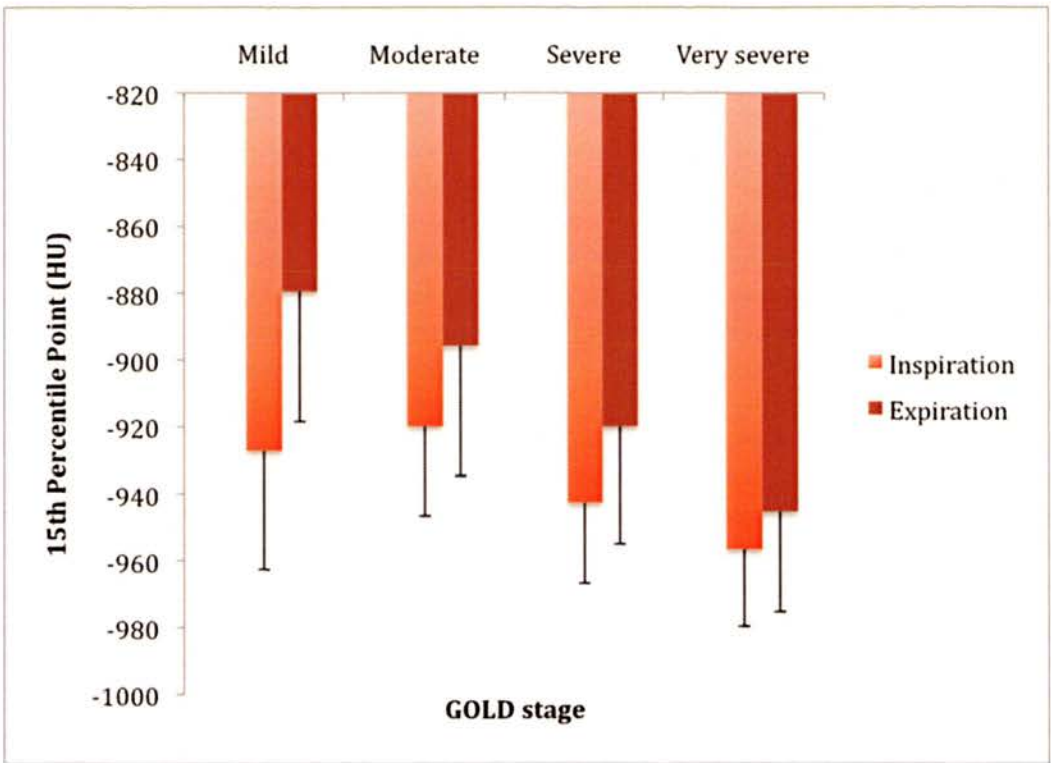
Number of subjects in each GOLD stage: I (mild) $n=13$, II (moderate) $n=70$, III (severe) $n=73$, IV (very severe) $n=26$.

Bonferroni post-hoc analysis on inspiration: GOLD I:IV $p=0.003$, II:III $p<0.001$, II:IV $p<0.001$.

Bonferroni post-hoc analysis on expiration: GOLD I:III $p<0.001$, I:IV $p<0.001$, II:III $p<0.001$, II:IV $p<0.001$.

Similar relationships were seen between GOLD stage, pixel index -950 and 15th percentile.

Figure 6.4. The relationship between airflow limitation (GOLD stage) and the quantity of emphysema on CT (15th percentile point) on inspiration and expiration in the COPD cohort. More severe airflow limitation is associated with lower lung density (more emphysema) on inspiration ($p<0.001$) and expiration ($p<0.001$, ANOVA). The histograms represent the mean and the error bars represent the standard deviation.



Number of subjects in each GOLD stage: I (mild) n=13, II (moderate) n=70, III (severe) n=73, IV (very severe) n=26.
 Bonferroni post-hoc analysis on inspiration: GOLD I:IV $p=0.056$, II:III $p<0.001$, II:IV $p<0.001$.
 Bonferroni post-hoc analysis on expiration: GOLD I:III $p=0.045$, I:IV $p=0.001$, II:III $p<0.001$, II:IV $p<0.001$.

CT parameters and markers of inflammation

Lung volume, pixel index and 15th percentile were analyzed with markers of pulmonary and systemic inflammation. There was no significant correlation between blood white cell count, neutrophils or C-reactive protein and the CT parameters in inspiration or expiration, and there were no significant correlations between induced sputum percentage neutrophils, IL-1 β , IL-6 or IL-8 and CT parameters on inspiration or expiration.

CT parameters and clinical features of COPD

Age and smoking pack years did not correlate significantly with CT lung volume or lung density values on inspiration or expiration (Tables 6.5). There was no difference in lung volumes or density in COPD patients who were current smokers compared to ex-smokers in inspiration or expiration (Table 6.6 and 6.7). As expected, lung volumes were significantly higher in men than women (inspiration $p=0.004$, expiration $p=0.015$) but there was no gender difference in lung density.

Body mass index and the BODE index correlated with all CT parameters (Table 6.5 and figures 6.6 and 6.7). Exacerbation frequency, MRC dyspnoea score, MRC chronic bronchitis score, oxygen saturations and 6 minute walking distance were not associated with CT lung volumes or lung density. SGRQ scores (total, activity, symptoms and impact) were not associated with CT lung volumes or lung density, except SGRQ activity score, which correlated significantly with the log expiratory pixel index -950 ($r=0.209$, $p=0.03$).

Table 6.5. Pearson's correlation coefficients are displayed for the relationships between clinical features (continuous variables) in the COPD cohort and CT volume and density indices on inspiration and expiration. Significant correlations are marked as * representing statistical significance at the $p < 0.05$ level.

CT	Age	Pack years	SGRQ total	SGRQ symp	SGRQ activity	SGRQ impact	BMI	6MW	BODE
Inspiration									
Volume	-0.08	-0.02	-0.09	-0.12	-0.02	-0.10	-0.23*	0.02	0.18*
Log PI -950	-0.04	-0.09	0.05	-0.08	0.17	0.01	-0.43*	0.07	0.33*
15 th perc	0.10	0.16	0.00	0.11	-0.11	0.03	0.52*	-0.09	-0.24*
Expiration									
Volume	-0.12	-0.11	0.05	0.04	0.11	0.02	-0.30*	-0.03	0.31*
Log PI -950	-0.09	-0.08	0.11	-0.02	0.21*	0.07	-0.43*	0.02	0.35*
15 th perc	0.11	0.08	-0.07	0.03	-0.17	-0.03	0.53*	-0.04	-0.32*

PI = Pixel Index.
15th perc = 15th percentile.
Pack years = smoking pack years.
SGRQ = St George's respiratory questionnaire.
Symp = symptoms.
BMI = body mass index (kg/m^2).
6MW = 6 minute walking distance (m).
BODE = BMI, airflow obstruction, dyspnoea and exercise index.

Table 6.6. CT lung volume and density indices on inspiration are displayed for categorical variables. CT parameters are displayed as mean \pm SD for each variable. Significance is shown as * representing statistical significance at the $p < 0.05$ level.

		Lung volume, L	Log PI -950	15 th percentile
Gender	Male	7.1 \pm 1*	5.4 \pm 1	-936 \pm 28
	Female	5.2 \pm 1	3.9 \pm 1	-930 \pm 29
Smoking status	Smoker	6.1 \pm 1	-3.2 \pm 2	-929 \pm 28
	Ex-smoker	6.6 \pm 1	-4.8 \pm 1	-936 \pm 29
Exacerbations	\leq 2 per year	6.3 \pm 1	-3.0 \pm 1	-933 \pm 27
	$>$ 2 per year	6.5 \pm 1	-2.9 \pm 2	-935 \pm 32
Oxygen saturations	\leq 93 %	6.5 \pm 1	-2.7 \pm 2	-939 \pm 34
	$>$ 93 %	6.3 \pm 2	-2.9 \pm 1	-934 \pm 28
MRC bronchitis	Yes	6.4 \pm 1	-2.7 \pm 1	-937 \pm 26
	No	6.4 \pm 2	-3.0 \pm 2	-932 \pm 29
MRC dyspnoea	0-2	6.4 \pm 1	-3.1 \pm 1	-931 \pm 29
	3	6.4 \pm 1	-2.8 \pm 1	-935 \pm 29
	4-5	6.2 \pm 0	-2.4 \pm 1	-941 \pm 21

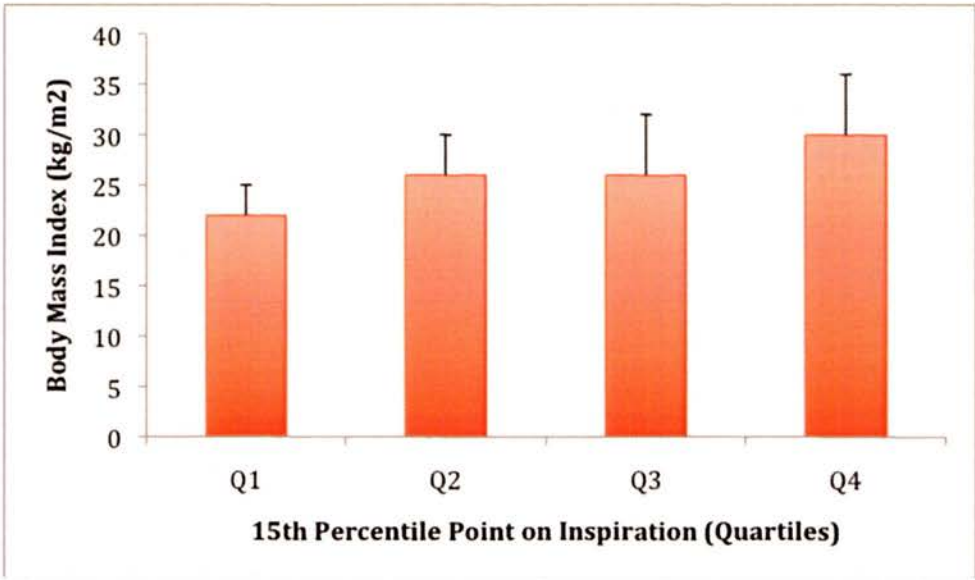
PI = Pixel Index

Table 6.7. Clinical features and CT volume and density indices on expiration are displayed for categorical variables. CT parameters are displayed as mean \pm SD for each variable. Significance is shown with * ($p < 0.05$).

		Lung volume, L	Log PI -950	15 th percentile
Gender	Male	5.4 \pm 1*	-3.5 \pm 2	-914 \pm 41
	Female	3.9 \pm 1	-4.1 \pm 2	-904 \pm 39
Smoking status	Smoker	4.8 \pm 1	-3.9 \pm 2	-906 \pm 37
	Ex-smoker	4.9 \pm 1	-3.6 \pm 2	-911 \pm 42
Exacerbations	\leq 2 per year	4.8 \pm 1	-3.8 \pm 2	-910 \pm 36
	$>$ 2 per year	4.9 \pm 1	-3.6 \pm 2	-911 \pm 48
Oxygen saturations	\leq 93 %	5.1 \pm 1	-3.3 \pm 2	-920 \pm 40
	$>$ 93 %	4.8 \pm 1	-3.6 \pm 2	-911 \pm 40
MRC bronchitis	Yes	4.8 \pm 1	-3.6 \pm 2	-914 \pm 38
	No	4.9 \pm 1	-3.9 \pm 2	-906 \pm 42
MRC dyspnoea	0-2	4.6 \pm 1	-4.0 \pm 2	-904 \pm 39
	3	5.0 \pm 2	-3.6 \pm 2	-914 \pm 42
	4-5	4.6 \pm 1	-3.3 \pm 2	-918 \pm 35

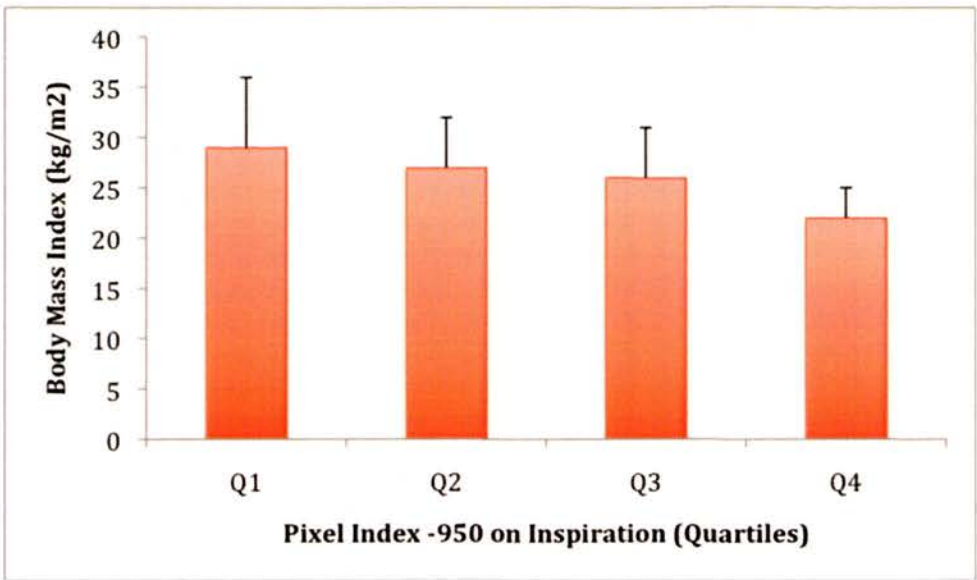
PI = Pixel Index

Figure 6.6. The relationship between lung density on inspiration measured by 15th percentile point and body mass index ($p < 0.001$, ANOVA) is displayed. The lowest 15th percentile quartile represents the lowest lung density (representing more emphysema). Significance taken as $p < 0.008$ due to multiple comparisons. The histograms represent the mean and the error bars represent the standard deviation.



Bonferroni post-hoc analysis of 15th percentile quartiles with BMI:
Q1:Q2 $p = 0.008$, Q1:Q3 $p = 0.008$, Q1:Q4 $p < 0.001$, Q2:Q4 $p = 0.02$, Q3:Q4 $p = 0.01$

Figure 6.7. The relationship between CT lung density (pixel index -950) on inspiration and body mass index ($p<0.001$, ANOVA) is displayed. The highest pixel index quartile represents the lowest lung density (representing more emphysema). Significance is taken as $p<0.008$ due to multiple comparisons. The histograms represent the mean and the error bars represent the standard deviation.



Bonferroni post-hoc analysis of Pixel Index -950 quartiles with BMI:
Q1:Q4 $p<0.001$, Q2:Q4 $p<0.001$, Q3:Q4 $p=0.015$.

Significant associations between lung density and clinical features in the univariate analysis were analyzed in a multiple regression model (Table 6.8). Pixel index -950 on expiration was the lung density parameter chosen as the dependent variable due to expiratory scans reflecting both small airways disease and emphysema. BMI, BODE index and SGRQ activity score were included in the model. BODE and BMI were analyzed in the model separately as they exhibited significant co-linearity due to the BMI component of the BODE index. BMI was independently associated with lung density after adjusting for airflow limitation (FEV₁/FVC ratio) but SGRQ activity score (p=0.8) and the BODE index (p=0.6) were not.

Table 6.8. Multiple linear regression of body mass index and SGRQ activity score with log pixel index -950 (on expiration) as the dependent variable, adjusting for post-bronchodilator FEV₁/FVC ratio.

	B	SE	t	p
Intercept	1.843	0.715	2.577	0.011
FEV₁/FVC ratio post-bronchodilator	-0.089	0.011	-7.845	<0.001
Body mass index	-0.051	0.023	-2.200	0.030
SGRQ activity	0.001	0.005	0.285	0.777

R squared = 0.517 (Adjusted R Squared = 0.504), p<0.001

Discussion

High lung volumes and low lung density were significantly associated with airflow limitation measured by FEV₁ and FEV₁/FVC ratio (post-bronchodilator % predicted values) and with low body mass index. FEV₁/FVC ratio showed the strongest correlations with CT lung volume and density. Expiratory CT parameters had a more pronounced association with airflow limitation than inspiratory parameters. After adjusting for the degree of airflow limitation, CT measurements of lung volume and density were not associated with SGRQ scores, MRC dyspnoea or chronic bronchitis scores, exacerbation frequency, oxygen saturations or the BODE index and there was no significant relationship between CT parameters and markers of pulmonary or systemic inflammation.

CT quantification of emphysema and lung function

Using an automated method of quantitative CT analysis, we found that different methods of measuring lung density – both a density mask at two different thresholds (-910 and -950 HU) and a pre-chosen percentile point (15th percentile) – were similarly associated with airflow limitation. We found the strongest correlations between CT lung density (log PI-910) and post-bronchodilator FEV₁/FVC ratio ($r=-0.7$). Previous studies reported similar results using a variety of techniques to measure lung density. (181, 182, 192, 203, 204) Early studies (192, 204) found that the lowest 5th percentile of the lung density histogram correlated with FEV₁/FVC ratio ($r=0.73$, $p<0.001$). Pathologically validated studies using a density threshold of -950 HU found that CT lung density was associated with FEV₁. (212, 392) Studies using semi-

quantitative assessment of whole lung density found a similar correlation between lung density and FEV₁. (203, 207) Nakano and co-workers (181) measured lung density in three CT slices and calculated the percentage of low attenuation areas (%LAA) at a threshold of -950 HU. They found that LAA% was associated with FEV₁ % predicted. Using fully automated analysis, Aziz and co-workers found that emphysema was significantly associated with FEV₁ ($r=-0.63$, $p=0.0005$, $n=101$). (182) Our results therefore confirm the results from previous work showing strong correlations between quantitative assessment of emphysema and airflow limitation.

The association between lung density and airflow limitation was more pronounced on expiration. Other studies also found better correlations between expiratory CT parameters and pulmonary function tests, correlations with FEV₁ improving from 0.5 to 0.7 between inspiration and expiration, which is consistent with our data. (213, 214) Interestingly, despite correlating well with FEV₁ and gas transfer factor, (207) lung density was not clearly associated with elastic recoil. (394) CT parameters on expiration may enable indirect measurement of some of the pathological processes occurring in COPD, such as gas trapping, altered elastic recoil and small airway disease. Yamashiro and co-workers (395) found that the ratio of expiratory to inspiratory lung volume was representative of expiratory to inspiratory mean lung density, reflecting gas trapping and airflow limitation ($n=46$). In contrast, they found evidence for more collapsible lungs, and therefore less gas trapping (increased inspiratory to expiratory volume ratio) in patients with milder airflow limitation. (395) We

found that the difference in lung volume and density between inspiration and expiration becomes smaller as airflow limitation progresses. Our data is supported by work by O'Donnell and co-workers. (328) This is a potentially useful phenotypic feature that is likely to be a reliable method of staging patients. Disease of the small airways (< 2 mm in diameter) is one of the defining features of COPD. Hogg and co-workers report a 100-fold reduction in airway lumen area in airways < 2 mm in diameter in COPD patients in micro-CT analysis of pathological samples and reported that this was likely to be one of the key mechanisms causing increased peripheral airway resistance and therefore gas trapping in COPD. (396) Micro-CT is not useful in a clinical context as it uses much higher radiation doses. Airways < 2 mm in diameter are difficult to measure on CT. (254) By providing a means to measure gas trapping, expiratory CT parameters may therefore provide an indirect measurement of small airway disease in addition to accurately assessing the extent of emphysema.

Lung volumes were significantly higher with worsening airflow limitation in our cohort. There was a clinically significant increase in lung volume with increasing GOLD stage, with a mean of 3.1 litres in expiration in GOLD stage I compared to a mean of 6.2 litres in GOLD stage IV. Our results are compatible with the findings of O'Donnell and co-workers who reported mean lung volumes on inspiration of 5.3L on CT. (397) This group reported that CT and helium dilution measurement of lung volumes gave similar results in patients with airflow limitation, whereas plethysmography tended to over-estimate lung

volumes in patients with more severe airflow limitation ($FEV_1 < 30\%$ predicted). (397) Quantification of lung volume on CT may be a useful staging adjunct in COPD patients.

CT quantification of emphysema and clinical features of COPD

Low body mass was significantly associated with lower lung density but not with lung volume in this COPD cohort, after adjusting for airflow limitation. Findings from previous studies using semi-quantitative measurement of emphysema support this conclusion. (205, 398, 399) Ogawa and co-workers (232) found a negative correlation between BMI and lung density ($r^2 = -0.557$, $p < 0.0001$) using three CT slices for quantitative lung density analysis. Lee and co-workers found the same relationship between BMI and lung density using whole lung quantitative CT in 34 subjects. (190) The extent of emphysema on CT has also been associated with fat-free mass, (233) and with a worsening BODE index score. (234) While we found an association between lung density and BODE index, this was predominantly due to the FEV_1 component of the BODE index.

BMI potentially confounds the measurement of lung density by causing artefact, such that a higher BMI may result in a higher measurement of lung density. The association between lung density and BMI is also potentially confounded by the effect of smoking. Current smoking is associated with a lower body mass index (as was the case in our cohort, see Chapter 5) but we found no association between CT lung volume or density and current smoking status or smoking

pack years. The relationship between BMI and lung density may also have been confounded by disease severity, as BMI was lower in subjects with more severe airflow limitation. But as with one smaller study, we found that BMI was significantly associated with lung density after adjusting for airflow limitation. Renvall and co-workers investigated a cohort of 40 COPD patients and found that the quantity of emphysema on CT was associated with BMI irrespective of the degree of airflow limitation (FEV₁ 20-80% predicted across the cohort), $r^2 = 0.171$, $p=0.01$. (400) They also found that BMI correlated inversely with nutritional intake, in that COPD patients with low BMI consumed more calories than their counterparts with a normal or high BMI, supporting a catabolic state in these patients. (400)

It is interesting that there was no association between CT lung density and health related quality of life (SGRQ scores), dyspnoea (MRC dyspnoea score), MRC chronic bronchitis scores, exacerbation frequency or oxygen saturations in this cohort. Previous studies of these clinical features with CT parameters yielded mixed results and our study is one of the few to use a fully automated technique to measure lung density in association with these features. Han and co-workers, using an automated quantitative assessment of emphysema, found that SGRQ score, the SF-12 symptom score, MRC dyspnoea score and 6-minute walk were associated with emphysema severity on CT, but found no association between the quantity of emphysema and exacerbation frequency. (401) Gas trapping on expiratory scans correlated with dyspnoea in 51 patients, using a technique to measure emphysema on three pre-allocated CT slices. (213) Using

a visual scoring method to assess emphysema in a cohort of 307 COPD patients, Makita and co-workers reported that patients with more emphysema had lower health related quality of life scores and lower BMI. (205) Our group reported an association between the distribution of emphysema on CT and MRC dyspnoea and SGRQ score. (222) The position of the CT slices chosen by Makita and co-workers may therefore have influenced their findings. Supporting our findings, they also found that symptoms of chronic bronchitis were unrelated to the degree of emphysema. (205) Previous studies suggest that thickening of the distal airways precedes the development of emphysema in many cases. (396) Symptoms of chronic bronchitis are presumed to co-exist with airway thickening and there is some evidence to support this. (262) Chronic bronchitis may therefore be more closely associated with airway measurements than with the degree of emphysema on CT. In fact, we found this was the case when analyzing airway data from this cohort. (262)

Inflammation and lung density

There are few previous studies investigating the relationship between inflammation and the quantity of emphysema. We found no association between blood or sputum markers of inflammation and lung density. The lack of a relationship between lung density and blood neutrophils or CRP seemed to indicate that emphysema was not related to systemic inflammation in this COPD cohort. Ogawa and co-workers measured serum CRP, cholesterol, albumin, triglycerides, total protein and cholinesterase and also found no association between any of these markers and either BMI or %LAA. (232) Our study adds to

these findings as we used whole lung density measurements whereas this group used only three CT slices for density analysis. Our cohort was composed of male and female subjects, whereas Ogawa and co-workers investigated only male subjects. (232) Another study found no association between the extent of emphysema on CT and systemic C reactive protein levels in a cohort of smokers. (233) These results support theories suggesting that emphysema develops through mechanisms not mediated by inflammation, but perhaps through vascular (402) or genetic mechanisms, (403) or through pathways involving elastin degradation by proteinases. (404) For example, emphysema has been associated with an increase in sputum MMP-9, and sputum ratio of MMP-9 to TIMP-1 (the main tissue inhibitor of matrix metallo-proteinases) in a cohort of COPD patients, although there was no difference in sputum, urinary or plasma desmosine (a product of elastin breakdown) between subjects with (n=10) and without (n=15) emphysema on CT in this study. (234) Increased severity of COPD in pathological studies has been associated with altered collagen deposition that may represent developing fibrosis. This may not be mediated through inflammatory pathways. (396) Hogg describes an overall reduction in bronchiolar tissue as COPD advances. (396) A lower volume of tissue capable of producing inflammatory mediators may provide an alternative explanation for the lack of association we found between pulmonary and systemic inflammation and the quantity of emphysema. Also, inflammation in the proximal airways may not be representative of inflammation in the more distal airspaces.

Interestingly, systemic inflammation (blood neutrophils) was associated with COPD severity measured by airflow limitation in this cohort but it was not associated with the quantity of emphysema. For this reason, characterizing COPD patients according to systemic inflammation, airflow limitation and lung density may be useful in further characterizing COPD for the purpose of treatment and prognosis.

Future use of quantitative CT scanning

CT scanning is increasingly utilized in the diagnosis and management of COPD in clinical practice and should perhaps become routine in the diagnostic work up for patients with suspected COPD. CT is often able to detect emphysema before an abnormality is seen on spirometry. In a cohort undergoing CT scanning for lung cancer screening, 78% of smokers had macroscopic emphysema but normal spirometry. (211) Early diagnosis may be useful in encouraging patients in smoking cessation. CT assessment of the quantity and distribution of emphysema is already used in treatment decision-making to help select potential candidates for lung volume reduction surgery. (224, 229, 230) In addition to measuring lung density on inspiration and expiration, the assessment of airway dimensions on CT may provide more information on the contribution of airway disease to the airflow limitation in COPD. Methods for the accurate assessment of airways using CT are currently under development. (236, 237, 405)

Quantitative CT may be of prognostic value. Our results suggest that lung volume and density measurements may be useful in staging COPD. Clinical trials in alpha-1 antitrypsin deficiency have pioneered the development of protocols for CT lung density quantification in a longitudinal setting for use in clinical trials. (193, 231) Work is still required to determine the changes in lung volumes and density over time in COPD patients with smoking related emphysema.

Limitations

There is some selection bias in the CT data as patients attending for CT were more likely to be ex-smokers and had significantly lower SGRQ and MRC dyspnoea scores. However, there was no significant difference in airflow limitation between these groups.

A number of technical factors may have influenced the results. Our scans were taken using a low radiation dose (20mAs). But Madani and co-workers reported that LAA% predicted macroscopic and microscopic emphysema just as accurately on low radiation dose scans (20mAs) as with a higher dose (120mAs). (406) A low radiation dose is certainly preferable in clinical practice.

Inspiratory scans were taken with patients asked to breathe to full inspiration, though this does not achieve total lung capacity when supine. All subjects should be equally affected by this type I error, but in overweight patients, some further restriction in inspiration could be expected, which might result in an

apparent increase in lung density in these subjects. Expiratory scans would not be so affected by this and yet similar relationships were seen between expiratory CT values and clinical features, partially controlling for this potential limitation. Patients with higher BMI also have more chest wall tissue, which might lead to a reduction in radiation dose to the lungs. This artefact would however result in an apparent increase in attenuation in these subjects that might account for our findings.

The 15th percentile is subject to variation depending on the level of inspiration achieved by a patient on the day of the scan. This was not controlled for in our study, which was cross-sectional in nature. It would become more important in a longitudinal analysis and a method to correct for this variation has been described by Parr (407) and Dirksen. (393)

We did not investigate the relationship between lung density and more detailed assessment of pulmonary function such as gas transfer as this has already been measured in previous work. (182, 192, 203, 212, 232, 392)

Conclusions

Lung density and volume can be accurately measured on CT scans and both are associated with the severity of airflow limitation in COPD. FEV₁/FVC ratio correlated better with lung density than FEV₁ or FVC. Expiratory CT parameters correlate more strongly with airflow limitation. Low lung density was associated with low body mass index independently of smoking history and airflow limitation. There was no association between inflammation in blood or sputum and CT lung density in this cohort. CT may be useful in the diagnosis, staging and characterization of COPD and should perhaps be considered part of the routine diagnostic work-up for these patients.

Chapter 7: Discussion

The purpose of this thesis was to investigate a cohort of stable COPD patients with a view to identifying potential markers to add to the disease characterization provided by FEV₁. Firstly markers of inflammation in blood and induced sputum were measured in COPD patients compared to healthy controls. Potential “phenotypes” of COPD were then explored using markers of inflammation, clinical features and the extent of emphysema.

These were the main findings:

- Markers of inflammation were increased in blood and sputum in COPD patients compared to healthy controls.
- Blood and sputum neutrophils were independently associated with lung function.
- Pulmonary and systemic inflammatory markers demonstrated different profiles according to different clinical features of COPD. After adjusting for potential confounding factors, health related quality of life and dyspnoea were associated with systemic inflammation, haemoglobin oxygen saturation was associated with blood neutrophils, and the BODE index was associated with blood neutrophils, CRP and sputum IL-8, but there was no association between inflammatory markers and exacerbation frequency or BMI.

- Emphysema, as assessed by CT lung density was associated with airflow limitation and body mass index, but not with markers of systemic or airway inflammation, and not with smoking history.
- Inflammatory markers and CT lung density each provided information about COPD that did not always reflect changes in lung function and may therefore be useful in characterizing COPD patients in addition to lung function alone.

The inflammatory markers measured in this cohort have not been previously investigated in both blood and induced sputum in a single cohort of COPD patients and healthy controls. An important strength of the study was the inclusion of a range of COPD disease severities, as research has often been limited to COPD patients with milder airflow limitation. (46) There is very little previous work investigating the relationship between lung density and inflammation in COPD and this is one of the few studies to characterize COPD patients using a quantitative automated method of lung density analysis.

Differentiating COPD from healthy subjects with inflammatory markers

The aim of this study was to investigate potential biomarkers for COPD. A biomarker has been defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic interventions.” (20) In COPD, there is a need to identify biomarkers in addition to FEV₁, which might be used to monitor disease severity, to predict prognosis or to define disease

phenotypes. (21) A COPD “phenotype” has been defined as one of “the outward physical manifestations of patients with COPD; comprising anything that is part of observable structure, function or behaviour.” (22) In this context, one of the aims of this research was to establish whether or not markers of inflammation would be useful in differentiating COPD patients from healthy subjects. In accordance with the generally accepted concept that COPD is characterized by an “abnormal inflammatory response in the lungs,” (2) all of the markers of systemic and pulmonary inflammation measured were significantly increased in COPD patients compared to healthy controls after controlling for age, gender and smoking status, except for induced sputum % neutrophils and IL-1 β . A wide range of inflammatory markers were measured, and not all COPD patients exhibited either airway or systemic inflammation according to the markers assessed. In order to consider the ability of these markers to differentiate COPD from non-COPD subjects, several additional factors need to be considered.

The systemic inflammatory markers measured - CRP, blood white cell and neutrophil counts - are not specific for COPD, but the finding that these markers were increased in COPD compared to controls is consistent with the established literature. (82, 104) There was a considerable overlap in the values for blood total white cell count and neutrophils in COPD patients compared to controls, and the mean values for both in stable COPD patients were within the clinically accepted normal range (mean blood neutrophils 4.9×10^{-9}). Total white cell count and neutrophils are not therefore able to specifically differentiate COPD from controls. But as levels are significantly higher in COPD patients even when

stable, they may be useful in defining “phenotypes” within the COPD population.

Highly sensitive CRP was significantly increased in COPD patients compared to healthy subjects. As there was an overlap in CRP levels in COPD patients compared to controls, CRP could not be defined as a sensitive or specific diagnostic tool for identifying COPD patients. However, the median level of CRP in our COPD patients (3.8 mg/L) was more than twice that of controls (1.4 mg/L), and is consistent with that reported in other COPD populations. (408) Although these values are both below the clinically accepted normal range of < 5mg/L, measurement of CRP using a highly sensitive assay yielded values for COPD patients which were significantly higher than healthy controls and this finding was independent of current smoking status. Again, this is consistent with previous work. (95) But further studies might focus on defining a useful cut-off point. From our data, this may be around 3mg/L. As CRP is a strong independent predictor of hospitalization and death in COPD, independently of FEV₁ and smoking history, (16, 98) it may be a useful addition to the diagnostic and prognostic work up for COPD. Raised CRP is associated with poor cardiovascular outcomes in the general population. (409) As COPD patients are at increased risk of cardiovascular disease, (168) measurement of baseline CRP may also be useful in guiding the management of cardiovascular risk in these patients.

Induced sputum % neutrophils, IL-6 and IL-8 were each significantly higher in COPD patients compared to healthy subjects, but after adjusting for age, gender

and smoking status, only IL-6 and IL-8 remained significantly different in COPD patients. Of the induced sputum markers measured, percentage neutrophils showed the greatest differentiation between COPD and healthy subjects with a median of 89% in COPD compared to 77% in controls. Although not specific for COPD, sputum % neutrophils are consistently increased in COPD in other studies, even compared to other airway diseases such as asthma. (23) From our results, sputum % neutrophils merit further investigation as a potentially useful method of phenotyping COPD, even although they are significantly affected by smoking history. In the case of sputum IL-8, our work confirms results of previous small studies that IL-8 is increased in induced sputum from COPD patients compared to healthy controls. (75, 281, 282) However, while increased induced sputum IL-6 has been clearly described during COPD exacerbations, (71, 72, 150, 376) published data on induced sputum IL-6 in stable COPD compared to healthy subjects is scarce. (46)

As far as the author is aware, this is the first study showing no difference in IL-1 β between COPD and controls. No studies investigating IL-1 β in induced sputum from healthy smokers have yet been published, (284) which may be due to positive publishing bias. Results from studies investigating levels of IL-1 β in BAL and EBC from COPD subjects have been contradictory. (285-289) Although we found that IL-1 β levels in induced sputum supernatant did not differentiate COPD from healthy subjects, it may still have an important pathogenic role in COPD. It is possible that IL-1 β found in induced sputum (generally accepted as a measure of inflammation in the more proximal airways) does not accurately

reflect the quantity or activity of IL-1 β in the distal airways or lung parenchyma. (294) It is possible that measurement of IL-1 β alone does not accurately represent its inflammatory effects, as there is some evidence that the increased pro-inflammatory action of IL-1 β in COPD results from a reduction in levels of IL-1 antagonists rather than an increase in IL-1 β *per se*. (293) Alternatively, the pathogenic role of IL-1 β in COPD may be mediated through mechanisms other than inflammation. For example, IL-1 β is known to affect airway remodeling and fibrosis and has been implicated in the induction of emphysema. (290-292)

The distribution histogram for sputum cytokines was highly skewed with levels below the detectable limit for the assay in many cases, particularly for IL-1 β and IL-6. In contrast, IL-8 levels were usually high requiring extra dilution to achieve levels fitting the standard curve for the assay. For the proper validation of a biomarker, reproducibility studies need to be performed, and the variability of cytokine levels and difficulty with detection may limit their usefulness as reliable diagnostic markers in COPD. Inflammatory marker levels are given as population based data and generally have wide variation. The real challenge is to develop a marker relevant to individual patients. The distribution histograms for IL-1 β and IL-6 were similar to that for CRP, indicating that further work to identify cut-off thresholds for these markers might be useful. For example, Conway Morris and co-workers (410) proposed threshold levels of IL-1 β and IL-8 in bronchoalveolar lavage fluid in a study aimed at identifying markers to aid the diagnosis of ventilator associated pneumonia in ICU patients. Such a

strategy may also be of value in COPD. No pre-defined thresholds indicating “normality” for these markers in induced sputum could be found in the literature. As a result, they were analyzed using simple non-parametric correlations, and also by defining cut-off points based on the 50th percentile of the distribution histogram. As with CRP, the development of more sensitive assays may be necessary to detect low levels of IL-1 β and IL-6.

The effect of current and cumulative smoking history on inflammation

Smoking has established pro-inflammatory effects and is an important potential confounding factor in this study. However, blood levels of CRP and blood neutrophils were increased in COPD patients compared to healthy controls irrespective of smoking history in our cohort. In contrast to CRP, circulating neutrophil levels were significantly affected by current cigarette smoking such that blood neutrophils were increased in COPD patients, but were also increased in current smokers with normal spirometry. Different profiles of blood leukocytes and CRP have previously been reported in response to smoking habit in the literature. Our results are consistent with previous studies reporting an increase in leukocytes in current smokers. (85, 295) Leukocytes are increased in the circulation of smokers as a result of chronic stimulation of the bone marrow (310) and systemic neutrophilia has been associated both with current smoking history and smoking pack years. However, a further increase in blood leukocytes and CRP has been reported in COPD in addition to the effects of smoking, which concurs with our results. (82, 85, 276) Neutrophils may be useful for characterizing smokers for the purpose of prognosis as raised

peripheral blood white count in current, but not ex- or non-smokers, has been associated with annual rate of decline in FEV₁. (96) While blood neutrophils were increased in current smokers in our cohort, there was a tendency towards an increase in blood CRP in ex-smokers, as both COPD ex-smokers and healthy ex-smokers showed a trend towards increased levels of CRP compared to current smoking groups. This finding is consistent with data from the Framingham cohort, which showed that CRP was significantly higher in ex-smokers compared to never-smokers. (298) There is evidence that CRP levels remain elevated in “healthy” ex-smokers for many years after quitting. (300) CRP and blood neutrophils therefore seem to provide complimentary information about the inflammatory status in COPD patients with reference to smoking status.

Our results showed that ex-smoking COPD patients had higher % neutrophils in sputum than COPD current smokers, and that healthy ex-smokers similarly had increased % neutrophils in sputum compared to healthy current smokers. While on one hand smoking cessation has been associated with a reduced rate of decline in FEV₁ in early COPD, (308) our findings concur with the results of prospective studies which described an increase in airway inflammation after smoking cessation in patients with COPD, which was not seen in healthy smokers. (275, 305, 411) The inflammatory effect of cigarette smoke exposure is undisputed, as smoking increases inflammation, through mechanisms such as the inhibition of histone deacetylation, resulting in an increased transcription of pro-inflammatory mediators. (33) But the finding that lung inflammation is

further increased in ex-smoking COPD patients' supports the hypothesis that COPD is responsible for a further increase in inflammation in addition to the effect of smoking.

Sputum % neutrophils were significantly affected by smoking status. Airway inflammation has previously been associated with cumulative smoking history. (295) Neutrophils in BAL were highly correlated both with smoking pack years ($r=0.65$) and with the number of cigarettes smoked per day ($r=0.41$), $n=53$. (303) A dose-response relationship with smoking pack years was found with neutrophils, macrophages, IL-1 β and IL-8 in BAL. (286) Although there were small numbers in the control groups when divided according to smoking status, sputum IL-6 and IL-8 remained elevated in COPD patients. However, the low sputum yield in "healthy" subjects (26%) is likely to limit the ability of sputum IL-6 and IL-8 to effectively differentiate COPD patients from healthy subjects. It should also be considered that the unexpectedly high levels (of for example sputum IL-8) seen in some of the "healthy" never-smokers might represent genuine inflammation in subjects with un-diagnosed underlying lung inflammation.

Inflammation and lung function

Neutrophils in blood and induced sputum were significantly related to the severity of airflow limitation after controlling for age, gender, smoking status and inhaled corticosteroid use, whereas blood CRP and sputum cytokines were not related to spirometry in our COPD cohort. Blood and sputum neutrophils

both correlated with post-bronchodilator FEV₁ ($r=0.26$ and $r=0.31$ respectively). Previous studies have shown that neutrophils in the small airways (250) and induced sputum (45, 325) increase with the severity of airflow limitation in COPD. But few studies have investigated the relationship between blood and sputum inflammation and airflow limitation in the same COPD cohort. Vernooy and co-workers found that sputum IL-8 and soluble TNF receptor 55 correlated with FEV₁ in 18 COPD patients (116) and Bizeto and co-workers found that sputum neutrophils were associated with airflow limitation in 22 COPD patients. (115) In a longitudinal study Donaldson and co-workers found that blood neutrophils and fibrinogen, sputum percentage neutrophils and sputum IL-6 increased over time and were associated with a faster rate of decline in FEV₁ (% predicted) after controlling for age, gender and smoking history. (72) This is consistent with our findings with respect to blood and sputum neutrophils. The increase in sputum IL-6 over time in this study was small, which may explain the discrepancy with our findings, and this was a longitudinal rather than a cross-sectional study.

CRP was significantly higher in COPD patients compared to controls, but was not associated with the severity of airflow limitation. Blood neutrophils and CRP correlated with each other, and yet neutrophils increased significantly with COPD severity by GOLD stage whereas CRP did not. A disparity in markers of systemic inflammation in relation to airflow limitation in COPD has previously been reported. Six systemic inflammatory markers were measured in a Norwegian COPD cohort. In this study, CRP was increased in COPD patients

(n=409) compared to healthy controls (n=231) but osteoprotegrin was significantly lower in COPD patients and other potential markers of systemic inflammation were not different between COPD and healthy subjects. Of the 6 markers measured in the Norwegian study, only soluble TNF-R1 and osteoprotegrin were associated with GOLD stage but CRP was not. (412)

Osteoprotegrin is a cytokine from the TNF receptor superfamily with an established role in the regulation of bone turnover and cell survival. It is also expressed in immune and vascular tissue and is thought to down-regulate the immune response by reducing T-cell mediated inflammation and reducing the survival of dendritic cells. There is some evidence that osteoprotegrin is instrumental in preventing processes involved in atherosclerosis progression. (413)

Sputum cytokines were not associated with airflow limitation in our COPD patients. This is in contrast to results from smaller studies using induced sputum and similar analytical methods. Keatings and co-workers found that sputum IL-8 and neutrophils were increased in COPD patients compared to healthy smokers (n=14), Yamamoto and co-workers found that IL-8 levels increased with the severity of airflow limitation (n=33) and Hacievliyagil and co-workers (n=24) found an inverse association between sputum IL-6 and IL-8 with FEV₁. (75, 281, 283) It is likely that the variability in detectable levels of induced sputum cytokines contributed to the disparity between our results and those found in smaller studies.

The degree of airflow limitation is the traditional method to assess disease severity in COPD, but it is not the only method. (2) The finding that sputum cytokines were not related to spirometry is important as it suggests that sputum inflammation reflects aspects of COPD not defined by lung function. However, this is a cross-sectional study and it is not possible to ascertain the relationship of sputum cytokines to disease progression. Some markers of pulmonary inflammation have been associated with changes in lung function over time. D'Armiento and co-workers investigated cell counts and cytokines in BAL and plasma from 27 COPD patients using a cytokine array technique. BAL eotaxin-1 levels were significantly higher in rapidly progressive COPD than in either the healthy control subjects, or than in COPD patients whose lung function was more stable over time. (414) Further longitudinal studies are required to determine the association between sputum inflammation and prognosis in COPD.

Pulmonary and systemic inflammation

The relationship between pulmonary and systemic inflammation was investigated in the COPD cohort. Markers of systemic inflammation (CRP and neutrophils) correlated significantly with each other, but neither was associated with markers of pulmonary inflammation. Blood and sputum neutrophils were associated with airflow limitation and yet did not correlate with each other. These findings suggest that lung and systemic inflammation may be regulated independently in COPD.

The mechanisms linking lung and systemic inflammation in COPD are yet to be established. It is hypothesized that systemic inflammation in COPD results from an “overflow” of inflammatory mediators from the lung into the systemic compartment (82) but this is not supported by our results as, contrary to expectation, while sputum markers correlated with each other, none of the sputum markers correlated significantly with either of the systemic markers measured. Therefore, although they were both increased in COPD patients compared to healthy subjects, and were both associated with the severity of airflow limitation, they were independent of each other, suggesting that they are not causally related. Our results and those of others demonstrate that there is a significant inflammatory response in the lungs of patients with COPD. (1) But the mechanisms for the development of systemic inflammation are not certain. COPD patients have significant systemic inflammation independent of current smoking status, evidenced by increased circulating neutrophils, CRP, fibrinogen and TNF α . (82) While the ability of pulmonary inflammation to induce a systemic inflammatory response has previously been reported, (108, 114) in COPD there is a notable lack of correlation between lung and systemic inflammatory markers. (72, 115, 116) Other mechanisms linking pulmonary and systemic inflammation must therefore be considered.

Smoking provides a potential causative link for lung and systemic inflammation. But while systemic and pulmonary inflammatory markers were not related to each other in our cohort, we found that blood and sputum inflammation was increased in COPD after controlling for smoking status and smoking history.

Smoking effects are known to influence cell function and immune regulation and may plausibly link pulmonary and systemic inflammation in this way. For example, leukotriene B4 (LTB4) release is significantly reduced *in vitro* in serum from cigarette smokers compared to non-smokers. LTB4 influences the immune response by affecting aggregation, cell margination and enzyme release from neutrophils. (415) Blood leukocytes and CRP have previously been noted to increase with cumulative smoking history in a population study. (309) But like Franciosi and co-workers, surprisingly we found no association between smoking pack years and disease severity measured by lung function in COPD patients. (46)

Oxidative stress may also be a factor linking smoking with systemic inflammation as current smoking results in an increased systemic oxidative burden. But reports on the relationship between systemic oxidative stress and the degree of airflow limitation in COPD are contradictory. (319)

As discussed in Chapter 5, hypoxaemia is implicated in a number of mechanisms potentially linking pulmonary and systemic inflammation.

Sputum microbiology and bacterial load were not investigated in our cohort. Bacterial colonization has been associated with pulmonary and systemic inflammation in the past, (416) though results are not entirely consistent. Bacterial load has been associated with induced sputum IL-8 and with the rate of decline in FEV₁ in subjects with COPD. (321) In contrast, *Haemophilus*

influenzae colonization was associated with increased sputum neutrophils, sputum IL-1 β and IL-12, but not with IL-6 or IL-8 in patients with moderate COPD. (320) Bacterial colonization should be taken into account as a potential confounding factor in further study of sputum inflammation in COPD.

Sixty-six percent of our COPD patients were taking inhaled corticosteroid treatment, but none of the patients were taking oral steroid therapy at the time of data collection. Inhaled corticosteroid use did not significantly affect levels of inflammatory markers in blood or induced sputum in our cohort. Inhaled corticosteroids have been associated with a reduction in systemic markers of inflammation in COPD, resulting in a 40% reduction in circulating CRP in one study. (111) However, other studies reported no change in systemic inflammation in response to inhaled corticosteroids. Combination inhaled corticosteroids and bronchodilators were associated with reduced pulmonary inflammation but not with reduced systemic inflammation in other studies. (110, 358) Perng and co-workers reported a reduction in sputum IL-8 and MMP-9 following treatment with inhaled fluticasone and salmeterol. But there was no change in induced sputum cell counts, serum CRP, lung function or quality of life. (358) Inhaled fluticasone and salmeterol were associated with an improvement in health status and lung inflammation (surfactant protein D) in a study of 289 patients, but did not alter levels of systemic inflammatory markers (CRP or IL-6). (110) Several studies have reported no change in sputum IL-8 or induced sputum neutrophils in response to inhaled corticosteroid treatment, (322-324, 333) which is supported by our findings.

Other treatment effects may impact on systemic inflammatory markers. The anti-inflammatory effects of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are increasingly recognized. Statins treatment has been associated with lower levels of circulating CRP. But in our cohort, there was no association between statin use and CRP or any of the other markers of systemic or pulmonary inflammation measured. If a greater number of patients with severe airflow limitation had also been taking statin therapy, this could potentially have influenced the lack of association seen between CRP and airflow limitation in our cohort. But we found no difference in the frequency of statin use by disease severity according to GOLD stage. In a Norwegian population study (n=3877) statin use was strongly associated with low CRP, whereas inhaled corticosteroids were not. (367) Importantly the authors report that this reduction in CRP in association with statin use was seen particularly in patients with cardiovascular disease and not in subjects with airflow limitation. Whereas a randomized, double-blind, placebo controlled trial (n=125) in COPD patients found that treatment with pravastatin was associated with a reduction in systemic inflammation (CRP) and improved exercise capacity. (368) Therefore, while statin use seems to have some effect on CRP levels, it did not impact on our findings in this cohort.

The markers of systemic inflammation measured in our cohort were unfortunately limited to CRP and blood leukocytes due to resource limitations. It would have been interesting to compare results for CRP and leukocytes with

other systemic inflammatory markers such as circulating fibrinogen, which has been associated with accelerated decline in FEV₁, (417) and with blood IL-6, which is increased significantly during COPD exacerbations. (418) In one COPD meta-analysis, blood IL-8 also showed a trend towards association with worsening airflow limitation, but data were lacking particularly in subjects with more severe disease. (46)

CT parameters and lung function

We measured lung volumes and whole lung density on CT scans in inspiration and expiration using an automated method. Lung volume and density were both associated with the degree of airflow limitation in the COPD cohort, and the relationships were more pronounced with volume and density measurements from the expiratory CT scans. High lung volumes and low lung density were significantly associated with airflow limitation measured by FEV₁ and FEV₁/FVC ratio (post-bronchodilator % predicted values). As there is no single established method for measuring lung density on CT, density was measured by 3 different methods: thresholds of -910 and -950 HU and the 15th percentile point. Each of these was significantly associated with post-bronchodilator spirometry but the strongest correlations were seen with FEV₁/FVC ratio. The change in lung volume with increasing COPD severity by GOLD stage was clinically significant such that subjects had a mean expiratory lung volume of 3.1 litres in GOLD stage I compared to a mean of 6.2 litres in GOLD stage IV.

Our results confirm the results from previous work showing strong correlations between quantitative assessment of emphysema and airflow limitation, (181, 182, 192, 203, 204, 207, 212, 392) and with studies showing a stronger correlation between spirometry and CT lung density on expiration. (213, 214) CT parameters on expiration may enable indirect measurement of some of the pathological processes occurring in COPD in addition to emphysema, such as gas trapping, altered elastic recoil and small airway disease. (395) Disease of the small airways (< 2 mm in diameter) in COPD is likely to be one of the factors resulting in increased peripheral airway resistance and therefore gas trapping in COPD. (396) As airways <2mm in diameter are difficult to measure on CT, (254) expiratory CT scans may provide a useful surrogate measurement for small airway disease and gas trapping. Quantification of lung volumes and density on CT may be useful for characterizing COPD phenotypes, and may also provide a useful staging adjunct in COPD.

Emphysema and inflammation

Emphysema as assessed by CT lung density was not related to systemic inflammation measured by total white blood count, neutrophils or CRP, or to markers of airway inflammation measured in induced sputum. There are few published studies investigating the relationship between inflammation and the quantity of emphysema on CT. In agreement with our findings, two recent studies also found no relationship between CRP and lung density in a cohort of smokers and in a cohort of COPD patients. (232, 233)

We have shown that airflow limitation is associated with lung density and with pulmonary and systemic inflammation. And yet, lung density and markers of inflammation were not related to one another. Although this was a cross-sectional study, and as such could not be used to determine causation, our results suggest that inflammation was not directly associated with emphysema. However, in concordance with others, we found significant associations between blood and sputum neutrophils and lung function. (28, 45) Previous studies investigating the relationship between the presence of emphysema and inflammation are contradictory. Hogg and co-workers found increased inflammation in the lung parenchyma in patients with severe emphysema, (250) and yet like us, others have found no association between inflammation and emphysema. (232, 233) It may be that we are assessing inflammation in the wrong compartment, as induced sputum does not effectively sample inflammation in the distal airspaces, (294) or perhaps we have not sampled the correct inflammatory mediator cell type. For example, while neutrophils are found in increased numbers in the large airways, they are found in lower numbers in the small airways and lung parenchyma in COPD. Instead, in these regions, the predominant cells are CD8⁺ lymphocytes and macrophages. (419, 420) Finkelstein and co-workers found that neutrophils in the alveolar space were not associated with the quantity of emphysema, whereas lung destruction was associated with the number of alveolar macrophages and T-lymphocytes. (56) While neutrophils induce elastolysis, they are not predominantly associated with parenchymal destruction in cystic fibrosis or bronchiectasis, so this does not fully explain their role in the development of emphysema in COPD.

(34) Smoking history is likely to be an important confounding factor here, as we have shown that neutrophils in induced sputum and blood are also significantly increased in smokers, but current smoking status and cumulative smoking history in pack years were not associated with lung density in our cohort. This is in concordance with the finding that emphysema develops only in susceptible smokers. (270, 271)

As our results showed no association between emphysema and either systemic inflammation or inflammation in the large airways, it may be that the key mechanisms for the development of emphysema are regulated not through inflammatory pathways, but perhaps through vascular (402) or genetic mechanisms. (403) Or perhaps inflammation occurs as a secondary effect following another underlying mechanism. It is possible that emphysema develops through pathways involving elastin degradation by proteinases, although this too seems to be mediated via inflammatory pathways. (404, 421) Emphysema severity may be more closely associated with molecular pathways involved in tissue remodeling rather than inflammation. Emphysema severity has been associated with biomarkers of tissue remodeling such as sputum MMP-9, and sputum ratio of MMP-9 to TIMP-1 (the main tissue inhibitor of matrix metallo-proteinases) in COPD patients. (234) Mast cells, which are also implicated in tissue remodeling, have been found in greater numbers in the mucosa and connective tissue in COPD subjects with more severe airflow limitation. (422) As statins inhibit the release of MMP-1, 3 and 9 and expression of mRNA from fibroblasts *in vitro*, they may counteract fibroblast mediated

remodeling, and may therefore provide a potentially useful intervention in this process in COPD. (423)

Clinical features

One of the aims of this thesis was to investigate the relationship between clinical parameters of COPD with respect to inflammation and lung density. When analyzed according to established clinical features, there were significant differences in the profiles of pulmonary and systemic inflammatory markers. Quality of life and dyspnoea were associated with systemic but not pulmonary inflammation, whereas BODE index scores were associated with systemic inflammation and sputum IL-8. Hypoxia was associated with increased blood neutrophils, but there were no associations between markers of inflammation and BMI or exacerbation frequency after adjusting for potential confounding factors including the severity of airflow limitation. Sputum IL-6 and IL-1 β were not significantly associated with any of the clinical parameters investigated. Emphysema on CT was associated with BMI but not with any of the other clinical features measured.

Quality of life and dyspnoea

SGRQ and MRC dyspnoea scores were associated with systemic inflammation but not with pulmonary inflammation or emphysema severity in our COPD patients. Raised levels of CRP have been associated with quality of life in other COPD cohorts, (105, 356) but as far as the author is aware, this is the first study to report a significant association between systemic leukocytosis and quality of life in COPD. The only reported association between lung inflammation and

quality of life in the COPD literature was the finding that increased induced sputum % macrophages was associated with SGRQ total and symptom scores in a cohort of 102 patients. However, this study found no relationship between SGRQ scores and sputum total or other differential cell counts including neutrophils. (117)

Improvements in quality of life have been reported in response to therapeutic interventions in COPD, even when this is not reflected in improved lung function. (110, 127) It may be that therapies aiming to reduce systemic inflammation will have the greatest impact on quality of life for these patients. However therapies that target lung inflammation alone, may have little impact on quality of life. For example, theophylline reduced lung inflammation in a small study, but had no effect on systemic inflammation or on quality of life scores. (357)

Lung density was not related to SGRQ scores, MRC dyspnoea score or the MRC chronic bronchitis score. And yet both the MRC dyspnoea score and the SGRQ total scores were associated with the distribution of emphysema on CT in our patients. (222) Previous studies have reported associations between quality of life scores and emphysema severity on CT (SGRQ score, SF-12 symptom score, MRC dyspnoea score and 6-minute walk) but only one of these studies used a quantitative method to measure lung density. (205, 213, 401) Parr and coworkers found an association between emphysema severity on CT and SGRQ

(n=74) in PiZ alpha-1 antitrypsin patients, and these patients generally had more severe emphysema in the lower lung regions. (240)

Symptoms of chronic bronchitis might be expected to be present in patients irrespective of the degree of emphysema as there is evidence that thickening of the distal airways precedes the development of emphysema in many cases. (396) In agreement with our results, Makita and coworkers reported that symptoms of chronic bronchitis were equally distributed irrespective of the degree of emphysema. (205)

Body mass index

Body mass index is associated with prognosis in COPD and is incorporated in the BODE index due to its prognostic value in addition to lung function alone. The relationship between BMI, inflammation and CT parameters was investigated in this thesis. Low BMI was associated with higher lung volumes on CT and more emphysema but not with markers of blood or sputum inflammation after adjusting for potential confounding factors.

There are little data on inflammation and BMI in COPD, which may be due to difficulty in extrapolating confounding factors like disease severity and smoking habit. But there is evidence to support the presence of increased systemic inflammation in COPD patients with low BMI. For example, circulating TNF α and IL-1 β have been associated with cachexia in COPD. (157, 293, 360) Our results showed an association between BMI and blood neutrophils after

adjusting for current smoking status, but not after controlling for airflow limitation. Increased inflammation in subjects with a low BMI is in contrast to a healthy population in which obesity has been identified as a cause of increased systemic inflammation including leukocytosis. (359) We found no relationship between low BMI and either blood neutrophils or CRP after controlling for confounding factors. This is in concordance with published data. (105, 365-367)

We found that BMI was associated with sputum neutrophilia but also with sputum IL-6 but this association did not persist after controlling for smoking status and airflow limitation. A population study found no association between BMI and sputum cell counts, $n=727$. (375) Two previous studies also found no association between induced sputum neutrophils, LTB-4 or $\text{TNF}\alpha$ with BMI. (154, 374)

Low body mass was significantly associated with greater lung volumes and lower lung density in our COPD cohort. Findings from previous studies using semi-quantitative measurements to quantify emphysema support this conclusion. (205, 398, 399) Ogawa and co-workers (232) found a negative correlation between BMI and lung density ($r^2=-0.557$, $p<0.0001$) using three CT slices for quantitative lung density analysis. Lee and co-workers found the same relationship between BMI and lung density using whole lung quantitative CT in 34 subjects. (190)

The association between lung density and BMI is potentially confounded by the effect of smoking. Current smoking has been associated with a lower body mass index (as was the case in our cohort, see chapter 6) but we found no association between CT lung volume or density and either current smoking status or smoking pack years. The relationship between BMI and lung density may also have been confounded by disease severity, as BMI was lower in subjects with more severe airflow limitation, but we found that BMI was significantly associated with lung density after controlling for airflow limitation. Renvall and co-workers investigated a cohort of 40 COPD patients and also found that the quantity of emphysema on CT was associated with BMI irrespective of the degree of airflow limitation. (400) BMI therefore seems to be an important phenotype of COPD in addition to airflow limitation and the extent of emphysema, which supports its inclusion as a prognostic indicator in COPD. (15, 118)

Exacerbation frequency

Frequent exacerbators had increased blood leukocytes and increased sputum IL-6 in our cohort but this was not significant after controlling for disease severity. Frequent exacerbations were not associated with lung volumes or emphysema on CT. COPD exacerbations have been extensively investigated with respect to inflammation but there is little data linking exacerbation frequency to lung density. While we found an increase in induced sputum IL-6 in stable COPD patients with > 2 exacerbations per year which has been previously reported, (71, 72, 150, 376) this was not the case after controlling for airflow limitation.

Aaron and co-workers (377) found an increase in IL-8 and TNF α in induced sputum during COPD exacerbations (n=14), with levels returning to baseline after the exacerbation but we found no association between IL-8 and exacerbation frequency in stable COPD subjects. We did not analyze sputum for bacteria or viruses, but sputum neutrophilia has been associated with microbial colonization. (320) Microbial colonization might be responsible for increased exacerbation frequency, but Aaron and co-workers (377) found no difference in induced sputum cytokine levels in patients with or without documented bacterial or viral infection, which suggests that bacterial colonization is not likely to be solely responsible for the increased sputum IL-6 described by others.

Systemic inflammation and inactivity are key mechanisms relating COPD to its associated co-morbidities. Exacerbations are associated with both of these factors and with severity of airflow limitation and may be a key link between pulmonary and systemic inflammation and the systemic consequences of COPD. (72, 91) And yet systemic inflammation was not significantly associated with exacerbation frequency in our subjects. We found a significant association between exacerbation frequency and blood white cell count (p=0.01) and a borderline association between exacerbation frequency and blood neutrophils (p=0.055), but this was not significant after controlling for airflow limitation. CRP was not associated with exacerbation frequency in our COPD patients. Exacerbation frequency has been associated with increased systemic inflammation in COPD. (72, 151, 152) But this has not been the case for CRP or

leukocytes. (153, 412) Blood white cell count and neutrophils are frequently elevated during COPD exacerbations. But our results do not support a chronic elevation of blood white cells in COPD patients with more frequent exacerbations, independent of disease severity and smoking status. In some studies CRP has been associated with COPD exacerbations, such that levels increased significantly during exacerbations compared to other systemic markers of inflammation. (151, 379) CRP levels have been shown to take longer to return to baseline following an exacerbation compared to other markers, with levels being persistently raised compared to baseline at 14 days post-exacerbation. (376) Our COPD patients were sampled at least 6 weeks post-exacerbation, when CRP is likely to have returned to its resting level.

Current smoking is associated with increased blood white cell count and could be a potential cause of more frequent exacerbations. However, there was no increase in exacerbation frequency in current compared to ex-smoking COPD patients in our study ($p=0.2$). We found no association between markers of inflammation and the extent of emphysema on CT in these subjects. However, in a subgroup of this cohort ($n=56$) there was a significant association between thicker airway dimensions in the proximal airways on CT and an increased exacerbation frequency in addition to an increased prevalence of chronic bronchitic symptoms. (262)

Hypoxia

Hypoxia provides a potential causative link between pulmonary and systemic inflammation as it induces the expression of inflammatory cytokines *in vitro* (381-383) through the activation of NFκB. (384) Yet human and animal studies report mixed associations between hypoxia and levels of inflammatory cytokines. (385-387)

Blood neutrophils were associated with hypoxia in our COPD cohort after controlling for potential confounding factors but CRP was not. Few studies have investigated the relationship between neutrophilia and hypoxia, and yet a few interesting associations are reported. For example, an influx of neutrophils and macrophages to the alveolar space occurred in response to moderate hypoxia in rats, (164) and neutrophils were present in increased numbers in muscle fibres from hypoxic COPD patients. (380) Tissue hypoxia was independently associated with CRP in one study of 50 patients, (388) and studies at altitude describe an increase in systemic inflammation (IL-1 receptor antagonist, IL-6 and CRP) that may be hypoxia induced. (166) Hypoxia is associated with increased platelet activation, the up-regulation of intracellular adhesion molecules (167) and the development of atherosclerosis. (385) These factors in addition to hypoxia induced haemodynamic stress may partly explain the increased incidence of cardiovascular disease in COPD. (168)

There is some evidence that hypoxia increases inflammation through mechanisms that alter vascular responses. (169, 170) Activated T cells from COPD patients secreted significantly more VEGF in response to hypoxia than cells from healthy subjects. (386) Hypoxic COPD patients with pulmonary

hypertension confirmed on right heart catheterization had increased levels of circulating IL-6 and an increase in IL-6 mRNA in response to hypoxia. (389)

Hypoxia inducible factor (HIF-1 α) has a key role in the regulation of pulmonary vascular remodeling in response to hypoxia. (81) Further work is required to investigate the role of neutrophils in response to hypoxia in COPD.

We found no relationship between oxygen saturations and lung volumes or lung density, which is consistent with the historical emphysematous “pink puffer” phenotype of COPD. Our results are consistent with findings from an early study, which reported that CT lung density and PaO₂ were not related to one another, (192) but there is little other published data linking whole lung density to oxygenation. There is some evidence that upper zone emphysema is associated with reduced oxygenation. Parr and co-workers have shown that upper zone emphysema was associated with a greater A-a gradient and therefore with more hypoxaemia in patients with alpha-1 anti-trypsin deficiency. (225)

Limitations

A number of limitations of this study need to be considered. This is a cross-sectional study, and therefore results cannot be extrapolated to make deductions about causation. Induced sputum is useful as a non-invasive technique for assessing airway inflammation, but the yield of induced sputum in this cohort was low, particularly in healthy non-smokers. The implications of this limitation have been fully discussed in the discussion section of Chapter 3. Induced sputum is thought to be mainly representative of inflammation in the

larger airways and may not closely represent inflammation in the lung parenchyma. (294, 345) Inflammation in induced sputum may therefore not be assumed to be representative of general lung inflammation. Numbers of induced sputum neutrophils increase with age. (332) This may have influenced our results, as our cohort was not age matched, but was controlled for in the analysis. The percentage of sputum neutrophils in our healthy subjects is consistent with results reported by Thomas and coworkers, who also reported that the sputum neutrophil differential count does not continue to increase after 50 years of age. (332) The detection of soluble mediators in induced sputum may be reduced by the use of the mucolytic agent dithiothreitol (DTT). While this agent improves cell yield by improving the homogenization of induced sputum, the yield of soluble factors like cytokines may be reduced. (337) However, the cytokines measured in our study (IL-1 β , IL-6 and IL-8) do not seem to be affected by DTT. (424)

It would have been interesting to assess a larger selection of markers of systemic inflammation. Blood levels of IL-1 β , IL-6 and IL-10 were analyzed in a subset of 39 COPD patients and controls from this cohort. Of these only IL-6 was significantly higher in COPD subjects ($p=0.049$). Due to resource limitations, blood leukocytes and highly sensitive C-reactive protein were chosen to measure systemic inflammation due to their important association with prognosis in COPD. (16, 95)

A number of technical factors may have influenced the CT results. Scans were taken using a low radiation dose, which results in reduced edge definition. But it has been demonstrated that even a very low radiation dose enables accurate measurement of lung density, and this has the added benefit of reduced radiation dose to study subjects. (406)

We did not investigate the relationship between lung density and more detailed assessment of pulmonary function such as gas transfer as this has already been measured in previous work. (182, 192, 203, 212, 232, 392) Spirometric gating was not used, but patients were coached to achieve as close to full inspiration as possible, which is known to give good results. (195) There is some evidence that spirometric gating helps to standardize CT data acquisition for a given lung volume, (425) but it is more suited to obtaining data for a number of CT slices, rather than for whole lung analysis. This consideration is likely to be more relevant to longitudinal studies. (198) In particular, the 15th percentile is subject to variation depending on the level of inspiration achieved by a patient on the day of the scan. This was not controlled for in our study, which was cross-sectional in nature. A method to correct for this variation has been described by Parr (407) and Dirksen. (393)

Future directions

The association between inflammation and prognosis, and between lung density and prognosis are yet to be established. Current features of COPD known to be associated with prognosis include FEV₁ and the other components of the BODE

index. Our results suggest that inflammation and lung density are able to further characterize COPD in addition to lung function, but whether these parameters give useful prognostic information will need to be tested in longitudinal studies. Of the inflammatory biomarkers measured, blood neutrophils and CRP, and induced sputum % neutrophils and IL-8 showed significant associations with clinical features in COPD and are likely to be worthy of further investigation. Analysis of inflammatory cytokines in sputum might benefit from the determination of useful clinical cut-off points. (410)

CT scanning is increasingly utilized in the diagnosis and management of COPD in clinical practice and should perhaps become routine in the diagnostic work up for patients with suspected COPD. CT is able to detect emphysema before an abnormality is seen on spirometry, (211) and early diagnosis may be useful in encouraging patients with smoking cessation. Emphysema distribution on CT and measurement of airway dimensions may help to further characterize the “emphysematous” and “bronchitic” phenotypes of COPD. CT data on the distribution of emphysema and airway dimensions has been associated with lung function parameters and with body mass index, (181, 182, 203, 205, 232) and assessment of emphysema using CT is already used in treatment decision-making, for example for lung volume reduction surgery. (224, 229, 230)

Our results suggest that lung volume and density measurements may be useful in the staging of disease in COPD. Results from alpha-1 antitrypsin research showed that CT yielded more sensitive outcome measures than physiological

parameters or health status in these patients. (393) But the value of lung density in ascertaining prognosis is not established. Clinical trials in alpha-1 antitrypsin deficiency have pioneered the development of protocols for CT lung density quantification in a longitudinal setting for use in clinical trials, (193, 231) but work is still required to determine the quantity of change over time in lung volumes and density in COPD patients with smoking related emphysema. There are some indications that CT parameters are likely to be useful in providing data on disease stage and prognosis in COPD. Han and co-workers suggested that CT lung density might complement the BODE index in predicting prognosis in COPD as they found a significant association between lung density and each of the BODE index components. (401) The results from the NETT trial found that CT quantity of emphysema was weakly but significantly associated with outcome after lung volume reduction surgery. (426)

Advances in imaging methods are likely to yield useful data for characterizing COPD and monitoring response to treatment. (172) Imaging can now provide structural and functional data on lung ventilation and perfusion *in vivo*. For example, functional imaging with ³He (hyperpolarized helium) MRI was able to distinguish COPD patients from healthy subjects, without the use of ionizing radiation. (427) Investigation of lung ventilation and perfusion using PET scanning demonstrated significantly increased ventilation/perfusion heterogeneity in COPD. (428) A study comparing ³He MRI with 3D-HRCT found that these imaging methods provided complimentary morphological and functional information in COPD. (429) Further research is required to

determine which of these methods is able to determine changes in COPD over time and in response to therapeutic interventions.

Chapter 8: Conclusions

The aim of this thesis was to investigate the role of inflammation in the airways and in the systemic compartment in COPD patients compared to healthy subjects, and to investigate the relationship between inflammation and clinical and anatomical features in COPD. The main findings were as follows:

- Systemic and pulmonary inflammation was increased in COPD patients compared to healthy controls.
- Blood and sputum neutrophils were associated with the degree of airflow limitation in COPD.
- Systemic inflammatory markers correlated with each other but blood markers did not correlate with sputum markers of inflammation, which suggests that lung and systemic inflammation may be regulated independently in COPD.
- Increased systemic inflammation was associated with reduced quality of life and more dyspnoea.
- Blood neutrophils were associated with hypoxia.
- BODE index score was associated with systemic inflammation and sputum IL-8.
- Quantitative CT assessment of lung density and volume were associated with the severity of airflow limitation and BMI, but not with blood or sputum inflammation.

- Expiratory CT parameters correlated better with lung function than inspiratory parameters.

Recommendations for future work

Classification of COPD patients according to inflammation and quantity of emphysema may have implications for response to treatment and prognosis.

Our results suggest that blood neutrophils, CRP, sputum IL-8 and CT quantification of emphysema merit further investigation in longitudinal studies to determine their association with disease progression in COPD.

Systemic and pulmonary inflammation were not related to each other in this cohort, which suggests that systemic inflammation does not result from an “overflow” from the pulmonary compartment. Further work is needed to investigate the mechanisms behind systemic inflammation in COPD. Therapies designed to target systemic inflammation may result in symptomatic improvement.

Smoking has an important effect on inflammation and particularly affects neutrophils in blood and sputum. Future studies should be designed with this effect in mind.

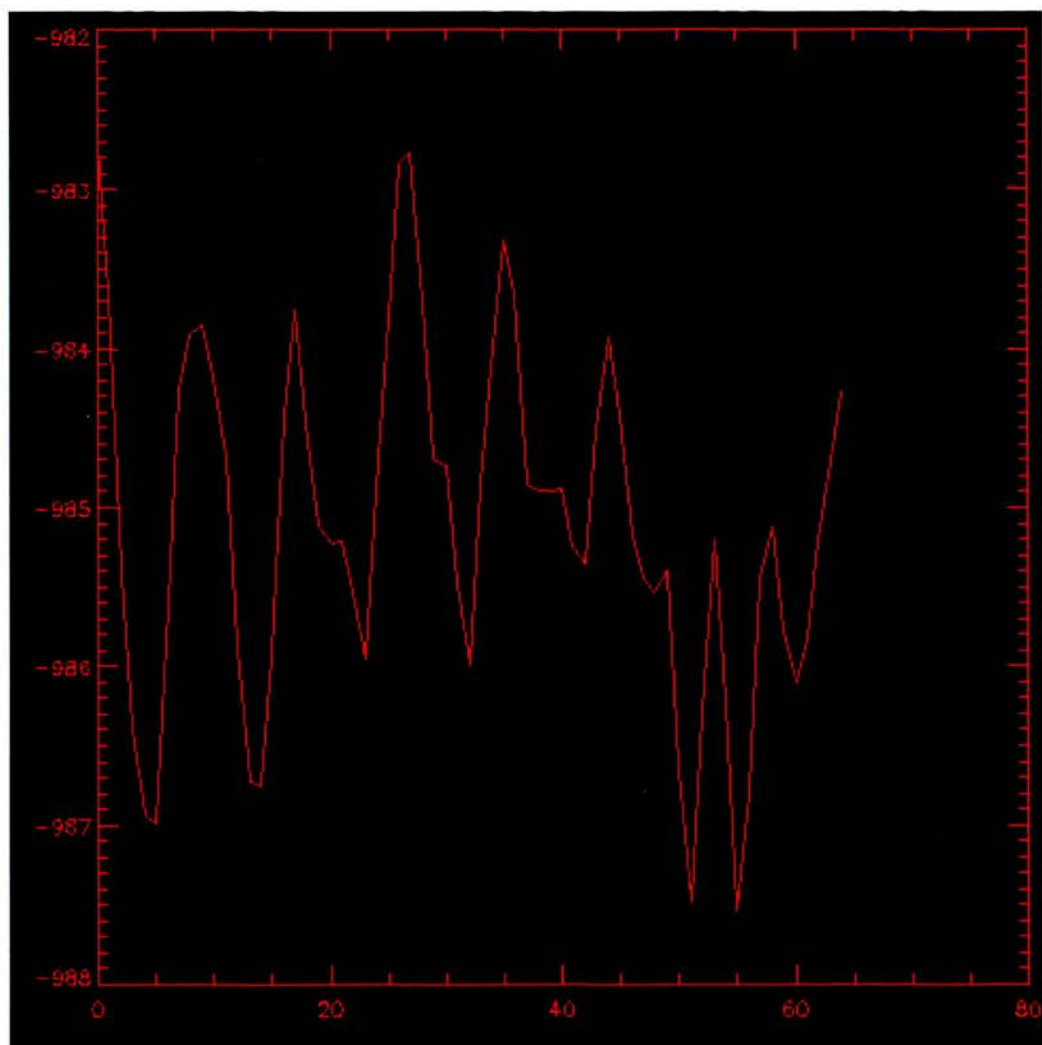
Appendix: CT air calibration method

A maximum intensity projection was calculated between selected slices near the apex of the lung and the base of the lung to allow an elliptical extra-thoracic region to be drawn avoiding clothing. The lower slice is sometimes restricted by lack of space above the chest wall (particularly in large patients) but is usually below the lower part of the sternum. The average value within this region between the superior and inferior slice limits is taken. Stoel and co-workers sample a region a constant distance from the chest wall, whereas the selected region in the Edinburgh software does not change location relative to the couch. (195) The average value for air calibration is used to modify the thresholds used for pixel index 950 ($\text{pixelindex950_corrected} = -950.0 \cdot \text{aircal} / -1000.0$) and pixel index 910 ($\text{pixelindex910_corrected} = -910.0 \cdot \text{aircal} / -1000.0$). The values used are recorded in the spreadsheets (CORRECTED_THRESHOLDS).

The mean air offset using this method depends on the filter, but is approximately -985 with the FC03 filter. Estimates of lung density were taken over a long range between the apex and base of the lung, and we suggest that the average extra-thoracic air value should be taken to at least the inferior end of the sternum if possible. Measurements were made in the region between clothing and the anterior limit of the image, and normally as far away as possible. Measurements were taken over a number of slices (see Figure 1).

The CT software was developed at Edinburgh University by Martin Connell, medical physicist. The software produced similar results to the Leiden software and to VIDA software when compared during site visits. The method we used to correct for the air offset in the Toshiba scanner is described in the paper by Berend Stoel and co-workers. (430) Toshiba stated that calibration using extra-thoracic air using filters with beam hardening correction was their recommended procedure around the time this document was published.

Figure 1. Example air calibration profile in Hounsfield Units over approximately 60 slices.



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