Novel mechanisms for increased cardiovascular risk in chronic obstructive pulmonary disease

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A thesis presented for the degree of Doctor of Medicine at the University of Edinburgh

December 2011

Abstract

Chronic obstructive pulmonary disease (COPD) is a significant burden on individuals with the disease and on healthcare resources. By 2020 it is projected to be the third leading cause of death worldwide. COPD is a condition that is characterised by chronic lung inflammation and damage. However, it is now known that COPD not only affects the lungs, but also has systemic associations, effects and consequences. These include osteoporosis, skeletal muscle wasting and dysfunction, depression, anaemia, systemic inflammation and cardiovascular disease. Population based studies have identified that COPD is a risk factor for cardiovascular morbidity and mortality, independent of traditional risk factors including cigarette smoking. The mechanisms responsible for this association have yet to be established.

In the studies in this thesis, I investigated a number of novel mechanisms that may contribute to the increased cardiovascular risk in COPD. It is thought that the low-grade systemic inflammation associated with COPD may have a role to play. In addition, the enhanced systemic inflammatory response in exacerbations of COPD may predispose these individuals to cardiovascular events. Activation of platelets and interaction between platelets and monocytes are early processes in the pathogenesis of atherothrombosis. I therefore measured markers of platelet activation in patients with COPD and healthy controls. In a second study, platelet activation was measured in patients admitted to hospital with an exacerbation of COPD and in convalescence. Patients with COPD had higher platelet-monocyte aggregation in comparison to controls matched for age and cigarette smoke exposure. This was further increased during exacerbations.

In addition to platelet activation, vascular dysfunction predisposes to cardiovascular morbidity and mortality. I undertook comprehensive assessments of vascular function (arterial stiffness, endothelial vasomotor function and endogenous fibrinolysis) in patients with COPD and healthy ex-smoking controls. I confirmed that patients with COPD have increased arterial stiffness independent of cigarette smoking. However,

contrary to prior popular assumption, endothelial vasomotor and fibrinolytic function were not impaired in comparison to healthy controls matched for smoking history.

We had previously reported an association between emphysema severity and arterial stiffness. I hypothesised that the mechanism for this association in COPD patients may be increased elastin degradation, not only in the lungs, but also in the large arteries. To test the hypothesis that COPD is a condition with systemic elastin degradation, I measured elastin degradation in skin biopsies from patients with COPD and healthy controls. There was increased cutaneous elastin degradation in COPD patients. In addition, there was increased expression of matrix metalloproteinases in COPD skin biopsies, which may be a mechanism for this observation. Furthermore, emphysema severity and arterial stiffness were associated with cutaneous elastin degradation.

These studies have identified platelet activation and arterial stiffness as novel mechanisms for the development of cardiovascular disease in COPD. Platelet inhibition and improvement of vascular function represent plausible targets for the prevention of cardiovascular events in this population. In addition, I have provided evidence for elastin degradation as a systemic effect of COPD and that systemic upregulation of matrix metalloproteinases may be the unifying mechanism for this. Focus on inflammatory pathways that result in this will provide more insight into the pathogenesis of COPD and help direct future therapies.

Declaration

This study represents original work carried at the Wellcome Trust Clinical Research Facility, Royal Infirmary of Edinburgh and in ELEGI Laboratories in the Centre for Inflammation Research, University of Edinburgh. All studies were conducted between August 2006 and August 2009.

This research was funded by a Chief Scientist Office grant and additional funding from a Scottish Society of Physicians small project grant. I solely undertook the vascular studies, with assistance in measuring arterial stiffness from the experienced nursing staff of the Clinical Research Facility as acknowledged below. I had shared responsibility for obtaining the blood samples for measurement of platelet activation in patients admitted to hospital with COPD with my colleague, Dr David McAllister. In addition I undertook the laboratory measures myself, with assistance from colleagues in the Centre for Inflammation Research as acknowledged. I am grateful to Dr Imran Haq from the Centre for Medical Research at the University of Cambridge for his assistance in performing gelatin zymography.

None of this work has been previously submitted for any other degree. All studies were performed following ethical approval by Lothian Regional Ethics Committee and written informed consent was obtained from each subject prior to entry into the studies.

John D Maclay

December 2011

Acknowledgements

This research was undertaken under the supervision of Professor William MacNee (Professor of Respiratory Medicine). I am grateful for his support and guidance over the past four years. He has allowed my good ideas to prosper while quickly and politely glossing over those with less potential. I am also thankful for the many opportunities he has given to me, enabling me to further myself and my career.

I would like to thank the nurses at the Wellcome Trust Research Facility. Their professionalism made every patient visit run smoothly and they were always available for help and advice. I would especially like to thank Finny Paterson for sharing her expertise in helping to perform the arterial stiffness measurements.

As a clinician entering a research laboratory, one relies on experienced scientists to lend a guiding hand. I am grateful to Dr Ellen Drost, Irene McGuinnes, Craig Poland and Dr Rodger Duffin for their general help within ELEGI labs, Shonna Johnston for her supervision in performing flow cytometry and Scott Maxwell for his input with immunohistochemistry. Dr Roberto Rabinovich guided my mRNA and protein extraction following his experience working with skeletal muscle in COPD patients and Steve Hartland made it possible to perform rtPCR. In addition, thanks to Imran Haq from the University of Cambridge who offered his expertise in performing gelatin zymography to me at the ERS Lung Science conference.

It is important to acknowledge Dr Nick Mills, as it was his drive and zeal that initially helped to get this project funded and become a realistic proposition. He has an incredible work ethic and his continued support in pushing projects forward invariably results in a successful outcome, and this success is reflected in his flourishing career.

In addition, I would like to thank my good friend Dr Robert Gray. His good company and unlimited stories made the significant mileage to Edinburgh much more straightforward. While some conversations were light hearted, a great many involved

discussing research ideas and strategies which undoubtedly advanced my research and provided me with future directions.

I would like to thank my friend and colleague Dr David McAllister. His enthusiasm was infectious and his work ethic unparalleled. In addition, his prodigious energy was matched by his knowledge and exponential development into an excellent researcher. More importantly his friendship was much appreciated in more difficult periods of research and his, and his wife Kate's hospitality will never be forgotten.

Finally I would like to thank my wife Jennifer. Throughout the trials and tribulations of this period of research she has always been a beacon of light. I thank her for understanding when hard work was required, her listening when hard work was difficult and her zest for life when the hard work was done.

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Abbreviations

AAT	alpha-1-antitrypsin deficiency
ABC	avidin-biotin complex
ACE	angiotensin converting enzyme
AIx	augmentation index
AP	augmentation pressure
ATS	American Thoracic Society
BCA	bicinchoninic acid
BMI	Body Mass Index
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CIIS	cardiac infarction injury score
CO	carbon monoxide
COPD	chronic obstructive pulmonary disease
CRP	c-reactive protein
CT	computerised tomography
C_T	threshold cycle
DAB	3,3-diaminobenzidine
DLco	diffusing capacity of the lung for carbon monoxide
dNTPs	deoxynucleotides
DTT	dithiothreitol
DVT	deep venous thrombosis
ECG	electrocardiogram
EDHF	endothelium-derived hyperpolarizing factor
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial NO synthase
ERS	European Respiratory Society
FBF	forearm blood flow
FEV_1	forced expiratory volume in one second
FITC	fluorescein isothiocyanate
FVC	forced vital capacity
G1	gate 1 for flow cytometry
G2	gate 2 for flow cytometry
GOLD	Global Initiative for Chronic obstructive lung disease
Hct	haematocrit
HDL	high density lipoprotein
hsCRP	high-sensitivity CRP
ICAM-1	inter-cellular adhesion molecule-1
ICS	inhaled corticosteroid
IL	interleukin
ISD	Information Services Division
L-NMMA	NG-Monomethyl-L-Arginine
LAA	low attenuation areas
LABA	long acting beta agonist
MMP	matrix metalloproteinase
MRC	Medical Research Council
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NHANES	National Health and Nutrition Examination Survey
NIH	National Institute of Health
NO	nitric oxide
PAI-1	plasminogen activator inhibitor
PCR	polymerase chain reaction
PE	pulmonary embolus
PE	phycoerythrin
Pi	protease inhibitor
PM	particulate matter
PWA	pulse wave analysis
PWV	pulse wave velocity
qPCR	quantitative PCR
ROS	reactive oxygen species
rtPCR	real-time reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SNS	sympathetic nervous system
t-PA	tissue plasminogen activator
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumour necrosis factor alpha
TORCH	Towards a Revolution in COPD Health
UPLIFT	Understanding Potential Long-term Impacts on Function with Tiotropium
VCAM-1	Vascular cell adhesion molecule-1

Chapter 1. Introduction

1.1 Definitions of chronic obstructive pulmonary disease

American Thoracic Society/European Respiratory Society consensus definition.
Having developed definitions for chronic obstructive pulmonary disease (COPD) separately, the ATS and ERS derived a global definition of COPD in their consensus statement in 2004 [Celli, 2004b]. This cemented the importance of obstructive spirometry for a diagnosis of COPD and clarifies the criteria of chronic airflow limitation – "not fully reversible". It acknowledges the most common aetiology and local pulmonary pathological changes as an "abnormal inflammatory response of the lungs to noxious particles or gases". Finally, reflecting current thinking, there is reference to the association with "systemic consequences".

The complete definition is as follows:
"Chronic obstructive pulmonary disease (COPD) is a preventable and treatable disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and is associated with an abnormal inflammatory response of the lungs to noxious particles or gases, primarily caused by cigarette smoking. Although COPD affects the lungs, it also produces significant systemic consequences."

Global Initiative for Chronic obstructive lung disease (GOLD) definition
This joint project by the National Heart, Lung and Blood Institute and the World Health Organisation originally published in 2001 was intended to reignite research interest and to encourage a focussed worldwide effort to diagnose and correctly manage COPD. This project provided a similar definition of COPD to that of the ATS/ERS with references to spirometric diagnosis, pathological response of the lungs to injury and systemic effects of this condition [Pauwels, 2001]. This project is dynamic and there have been regular updates to these guidelines on the internet but with little change in the definition of the disease.

The complete definition follows:
"Chronic obstructive pulmonary disease (COPD) is a preventable and treatable disease with some extra-pulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation

that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases."

These guidelines allow clinicians to diagnose patients under an umbrella term of COPD, partly in an attempt to discourage the use of the terms chronic bronchitis and emphysema which can be confusing to both patients, families and healthcare professionals alike. However, while terms such as 'pink puffer 'and' blue bloater' are now historical, they do highlight the wide spectrum of disease that these definitions of COPD encompass. Recently research studies have attempted to again describe subtypes or phenotypes of the condition according to clinical, radiological and pathological characteristics that may reflect differences in pathogenesis or responses to treatment between groups [Han, 2010]. Interestingly recent work has shown that patients with symptoms of chronic bronchitis (presence of expectoration of bronchial secretions most days for 3 months a year, for two consecutive years) have increased airway wall thickness demonstrable on CT scanning in comparison to those without [Patel, 2008], and that individuals with emphysema have a lower body mass index than non-emphysematous patients [Ogawa, 2009] – increasing the likelihood that we will revisit the pink puffer and blue bloater again.

1.2 Burden of disease

COPD is a major cause of mortality both in the UK and worldwide. It accounts for 4% of deaths globally and these figures are projected to increase in the next 10 years [Murray, 1997]. By 2020, it is thought that COPD will be the third leading cause of death worldwide [World Health Statistics, 2008]. Currently, of the top five commonest causes of death, COPD alone is increasing in morbidity and mortality. In Scotland however, the annual number of deaths among males, where COPD was the primary underlying cause of death, fell 34% from 79.1 in 1981 to 52.6 per 100,000 in 2006 (Figure 1.1) [Scottish Public Health Observatory, 2010a].

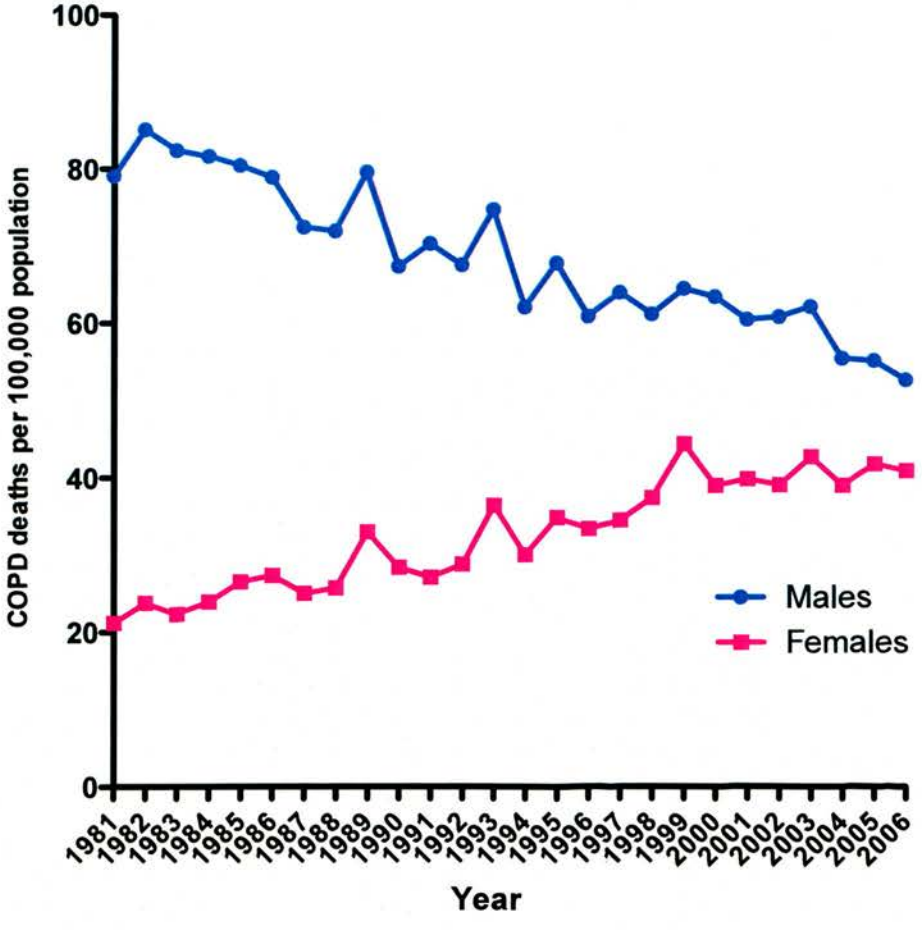

Figure 1.1 Mortality rate for COPD as primary underlying cause of death, by gender, Scotland 1981 to 2006

(adapted from Scottish Public Health Observatory, 2010)

During the same period however, the rate for females increased by 93% from 21.2 to 40.9. In the UK, COPD accounted for 27,000 deaths in 2005, and was an important comorbid feature, in nearly twice this number, in individuals dying of other causes. For patients admitted to hospital with an exacerbation of COPD, the 90-day mortality is 15% [Price, 2006].

COPD is a major burden on healthcare services worldwide. The National Institute for Clinical Excellence (NICE) estimates that COPD accounts for £800 million in health care costs and is the cause of 90,000 admissions per year, with an average length of stay of 11 days [Burney and Jarvis, 2006]. Furthermore, 31% of patients will be readmitted within 90 days of their discharge.

1.3 Causes of COPD

1.3.1 Active and passive cigarette smoking

Cigarette smoking is responsible for at least 90% of cases of COPD. Fifteen to twenty percent of cigarette smokers develop clinically significant COPD [Lundbäck, 2003]. Despite health promotion campaigns worldwide, the prevalence of cigarette smoking globally continues to increase. Stopping smoking is the single most effective way of improving life expectancy and preventing an accelerated decline in lung function and this is particularly the case in early quitters (ie prior to age 30) in comparison to late quitters [Kohansal, 2009]. Additionally it reduces frequency of exacerbations of COPD, which in turn reduces the burden on healthcare services [Godtfredsen, 2002].

The effect of smoking on lung function was described by Fletcher and colleagues in the 1960's [Fletcher, 1977]. They described an increase in the decline of airflow limitation specifically in working men of 18mls/year in smokers in comparison with non-smokers. A contemporary study that investigated this in over 4000 male and female subjects from the Framingham offspring study [Kohansal, 2009], has shown similar differences in lung function decline as those described by Fletcher. Male smokers have an increased rate of decline of FEV_1 of 38mls per year and female smokers have an increased rate of decline of 23mls per year in comparison to non-smokers with 20mls and 18mls respectively. As a proportion, 33% of male and 24% of female smokers developed airflow limitation, in comparison with 7 and 6% respectively in the never smoker cohort indicating a specific susceptibility to cigarette smoke.

The majority of research investigating the aetiology of COPD overwhelmingly identifies personal active smoking as the primary causal factor. Considerably less has been published regarding passive smoking as a risk factor in the development of COPD [Eisner, 2006]. Smoking is thought to be the major cause of indoor particulate pollution in the developed world. Indoor levels of particulate matter with a diameter less that 2.5 microns (PM 2.5) have been shown to be ten times higher in homes where cigarette smoking is allowed in comparison to residences where it is not allowed [Osman, 2007]. Additionally, the presence of continued indoor smoking was

associated with greater respiratory symptoms even in ex-smokers with COPD. With the introduction of indoor smoking bans across Europe and the concurrent improvement of respiratory ailments in workers previously exposed to a smoky environment, there is likely to be a reduction in COPD in individuals previously exposed passively to cigarette smoke. Indeed the reduction in PM2.5 levels in Irish bars has resulted in improvements in respiratory symptoms and lung function in non-smoking bar staff [Goodman, 2007].

1.3.2 Other inhalational exposures

Smoking cannabis is known to cause airflow limitation, and its effects are thought to be significantly greater than cigarette smoke. One study from New Zealand reported that one joint had the same effect on lung function as 2.5-5 cigarettes. Interestingly, this seems likely to be due to small airway changes as cannabis inhalation was not associated with emphysematous changes in comparison to cigarette smoking [Aldington, 2007]. However an Australian group describe early bullous disease in cannabis smokers that occurs much earlier than in cigarette smokers [Hii, 2008].

As noted above, the major contributor of indoor particulate air pollution in the developed world is smoking. In the developing world, indoor wood and other biomass fuel burning stoves are a significant causal factor in the development if COPD, particularly in women [Orozco-Levi, 2006; Ramírez-Venegas, 2006].

There is now an abundant literature linking COPD to exposure to dusts in the workplace. An American Thoracic Society document investigating occupational exposures and the development of respiratory conditions states that 15% of COPD is likely to be work related [Eisner, 2006]. Groups at particular risk are coal miners, hard rock miners, as well as workers exposed to mineral dusts and fine metal particulate such as cadmium and vanadium.

The role of outdoor air pollution in the development of COPD remains unclear. However, it does appear that air pollution increases morbidity and healthcare utilisation due to exacerbations of both asthma and COPD [Halonen, 2008; Anderson, 1997].

1.3.3 Socioeconomic class

Social class is an important determinant of healthcare utilisation, morbidity and mortality. Lower socioeconomic class is associated with reduced lung function (both FEV_1 and FVC) as well as DLco, independent of potential confounders including anthropometric data (such as height and weight), smoking, race, sex and respiratory illness [Hegewald, 2007; Welle, 2004]. Large population-based studies have shown associations between mortality from adult respiratory diseases and socioeconomic class. One study from Denmark showed an association between the level of education and respiratory mortality and an association between employment grade, household income, housing conditions and cohabitation and respiratory mortality in males [Prescott, 2003].

This is reflected in audit work performed by the Information Services Division (ISD) in Scotland, a national organisation for health information [Information Services Division (ISD) in Scotland, 2011]. In Scotland, there is a strong association between COPD and deprivation, with the rate of emergency admissions for COPD increasing with increasing levels of deprivation in Scotland (Figure 1.2) [Scottish Public Health Observatory, 2010b].

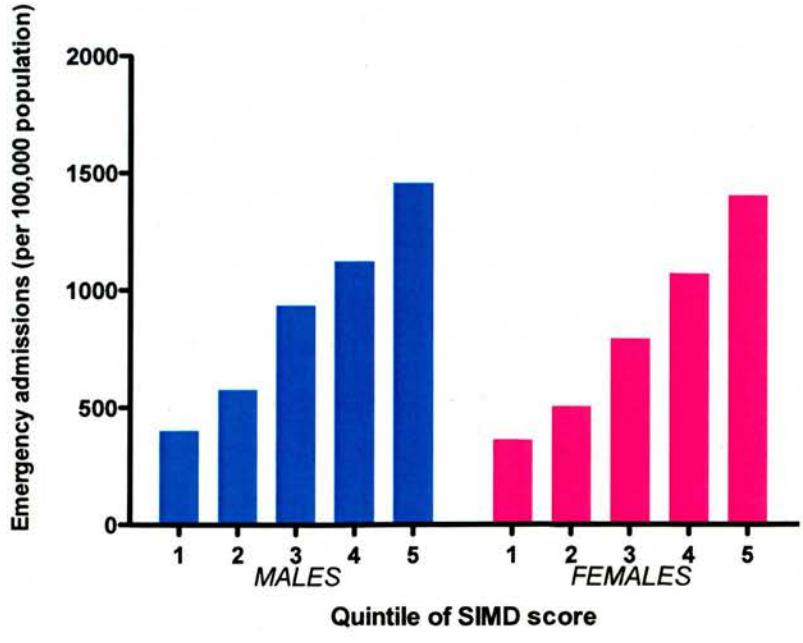

Figure 1.2 Emergency admissions for COPD by age and deprivation, Scotland, 2006.
SIMD=Scottish Index of Multiple Deprivation – 1 is least deprived
(adapted from Scottish Public Health Observatory 2010)

1.3.4 Genetics including alpha-1-antitrypsin deficiency

While cigarette smoke causes COPD, not all smokers develop this condition. Thus it is thought that some individuals have a susceptibility to lung damage in response to this insult. In addition, there is marked individual variability in the severity of disease amongst cigarette smokers. Twin studies have enabled quantitative estimates of the heritability of traits such as the FEV_1 and have suggested that approximately 50% of the variation in FEV_1 is related to genetic influences. This is also the case regarding the development of COPD [Zhai, 2007; Chen, 1999; Ingebrigtsen, 2010]. Furthermore, there is evidence of an increased risk of the development of COPD in first degree relatives of early-onset cases in comparison with controls [Silverman, 1998]. Familial studies show independent aggregation of airway wall thickening and emphysema severity, the two major pathological abnormalities contributing to airflow limitation in COPD [Patel, 2008].

Finally, in a minority of cases of COPD, deficiency of the antiprotease α-1-antitrypsin (AAT) is the causative factor [Lomas, 2004]. This autosomal recessive condition is the best-defined genetic abnormality causing COPD. In line with other genetic associations with COPD however, the rate of decline in lung function in individuals with Pi (protease inhibitor)-ZZ genotype display marked variability [Black, 1978], suggesting that other genetic factors and an interaction with environmental factors may have a role. This will be dealt with in section 1.4.3.

Several specific loci have been identified as conferring increased susceptibility to COPD such as SERPINA 1 and 3 and SERPINE 2 [Hersh, 2008], and a mutation in the terminal exon of the elastin gene has been associated with early onset emphysema [Kelleher, 2005]. However none of these genes have been shown to be consistently associated with COPD, and are generally seen in only a small proportion of affected individuals. Furthermore, a large study investigating single nucleotide polymorphisms in the terminal exon of the elastin gene found no association with early onset emphysema [Cho, 2009].

1.3.5 Childhood factors

As mentioned previously, an analysis of lung function decline in the Framingham Offspring cohort demonstrated that a third of male smokers and a quarter of female smokers had accelerated lung function decline in comparison with non-smokers [Kohansal, 2009]. The decline in lung function was even more pronounced in individuals with underlying respiratory symptoms or a respiratory diagnosis prior to enrolment in the study (mean age of individuals at exam 1 was 36 years).

Many studies have associated early life factors with respiratory morbidity and mortality in later life. Barker and colleagues reported an association between low birth weight and adult respiratory function, and this relationship appeared to be present in all socioeconomic classes [Barker, 1991]. Low birth weight was also associated with death from COPD. Bronchitis, pneumonia, or whooping cough in infancy also was associated with decreased adult respiratory function, but not mortality due to COPD. Associations between lung function and COPD mortality and both low birthweight and childhood respiratory infections have since been reported with statistical adjustment for potential confounders such as smoking and socioeconomic class [Tennant, 2008; Galobardes, 2008]. In addition, exposure to one or more 'childhood disadvantage factor' ie parental asthma, childhood asthma, maternal smoking and childhood respiratory infections predispose to a lower FEV_1 and future COPD [Svanes, 2010]. Lung function in infancy has been shown to predict airflow limitation at age 22, suggesting very early life factors influence future airways disease [Stern, 2007].

1.3.6 Other causes

Further to studies investigating the protective effects of an antioxidant rich Mediterranean diet in cardiovascular disease, recent work has suggested that diet may be associated with development of COPD. A large epidemiological study reported that a healthy diet of regular fruit, vegetables, fish and whole grain products was potentially protective in comparison to an unhealthy "Western" diet (consisting of refined grains, French fries and cured and red meats) [Varraso, 2007]. The same group extended their interest in the contribution of diet to COPD by theorising that

cured meat consumption, rich in oxidant nitrites could influence development of COPD [Jiang, 2007]. They investigated this in two cohorts and found that cured meat consumption may influence development of COPD, while it does not exacerbate development of chronic asthma. They suggest that the combination of smoking plus consumption of cured meats may have a synergistic effect on the development of COPD.

1.4 Pathogenesis of COPD

1.4.1 Pulmonary and Systemic Inflammation

COPD is characterised by chronic inflammatory changes in the lungs. However, it is unclear whether lung inflammation in COPD is a causative factor in the development of COPD or a consequence of the disease. Given the enhanced inflammatory response in the lungs of smokers who develop COPD and the fact that this persists in COPD patients despite ceasing smoking [Willemse, 2005; Lapperre, 2006], it seems likely to be both a cause and a consequence of lung damage.

Within the airway lumen in COPD, neutrophils predominate and their increased activation results in secretion of cytotoxins including proteinases such as neutrophil elastase and oxidants. Neutrophil elastase and reactive oxygen species upregulate epithelial mucin gene expression and are likely to result in the symptoms of chronic bronchitis [Fischer, 2002]. Several studies have shown an association between airway neutrophil burden and disease severity and progression measured by spirometry [Gianetti, 2006; Parr, 2006; Stănescu, 1996].

Within the airway wall, macrophages and T-lymphocytes are increased in COPD patients [Tetley, 2002; O'Donnell, 2006]. Indeed small airway narrowing and occlusion is thought to play a key role in the development of airflow limitation and the degree of airway narrowing is associated with mortality [Cosio, 1978; Hogg, 2007].

Within the lung parenchyma, COPD patients have increased numbers of lymphocytes, particularly CD8+ [Cosio, 2002]. Lymphocytes are associated with emphysema severity as well as airflow limitation and are found in increased numbers in the sputum of COPD patients and thus may have an important role in the pathogenesis of COPD [Chrysofakis, 2004; Freeman, 2010].

As well as pulmonary inflammation, COPD is associated with increased systemic inflammation. In a systematic review of fourteen original studies, Gan and co-workers demonstrated that peripheral blood leukocyte count and C-reactive protein (CRP)

levels were raised in COPD patients, compared to smokers without COPD [Gan, 2004]. Systemic inflammation may also contribute to the extra-pulmonary features associated with COPD, such as skeletal muscle dysfunction, osteoporosis, and an increased risk of cardiovascular disease [Agustí, 2007]. In recent years, clinical parameters such as reduced exercise capacity, high MRC dyspnoea score and low BMI have been found to be important prognostic factors, along with the severity of airflow limitation, in patients with COPD [Celli, 2004a].

1.4.2 Oxidative stress

There is now substantial evidence that oxidative stress, due to an imbalance of oxidants and antioxidants, may underpin some of the pathogenic mechanisms associated with COPD. Cigarette smoke contains many oxidants [Pryor, 1993]. Multiple studies have shown increased markers of oxidative stress in the lungs of patients with COPD [Rahman, 1996; Ichinose, 2000], less so in comparison to healthy smokers [Pierrou, 2007]. Oxidants may cause direct damage to the lung parenchyma but also may activate proteases, inactivate antiproteases and interfere with lung repair mechanisms [Strack, 1996; Cohen, 1982].

Increased levels of oxidants and breakdown products of oxidative stress have been measured in exhaled breath condensate (hydrogen peroxide, nitrosothiol) [Corradi, 2001; Nowak, 1998], induced sputum (nitrotyrosine) [Ichinose, 2000] and broncho-alveolar lavage (reactive oxygen species - ROS) in COPD [Verhoeven, 2000]. Oxidative stress increases with severity of airflow obstruction as measured by lipid peroxidation products in induced sputum from COPD patients [Paredi, 2000].

There is also evidence of systemic oxidative stress which may lead to development of the systemic effects associated with COPD, including muscle wasting and cachexia [Rahman, 1996; MacNee, 2005]. Isoprostanes are produced by peroxidation of arachidonic acid by free radicals, and are thus used as a marker of ROS activity. Elevated F2$\propto$-isoprostane has been observed in the urine of stable COPD patients and during acute exacerbations of COPD [Practico, 1998]. Furthermore, peripheral blood neutrophils from COPD patients have been shown to produce more ROS than normal

subjects, and are associated with increased plasma levels of nitrotyrosine and products of lipid peroxidation [Rahman, 1996; Clini, 1998].

1.4.3 Protease/antiprotease imbalance

It seems likely that an imbalance between proteases and antiproteases plays a key role, not only in the pathogenesis of COPD, but also in other chronic lung diseases. Indeed, an inherited deficiency of the antiprotease ∝1-antitrypsin (AAT) causes COPD, even in never smokers. However, a number of specific proteases have been identified in cigarette smoke induced COPD and the individual proteolytic pathways as yet are not well described.

The main role of AAT within the lung is to protect tissue from damage due to neutrophil elastase [Lomas, 2004]. AAT is produced in the liver and individuals with the most severe Z variant develop aggregations of AAT molecules resulting in slow migration of AAT out of the liver and consequent lung deficiency. The consequences of this deficiency are parenchymal destruction due to unprotected proteolytic damage to the alveolar walls and subsequent pan-acinar emphysema. The resultant accumulation of excess AAT in the liver can result in hepatocellular damage and cirrhosis.

Neutrophil elastase is thought to be one of the key factors causing lung damage, specifically emphysema in COPD. Several different studies support this view. Instillation of neutrophil elastase in dogs causes emphysema [Janoff, 1977], neutrophil elastase knockout mice are protected against emphysema when exposed to cigarette smoke [Shapiro, 2010] and neutrophil elastase inhibitors prevent emphysema in guinea pigs [Wright, 2002]. Although neutrophil elastase inhibitors have been shown to be safe in humans, their protective effects demonstrated in animal models have not been replicated in humans [Luisetti, 1996].

It is now thought that matrix metalloproteinases (MMPs) are important in the pathogenesis of COPD. MMPs comprise 24 zinc-dependent endopeptidases that play a role in tissue remodelling and repair associated with development of inflammation

by degrading collagen, laminin, and elastin [Yoshida, 2007]. The MMP family can be classified into distinct subclasses – the collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), membrane-type MMP (MMP-14 to MMP-25), matrilysin (MMP- 7), and macrophage metalloelastase (MMP-12). The major physiological inhibitors of MMPs in vivo are beta-2 macroglobulin and the tissue inhibitor of metalloproteinases (TIMP) family, which are naturally occurring proteins specifically inhibiting these proteases, produced by different cell types. The TIMP family comprises four structurally related members, TIMP-1, -2, -3 and -4. In COPD pathogenesis, there has been particular interest in the gelatinases (MMP-2 and -9) and macrophage metalloproteinase (MMP-12).

Several studies have linked MMP-2 with emphysema. Cigarette smoke increased MMP-2 content in mice bronchoalveolar lavage fluid [Seagrave, 2004]. Furthermore, in human studies, MMP-2 protein expression, assessed using immunohistochemistry, was increased in peripheral human lung tissue in COPD patients in comparison with healthy smokers and non-smokers [Baraldo, 2007]. MMP-2 expression was also increased in alveolar macrophages in severe COPD in comparison with individuals with mild/moderate disease, and this expression was also associated with emphysema severity and airflow limitation. Finally increased MMP-2 activity has been demonstrated in the sputum of COPD patients in comparison to healthy controls [Cataldo, 2000].

Knockout models in mice do not support the hypothesis that MMP-9 contributes to parenchymal destruction leading to emphysema [Atkinson, 2010]. Furthermore, in pathological specimens of human lung, the concentration of macrophage MMP-9 mRNA were similar in areas of lung with and without emphysema [Atkinson, 2010]. However, transgenically altered mice that overexpress MMP-9 in alveolar macrophages develop airspace enlargement [Foronjy, 2008]. In guinea pigs, a dual MMP-9/MMP-12 inhibitor reversed smoke-induced airspace enlargement [Churg, 2007] and MMP-9 promoter polymorphisms in humans are associated with upper lung dominant emphysema [Ito, 2005]. In addition, peripheral blood monocytes of COPD patients release 2.5 times greater MMP-9 than controls [Aldonyte, 2003], and COPD patients also have higher circulating levels of MMP-9 [Bolton, 2009].

Therefore, there may also be a role for MMP-9 outwith the lung compartment in COPD.

MMP-12 has also been implicated in the pathogenesis of COPD. MMP-12 knockout mice are protected from emphysema despite prolonged cigarette smoke exposure [Hautamaki, 1997]. A single nucleotide polymorphism in the gene coding for MMP-12 is associated with improved lung function in children and adults who smoke and a reduced risk of COPD in adult smokers [Hunninghake, 2009]. MMP-12 was increased in the sputum of patients with COPD in comparison to controls [Demedts, 2006]. There is compelling data suggesting that genetic variation in genes coding for MMP-12 can influence susceptibility to or protection against the development of COPD [Haq, 2010].

1.4.4 The autoimmune hypothesis

The hypothesis that COPD has an autoimmune component that contributes to its pathogenesis has been mooted in the last decade [Agusti, 2003]. It is an attractive theory as it accounts for airway inflammation that develops with exposure to cigarette smoke persisting for subsequent years [Willemse, 2005]. Furthermore, it may explain why a minority of smokers are susceptible to lung damage on exposure to cigarette smoke. Finally, the autoimmune theory helps us understand the development of COPD as a condition with extra-pulmonary effects, in a similar way to connective tissue disorders such as rheumatoid arthritis.

Autoimmune conditions are characterised by B- and T-cell responses to self-epitopes and thus the demonstration of B-cell immunoglobulin production and/or T-cell reactivity against autologous antigens is a defining feature of autoimmunity. Lee and colleagues described anti-elastin antibodies in a cohort of emphysematous patients in comparison with a non-emphysematous control group [Lee, 2007]. In addition, they reported enhanced T-helper cell responses in the emphysema cohort to elastin peptides in comparison with healthy controls and an asthma control population, with the production of both interferon-gamma and IL-10. Both cytokines were closely associated with CT-quantified emphysema severity. The authors suggest that exposure to cigarette smoke leads to proliferation and activity of B- and T-cells

against elastin that propagates inflammation after withdrawal of a cigarette smoke stimulus.

However, other groups have discordant results. Cottin et al examined a cohort of patients with combined pulmonary fibrosis and emphysema (CPFE) and found no differences in the presence of anti-elastin antibodies in comparison to control subjects [Cottin, 2009]. Although this was in a different specific condition, it seems likely that patients with CPFE with CT-defined emphysematous changes would have been expected to have some evidence of enhanced B-cell activity. Another group found increased levels of circulating anti-elastin antibodies in controls compared to patients with usual COPD and COPD due to AAT deficiency in comparison to healthy controls [Wood, 2011]. The authors suggest that local measurement of antibody complexes rather than circulating levels may be more enlightening. In these negative studies, a specific comparison between patients with and emphysematous phenotype and non-emphysematous phenotype was not performed, but the lower levels in a group of COPD patients compared to healthy controls does question the validity of the conclusions of the initial study.

Another recent study reported increased titres of anti-nuclear antibodies and anti-tissue antibodies in COPD patients in comparison with controls [Nunez, 2011]. In addition, anti-tissue antibodies were associated with the severity of airflow limitation and reduced DLco. Antibodies to primary pulmonary epithelial cells were also prevalent in patients with COPD, compared to healthy controls and a recent manuscript described increased anti-endothelial cell antibodies in comparison to controls [Karayama, 2010; Feghali-Bostwick, 2007]. It is difficult to know whether these antibodies are associated with a generalised enhanced immune response rather than having a specific role in the pathogenesis of COPD.

1.5 Causes of death in COPD

Traditional methods of assessing cause of death in COPD such as mortality data from death certificates are unreliable, particularly when making international comparisons. This should improve with standardisation of the definitions of COPD and improvement in international communication.

A coding analysis in 2003 showed that ischaemic heart disease and lung cancer were the commonest causes of death in COPD patients [Hansell, 2003]. In general it is thought that COPD is under-reported on death certificates which makes large epidemiological studies investigating cause of death unreliable [Jensen, 2006; Hansell, 2006]. COPD is under-diagnosed [Mannino, 2006], and airways disease may be misclassified – which results in lack of reporting of COPD as a contributing factor to mortality [Hansell, 2006].

There is more reliable, recently available evidence from large randomised controlled trials that examine mortality specifically as an end-point. TORCH (Towards a Revolution in COPD Health) is a randomised placebo controlled trial investigating the use of inhaled steroids alone or in combination with long-acting beta agonists in moderate to severe COPD [Calverley, 2007]. This study of 6184 subjects over three years yielded 911 deaths. A clinical endpoint committee examined each death "using the death certificate, medical records including emergency department and hospital records, x-ray reports, laboratory reports, operative and procedure reports, histological reports from biopsy specimens and necropsy reports". For deaths out with hospital, the committee "attempted to obtain witness interviews to describe the circumstances of the death, when the participant was last known to be alive and whether symptoms were known to precede the death". Twenty-six percent of deaths were attributed to cardiovascular causes, 35% to respiratory causes such as COPD and pneumonia and 21% to cancer (two-thirds of these were lung cancer). There were also disease-specific mortality figures from UPLIFT (Understanding Potential Long-term Impacts on Function with Tiotropium), another large randomised placebo controlled trial investigating the long acting anti-cholinergic tiotropium and its effect on mortality [Celli, 2009]. In this study only 16% of deaths were attributable to cardiovascular

causes, 40% were due to respiratory causes and 33% to cancer (just over half were lung cancer).

A further potentially reliable source of information is post-mortem studies. One such study revealed that of those individuals with COPD who underwent post-mortem, 37% died of heart failure, 28% of pneumonia, 14% from respiratory failure secondary to COPD and 9% of pulmonary embolism [Zvezdin, 2009]. The difficulty with these data is that this is not a consecutive series and thus not reflective of all patients dying in a hospital setting with COPD, but merely those in which it was felt a post-mortem was required ie if the cause of death was not established or those in which a post-mortem was permitted by the patient's family.

1.6 COPD and comorbidity

COPD is now recognised to be a condition with systemic effects and is associated with increased prevalence of other conditions. Cardiovascular disease, osteoporosis, muscle wasting and dysfunction and anaemia are all more common in patients with COPD. It is as yet unclear whether these associations are a consequence of symptoms such as breathlessness, reduced exercise tolerance and treatment including oral corticosteroids, or that COPD is an independent causal factor.

1.6.1 Osteoporosis

Patients with COPD have multiple risk factors for osteoporosis – these include advanced age, limited exercise, corticosteroid use as well as certain sub-groups of COPD with low BMI. Thus it is unsurprising that COPD is associated with osteoporosis. A recent systematic review calculated an overall mean prevalence of 35% from 14 papers measuring bone mineral density in COPD [Graat-Verboom, 2009]. These individuals had a mean FEV_1 percent predicted of 47% and age of 63. Consequently COPD patients are at increased risk of fractures. In a Norwegian study, COPD patients had significantly higher risk of vertebral deformity than an age, sex and BMI matched population-based control population [Kjensli, 2009]. Several studies have shown bone mineral density to be associated with airflow limitation, in some cases corrected for oral corticosteroid consumption [Vrieze, 2007; Kjensli, 2009] and BMI [Bolton, 2004; Incalzi, 2000].

However, recent studies have suggested that COPD is a risk factor for osteoporosis independent of corticosteroid therapy. A study of Japanese men showed that the severity of emphysema on computed tomography (CT) scanning and low body mass index were independent predictors of reduced bone mineral density after adjusting for age, sex, and smoking history, implying that COPD itself may be a risk factor for osteoporosis. It is important to note, however, that this was a slightly unusual cohort, naive to both inhaled and oral corticosteroids [Ohara, 2008].

1.6.2 Muscle wasting and dysfunction

Reduced exercise tolerance is a cardinal measure of functional status in the assessment of individuals with COPD. It is also a primary issue affecting quality of life. One of the major factors limiting exercise tolerance is skeletal muscle wasting and dysfunction. Indeed, BMI and fat free mass index (FFMi) are predictors of overall mortality in COPD independent of the traditional spirometric assessment of the severity of disease [Schols, 1998; Vestbo, 2006]. In combination with other simple measures of severity, BMI is one of the components of the BODE index, a simple multifactorial tool that predicts mortality in COPD [Celli, 2004a].

The mechanism of skeletal muscle dysfunction in COPD is unclear. There is evidence that oxidative stress, systemic inflammation, mitochondrial dysfunction and deconditioning may all play a part [Man, 2009].

1.6.3 Anaemia

In common with other chronic diseases, patients with COPD are susceptible to anaemia [Similowski, 2006]. However, COPD, and in particular severe COPD, is associated with polycythaemia and therefore a raised haematocrit. The WHO recognises anaemia as a disease associated with a low haemoglobin (males<13.0g/dl and females<12g/dl) [Butterworth, 1968]. Two studies used the WHO definition of reduced haemoglobin as a marker of anaemia. This showed a prevalence of 23% in 312 patients admitted to a German hospital with COPD as a diagnosis [John, 2006]. In another study, 17% of 677 predominantly male patients admitted to a Veterans facility had a haemoglobin<13g/dl [Cote, 2007]. Anaemic patients had reduced functional capability (lower exercise capacity and higher MRC dyspnoea score) independent of lung function as well as increased mortality, but this was not independent of other predictors of mortality (age, comorbidities and BODE index).

However, anaemia can also be defined by a haematocrit, <39% in men and <36% in women. In severe COPD, requiring long-term oxygen therapy, a reduced haematocrit was associated with increased mortality, whereas a raised haematocrit was protective,

independent of other markers of mortality [Chambellan, 2005]. This indicates that haematocrit may be a better measure of anaemia in COPD populations.

1.6.4 Pulmonary embolism (PE) and deep venous thrombosis (DVT)

Patients hospitalised with COPD have an increased risk of developing PE for a number of reasons: sedentarism, heart failure, age and acute infection. COPD patients may also have an increased thrombotic tendency – COPD is associated with raised levels of β-thromboglobulin, soluble P-selectin and P-selectin glycoprotein ligand (markers of platelet activation) and thrombin-antithrombin III complexes (a marker of a hypercoagulable state) [Ashitani, 2002; Ferroni, 2000; Schumacher, 2005]. The prevalence of DVT in exacerbations of COPD has also been examined. A prospective study showed that 10% of patients admitted with an exacerbation of COPD had evidence of DVT [Schönhofer, 1998]. In contrast, there is a DVT prevalence of 2.6% in asymptomatic general medical admissions [Lawall, 2007].

1.6.5 Lung cancer

With the shared risk factor of cigarette smoking, it is hardly surprising that lung cancer accounts for a significant proportion of the mortality associated with COPD. Thirty-eight percent of individuals with asymptomatic airflow limitation recruited for the Lung Health Study died of lung cancer [Anthonisen, 2002]. Interestingly, recent work has suggested that emphysema and airflow limitation are risk factors for lung cancer, independent of cigarette smoke exposure [Wilson, 2008]. The pathogenesis for this is unclear: chronic inflammation and associated lung damage may contribute along with impaired lung repair mechanisms, while there are potential genetic links between lung cancer and COPD and certain specific candidate gene loci [Schwartz, 2006]. Indeed there are retrospective studies suggesting that reducing pulmonary inflammation with inhaled corticosteroids or systemic inflammation with statin therapy may reduce the risk of lung cancer in COPD [Parimon, 2007; van Gestel, 2009].

1.6.6 Cardiovascular disease

The most significant non-respiratory contributor to both morbidity and mortality in COPD is cardiovascular disease. The TORCH study found cardiovascular disease accounted for 26% of the total deaths [Calverley, 2007]. The increased cardiovascular morbidity and mortality in COPD will be dealt with later.

There is an increased prevalence of heart failure and cardiac arrhythmias in COPD. Heart failure is common in COPD and COPD is common in heart failure patients. A study of 186 consecutive patients with left ventricular systolic dysfunction in a heart failure clinic found that 39% had COPD diagnosed on spirometry, and those patients with heart failure and severe COPD had a worse prognosis than patients with mild to moderate COPD or normal lung function [Mascarenhas, 2008]. Higher mortality was again reported among patients with COPD compared with individuals without lung disease in a study of 4132 patients hospitalized with cardiac failure in Norway [De Blois, 2010]. Patients with COPD also have increased risk of cardiac arrhythmias [Shih, 1988]. Following surgery for non-small cell lung carcinoma, patients with spirometric evidence of COPD have increased risk of supraventricular tachycardia, and they tend to be refractory to first–line treatment [Sekine, 2001]. Atrial fibrillation is also more common in COPD following coronary artery bypass graft [Mathew, 2004].

1.7 COPD and cardiovascular disease

1.7.1 Airflow limitation and cardiovascular risk

The association between FEV_1 and cardiovascular risk was established over 15 years ago [Hole, 1996]. Subsequently this relationship has been confirmed in large population-based studies, even in healthy individuals, and after adjustment for well established risk factors for cardiovascular disease including age, sex, smoking, cholesterol and education level/social class [Truelsen, 2001; Sorlie, 1989; Lee, 2010]. The Third National Health and Nutrition Examination Survey (NHANES III) comprising healthy individuals aged 40 to 60, reported a 5-fold increase in cardiovascular mortality in the quintile with the lowest FEV_1 in comparison with the highest [Sin, 2005]. The authors also performed a systematic review of population studies and reported the same findings (Figure 1.3).

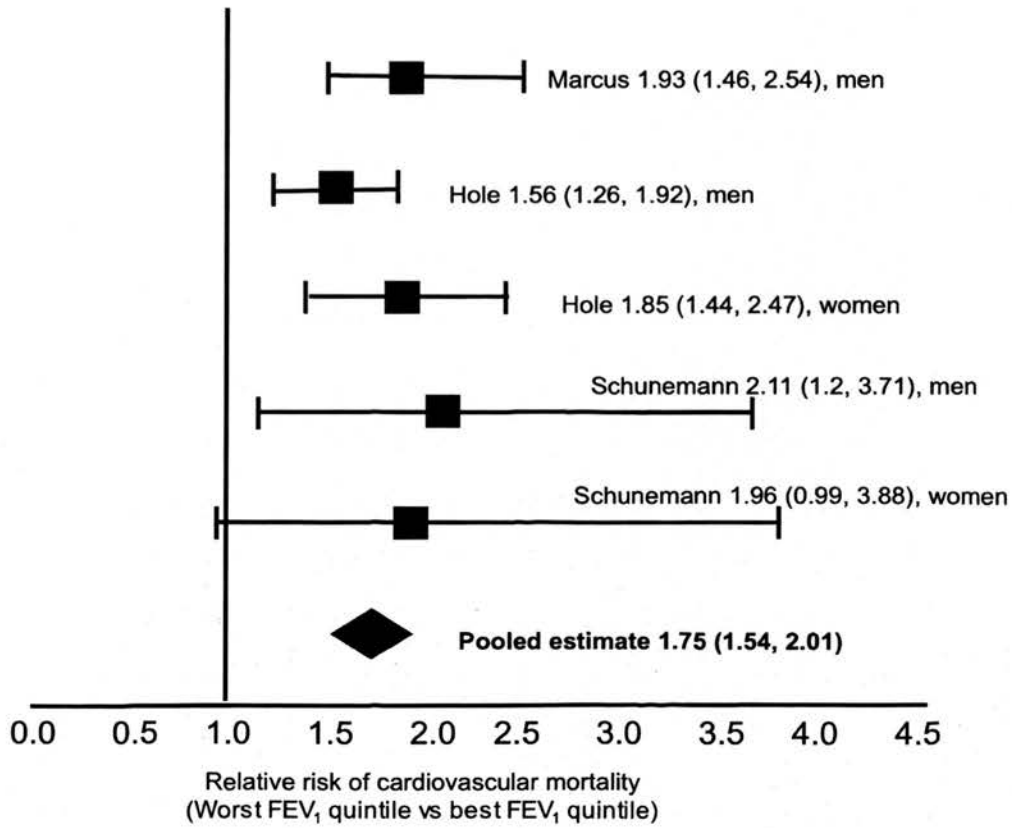

Figure 1.3 Metanalysis of studies that reported relative risk of cardiovascular mortality based on FEV_1

Adapted from Sin, 2005; boxes represent relative risk and bars represent 95% confidence intervals.

In addition, decline in FEV_1 is associated with increased cardiovascular mortality. The Baltimore Longitudinal Study of Ageing showed that those individuals who had the most rapid deterioration in FEV_1 over a follow up period of 16 years were three to five times more likely to die from a cardiac cause than those with the slowest decline in FEV_1 [Tockman, 1995]. Both FEV_1 and decline in FEV_1 predict cardiovascular risk even in never smokers, suggesting this association is independent of cigarette smoking.

FEV_1 is also an independent predictor of cardiovascular mortality in COPD. The Lung Health Study reported that for every 10% decrease in FEV_1, there was an increase of approximately 28% in fatal coronary events, and 20% in non-fatal coronary events, amongst subjects with mild to moderate COPD [Anthonisen, 2002].

However, low FEV_1 is not specifically associated with increased risk of cardiac mortality. FEV_1 predicts stroke mortality [Truelsen, 2001], as well as all-cause cancer mortality [Eberly, 2003], and death from non-respiratory, non-cardiovascular causes [Hole, 1996]. Therefore FEV_1 may be a measure representing exposure to a wide range of determinants of health which are difficult to adjust for statistically, such as poor nutrition, and exposure to environmental pollution (including passive smoke). However, another possibility is that individuals with lower FEV_1 might have an enhanced inflammatory response or impaired healing to such stimuli. Only a proportion of individuals, even with significant cigarette smoke exposure develop COPD [Fletcher, 1977], and similar hypotheses have been suggested to explain this observation [Young, 2007].

1.7.2 Cardiovascular mortality and morbidity in COPD

Cardiovascular disease contributes significantly to mortality in patients with COPD. The Tucson Epidemiological Study of Obstructive Airways Disease examined cause of death from death certification and reported that nearly 50% of patients with obstructive airways disease as a contributing cause of death had a cardiovascular event as the primary cause [Camilli, 1991]. Furthermore, a retrospective study of Canadian healthcare databases including 11,493 patients with COPD found a two to threefold increase in cardiovascular mortality in comparison with age and sex

matched control subjects (relative risk 2.07; CI 1.82-2.36) [Curkendall, 2006]. A more cause-specific analysis found increased risk of congestive cardiac failure (RR 4.09), arrhythmia (RR 2.81) and acute myocardial infarction (RR 1.51)

In individuals with established cardiovascular disease, COPD is a risk factor for cardiac death. In a three year follow up study of over 4000 patients treated for cardiovascular disease, there was a mortality of 21% in patients with COPD compared to 9% in those without [Berger, 2004]. In a prospective study examining the year post-myocardial infarction, mortality was significantly higher in subjects with COPD (15.8 vs. 5.7%) [Salisbury, 2007].

The economic burden of COPD was addressed above, but the in-patient cost is not limited to exacerbations of airways disease. In a retrospective matched cohort study from the Northern California Kaiser Permanent Medical Care Programme involving 40,966 patients with COPD diagnosed between 1996 and 1999, the risk for hospitalisation with cardiovascular disease was higher in COPD patients (RR 2.09; CI 1.99-2.20), than in age and sex matched control subjects [Sidney, 2005].

In these studies morbidity and mortality data were obtained from routine data sources such as death certificates, which can lead to diagnostic misclassification. However, in the TORCH trial, cause of death was accurately assessed by an adjudication panel. Thirty-five percent of deaths were due to pulmonary causes and 26% to cardiovascular disease [Calverley, 2007]. Similarly in the Lung Health Study, an independent mortality and morbidity review board established cause of death and hospitalization in 5,887 COPD patients aged 35-60 with mild to moderate airways obstruction over five years [Anthonisen, 2002]. This cohort had a five year mortality of 2.5% of which 25% died of a cardiovascular event, and cardiovascular disease accounted for 42% of the first hospitalisation and 44% of the second hospitalisation over a follow up period of 5 years in patients with relatively mild COPD, compared with 14% of hospitalisations from respiratory causes.

Thus a range of general population studies and studies in COPD patients suggest that airflow limitation and a diagnosis of COPD may be an important risk factor for ischaemic heart disease and sudden cardiac death. The mechanism responsible for the

increased risk of cardiovascular disease in COPD patients is not known, however a number of hypotheses have been proposed.

1.7.3 Mechanisms of cardiovascular disease in COPD

Cardiovascular disease and COPD share a number of common risk factors. These include smoking, sedentarism and low socio-economic class. There are a number of other factors that may contribute to the increased risk.

1.7.3.1 Traditional risk factors

COPD is said to be associated with an increased risk of cardiovascular disease independent of cigarette smoking. However, smoking is the causative factor in the majority of individuals who develop COPD and these individuals are particularly susceptible to cigarette smoke. It is therefore difficult to show in a COPD population that any effect is due to COPD alone, as COPD and smoking are inextricably linked and it is very difficult to fully correct for cigarette smoke exposure in studies by statistical means. It seems likely that cigarette smoke plays an important role in the development of cardiovascular disease in COPD. Interestingly, carotid intimal medial thickness (a surrogate measure strongly associated with atherosclerotic plaque burden) was measured using ultrasound in a group of healthy Japanese men, and was significantly increased in individuals who smoke and have airflow limitation in comparison to matched smokers and non-smokers [Iwamoto, 2008]. This suggests that smokers with a spirometric diagnosis of COPD have evidence of subclinical atherosclerosis, independent of cigarette smoking.

Other traditional risk factors are common in COPD. Investigators reported an increased prevalence of diabetes and hypertension in patients with COPD in comparison with healthy individuals and this increased prevalence was even more evident in GOLD stages 3 and 4 in the Atherosclerosis Risk in Communities Study (ARIC) population [Mannino, 2008].

1.7.3.2 Novel risk factors

Traditional risk factors for cardiovascular disease have long been established. However, these do not fully explain all of the cardiovascular risk. More recently, alternative novel, more mechanistic risk factors have been mooted and each of these may play a role in the increased cardiovascular risk associated with COPD (Figure 1.4).

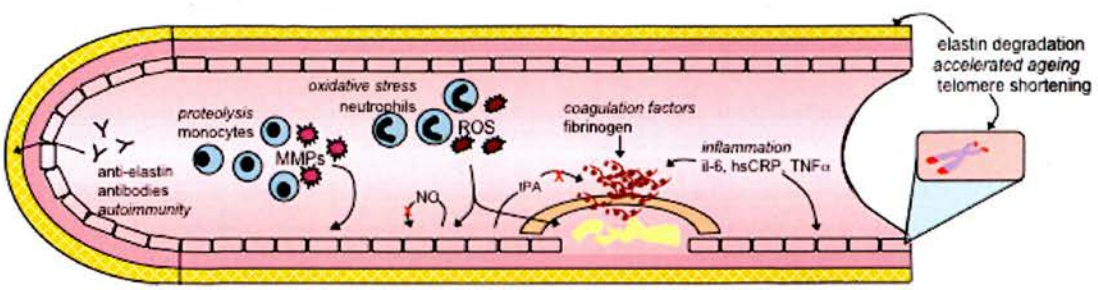

Figure 1.4 Putative mechanisms for cardiovascular disease in COPD: systemic inflammation, oxidative stress, protease/antiprotease imbalance, endothelial dysfunction, elastin degradation and autoimmune causes.

1.7.3.2.1 Inflammation

For over 20 years, systemic inflammation has been related to cardiovascular morbidity and mortality. Measurement of CRP is known to predict cardiovascular events not only in high risk, post-acute coronary syndrome populations, but also in healthy individuals. The Framingham Risk Score, the most widely used tool for predicting risk of cardiovascular events, is improved by adding CRP to prediction models comprising of traditional risk factors [Wang, 2006]. Recent work has shown that intervening with statins may reduce cardiovascular events in individuals with high levels of circulating inflammation but normal cholesterol levels [Ridker, 2008].

The pathophysiology of atherosclerosis and the role of inflammatory pathways in this process is complex [Libby, 2002]. The contribution of lipid metabolism to atherosclerosis is long established. However, more recent studies have revealed the importance of inflammation in plaque initiation, development, and rupture [Libby, 2002; Libby, 2005]. The atherosclerotic process starts with injury to the vascular

endothelium, which is made more permeable by a variety of factors, including systemic inflammation and oxidative stress. Lipoproteins then enter the intima via the vascular endothelium. Modified lipoproteins and systemic oxidative stress and inflammation induce cytokine production and increase the expression of cell adhesion molecules, such as ICAM-1 and VCAM-1, on the vascular endothelium, allowing circulating leukocytes to adhere to damaged endothelial surfaces. The release of chemotaxins directs migration of these leukocytes to the vascular intima. In this inflammatory environment, there is increased expression of scavenger receptors on monocytes/macrophages that ingest modified lipid lipoprotein particles, promoting the development of foam cells. Vascular smooth muscle cells then proliferate and may migrate from the media into the intima. These muscle cells produce extracellular matrix, which accumulates in the plaque with the formation of fibro-fatty lesions. This results in vessel wall fibrosis and consequent smooth muscle cell death. Calcification may occur, producing a plaque with a fibrous cap surrounding a lipid-rich core.

A number of cells and molecules can both promote and amplify this inflammatory process. Activated T-lymphocytes and macrophages can stimulate the release of cytokines, resulting in endothelial activation. In addition to an increased expression of adhesion molecules on activated endothelium, cytokines such as interleukin (IL)-1, IL-6, and tumour necrosis factor alpha (TNF-α) can facilitate the deposition of components of atheromatous plaque formation. C-reactive protein (CRP) is an acute phase protein primarily produced by hepatocytes under the stimulation of IL-6 that is released after vascular damage. CRP, when released into the circulation, can up-regulate other inflammatory cytokines, activate complement, and promote the uptake of low density lipoproteins by macrophages. CRP also interacts with endothelial cells to stimulate the production of IL-6 and endothelin-1 [Verma, 2002; Yeh, 2001]. CRP is found in atheromatous lesions and may therefore have a causal role in atherogenesis [Torzewski, 2000]. Studies in vitro have shown that CRP may adversely affect vasomotor endothelial function through the inhibition of endothelial nitric oxide synthase and consequently the production of nitric oxide (NO). Endothelial fibrinolysis is also impaired by CRP, which induces the production of PAI-1 (plasminogen activator inhibitor), an inhibitor of tissue plasminogen activator (t-PA) [Venugopal, 2002; Devaraj, 2003]. A number of other inflammatory biomarkers have

also been implicated in plaque formation, such as IL-6, IL-8, and fibrinogen [Luc, 2003; Boekholdt, 2004; Ridker, 2000a].

There is considerable evidence of increased systemic inflammation, both activated circulating leukocytes and increased inflammatory mediators, in COPD [Wouters, 2005]. The origin of the systemic inflammatory response in COPD has not been clearly established, but a number of mechanisms have been proposed. These include direct ''spillover'' of lung inflammation to the systemic circulation, an effect of lung hyperinflation, tissue hypoxia, muscle dysfunction, and bone marrow stimulation. Peripheral blood neutrophils are activated in patients with COPD to release reactive oxygen species [Noguera, 2001], have increased expression of adhesion molecules [Noguera, 1998], and demonstrate enhanced chemotaxis and extracellular proteolysis [Burnett, 1987], mechanisms that are involved in the pathogenesis of atherosclerosis. These include release of 2.5-fold greater amounts of matrix metalloproteinase-9 (MMP-9) from circulating monocytes of patients with COPD in comparison with control subjects [Aldonyte, 2003], and MMP-9 has been implicated in the pathogenesis of arteriosclerosis and in plaque rupture [Libby, 2005]. CRP is a biomarker of systemic inflammation and is also a marker of increased cardiovascular risk, while in COPD it is a marker of increasing severity of disease measured by airflow limitation, body mass index and exercise capacity and increased mortality. In a cohort of 1,302 individuals with airflow limitation selected from the Copenhagen City Heart Study, individuals with baseline CRP greater than 3mg/L had a higher risk of hospitalization and death from COPD (hazard ratios 1.4, 25% CI, 1.0–2.0; and 2.2, 25% CI, 1.2–3.9, respectively), compared with individuals with a baseline CRP less than or equal to 3 mg/L adjusted for age, sex, FEV_1% predicted, tobacco consumption, and ischaemic heart disease [Dahl, 2006]. However, it may be that CRP is merely a marker and does not play an active role in formation of atheromatous plaques since recent work with rabbits suggest that CRP does not promote atherogenesis [Koike, 2009].

In an attempt investigate the link between inflammation and cardiovascular disease in COPD, a Canadian group used the NHANES cohort to study the relationship between the severity of airflow limitation, CRP concentrations and their relationship to the cardiac infarction injury score (CIIS) [Albert, 2003]. The CIIS is an

electrocardiographic coding scheme to assess cardiac injury and is related to cardiovascular mortality [Rautaharju, 1981]. In this study the cohort was divided according to the degree of airflow limitation (none, mild, moderate, and severe) and the groups matched for lipid profile, although blood pressure and smoking history were higher in those with severe airflow obstruction. Patients with more severe airflow limitation had both a higher CRP level and a higher CIIS. The presence of severe airflow limitation and high CRP were associated with an even higher CIIS [Sin, 2003]. However, this ECG scoring system has not been validated for a COPD population, and so the results should be interpreted with caution.

1.7.3.2.2 Oxidative stress

COPD has been associated with both local pulmonary and systemic oxidative stress as described above. No studies have specifically addressed the hypothesis that increased oxidative stress in COPD increases cardiovascular risk. However, a number of studies have been published on oxidative stress in CVD, and rather more have been published on oxidative stress in COPD. Oxidative stress is an imbalance between the production of reactive oxygen species (ROS, including free radicals, reactive oxygen and nitrogen species) and protective antioxidants (such as superoxide dismutase and glutathione peroxidase). Unopposed oxidation causes apoptosis, cell destruction and necrosis, but also enhances inflammation through the activation of gene expression for inflammatory mediators and adhesion molecules.

A major source of endogenous ROS is leakage from mitochondria along with other cellular processes involving enzymes such as xanthine oxidase, ROS released from inflammatory leukocytes via the NADPH oxidase system and cytochrome P450 [Harrison, 2003; Chen, 2003]. Exogenous sources of oxidants include inhalation of tobacco smoke and air pollution [MacNee, 2005].

Ischaemic heart disease has also been associated with systemic oxidative stress. Several traditional risk factors including hypertension, hypercholesterolaemia, smoking and diabetes are associated with increased production of oxygen free radicals from the vascular endothelium and smooth muscle cells. ROS have been shown to cause atherosclerosis by a number of mechanisms - up-regulation of cell adhesion

molecules, proliferation of vascular smooth muscle, apoptosis of endothelium, lipid oxidation, activation of matrix metalloproteinases and altered vasomotor activity [Lee, 2001; Griendling, 1998; Aoki, 2001; Kameda, 2003]. NADPH oxidase is particularly important in production of ROS from vascular cells. Angiotensin II activates this enzyme and exacerbates the oxidative stress [Landmesser, 2003]. This may be one of the mechanisms by which angiotensin converting enzyme (ACE) inhibitors confer vascular protection.

Measurement of ROS is problematic and addressing the issue of increased oxidative stress in COPD and its effect on cardiovascular risk is therefore difficult. Nonetheless, interesting recent studies suggest that inhaled particulate matter may cause abnormal endothelial function by the effects of ROS on nitric oxide and similar mechanisms may be present in COPD [Mills, 2005].

1.7.3.2.3 Physiological stresses

Patients with COPD are subject to hypoxia—either sustained hypoxia in patients with severe disease and respiratory failure, or intermittent hypoxia, for example during exercise or exacerbations. However, there is a threshold effect for hypoxia in patients with COPD related to the severity of the airflow limitation, which does not seem to apply to the relationship between pulmonary function and cardiovascular risk. Hypoxia has been shown to have a number of effects that influence atherogenesis. These include increasing systemic inflammation and oxidative stress, up-regulating cell adhesion molecules, and inducing haemodynamic stress [Lattimore, 2005; Ichikawa, 1997; Hartmann, 2000]. Increased foam cell production, a critical constituent of unstable atherosclerotic plaques, is also stimulated when macrophages are exposed to hypoxic conditions [Lattimore, 2005]. The cellular adhesion molecules ICAM-1 and P-selectin have been shown to be up-regulated by hypoxic challenge in human umbilical endothelial cells [Ichikawa, 1997], and CRP also increases in response to hypoxia [Hartmann, 2000]. Hypoxia can also induce increased oxidative stress. In an animal model, hypoxia produced atherosclerosis in the presence of dyslipidemia and increased lipid peroxidation, a marker of oxidative stress [Savransky, 2007], and reduced levels of the antioxidant superoxide dismutase are found in the myocardial tissue of rats exposed to hypoxic environments [Chen, 2005].

Hypoxia also induces haemodynamic stress [Thomson, 2006]. Normal subjects exposed to a hypoxic challenge to reduce oxygen saturations to 80% for 1 hour developed an increased heart rate and cardiac index. These effects of acute and intermittent hypoxia may have relevance for individuals with COPD, who are subjected to intermittent hypoxic episodes during exertion and exacerbations. Hypoxia also affects the renal circulation, reducing renal blood flow and activating the renin-angiotensin system, resulting in increased peripheral vasoconstriction and oxidative stress [Skwarski, 1998].

Activation of the sympathetic nervous system (SNS) is associated with increased risk of cardiovascular disease [Curtis, 2002], which, given that both COPD and chronic respiratory failure are associated with SNS activation [Heindl, 2001], may contribute to the cardiovascular morbidity and mortality observed in patients with COPD. Several studies have found that a high resting heart rate is an independent risk factor for cardiovascular morbidity and mortality in the general population, and resting tachycardia is common in COPD [Cook, 2006]. Furthermore, COPD is also associated with reduced heart rate variability, a marker of abnormal cardiac autonomic regulation, which has been found to predict mortality in the elderly [Tsuji, 1994; Volterrani, 1994]. In view of the potential adverse effects of sympathetic stimulation, and the beneficial effects of β-receptor antagonists in heart failure, atrial fibrillation, and myocardial infarction, several observational studies have examined the effects of β-agonists on cardiovascular morbidity and mortality, with conflicting results [Suissa, 1996; Suissa, 2003; Au, 2002]. However, the TORCH study found no increase in all-cause or cardiovascular mortality in 1,521 patients treated with salmeterol (a long-acting β-agonist), compared with the 1,524 patients allocated to placebo [Calverley, 2007].

In a meta-analysis including 131 patients with COPD randomised to either a cardioselective β-blocker or placebo, FEV_1 was not significantly different in patients treated with β-blockers [Salpeter, 2005]. Moreover, evidence from observational studies suggests that cardioselective β-blockers reduce mortality in patients with COPD after vascular surgery, myocardial infarction, or admission to hospital with

acute exacerbations of COPD [Dransfield, 2008; Gottlieb, 1998; van Gestel, 2008], although in such studies it is difficult to avoid residual confounding by disease severity. Nevertheless, there remains a reluctance to use β-blockers in patients with COPD [Egred, 2005], despite the joint recommendation of the American College of Cardiology and American Heart Association that β-blockers should not be routinely withheld in patients with COPD who have heart failure or a recent non ST-elevation myocardial infarction and a Cochrane systematic review advocating their safety [Hunt, 2005; Anderson, 2007].

1.7.3.2.4 Endothelial dysfunction

The endothelium is a single celled layer that acts as the inner lining of all blood vessels. As well as being a structural component of vascular walls, the endothelium has a number of important active roles in maintaining vascular homeostasis. These include control of vasodilatation, production of fibrinolytic factors and regulation of both platelet activation and inflammation around the vessel wall.

The endothelium controls vasodilatation on a minute-to-minute basis, producing powerful vasoactive mediators from the endothelium including nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclins. Furchgott first established that an intact endothelium produced molecules that cause vasodilation following administration of acetylcholine and bradykinin [Furchgott, 1980]. This factor was later recognised to be NO. NO is generated from L-arginine by the action of endothelial NO synthase (eNOS) in response to shear stress related to cardiac output and to signaling molecules such as bradykinin, adenosine, vascular endothelial growth factor, and serotonin. This gas diffuses to the vascular smooth muscle cells and activates guanylate cyclase, which leads to cGMP-mediated vasodilatation [Deanfield, 2007]. However, the endothelium continues to cause vasodilatation even with administration of NO-antagonists and in mouse models that knock out production of eNOS. This established the presence of a further factor, endothelium-derived hyperpolarizing factor, EDHF. The exact mechanism of its actions remain unclear [Luksha, 2009].

Stable atherosclerotic plaques are characterised by a thick fibrous cap and relatively little lipid accumulation. They progress relatively slowly and occlude the vessel lumen, resulting in angina pectoris. Vulnerable plaques, which contain large amounts of lipid and inflammatory cells, have a thin fibrous cap and are prone to rupture, resulting in myocardial necrosis. After plaque rupture, lipids leak on to the arterial lumen and produce vasoconstriction and thrombus formation. Acute release of tissue type plasminogen activator (t-PA) from the endothelium in response to thrombus formation causes fibrinolysis, dissolving the thrombus. t-PA causes proteolytic degradation of fibrin to soluble fibrin degradation products by catalysing the conversion of plasminogen to plasmin. The conversion of plasminogen to plasmin by t-PA is accelerated in the presence of fibrin at the endothelial cell surface, ensuring focused, localised action. The exact intracellular pathways are however still not clear [Oliver, 2005].

Endothelial damage results in local activation of platelets by the release of a variety of inflammatory mediators. This in turn causes platelet adhesion and recruitment of other inflammatory cells including monocytes [Ramos, 1999]. This is thought to be the first step in the formation of an atherosclerotic plaque. Thus maintenance of the endothelium is important in preventing progressive vascular disease.

There is now considerable evidence that impaired endothelial function is associated with cardiovascular morbidity and mortality. Several studies have evaluated coronary endothelial function in patients with coronary disease during coronary angiography using acetylcholine, an endothelium-dependent vasodilator, showing that individuals with endothelial dysfunction have increased risk of cardiac events [Widlansky, 2003]. Using the cold pressor test, this has also been shown in individuals with normal coronary arteries [Schindler, 2003]. Other aspects of endothelial dysfunction also predict cardiovascular events. Impaired fibrinolysis, measured by stimulated release of t-PA has also been shown to be a determinant of cardiovascular risk in patients with coronary disease [Robinson, 2007].

It is thought that endothelial dysfunction is one of the mechanisms by which traditional risk factors predispose individuals to cardiovascular events. There is a growing body of evidence that smoking causes endothelial dysfunction. Smoking is known to impair release of t-PA in healthy smokers in comparison to matched non-smokers [Newby, 2001; Jatoi, 2007]. These abnormalities have also been seen in passive smoking [Celermajer, 1996]. Hypercholesterolaemia causes impairment of endothelium-dependent vasodilatation [Chowienczyk, 1992] and this is further exacerbated by smoking [Heitzer, 1996]. Essential hypertension is also known to be associated with abnormal endothelium-dependent vasorelaxation [Panza, 1990] and a recent study has shown this to be subsequent to the development of hypertension, not a precursor to it [Shimbo, 2010]. There is significant evidence reporting vasomotor dysfunction in type 1 diabetes and it is thought that endothelial function contributes to this [Chan, 2003].

As well as traditional risk factors, more novel risk factors for cardiovascular disease are also associated with endothelial dysfunction. It is difficult to ascertain whether systemic inflammation has an active role in causing endothelial dysfunction or is simply a marker of active vascular disease. As mentioned above, local inflammatory cells and cytokines have a key role to play following endothelial denudation in atherothrombosis. The same is true for reactive oxygen species. Thus, if patients with COPD do have endothelial dysfunction it is likely to be due to a combination of both traditional and novel risk factors in COPD.

1.7.3.2.5 Arterial stiffness

Mechanical changes in the large arteries may predispose to cardiovascular events. The assessment of the gold standard of aortic pulse wave velocity (PWV) and more peripheral measurement of brachial pulse wave velocity are non-invasive, robust and reproducible measurements of arterial stiffness that are often used as a surrogate measures of endothelial function [Laurent, 2006]. Aortic PWV is associated with endothelial function [McEniery, 2006].

However, on administration of L-NMMA, a nitric oxide synthase inhibitor, changes in pulse wave velocity can be explained by changes in mean arterial pressure and thus

nitric oxide derived from the endothelium is not thought to play a major role in changes in aortic PWV, although it may influence another measure of arterial stiffness, augmentation index [Stewart, 2003; Wilkinson, 2002; Wilkinson, 2004]. Furthermore, studies in the elderly have shown a lack of relationship between arterial stiffness and endothelial function [Lind, 2008]. Indeed, arterial stiffness is also influenced by vascular smooth muscle and the extracellular matrix comprising of elastin and collagen.

In order to calculate aortic (carotid-femoral) pulse wave velocity, the transit time for the pulse wave to move from the heart to femoral artery is recorded using ECG gating and a manometer (described in detail in Chapter 3).

Arterial stiffness is associated with all of the traditional cardiovascular risk factors and it has been shown that increased arterial stiffness is associated with future cardiovascular events and all-cause mortality [Vlachopoulos, 2010]. Furthermore low-grade systemic inflammation, measured by CRP, is associated with increased arterial stiffness in healthy individuals [Yasmin, 2004].

There has been some research examining vascular function in COPD to try and elicit the mechanisms for the increased cardiovascular risk established in population-based studies. One initial manuscript reported increased arterial stiffness in COPD [Sabit, 2007]. This case-control study described differences in aortic pulse wave velocity between patients with COPD and healthy controls. However, these vascular measures are affected by acute and chronic smoking [Jatoi, 2007] and the COPD population had a higher proportion of current smokers and a significantly higher lifetime cigarette smoke exposure which may, in part explain these differences. Another case-control study showed increased augmentation pressure, another measure of arterial stiffness [Mills, 2008]. However, these changes were not independent of blood pressure (which influences arterial compliance) and used a less reliable measure of arterial stiffness. Our group also published a study describing an independent association between brachial (carotid-radial) PWV and emphysema severity quantified by CT scanning [McAllister, 2007b]. Although this method of assessing arterial stiffness is not the gold standard, the fact that this less sensitive test demonstrated a good correlation

with emphysema severity, suggests that there may be an even closer relationship using aortic PWV.

1.8 Summary

COPD is a disease that is recognised to have associations with other comorbidities and systemic effects. Cardiovascular disease contributes significantly to the morbidity and mortality associated with COPD, and COPD is a risk factor for cardiovascular disease independent of traditional risk factors. The cause of this is currently unclear. Recent studies have implied that patients with COPD have abnormalities of systemic vascular function which may predispose them to cardiovascular events. Additionally, there may be a role for systemic inflammation and oxidative stress that are present in COPD and may contribute to vascular damage.

1.9 Aims and hypotheses

The principle aim of this thesis is to improve understanding of the systemic vascular abnormalities associated with COPD. I will examine platelet activation in COPD which may play a role in the pathogenesis of cardiovascular disease in this condition. Furthermore, I will perform a comprehensive vascular assessment, particularly assessing arterial stiffness and the contribution of endothelial dysfunction to this in COPD. Finally I will determine whether there is evidence of systemic elastin degradation in COPD. The following hypotheses will be addressed:

1. Patients with COPD have increased markers of platelet activation in comparison to healthy subjects (Chapter 2)
2. Platelet activation is increased in patients with exacerbations of COPD in comparison to convalescence (Chapter 2)
3. Patients with COPD have increased arterial stiffness in comparison to healthy subjects (Chapter 3)
4. Patients with COPD have impaired endothelial vasomotor endothelial function causing increased arterial stiffness (Chapter 3)
5. Patients with COPD have impaired endothelial fibrinolytic function contributing to increased cardiovascular risk (Chapter 3)
6. Patients with COPD have increased skin elastin degradation in comparison to healthy subjects (Chapter 4)
7. Patients with COPD have increased cutaneous matrix metalloproteinase expression (Chapter 4).

Chapter 2. Increased Platelet Activation in Patients with Stable and Acute Exacerbation of Chronic Obstructive Pulmonary Disease

Maclay JD, McAllister DA, Johnston S, Raftis J, McGuinnes C, Deans A, Newby DE, Mills NL and MacNee W. *Thorax.* 2011;66:769-74

2.1 Summary

Chronic obstructive pulmonary disease (COPD) is associated with systemic inflammation and cardiovascular disease. Interactions between inflammatory cells and activated platelets are important in the pathogenesis of atherothrombosis and may contribute to cardiovascular risk in patients with COPD. Thus the aim of this study was to assess platelet-monocyte aggregation in patients with COPD and matched healthy controls, and in patients with an acute exacerbation of COPD. Eighteen men with COPD and 16 healthy male controls matched for age and cigarette smoke exposure were recruited. A further 12 patients were investigated during and at least 2 weeks following hospitalisation for an acute exacerbation. Platelet-monocyte aggregation and platelet P-selectin expression were determined using flow-cytometry. Patients with COPD had increased circulating platelet-monocyte aggregates, compared with controls [mean(standard deviation); 25.3(8.3)% *versus* 19.5(4.0)%, p=0.01]. Platelet-monocyte aggregation was further increased during an acute exacerbation compared with convalescence [32.0(11.0)% *versus* 25.5(6.4)%, p=0.03]. Platelet P-selectin expression and soluble P-selectin did not differ between groups. In conclusion, patients with stable COPD have increased circulating platelet-monocyte aggregates compared to well-matched healthy controls. Platelet activation is further increased in COPD patients during acute exacerbation. These findings identify a novel mechanism to explain increased cardiovascular risk in COPD and suggest platelet inhibition as a plausible therapeutic target.

2.2 Introduction

Chronic obstructive pulmonary disease (COPD) is an independent risk factor for cardiovascular disease [Curkendall, 2006], although the mechanisms responsible for this association remain unclear. Systemic inflammation is recognised as an important determinant of atherosclerosis [Ridker, 1997; Ridker, 2000b], and COPD is characterised by both pulmonary and systemic inflammation. It has been postulated that low-grade systemic inflammation in patients with COPD may explain this increase in cardiovascular risk [MacNee, 2008]. Inflammatory pathways are upregulated further during an acute exacerbation and may plausibly precipitate an acute cardiovascular event [McAllister, 2007a].

Inflammatory cells and cytokines have been implicated in atheromatous plaque formation and coronary thrombosis [Libby, 2002]. Following vascular injury and endothelial denudation, circulating platelets become activated, upregulating expression of cell surface receptors such as P-selectin and CD40 ligand to facilitate adhesion to the arterial wall. Activated platelets release inflammatory chemokines and recruit inflammatory cells to form platelet-monocyte aggregates, an early process in atherothrombosis [Davì, 2007]. As such circulating platelet-monocyte aggregates are considered a sensitive measure of platelet activation, and are raised in patients with acute coronary syndromes, smokers and in rheumatoid arthritis [Sarma, 2002; Harding, 2004a; Joseph, 2001].

We hypothesised that platelet activation will be increased in patients with stable COPD and acute exacerbations, and may represent a link between inflammation and cardiovascular disease in these patients. We therefore measured markers of platelet activation, including platelet-monocyte aggregates, in patients with stable COPD and matched controls, and in patients during an acute exacerbation and in convalescence.

2.3 Methods

We compared measures of platelet activation between patients with COPD and matched controls (study 1) and examined the effect of acute exacerbation by comparing platelet activation in patients during an exacerbation and in convalescence (study 2). These studies were approved by Lothian Regional Ethics Committee and conducted with the written informed consent of all participants.

2.3.1 Recruitment, inclusion and exclusion criteria

Study 1: Patients with COPD and matched controls

Eighteen male patients with COPD and 16 male controls were recruited from primary care and a hospital respiratory outpatient clinic at the Royal Infirmary of Edinburgh, Scotland, and matched for age and prior smoking habit. Ex-smokers of at least 6 months with a smoking history of $\geq$10 pack years were included. Control subjects had normal spirometry and no history of respiratory symptoms. Subjects with COPD had a history consistent with the disease, chronic airflow limitation on spirometry (post-bronchodilator FEV_1/FVC ratio $\leq$0.7), stable disease (no exacerbation of COPD within the previous 6 weeks) and were not prescribed regular oral steroid therapy or long-term oxygen therapy. Exclusion criteria in both patients and controls included other respiratory disease, coronary artery disease, diabetes mellitus, hepatic and renal failure, and any systemic inflammatory condition, such as rheumatoid arthritis or psoriasis, or use of medication known to affect vascular and platelet function (including statins, angiotensin-converting enzyme inhibitors and clopidogrel). Our strict inclusion and exclusion criteria were used to allow us to try and separate the effects of COPD on platelet activation from those of comorbid conditions known to influence platelet function.

Subjects were fasted overnight and blood sampled between 08.00 and 10.00. Subjects abstained from caffeine and alcohol for 24 hours prior to the study and avoided all medications for at least 12 hours prior to attendance. Exhaled carbon monoxide measurements (<5 ppm) ensured no acute cigarette smoke exposure. Height, weight, and post-bronchodilator spirometry were measured (Alpha Spirometer; Vitalograph,

Buckingham, UK) according to American Thoracic Society/European Respiratory Society standards following venesection [Miller, 2005].

Study 2: Acute exacerbations of COPD

Twelve patients admitted to the Royal Infirmary of Edinburgh, Scotland, UK, with an acute exacerbation of COPD were studied within 24 hours of admission and at least two weeks following discharge from hospital when their condition was considered to be clinically stable. The diagnosis of acute exacerbation of COPD was made by the admitting respiratory physician. All patients had documented chronic airflow limitation on spirometry when stable (post-bronchodilator FEV_1/FVC ratio ≤ 0.7 and FEV_1 percent predicted <80%), and a smoking history of ≥ 10 pack years. Subjects with a suspected or proven alternative diagnosis for the acute deterioration in symptoms, such as pneumonia, pulmonary embolism, or heart failure were excluded. Patients were seen for their follow up visit at least 2 weeks post treatment for exacerbation with improvement in their symptoms. No patients included in study 1 were enrolled in study 2.

2.3.2 Blood collection

Blood was drawn by clean venepuncture of a large antecubital vein using a 19-gauge needle with care taken to ensure a smooth blood draw. Samples were collected into tubes containing the direct thrombin inhibitor, d-phenylalanine-l-prolyl-l-arginine chloromethyl (PPACK, Cambridge Biosciences, Cambridge, UK) as previously described [Harding, 2007]. Tubes were gently inverted to ensure mixing of whole blood with anti-coagulant. Further blood samples were collected for the measurement of haemoglobin, haematocrit, and differential leukocyte count (Sysmex, Norderstedt, Germany), and for the measurement of blood glucose (fasting in the study 1, random in study 2) and lipid profiles (Olympus Analyzers, Brea, CA, USA) in the regional clinical laboratories at the Royal Infirmary of Edinburgh. Arterial blood gases were measured at rest in study 1 (Bayer Rapidlab, Morristown, NJ, USA), as were d-dimer (VIDAS assay; bioMérieux, Basingstoke, UK) and fibrinogen levels (ACL TOP analyser, Instrumentation Laboratory, Warrington, UK). Soluble P-selectin was

determined by an ELISA (R&D, Abingdon, UK) in platelet poor plasma. Serum C-reactive protein (CRP) concentrations were measured using a highly sensitive immunonephelometric assay (Behring BN II nephelometer, Hattersheim am Main, Germany).

2.3.3 Immunolabelling and flow cytometry

Flow cytometry is a technique used to examine characteristics of individual cells using the photons of light they emit or scatter as they pass through a light source. It allows identification of specific cell types and analysis of sub-sets in conjunction with monoclonal antibodies with attached fluorochromes [Michelson, 2000; BD Biosciences, 2010].

A cell suspension is passed through a flow cell which focuses cells into single file, passing through one or more light sources. Light is then scattered by diffraction, reflection/refraction or emitted, and then collected by a series of detectors (typically photomultiplier tubes) giving information about individual cells. This signal is processed and analysed. Forward scatter (measured within 5^{o} in-line with the light source) collects light scattered by diffraction and is an approximation of cell size, whereas side scatter collects refracted or reflected light and is a measure of cell granularity. In addition, side scattered light is separated by wavelength using a series of filters to identify light that is emitted from a single fluorochrome. This enables selection of specific groups within a population of cells (gating).

In this study, five minutes following sample collection whole blood was immuno-labelled at room temperature for subsequent flow cytometric analysis of platelet-monocyte aggregates using monoclonal antibodies to phycoerythrin (PE)-conjugated CD14 (specifically binds to monocytes), fluoroscein isothiocynate (FITC)-conjugated CD42a (specifically binds to platelets) and isotype matched controls (Biosource, Renfrew, UK). After 20 minutes of incubation samples were fixed and red cells lysed with FACS-Lyse solution (Becton Dickinson, Oxford, UK).

In addition, whole blood was immunolabelled with FITC-conjugated CD42a, PE-conjugated CD62P (specifically binds to P-selectin) and isotype matched controls (Biosource, Renfrew, UK), fixed with paraformaldehyde for subsequent analysis with flow cytometry to determine platelet surface expression of P-selectin.

Samples were analysed using a BD FACScan Flow Cytometer and data analysed using FlowJo software (Treestar, Oregon, USA). A medium flow setting was used to minimize leukocyte-platelet coincident events. Platelet-monocyte aggregates were defined as monocytes positive for CD42a (Figure 2.1) and platelet expression of p-selectin was defined as the percentage of platelets positive for CD62P. In our laboratory, the mean coefficient of variation for the percentage of platelet-monocyte aggregates is 7.8% [Din, 2008].

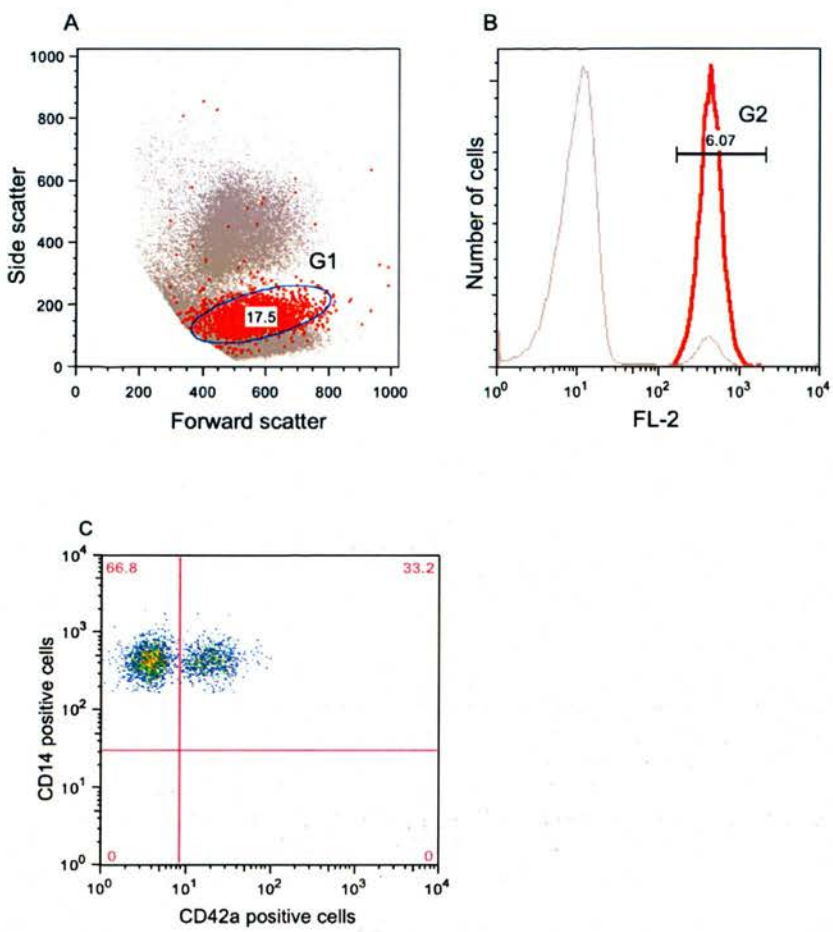

Figure 2.1 Flow cytometric analysis of platelet-monocyte aggregates in whole blood.
Monocytes are gated according to size and granularity and a gate (G1) is placed (*panel A*). Monocytes positive for CD14 are gated (G2 – *panel B*) and a quadrant plot separates CD14-positive monocytes and platelet-monocyte aggregates positive for both CD14 and CD42a (*panel C*).

2.3.4 Data analysis

Results are presented as mean (standard deviation). Unpaired *t*-tests were used to compare measures of platelet activation and haematological and biochemical indices between patients and controls (study 1), and paired *t*-tests were used for within-subject comparisons (study 2). C-reactive protein was log-transformed for positive skewness and the data were presented as median (interquartile range). There was no evidence of inhomogeneity of variance or departures from normality in any of the other data.

In exploratory analyses, associations between platelet-monocyte aggregates, age, markers of inflammation (blood neutrophils, blood leukocytes and highly sensitive c-reactive protein), and markers of disease severity (post-bronchodilator FEV_1 and arterial oxygen tension) were determined using Pearson's correlations. Statistical significance was taken at $p<0.05$.

2.4 Results

2.4.1 Study 1

Patients with COPD and healthy controls were well matched for age and smoking history with a median pack year history of 36 and 35 respectively (Table 2.1). Patients with COPD had moderate to severe airflow limitation (GOLD [Global Initiative in Obstructive Lung Disease] stage 2-4) with a mean FEV_1 of 1.5 L and FEV_1/FVC ratio of 0.42.

Platelet-monocyte aggregates were increased in patients compared to matched healthy controls [mean(standard deviation); 25.3(8.3)% vs 19.5(4.0)%, p=0.01] (Table 2.2, Figure 2.2A). Platelet expression of P-selectin was higher in patients with COPD than controls, but the difference was not statistically significant [1.6(1.2)% vs 1.1(0.8)%, p=0.16]. Similarly, there was no difference in plasma soluble P-selectin concentrations between patients and matched healthy controls. Interestingly there was a close association between platelet monocyte aggregation and platelet-surface P-selectin expression (r=0.59, p<0.001), but not with soluble P-selectin (r=0.30, p=0.10; Figure 2.3).

Platelet-monocyte aggregates were compared with markers of inflammation and disease severity across all subjects in study 1. Platelet-monocyte aggregates correlated with total blood leukocyte count (r=0.45, p=0.007) and neutrophil count (r=0.36, p=0.03). There was a weak association with FEV_1 (r=0.31, p=0.07). There were no significant associations between platelet-monocyte aggregates and age, arterial oxygen tensions or serum CRP concentration (Figure 2.4).

Table 2.1 Baseline characteristics of patients with COPD and matched controls

	Study 1		Study 2
	Controls	COPD	COPD
n	16	18	12
Age, yrs	63 (6)	65 (5)	68 (12)
Male, n (%)	16 (100)	18 (100)	5 (42)
Body mass index, kg/m^2	28 (4)	26 (4)	24 (6)
Smoking history, pack/years*	35 (26-48)	36 (35-51)	45 (39-68)
Current Smoking	0	0	3 (25)
Medications when stable			
Short acting beta agonist	0	17 (94%)	8 (67%)
Long acting beta agonist	0	3 (17%)	7 (58%)
Inhaled corticosteroids	0	1 (6%)	8 (67%)
Oral corticosteroids	0	0	2 (17%)
Combined LABA/ICS	0	13 (72%)	7 (58%)
Anti-cholinergics	0	11 (61%)	7 (25%)
Oxygen Therapy	0	0	1 (8%)
Aspirin	1 (6%)	2 (11%)	1 (8%)
Pulmonary function			
FEV_1, litres	3.4 (0.5)	1.5 (0.7)	0.81 (0.3)
FVC, litres	4.2 (0.6)	3.5 (0.6)	2.0 (0.7)
FEV_1% predicted	102 (10)	48 (20)	39 (17)
FVC% predicted	100 (11)	85 (15)	90 (22)
FEV_1/FVC ratio	0.79 (0.05)	0.42 (0.13)	0.42 (0.12)

Mean (standard deviation) or n (%) except where * indicates median (interquartile range); LABA=Long acting beta-2 agonist; ICS=inhaled corticosteroid; FEV_1=forced expiratory volume in 1 second; FVC=forced vital capacity

2.4.2 Study 2

Patients with an acute exacerbation had a mean age of 68, with lung function measured when stable similar to patients in study 1 (Table 2.1). However, other indicators of disease severity, such as the long-term use of nebuliser therapy, oxygen therapy, and oral corticosteroid were more prevalent in this group. During an acute exacerbation, all subjects received controlled oxygen, nebulised bronchodilators, oral corticosteroids and prophylactic low molecular weight heparin (enoxaparin 40mg), for a median of six days following their first symptom. Clinical parameters were consistent with an acute exacerbation and had returned to normal values by the follow up visit.

Platelet-monocyte aggregates were increased during the acute exacerbation compared to follow up [mean(standard deviation); 32.0(11.0) % vs 25.5(6.4) %, p=0.03] (Table 2.2, Figure 2.2B). Platelet expression of P-selectin was higher during the acute exacerbation compared to follow up, but the difference was not statistically significant [1.4(2.1) % vs 0.8(1.2) %, p=0.40], while there was no difference in soluble P-selectin concentrations.

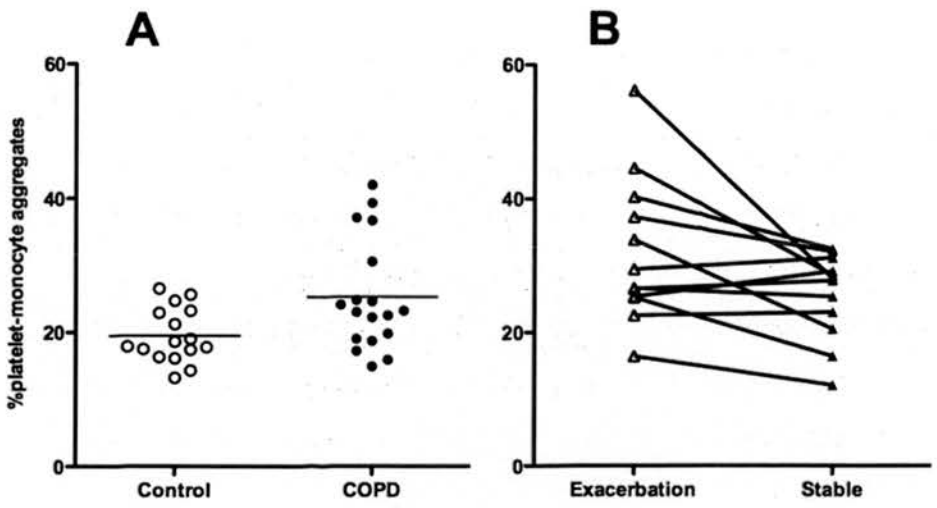

Figure 2.2 Platelet-monocyte aggregates in healthy controls, stable COPD patients and during exacerbations of COPD

Panel A: Patients with COPD have increased platelet-monocyte aggregates in comparison with matched controls (p=0.01); *Panel B*: patients with an exacerbation of COPD have increased platelet-monocyte aggregation in comparison to when their disease is stable (p=0.03). Lines represent the median values of the groups.

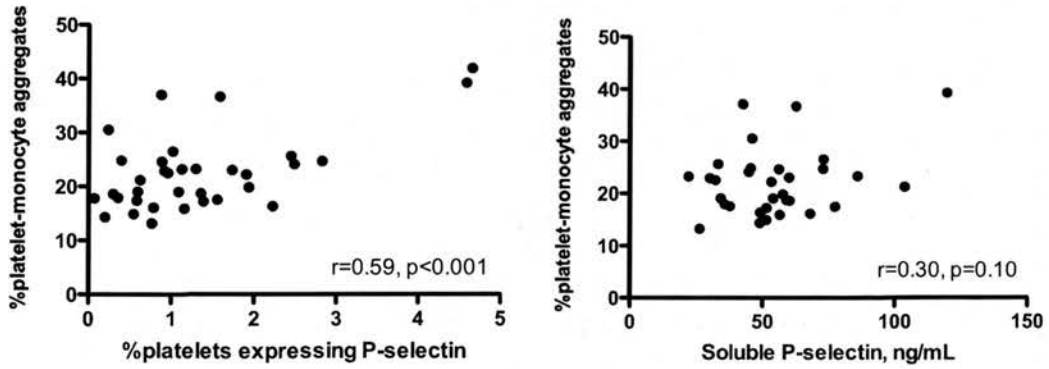

Figure 2.3 Associations between markers of platelet activation: platelet-monocyte aggregation and two measurements of p-selectin expression

Associations analysed using Pearson's correlation

Table 2.2 Haematological and biochemical indices in patients with COPD and controls

	Study 1			Study 2		
	Controls	**COPD**	**P value**	**Stable**	**Exacerbation**	**P value**
Measures of platelet activation						
Platelet-monocyte aggregates, %	19.5 (4.0)	25.3 (8.3)	0.01	25.5 (6.4)	32.0 (11.0)	0.03
P-selectin expression, %	1.1 (0.8)	1.6 (1.2)	0.16	0.8 (1.2)	1.4 (2.1)	0.40
Soluble P-selectin, ng/mL	53.0 (22.2)	56.5 (20.3)	0.65	53.5 (17.8)	61.5(20.8)	0.56
Haematological indices						
Leukocytes, cells $x10^9/L$	5.3 (1.3)	7.0 (1.4)	0.23	7.9 (1.6)	14.8 (3.2)	<0.001
Monocytes, $x10^9/L$	0.5 (0.2)	0.6 (0.2)	0.001	0.7 (0.3)	1.0 (0.3)	0.001
Haemoglobin, g/L	139 (11)	141 (13)	0.03	136 (9)	138 (12)	0.40
Haematocrit	0.4 (0.03)	0.4 (0.03)	0.004	0.4 (0.02)	0.4 (0.04)	0.41
Platelets, $x10^9/L$	196 (31)	262 (68)	0.001	290 (54)	333 (127)	0.09
D-dimer, ng/mL	341 (202)	373 (137)	0.63			
Fibrinogen, mg/dl	2.7 (0.5)	2.8 (0.5)	0.49			
Biochemistry						
Total cholesterol, mmol/L	4.9 (0.9)	5.5 (0.7)	0.03	5.4 (1.4)		
Glucose, mmol/L	5.2 (0.6)	5.1 (0.6)	0.71	5.5 (1.7)		
C-reactive protein, mg/L*	1.0 (0.5-3.2)	2.1 (1.3-5.4)	0.03	2.8 (1.6-8.2)	41 (4-103)	0.01
Arterial oxygenation, kPa	12.3 (1.6)	10.5 (1.7)	0.004			

Mean (standard deviation) except where * indicates median (interquartile range). P-values calculated using independent samples t–test in study 1 and paired t-tests in study 2. C-reactive protein was log-tranformed for analysis.

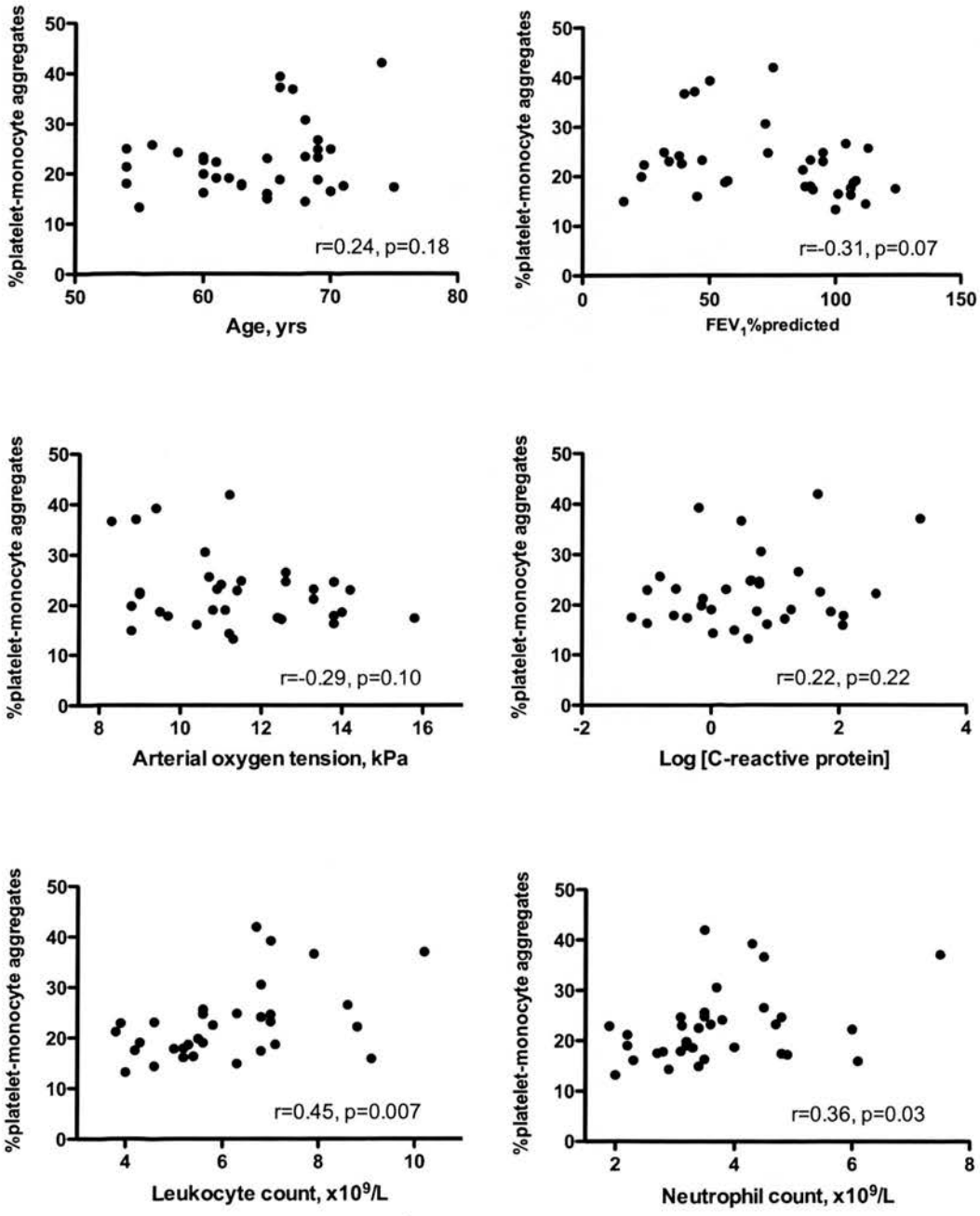

Figure 2.4 Associations between platelet-monocyte aggregation and markers of inflammation and disease severity in study 1

Associations analysed using Pearson's correlation

2.5 Discussion

Using a highly sensitive marker of platelet function we demonstrate that platelet activation is increased in patients with stable COPD, compared to controls matched for age and previous cigarette smoke exposure. Moreover, platelet activation is further increased in COPD patients during acute exacerbation. Taken together these findings suggest that platelet function may be modified as a consequence of COPD.

We suggest that platelet activation represents a novel mechanism linking COPD, inflammation, and cardiovascular disease. Platelet activation is known to predict adverse outcome in patients with stable coronary disease [Christie, 2008] and identify those patients likely to have recurrent cardiovascular events following percutaneous coronary intervention [Gianetti, 2006]. The interaction between platelets and inflammatory cells stimulates release of chemokines and further recruitment of immune mediators which are central to the development of atherosclerotic plaque.

Platelet activation has also been implicated in structural remodelling of the pulmonary vasculature, and is thought to play a role in the pathogenesis of all forms of pulmonary arterial hypertension [Humbert, 2004]. Therefore, our findings are potentially of relevance to the *pulmonary* vascular as well as systemic vascular features of COPD. We discuss abnormal systemic vascular function in COPD in Chapter 3. In this study we have looked at a distinct but equally important aspect of atherothrombosis and cardiovascular risk: platelet activation.

Previous studies have suggested that COPD is associated with a pro-thrombotic and hypercoagulable state [Ashitani, 2002; Davì, 1997]. Few studies have measured platelet activation, and none have employed a direct measure of platelet function. Soluble P-selectin was increased in patients with COPD [Ferroni, 2000] and inversely related to FEV_1 [Walter, 2008]. However, soluble P-selectin is not a direct measure of platelet activation, and may reflect P-selectin release from the endothelium and platelet surface [Fijnheer, 1997]. Additionally, concentrations of soluble P-selectin may not reflect platelet surface P-selectin expression [Gurbel, 2000; Kamath, 2002]. Our study adds to the literature by demonstrating increased platelet activation using whole blood flow cytometry, which is both a sensitive and specific technique, in

groups of COPD patients and controls well-matched for both age and, importantly, for smoking history. Furthermore, our findings are consistent across two separate cohorts with levels of platelet-monocyte aggregation identical in stable COPD patients from both studies.

The effects of cigarette smoking on markers of platelet activation and platelet-monocyte aggregates are well established [Harding, 2004a]. In our case-control study all patients and controls were ex-smokers, with normal exhaled CO levels, and were matched for smoking history, yet we demonstrate that platelet-monocyte aggregates were increased in COPD patients by approximately 30% compared to controls. The magnitude of this difference was comparable to differences previously reported between smokers and non-smokers [Harding, 2004a]. This implies that platelet function may be modified as a direct consequence of COPD, and that this potentially pro-thrombotic manifestation may be as important as cigarette smoking in determining cardiovascular risk in these patients. Our results are consistent with differences that are seen in other inflammatory conditions associated with increased cardiovascular risk, such as rheumatoid arthritis, where platelet-monocyte aggregate levels were around 20% higher than matched controls [Joseph, 2001]. Higher platelet-monocyte aggregation is found in acute coronary syndromes, with a 30-50% increase in platelet-monocyte aggregation in comparison to patients with non-cardiac chest pain, but these levels were found during acute arterial thrombotic events [Sarma, 2002; Michelson, 2001].

We have not identified the precise mechanism of platelet activation in patients with COPD, but a number of variables, such as increased systemic inflammation, hypoxaemia and haemodynamic stress that differed between patients and controls or were enhanced during acute exacerbation, may be implicated.

Platelet activation is inextricably linked to local vascular inflammation, with activated platelets causing release of chemokines, together with up-regulation of cell surface adhesion molecules that drive monocyte recruitment, platelet-monocyte interaction and adherence to denuded endothelium [Gawaz, 2005]. Furthermore, leukocytes recruited by chemotaxis cause activation of platelets [Del Maschio, 1990]. We identified increases in the number of peripheral blood leukocytes and neutrophils as

well as higher serum concentrations of CRP in patients with COPD compared to control subjects. Circulating leukocyte and neutrophil concentrations were associated with platelet-monocyte aggregates, supporting the hypothesis that systemic inflammation in COPD may contribute to platelet activation in this condition. This mechanism is thought to be important in other chronic inflammatory conditions with increased platelet-monocyte aggregates, such as rheumatoid arthritis and type 1 diabetes mellitus. [Joseph, 2001; Harding, 2004b]. Although there was not a significant relationship between CRP and platelet-monocyte aggregates, there was a positive association, and a significant relationship may have been revealed in a study of larger numbers.

Alternative mechanisms through which platelet activation may occur in patients with COPD include hypoxia, tachycardia, and hyperglycaemia [Diodati, 1992; Vaidyula, 2006]. These factors may cause further platelet activation during acute exacerbation and explain the association between lower respiratory tract infection and acute myocardial infarction [Smeeth, 2004]. Further studies using cellular and animal models are necessary to elucidate the relative importance of these mechanisms.

Interestingly, patients with COPD had higher platelet counts than healthy controls although levels remained within the normal range. An increase in platelet count *per se* has been associated with adverse cardiovascular outcomes in both healthy persons and patients with acute myocardial infarction [Thaulow, 1991; Ly, 2006]. Previous studies have suggested anaemia is associated with an increased morbidity and mortality in COPD patients independent of disease severity [Cote, 2007; Similowski, 2006], but the relationship between platelet count and clinical outcome has not been examined. Thus in comparison with controls, patients with COPD not only have greater platelet activation, but also increased numbers of platelets, and both may increase cardiovascular risk.

Platelet counts and platelet-monocyte aggregates may be more than simply markers of cardiovascular risk, with platelet activation a potential target for therapy. Platelet-monocyte aggregates form independently of the cyclooxygenase pathway and thus are not modified by aspirin therapy [Klinkhardt, 2003]. Population studies and controlled trials are necessary to determine whether aspirin is an effective anti-platelet therapy in

COPD and whether the regular use of anti-platelet agents could prevent cardiovascular events in patients with COPD.

2.5.1 Limitations

Although selection bias is possible in all case-control studies, the groups in study 1 were well matched for age, smoking, and other clinical characteristics, and as such we do not think this can explain the reported differences in platelet activation. We did not match on weight or BMI as we were interested in the systemic effects of COPD, and since reduced weight is a systemic effect of COPD, we did not want to over-match patients and controls. We did not perform any post-hoc exploratory multivariate analyses because this is problematic in case-control designs, and because the sample sizes were comparatively small for such methods. In study 2, we examined platelet activation within 24 hours of admission with an exacerbation of COPD, and at least two weeks post exacerbation, while there is some evidence that the effects of acute exacerbations may persist beyond 90 days [Seemungal, 2000]. However, this would likely cause an underestimation of an effect of exacerbation on platelet activation. Additionally, we were unable to impose the same restrictions on patients with acute exacerbations as we could in the stable condition, thus differences in medication, dietary intake or other environmental factors may have contributed to the platelet-monocyte aggregation observed during exacerbations. However, imposing such restrictions is impractical in these patients, and medications used during acute exacerbations, such as steroids and prophylactic low molecular weight heparin do not influence platelet activation [Schuerholz, 2007; Harding, 2006].

2.6 Conclusion

Using a highly sensitive marker of platelet activation we demonstrate that platelet-monocyte aggregates are increased in patients with stable COPD independent of cigarette smoke exposure. Platelet activation was further increased in patients during an acute exacerbation. Our findings suggest that platelet function may be modified as a direct consequence of COPD, and identify platelet activation as an important prothrombotic manifestation of the disease, which may be a useful therapeutic target in COPD.

Chapter 3. Vascular Dysfunction in Chronic Obstructive Pulmonary Disease

Maclay JD, McAllister DA, Mills NL, Paterson FP, Ludlam CA, Drost EM, Newby DE and MacNee W. Vascular dysfunction in COPD. *American Journal of Respiratory and Critical Care Medicine* 2009; 183:513-20

3.1 Summary

Cardiovascular disease is a major cause of morbidity and mortality in patients with chronic obstructive pulmonary disease (COPD), which may in part be attributable to abnormalities of systemic vascular function. It is unclear whether such associations relate to the presence of COPD or prior smoking habit. Thus the aim of this study was to perform a comprehensive assessment of vascular function in patients with COPD and healthy controls matched for smoking history. Eighteen men with COPD were compared with 17 healthy male controls matched for age and lifetime cigarette smoke exposure. Participants were free from clinically evident cardiovascular disease. Pulse wave velocity (PWV) and pulse wave analysis were measured via applanation tonometry at carotid, radial and femoral arteries. Blood flow was measured in both forearms using venous occlusion plethysmography during intra-brachial infusion of endothelium-dependent vasodilators (bradykinin, 100-1000 pmol/min; acetylcholine, 5-20 μg/min) and endothelium-independent vasodilators (sodium nitroprusside, 2-8 μg/min; verapamil, 10-100 μg/min). Tissue plasminogen activator (t-PA) was measured in venous plasma before and during bradykinin infusions. Patients with COPD had greater arterial stiffness [PWV, 11(2) vs 9(2) m/s; p=0.003: augmentation index, 27(10) vs 21(6) %, p=0.028], but there were no differences in endothelium-dependent and –independent vasomotor function or bradykinin-induced endothelial t-PA release (p>0.05 for all). These findings show that COPD is associated with increased arterial stiffness independent of cigarette smoke exposure. However, this abnormality is not explained by systemic endothelial dysfunction. Increased arterial stiffness may represent a mechanistic link between COPD and the increased risk of cardiovascular disease associated with this condition.

3.2 Introduction

Cardiovascular disease is a major cause of morbidity and mortality in patients with COPD [Calverley, 2007; Camilli, 1991; Curkendall, 2006; Huiart, 2005; Sidney, 2005]. This interrelationship partly reflects common risk factors, such as cigarette smoke exposure, low socio-economic class, and sedentarism. However, reduced forced expiratory volume in one second (FEV_1; most commonly caused by COPD) independently predicts cardiovascular risk in the general population [Hole, 1996]. Moreover, individuals with COPD have an increased risk of cardiovascular morbidity and mortality that is independent of traditional risk factors, including smoking [Curkendall, 2006]. Recent studies have identified increased arterial stiffness [Sabit, 2007] and altered vasomotor function [Eickhoff, 2008; Barr, 2007] in patients with COPD, perhaps suggesting a role for systemic vascular dysfunction in mediating this cardiovascular risk [Maclay, 2007], particularly as arterial stiffness predicts cardiovascular events in the general population [Willum-Hansen, 2006]. The structural components of the vessel wall all contribute to central arterial stiffness – the extracellular matrix largely comprising elastin and collagen, vascular smooth muscle and the endothelium [Zieman, 2005].

The endothelium plays a vital role in the control of blood flow, coagulation, fibrinolysis and inflammation. In particular, release of the endogenous fibrinolytic enzyme tissue plasminogen activator (t-PA) is vital for maintaining vessel patency by preventing persistent thrombotic occlusion. Vasomotor dysfunction is associated with atherosclerosis, traditional cardiovascular risk factors and, like arterial stiffness, independently predicts adverse cardiovascular events [Halcox, 2002; Perticone, 2001; Celermajer, 1992; Celermajer, 1996; Fichtlscherer, 2004; Mills, 2007].

Arterial stiffness, vasomotor dysfunction and impaired endogenous fibrinolysis are all features of cigarette smoking [Jatoi, 2007; Newby, 1999; Pretorius, 2002; Lang, 2008] and it remains unclear whether the observations of vascular dysfunction in patients with COPD are attributable to cigarette smoking only or are a consequence of COPD itself. We hypothesised that patients with COPD would have increased arterial stiffness as a consequence of systemic endothelial dysfunction, impaired endogenous

fibrinolysis and that this vascular dysfunction would be independent of their smoking habit.

3.3 Methods

3.3.1 Study Subjects

Eighteen men with COPD and 17 healthy male controls were recruited from primary care and a hospital respiratory out-patient clinic. Subjects were matched for age and prior smoking habit. Men aged 40-80 years who were ex-smokers (smoking cessation for at least 3 months) but with a smoking history of ≥10 pack years were included. Exclusion criteria were a history of pulmonary fibrosis, tuberculosis, bronchiectasis, lung cancer or lung resection, conditions known to affect vascular function including obstructive sleep apnoea, cardiovascular, cerebrovascular, and peripheral vascular disease, uncontrolled hypertension, diabetes, inflammatory conditions such as rheumatoid arthritis or psoriasis, or taking drugs that affect vascular function including statins, angiotensin-converting enzyme inhibitors and beta-blockers. Healthy control subjects had normal spirometry and no history of respiratory symptoms. Subjects with COPD had a history consistent with the disease, chronic airflow limitation on spirometry (post-bronchodilator FEV_1/FVC ratio ≤0.7), stable disease (no exacerbation of COPD within the previous 6 weeks, defined as a sustained change in symptoms requiring antibiotic or steroid therapy) and were not prescribed regular oral steroid therapy or long-term oxygen therapy.

All studies were conducted at the Wellcome Trust Clinical Research Facility, Royal Infirmary, Edinburgh. Height, weight, and pre- and post-bronchodilator spirometry were measured (Alpha Spirometer; Vitalograph, Buckingham, UK) according to American Thoracic Society/European Respiratory Society standards. All studies were approved by Lothian Regional Ethics Committee and conducted with the written informed consent of all participants.

3.3.2 Arterial stiffness measurements

3.3.2.1 Subject preparation and ambient conditions

Subjects had fasted overnight and abstained from coffee, tea and alcohol for the 24 hours prior to the study. All medications were withheld on the morning of the study. Exhaled carbon monoxide measurements (<5 ppm) ensured no acute cigarette smoke exposure. Following 30 minutes of supine rest, peripheral systolic and diastolic blood pressures were measured using an automated non-invasive oscillometric sphygmomanometer (Omron 705IT; Milton Keynes, UK). Studies were performed in the morning in a quiet, dimly lit, temperature-controlled room (22-25°C).

3.3.2.2 Pulse wave velocity and analysis

Arterial stiffness can be measured using several techniques all of which employ similar equipment. The gold standard method is pulse wave velocity [Laurent, 2006; Mackenzie, 2002]. Carotid-femoral pulse wave velocity measures the speed of the pulse wave across the aorta which is responsible for most of the pathophysiological effects of increased arterial stiffness. Large vessels cushion the pressure created in systole by ventricular contraction and this function is reduced in stiff arteries, resulting in a faster pulse wave. Carotid-femoral (aortic) pulse wave velocity (PWV) is increased with increased arterial stiffness. This measure is predictive of cardiovascular events in healthy individuals, as well as an associated with mortality in patients with ischaemic heart disease [Vlachopoulos, 2010; Willum-Hansen, 2006]. It is also possible to measure carotid-radial (brachial) pulse wave velocity across the brachial artery. However, unlike aortic PWV, measurement of arterial stiffness across this muscular artery is not associated with carotid intimal medial thickness, a non-invasive measure of atherosclerotic plaque burden. In addition, it is neither associated with cardiovascular events nor mortality [Tillin, 2007; Laurent, 2006].

Pulse wave velocity (PWV) is calculated from distance (D)/time (Δt). The distance is the surface distance between the two recording points (ie the carotid pulse and the femoral pulse). Using applanation tonometry at the carotid and femoral arteries a pressure waveform is recorded. The Sphygmocor system uses software which applies

an intersecting tangent method to identify the onset of the wave and the difference in transit time to the femoral and carotid arteries is taken as Δt (Figure 3.1).

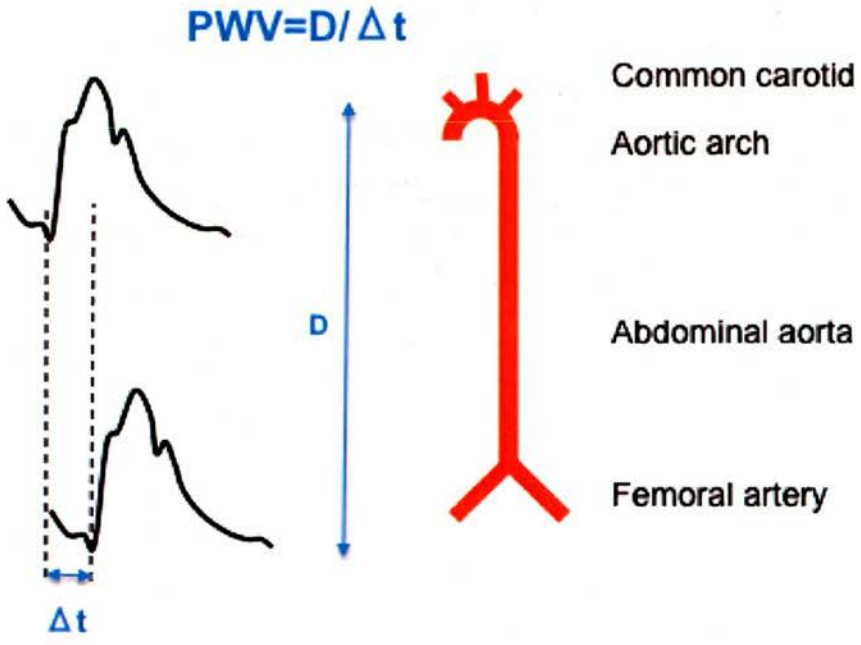

Figure 3.1 Carotid-femoral pulse wave velocity.

Measurement of carotid-femoral pulse wave velocity using the intersecting tangent method.

Pulse wave analysis is another non-invasive measure of arterial stiffness that can be assessed using the Sphymocor kit [O'Rourke, 2001]. This relies on wave reflection from bifurcating vessels. In compliant blood vessels, the pulse wave is reflected back to the aortic root in diastole. In stiff blood vessels, the wave is reflected earlier often during systole, augmenting the systolic pressure. Time to wave reflection (Tr) is reduced in the presence of stiff arteries. Using a radial pressure waveform measured by applanation tonometry, the difference in the systolic peak from the peak caused by the inflected waveform is the augmentation pressure (AP) (Figure 3.2). This expressed as a percentage of pulse pressure is called the augmentation index (AIx). AP and AIx are increased in the presence of stiff arteries. The main determinants of AIx are age, heart rate (it is often corrected for this), diastolic blood pressure and aortic pulse wave velocity. AIx-75 is the standard measure of augmentation index corrected for a heart rate of 75 and is calculated using a transfer function. Pulse wave analysis does not have the same predictive value as aortic PWV but it is still a useful measure of arterial stiffness as it requires little technical expertise. AIx does predict

mortality in end stage renal disease and cardiovascular events in hypertension [London, 2001; Williams, 2006].

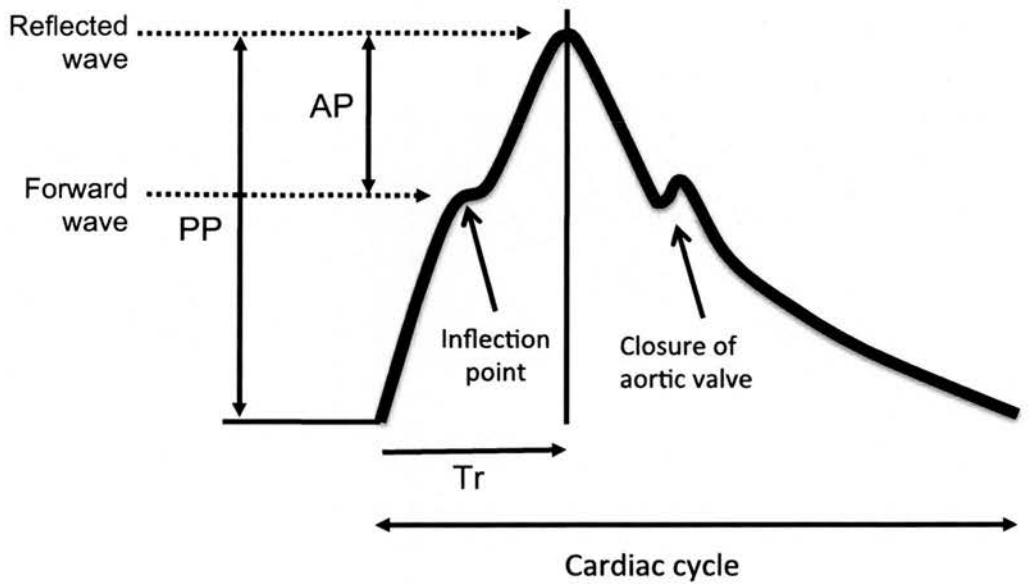

Figure 3.2 Augmentation index.

An example of a radial pressure waveform. Augmentation index is calculated as AP (augmentation pressure)/PP (pulse pressure) x100.

These studies were conducted as per the Expert Consensus Document on Arterial Stiffness, assessing both pulse wave velocity and pulse wave analysis using a high-fidelity micromanometer (Millar Instruments, Texas) and the SphygmoCorTM system (AtCor Medical, Sydney) [Laurent, 2006].

3.3.3 Venous occlusion plethysmography

Forearm venous occlusion plethysmography is a relatively inexpensive and minimally invasive technique used to study endothelial function in humans. Using this technique, understanding of the physiology of vasomotor function has been greatly enhanced. In addition, impairment of endothelial function has been demonstrated in many conditions including stable ischaemic heart disease, cigarette smokers, hypercholesterolaemia and type 1 diabetics [Heitzer, 2001; Newby, 1999; Chowienczyk, 1992; Johnstone, 1993].

3.3.3.1 Measurement of forearm blood flow

This technique uses a mercury-in-sialastic strain gauge that encircles the widest part of the forearm (Figure 3.3) [Wilkinson, 2001]. A pressure cuff is attached to the upper arm and this is set to inflate above venous pressure. A further cuff is attached to the wrist and is set to inflate higher than arterial pressure, excluding the hand circulation from the analysis. For three minutes, the lower cuff is continuously inflated. Also during this time, the upper cuff is set to inflate for nine seconds and deflate for four on a continuous cycle. Thus venous drainage is prevented and arterial inflow continues unchecked. The strain gauge records a linear increase in forearm volume (as there is an increase in resistance as the gauge stretches with forearm circumference), which is proportional to arterial inflow (Figure 3.4). This change in volume is predominantly due to blood flow through skeletal muscle in the forearm.

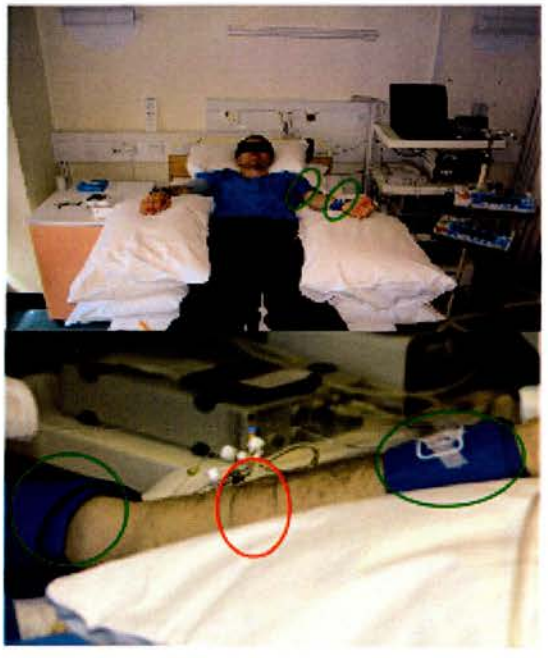

Figure 3.3 Venous occlusion plethysmography
A cuff is placed around the wrist and inflated to greater than systolic blood pressure to exclude the hand circulation. A second cuff is placed on the upper arm and intermittently inflated to greater than venous pressure to prevent venous return resulting in a linear increased in forearm volume. Pressure cuffs are highlighted in green and mercury-in-sialastic strain gauge in red.

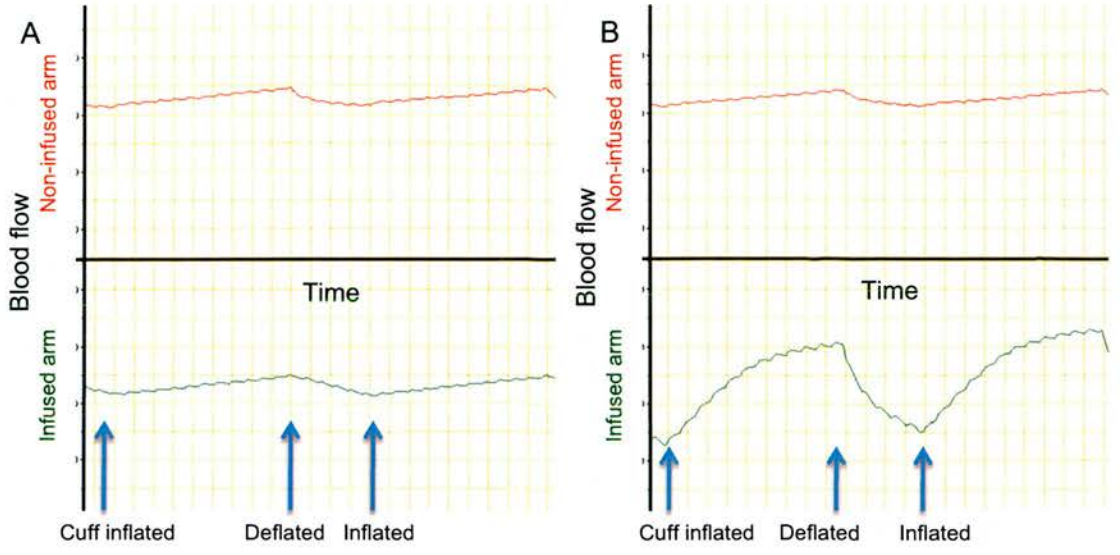

Figure 3.4 Forearm blood flow measurements.

Panel A is a baseline measurement with forearm volume increasing in a linear fashion with inflation of the upper arm cuff. Panel B shows increased forearm blood flow in response to bradykinin infusion. Note there is no change in blood flow in the non-infused arm.

3.3.3.2 Local drug administration

Administration of vasoactive drugs into the brachial artery allows assessment of vasomotor function in the forearm vasculature. Drugs are given through a 27-gauge cannula inserted under local anaesthesia. This cannula remains in situ for the length of the study. This technique is relatively safe but vigilance is advised as occasionally the needle becomes dislodged and requires to be replaced. Reassuringly, vasodilators can be used at very low concentrations, and thus have only local action and do not have any systemic effects, reducing risk.

In order to assess endothelial function, vasodilators are used which stimulate the endothelium to produce vasoactive mediators like nitric oxide to cause vasodilation, such as bradykinin and acetylcholine (Figure 3.4; Figure 3.5). In addition, to distinguish any dysfunction of the endothelium from other structures affecting vascular function, vasodilators that bypass the endothelium, acting directly on the vascular smooth muscle are used such as the nitric oxide donor sodium nitroprusside, and the calcium channel blocker verapamil.

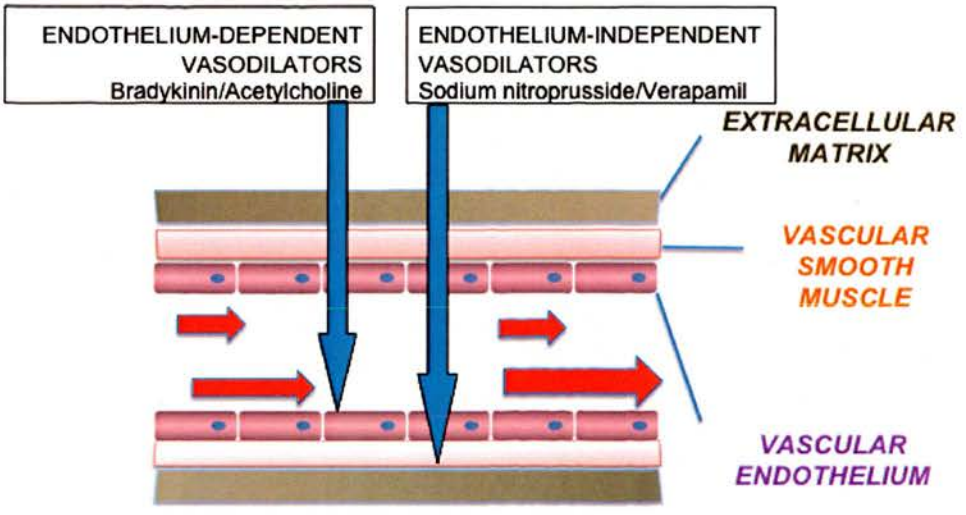

Figure 3.5 Vasodilators.

Endothelium-dependent vasodilators stimulate production of vasoactive mediators eg nitric oxide to cause vasodilation. Endothelium-independent vasodilators bypass the endothelium, acting directly on the smooth muscle to cause vasodilation.

In these studies, under the same ambient conditions and subject restrictions for the arterial stiffness measures, bilateral forearm blood flow was measured using venous occlusion plethysmography. The endothelium-dependent vasodilators, bradykinin (100, 300 and 1,000 pmol/min) and acetylcholine (5, 10 and 20 μg/min) and the endothelium-independent vasodilators, sodium nitroprusside (2, 4 and 8 μg/min) and verapamil (10, 30 and 100 μg/min) were infused via a 27-gauge intrabrachial needle incrementally for 6 minutes at each dose. Vasodilators were separated by 15-min saline infusions and given in random order except for verapamil, which was administered last due to its slow offset of action. Plethysmograph traces were recorded using Chart™ 5 software (ADinstruments) and were analysed by one investigator who was blinded to subject identity. The mean of the last five waveforms from each set of readings were used to calculate forearm blood flow.

3.3.4 Blood collection and assays

Venous cannulae (17-gauge) were inserted into both antecubital fossae. Baseline blood samples were obtained for hemoglobin and hematocrit, and fasting blood samples were obtained for both glucose and lipid profile measured in the clinical

laboratories of the Royal Infirmary of Edinburgh (Sysmex, Germany and Olympus Analyzers, USA). Arterial blood gases were measured at rest (Bayer Rapidlab, USA). Blood was sampled at baseline, centrifuged and serum stored at -80°C for subsequent analysis. Serum C-reactive protein (CRP) concentrations were measured using a highly sensitive immunonephelometric assay (Behring BN II nephelometer, Germany).

Infusion of intra-brachial bradykinin (100, 300 and 1,000 pmol/min) not only causes endothelium-dependent vasodilatation but also stimulates endothelial tissue plasminogen activator (t-PA) release [Newby, 1997; Wilkinson, 2001]. Venous blood (10 mL) was collected at baseline and during each dose of bradykinin into acidified buffered citrate (Stabilyte tubes, Biopool International) for t-PA antigen, and into citrate (Monovette, Sarstedt) for plasminogen activator inhibitor type 1 (PAI-1) antigen estimation. Samples were collected onto ice, centrifuged at 2000*g* for 30 min at 4°C and plasma was stored at -80°C until analysed. Plasma t-PA and PAI-1 antigen concentrations were determined by enzyme-linked immunosorbent assays (TintElize t-PA, Biopool EIA and Elitest PAI-1, Hyphen Biomed respectively).

3.3.5 Data analysis

Absolute t-PA release was calculated as the difference in the t-PA antigen concentration measured in the infused and non-infused arms. Estimated net release of t-PA antigen was calculated as previously described [Newby, 1997] as the product of the infused forearm plasma flow (based on the mean haematocrit, [Hct], and the infused forearm blood flow, [FBF]) and the concentration difference between the infused ($[t\text{-}PA]_{Inf}$) and non-infused ($[t\text{-}PA]_{Noninf}$) arms: Estimated net t-PA release = $FBFx(1\text{-}Hct)x([t\text{-}PA]_{Inf} - [t\text{-}PA]_{Noninf})$. Data were analyzed using two-way analysis of variance with repeated measures and Student's or Welch's (for groups with unequal variances) unpaired *t*-tests as appropriate. Univariate comparisons were analyzed using Pearson's correlations. C-reactive protein and PAI-1 were log transformed to correct for positive skewness and were presented as median (interquartile range). All analyses were performed using SPSS version 16.0 (Chicago, USA). Statistical significance was taken at $p<0.05$.

3.4 Results

Men with COPD and healthy controls were well matched for age and smoking history. As expected given the range of COPD severity (GOLD stage 1-4), patients with COPD had lower mean arterial oxygenation and higher mean heart rate, peripheral blood white cell count and hsCRP (Table 1). Forearm blood flow measurements could not be completed in one subject, and venous samples for t-PA analysis could not be obtained in a second subject. We were unable to measure carotid-femoral pulse wave velocity in two subjects.

3.4.1 Arterial Stiffness

Measures of arterial stiffness were higher in patients with COPD (Table 2; Figure 1). Carotid-femoral pulse wave velocity was higher in subjects with COPD compared with controls [mean (standard deviation); 11(2) vs 9(2) m/s, p=0.003]. When corrected for differences in heart rate, augmentation index was similarly increased in patients with COPD [27(10) vs 21(6) %, p=0.028]. There was no difference in time to wave reflection [142(21) vs 150(12) ms, p=0.13].

In all subjects, aortic pulse wave velocity correlated significantly with systolic blood pressure (r=0.62, p<0.001) and measurements of airflow obstruction (FEV_1, r=-0.38, p=0.03; FEV_1/FVC, r=-0.45, p=0.008), but there was no association with systemic inflammation (circulating leukocytes, r=0.21, p=0.24; hsCRP, r=0.30, p=0.10) or arterial oxygen tension (r=-0.05, p=0.80).

Table 3.1 Subject demographics

	COPD	CONTROL	p-value
n	18	17	
Age, years	65 (5.4)	63 (6.0)	0.40
Body mass index (kg/m^2)	26.4 (3.6)	28.4 (3.9)	0.13
Lung function			
FEV_1 % predicted	47.6 (20.1)	101.6 (10.0)	<0.001
FVC % predicted	85.3 (15.4)	100.6 (10.7)	0.002
FEV_1/FVC	0.42 (0.13)	0.79 (0.53)	<0.001
Inhaled medications, number of subjects (%)			
Short acting beta agonist	17 (94%)		
Anticholinergic	11 (61%)		
Long acting beta agonist	3 (14%)		
Inhaled corticosteroid	1 (6%)		
ICS/LABA combination	13 (72%)		
Traditional risk factors			
Total cholesterol, mg/dL	215 (27)	191 (35)	0.04
Cholesterol:HDL ratio	4.0 (0.8)	3.8 (1.0)	0.66
Fasting glucose, mg/dL	92 (11)	95 (11)	0.48
Pack years smoking*	35 (35-48)	34 (28-46)	0.13
Physiological parameters			
Heart rate, bpm	68 (12)	58 (8)	0.002
Systolic blood pressure, mmHg	127 (14)	126 (18)	0.86
Diastolic blood pressure, mmHg	75 (8)	78 (9)	0.33
PaO_2, kPa	10.5 (1.8)	12.2 (1.6)	0.005
Haematological measures			
Haemoglobin, $x10^9/L$	140.8 (13.5)	138.4 (10.9)	0.57
Haematocrit	0.42 (0.03)	0.41 (0.03)	0.16
Inflammatory markers			
C-reactive protein, mg/L*	2.1 (1.4-5.3)	1.0 (0.6-2.4)	0.03
Leukocytes, cells $x10^9/L$	7.0 (1.4)	5.3 (1.3)	<0.001

Definition of abbreviations: ICS/LABA=Inhaled corticosteroid/long acting beta agonist. Data presented as mean (standard deviation), except * median (interquartile range).Data compared with Student's t-test or Welch's t-test (hsCRP log transformed), except pack years smoking (Mann-Whitney U).

Table 3.2 Haemodynamic measures including arterial stiffness in COPD patients and matched controls

	COPD	Control	*p-value*
Heart rate (beats/min)	68 (12)	58 (8)	0.002
Peripheral systolic blood pressure (mmHg)	128 (18)	130 (14)	0.673
Peripheral diastolic blood pressure (mmHg)	77 (11)	77 (9)	0.921
Peripheral pulse pressure (mmHg)	43 (14)	45 (11)	0.618
Mean arterial pressure (mmHg)	95 (13)	96 (10)	0.894
Central systolic blood pressure (mmHg)	120 (18)	123 (14)	0.690
Central diastolic blood pressure (mmHg)	78 (18)	78 (9)	0.985
Augmentation pressure (mmHg)	14 (7)	14 (6)	0.793
Augmentation index (%)	31 (9)	30 (7)	0.524
Augmentation index-75 (%)	27 (10)	21 (6)	0.028
Time to wave reflection (ms)	142 (21)	151 (12)	0.13
Aortic pulse wave velocity (ms^{-1})	11 (2)	9 (2)	0.003

Compared using Student's t-test or Welch's t-test.

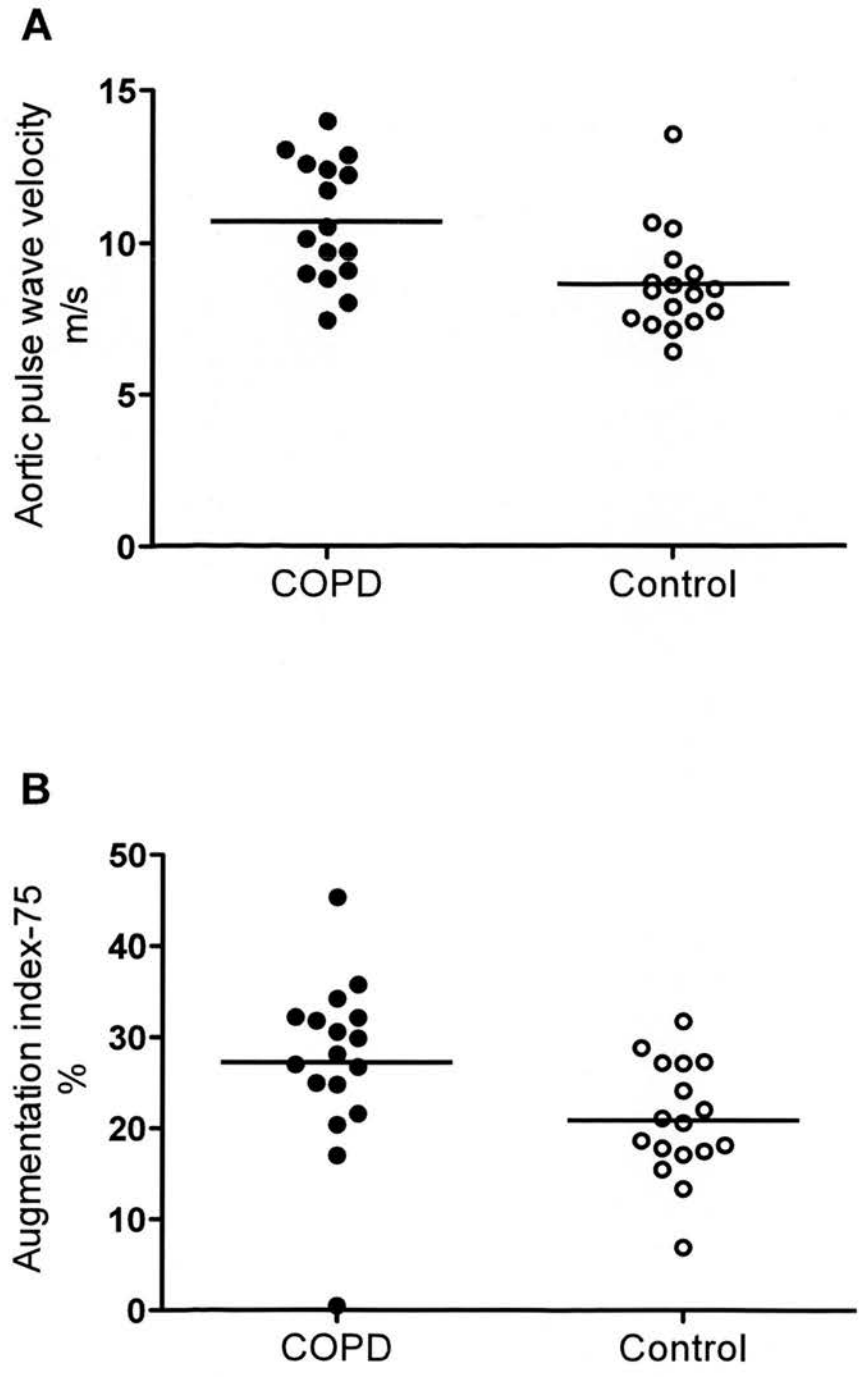

Figure 3.6 Patients with COPD have increased measures of arterial stiffness in comparison to matched controls

Aortic pulse wave velocity (A) and augmentation index (B) are higher in patients with chronic obstructive pulmonary disease (COPD) when compared with age and smoking habit matched controls (t-test, p=0.003 and p=0.03). Symbols represent individual values and lines the means.

3.4.2 Vasomotion

Baseline blood flow was similar at the start (p=0.43; table 3) and prior to infusion of each vasodilator, and blood pressure and heart rate were unchanged throughout the studies in both groups. All vasodilators caused a dose-dependent increase in forearm blood flow ($p<0.001$ for all; Figure 2) that was similar in both groups (bradykinin, p=0.73; acetylcholine, p=0.72; sodium nitroprusside, p=0.31; verapamil, p=0.80).

3.4.3 Fibrinolysis

Baseline plasma t-PA antigen concentrations were similar in patients with COPD and controls [mean±SD; 13.2(5.0) vs 13.0(4.0) ng/mL, p=0.92; Table 3]. Bradykinin caused a dose-dependent increase in plasma t-PA antigen concentrations in both groups ($p<0.001$ for both). There were neither differences in absolute t-PA antigen release nor stimulated net t-PA release following bradykinin infusion in subjects with COPD in comparison with controls [net t-PA release 63.2(102.7) vs 69.4(71.9) ng/100mL of tissue/min at 1,000 pmol/min, p=0.90; Figure 3]. Plasma PAI-1 antigen concentrations were similar in both groups [median (interquartile range), baseline 30.9 (24.4 to 41.6) ng/ml vs 28.4 (18.1 to 53.9) ng/ml; p=0.38] and were unchanged by bradykinin infusion (p=0.99).

Table 3.3 Tissue plasminogen activator (t-PA) release and forearm blood flow in response to increasing doses of bradykinin

	Bradykinin infusion (pmol/min)				
t-PA	0	100	300	1000	
Infused					p-value
COPD	13.2 (5.0)	16.9 (5.3)	19.3 (6.1)	23.6 (7.7)	0.92
Control	13.0 (4.0)	15.5 (5.2)	18.5 (6.5)	25.3 (8.9)	
Non-infused arm					
COPD	12.3 (4.5)	13.8 (4.2)	15.1 (4.4)	17.8 (5.0)	0.56
Control	12.0 (4.5)	13.5 (5.5)	13.7 (4.8)	16.3 (5.1)	
Difference between infused and non-infused arms					
COPD	1.0 (1.6)	3.1 (2.8)	4.1 (3.5)	5.7 (8.5)	0.60
Control	0.9 (2.0)	2.0 (4.2)	4.8 (6.2)	9.0 (9.2)	
Net t-PA release, ng/100 mL					
COPD	1.5 (3.1)	14.8 (11.3)	27.0 (20.8)	63.2 (102.7)	0.90
Control	0.8 (4.8)	5.9 (12.9)	25.2 (26.3)	69.4 (71.9)	
Forearm blood flow, mL/100mL					
COPD	2.7 (1.1)	9.0 (4.9)	11.9 (7.4)	16.0 (9.0)	0.73
Control	2.5 (1.3)	8.1 (3.1)	11.3 (4.4)	15.6 (6.4)	

Values represent mean (standard deviation).

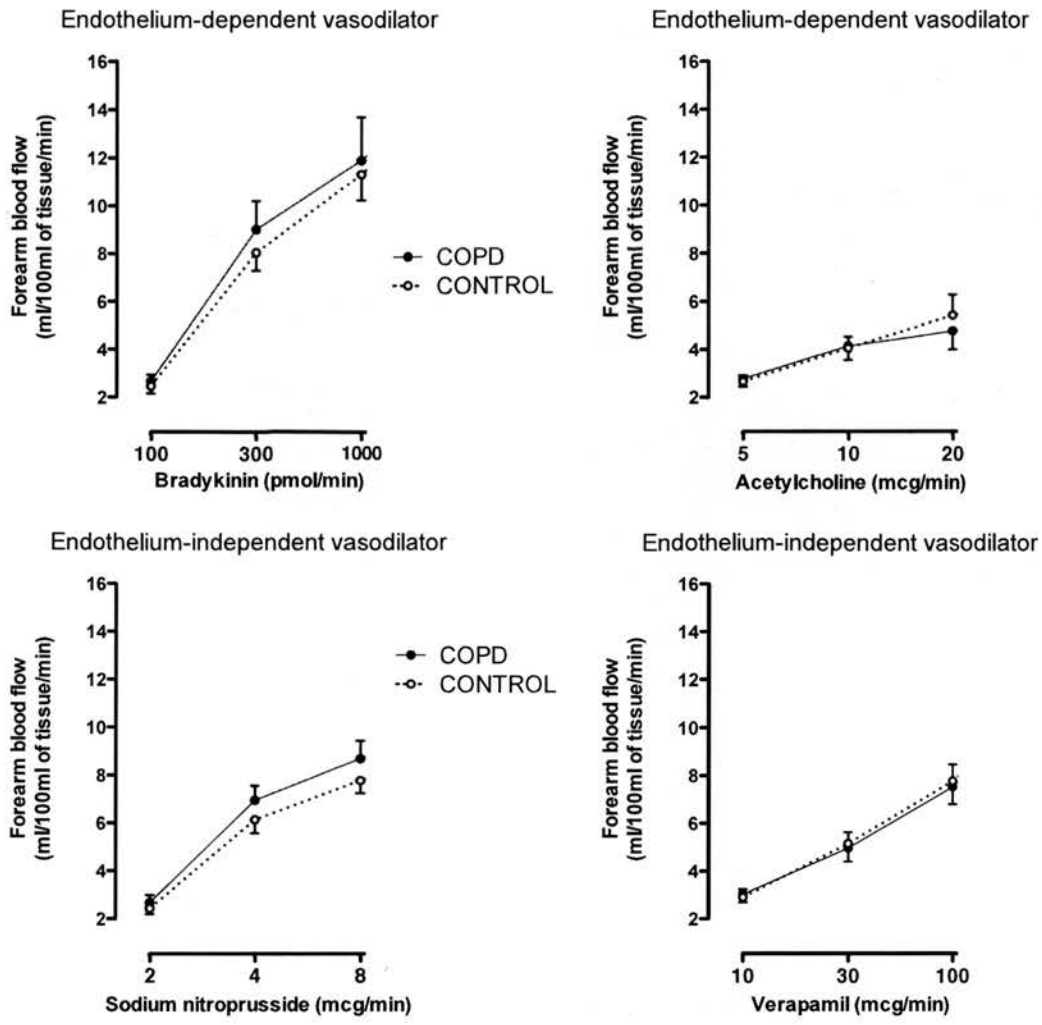

Figure 3.7 Forearm blood flow in response to endothelium-dependent and -independent vasodilators is similar in COPD patients and healthy controls

Vasomotor function in response to two endothelial-dependent and independent vasodilators is preserved in patients with chronic obstructive pulmonary disease (COPD; closed circles) in comparison to age and smoking habit matched controls (open circles; repeated measures two-way ANOVA; bradykinin, p=0.73; acetylcholine, p=0.72; sodium nitroprusside, p=0.31; verapamil, p=0.80). Symbols represent the means and bars the standard errors of the means.

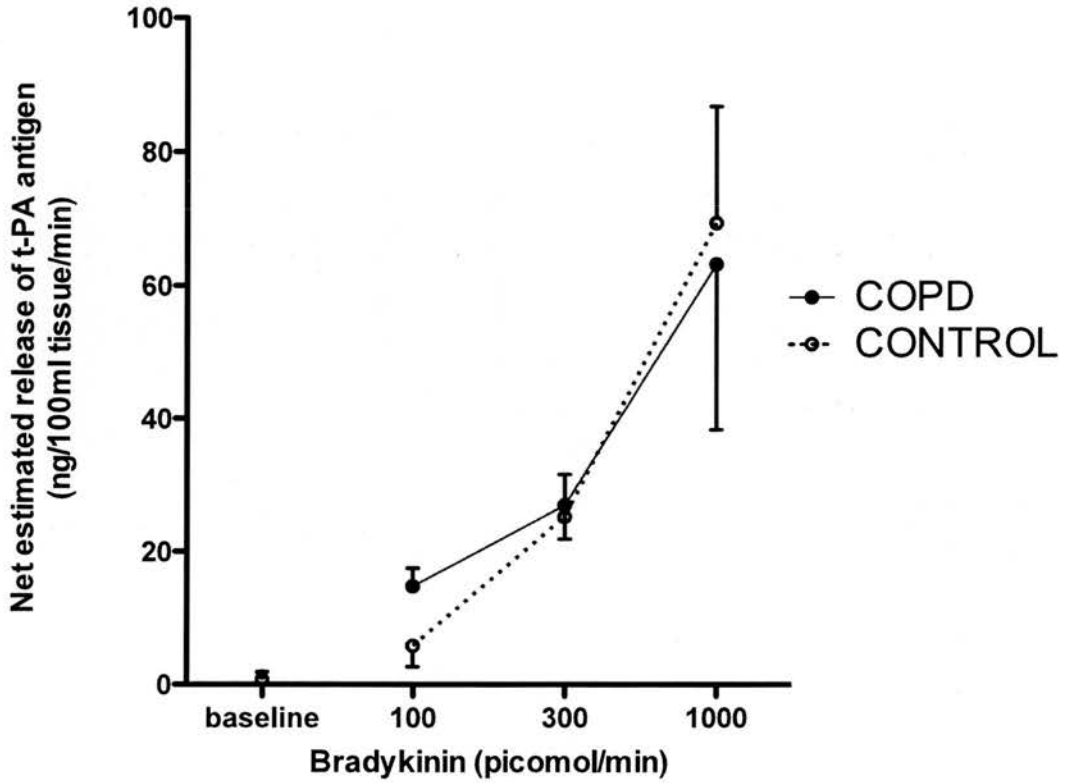

Figure 3.8 Acute endothelial release of tissue plasminogen activator (t-PA) in response to bradykinin in patients with chronic obstructive pulmonary disease in comparison to age and smoking habit matched controls

COPD - closed circles, healthy controls - open circles; repeated measures two-way ANOVA, p=0.90. Symbols represent the means and bars the standard errors of the means.

3.5 Discussion

In order to explore the mechanisms of increased cardiovascular risk associated with COPD, we performed a comprehensive panel of systemic vascular studies. We compared endothelial vasomotor and fibrinolytic function as well as arterial stiffness in men with COPD to a healthy male control group who were closely matched for smoking history. Men with COPD had increased arterial stiffness, but two major components of endothelial function, forearm vasodilatation and endogenous fibrinolytic function, were similar in COPD and matched control subjects. These data suggest that the increased arterial stiffness is an independent systemic manifestation of COPD and is *not* due to endothelial dysfunction. We hypothesise that there may be similar pathogenic processes involving breakdown of the extracellular matrix in the lung and vasculature in patients with COPD that results in increase arterial stiffness.

3.5.1 Arterial Stiffness

Increased large artery stiffness results in greater central aortic systolic pressures, increased left ventricular after-load and reduced diastolic coronary artery filling [Safar, 2003], and as such may be an important determinant of cardiovascular risk in patients with COPD. Previously, both Mills et al and Sabit et al have shown increased aortic pulse wave velocity, elevated augmentation pressure and reduced time to wave reflection in patients with COPD [Mills, 2008; Sabit, 2007]. However, these studies were limited by use of suboptimal measures of arterial stiffness [Mills, 2008] and inadequate matching of smoking exposure between study groups [Sabit, 2007]. In contrast, using recommended gold-standard measures of arterial stiffness (carotid-femoral pulse wave velocity), we have demonstrated that arterial stiffness is increased in patients with COPD compared to controls with similar cigarette smoke exposure. This suggests an association between arterial stiffness and COPD that is independent of the effects of cigarette smoke.

3.5.2 Endothelial Vasomotor Function

While arterial stiffness of a conduit artery, such as the aorta, is influenced by the extracellular matrix, vascular smooth muscle and the endothelium [Zieman, 2005], regulation of blood flow in resistance vessels is governed primarily by vascular smooth muscle and the endothelium. We have performed detailed studies of endothelial function across resistance vessels in the forearm vascular bed using two endothelium-dependent vasodilators (bradykinin and acetylcholine) and two endothelium-independent vasodilators (sodium nitroprusside and verapamil). Using the robust and well-validated technique of forearm plethysmography, we found no differences in endothelium-dependent or -independent vasomotor function in patients with COPD when compared to controls matched for smoking status. This is not to say that patients with COPD do not have endothelial dysfunction. We have previously demonstrated marked endothelial dysfunction in smokers [Newby, 1999] and it is possible that the effects of chronic smoking or aging may dominate any effects of COPD on resistance vessel vasomotor function.

In a previous study of vascular function in COPD, Barr et al [Barr, 2007] found that flow-mediated dilation (FMD), a non-invasive measure of arterial vasomotor function, was associated with both airflow obstruction and emphysema severity in former smokers with and without COPD. FMD measures vasodilatation of the brachial artery following reactive hyperaemia of the forearm [Corretti, 2002] which is partly endothelium- and nitric oxide-dependent [Doshi, 2001]. However, as the authors concede, no endothelium-independent vasodilator (e.g. nitroglycerine) was used as a control in this study, and therefore the abnormality described cannot be definitively localised to the endothelium, and may be due to dysfunction of other components of the arterial wall, such as the vascular smooth muscle or the extracellular matrix. Indeed, a subsequent case-control study did employ a nitroglycerine control and found that nitroglycerine-mediated dilatation was impaired in the COPD group [Eickhoff, 2008]. This finding implies that the vascular abnormality in COPD is not restricted to the endothelium. In addition, there is evidence that FMD is an unreliable measure of endothelial function in the presence of stiff arteries [Lind, 2007; Witte, 2005]. Interestingly, a pattern of vascular abnormalities similar to that presented in this study is seen in patients with Marfan's

syndrome who have large artery stiffness and preserved agonist-mediated vasodilatation despite impairment of FMD [Wilson, 1999].

Both FMD and venous occlusion plethysmography are well established techniques for assessing endothelial vasomotor function. Although the former technique examines vasomotion in a conduit vessel and the latter in resistance vessels, endothelial dysfunction is thought to be a systemic process and abnormalities are unlikely to be restricted to one vascular bed. Assessments of vascular function in the peripheral circulation, using either technique, closely relate to assessments of vasomotor function in the coronary circulation [Anderson, 1995; Monnink, 2002] and are predictive of cardiovascular events, even in individuals with no known atherosclerosis [Modena, 2002; Perticone, 2001]. Whilst there is evidence of selected abnormalities of either resistance or conduit vessels in specific circumstances such as rare hereditary arteriopathies [Stenborg, 2007], we think it unlikely that COPD preferentially affects endothelial function in conduit vessels.

3.5.3 Endogenous fibrinolysis

In addition to endothelial vasomotor function, we measured release of the endogenous fibrinolytic enzyme, tissue plasminogen activator (t-PA). Release of t-PA may be more sensitive than vasodilatation as a marker of endothelial function [Robinson, 2007]. One third of patients with acute coronary events undergo spontaneous reperfusion of the occluded vessel within 12 hours of symptom onset [Armstrong, 1989; DeWood, 1980; Rentrop, 1989], and t-PA release is thought to be the mechanism underlying this phenomenon. Impaired t-PA release has previously been described in cigarette smokers, in hypertension, and following acute exposure to air pollution [Hrafnkelsdottir, 1998; Mills, 2005]. However, consistent with our assessment of endothelial vasomotor function, we found that bradykinin-induced release of t-PA was similar in patients with COPD and in matched controls. This again suggests that COPD does not confer additional endothelial dysfunction above that observed with smoking.

3.5.4 Mechanisms

We found that COPD caused no impairment of endothelial vasomotor or fibrinolytic function in addition to any abnormality that may be caused by age and cigarette smoke exposure. However, COPD was associated with increased large elastic artery stiffness, having controlled for age and smoking. This suggests that arterial stiffness in COPD may be due to a structural defect in the extracellular matrix in the vascular wall rather than a functional deficit in the endothelium.

The development of arterial stiffness is a complex and incompletely understood process wherein endothelial and smooth muscle cells interact with the extracellular matrix to modify vessel wall structure and function [Vlachopoulos, 2006]. There are a number of mechanisms that may contribute to increased arterial stiffness in COPD. Our group previously reported that arterial stiffness was associated with emphysema severity, and proposed that this may represent a systemic susceptibility to connective tissue degradation [McAllister, 2007b]. Others have proposed that there may be systemic susceptibility to elastin degradation in COPD. Lee et al [Lee, 2007] demonstrated that subjects with emphysema have increased anti-elastin antibodies compared to non-emphysematous subjects. In addition, skin wrinkling (characterised by elastin breakdown in the skin) is also associated with CT emphysema severity, suggesting a common mechanism of lung and systemic elastin degradation in COPD [Patel, 2006].

Chronic systemic inflammation may be an important determinant of the increase in large arterial stiffness in COPD. Systemic inflammation is an important risk factor for cardiovascular disease [Ridker, 1997], and has been implicated as a contributing factor to the increased cardiovascular risk associated with COPD [Sin, 2003]. Furthermore, arterial stiffness is positively associated with CRP in healthy individuals [Yasmin, 2004] and circulating interleukin-6 levels are independently associated with pulse wave velocity in a COPD population [Sabit, 2007]. Although our study was not powered to examine associations between arterial stiffness and inflammatory variables, patients with COPD had higher circulating leukocytes and levels of CRP than controls.

There were also differences in both heart rate and arterial oxygen tension between the COPD patients and controls. Sympathetic activation and subclinical autonomic dysfunction are established features of COPD, and have been associated with reduced arterial compliance [Boutouyrie, 1994]. However, if autonomic dysfunction was the principle cause of increased arterial stiffness in COPD, we would expect to have observed differences in basal vascular tone and vasodilatation. Hypoxemia has variable effects on vascular function [Vedam, 2009; Reboul, 2005] and within a COPD population it is difficult to separate the roles of hypoxaemia and severity of lung disease on vascular function.

3.5.5 Study Limitations

Aging is associated with endothelial dysfunction [Celermajer, 1994; Vanhoutte, 2002], and our subjects had a mean age of 65 years. Therefore the effects of age may have superseded any effect of COPD on endothelial activity. It is possible that studies of younger patients with COPD may have identified abnormalities in endothelial function. Furthermore, we were not powered to look at the effects of disease severity within the COPD group. Given our subjects had a mean FEV_1 percent predicted of 47% we think it is unlikely that we would have missed an abnormality of endothelial function associated with mild to moderate COPD. It is possible that there may be abnormalities of endothelial function in patients with more severe disease.

Additionally, we limited our study to male patients. Although vascular function differs between the sexes, the difference almost disappears after the menopause, which is thought to result from the loss of the protective effects of estrogens [Bush, 1998; Celermajer, 1994; Jensen-Urstad, 2001; Lieberman, 1994]. However, given apparent differences in the natural history of COPD between genders [Dransfield, 2007; Martinez, 2007], caution must be exercised when extrapolating these results to women. We did not include an age-matched healthy life-long non-smoking control group to confirm the presence of endothelial dysfunction in the patient and matched control subjects. However, vascular dysfunction in smokers has been widely described [Jatoi, 2007; Lang, 2008; Newby, 1999; Pretorius, 2002]. The aim of our study was not to replicate previous work but to establish whether vascular

abnormalities are attributable to COPD independent of other confounding factors such as smoking.

3.6 Conclusion

We have shown that men with COPD have significantly increased arterial stiffness, but no impairment of systemic vasomotor or fibrinolytic endothelial function, in comparison to control subjects well matched for age and smoking. We therefore conclude that whilst abnormal endothelial function may be present in COPD, it is likely due to the effects of age and smoking, whereas increased arterial stiffness in COPD is independent of these factors. Increased arterial stiffness may represent the mechanistic link between COPD and increased risk of cardiovascular disease associated with this condition.

Chapter 4. Systemic elastin degradation in Chronic Obstructive Pulmonary Disease

Submitted for publication by Maclay JD, McAllister DA, Rabinovich R, Maxwell S, Hartland S, Connell M, Murchison J, Gray RD, Mills NL, and MacNee W. 2011

4.1 Summary

COPD is associated with increased arterial stiffness which may contribute to the increased risk of cardiovascular disease reported in this condition. Arterial stiffness correlates with the severity of emphysema. We hypothesised that the mechanism of the increased vascular stiffness in COPD is due to increase elastin degradation in the extracellular matrix of large arteries. To look for evidence of systemic elastin degradation, we examined both sun-exposed (exposed) and non-sun-exposed (non-exposed) full thickness skin biopsies in 16 men with COPD and 15 healthy ex-smokers matched for age and cigarette smoke exposure. Elastin degradation was assessed by immunohistochemistry as the percentage area covered by elastin fibres in the reticular dermis. Quantitative PCR of mRNA coding for MMP-2, -9, -12 and TIMP-1 was performed on homogenised skin and zymography for protein expression of MMP-2 and -9. Arterial stiffness was assessed as the carotid-femoral pulse wave velocity and emphysema severity was measured using quantitative CT scanning. We found that skin elastin degradation was greater in both exposed and non-exposed skin of patients with COPD in comparison to controls (exposed COPD vs controls, mean (SD); 43.5 (12.1)% vs 26.3 (6.9)%, $p<0.001$; non-exposed 22.4 (5.2)% vs 18.1 (4.3)%, $p=0.02$). Additionally, cutaneous expression of MMP-9 mRNA and proMMP-9 protein concentrations were increased in the exposed skin of COPD patients ($p=0.004$ and $p=0.02$ respectively), and these were associated with increased skin elastin degradation (MMP-9 mRNA $r=0.62$, $p<0.001$). In exploratory analyses in the entire cohort of ex-smokers, we showed associations between cutaneous elastin degradation and FEV_1 (exposed $r=-0.56$, $p=0.001$, non-exposed $r=-0.45$, $p=0.01$), emphysema severity (exposed $r=0.51$, $p=0.006$, non-exposed $r=0.48$, $p=0.01$) and pulse wave velocity (non-exposed $r=0.42$, $p=0.02$). To summarise, patients with COPD have increased skin elastin degradation in comparison to healthy ex-smoking controls and this is related to emphysema severity. Thus we describe systemic elastin degradation in patients with COPD. The increased expression of matrix metalloproteinases may represent a shared mechanism for both pulmonary and cutaneous elastin degradation.

4.2 Introduction

Chronic obstructive pulmonary disease (COPD) is defined as a condition with extra-pulmonary effects and systemic consequences, including cardiovascular disease [Pauwels, 2001; Celli, 2004b]. Systemic vascular dysfunction is emerging as a specific mechanism that may contribute to the increased cardiovascular risk in COPD [Maclay, 2009; Sabit, 2007; Eickhoff, 2008]. We have demonstrated that systemic arterial stiffness correlates with the severity of emphysema in COPD patients [McAllister, 2007b]. Thus we postulated that elastin degradation in the lung parenchyma and systemic arterial walls is the mechanistic link between the pulmonary and systemic vascular manifestations of COPD.

Elastin is an essential structural protein in the lungs, maintaining airway patency and ensuring elastic recoil. Emphysematous changes are, in part, due to elastin fibre breakdown which causes parenchymal destruction, reduced lung compliance and airway collapse [Shifren, 2006]. An imbalance of proteases and anti-proteases in COPD, with a net increase in elastolytic activity is thought to play an important role in the pathogenesis of emphysema[Turino, 2007], and recent work has focussed on the role of matrix metalloproteinases (MMPs) and their equivalent anti-proteases [Hunninghake, 2009; Atkinson, 2010]. MMPs have also been implicated as a pathogenic mechanism for the increased arterial stiffness that occurs with aging, caused in part by elastin breakdown in the arterial wall [Zieman, 2005; Labella, 1963].

The hypothesis that COPD may be associated with both local lung and systemic abnormalities in connective tissue was addressed by Smith et al in 1967. Having shown abnormalities in the dermis of patients with obstructive lung disease, primarily in collagen, the authors postulate that these changes may 'reflect a primary defect of connective tissue in the body as a whole' [Smith, 1967]. However, the smoking history of the subjects not reported. More recent studies have suggested that elastin degradation may occur outside the pulmonary compartment in COPD. Lee et al have reported increased circulating elastin antibodies in the plasma of emphysematous compared with non-emphysematous subjects [Lee, 2007]. Furthermore, Patel et al

reported that skin wrinkling was associated with emphysema in smokers, and postulated that this may be due to changes in collagen and elastin [Patel, 2006].

We hypothesise that the increase in elastin degradation and protease-anti-protease imbalance responsible for emphysema is present in systemic tissue, causing increased arterial stiffness and skin wrinkling. We therefore examined skin biopsies from patients with COPD and matched control ex-smokers for evidence of elastin degradation.

4.3 Methods

Of the thirty-five male ex-smokers recruited for the vascular studies reported in chapter 3, 16 men with COPD and 15 male controls agreed to undergo skin biopsies. The inclusion and exclusion criteria were identical to those listed in chapter 3. In brief, the subjects with COPD had a history consistent with the disease and post-bronchodilator spirometric evidence of airflow limitation (FEV_1/FVC ratio<0.7), while the controls had normal spirometry. All subjects were ex-smokers of at least 6 months and had a greater than ten pack year smoking history. They had no history of vascular disease or any other systemic inflammatory condition. Pre- and post-bronchodilator spirometry were measured (Alpha Spirometer; Vitalograph, Buckingham, UK) according to American Thoracic Society/European Respiratory Society standards [Miller, 2005]. Serum C-reactive protein (CRP) concentrations were measured using a highly sensitive immunonephelometric assay (Behring BN II nephelometer; Global Medical Instruments, Ramsay, MN). All studies were conducted at the Wellcome Trust Clinical Research Facility, Royal Infirmary, Edinburgh, approved by Lothian Regional Ethics Committee and conducted with the written informed consent of all participants.

4.3.1 Skin biopsy processing and staining

Two 4 mm punch biopsies were obtained from the sun-exposed skin of the dorsal surface of the forearm and non-sun-exposed skin of the buttock. Tissue was fixed in formalin and embedded in paraffin for morphometric analysis, snap-frozen and stored at -80°C for protein extraction and stored in RNAlater solution (Applied Biosystems, Carlsbad, CA) at -20°C for RNA extraction.

Histological evaluations were initially made in 3 μm thick elastic van gieson-stained sections. Following rehydration of samples, antigen retrieval was performed by microwaving in a pressure cooker in dilute citrate solution. Immunohistochemistry was performed using a monoclonal primary antibody to elastin (anti-human, Novocastra, Newcastle, UK) and a dextran polymer secondary antibody (Envision,

Dako, Cambridge, UK - Figure 4.1). One sun-exposed skin biopsy from a COPD patient was unsuitable for analysis.

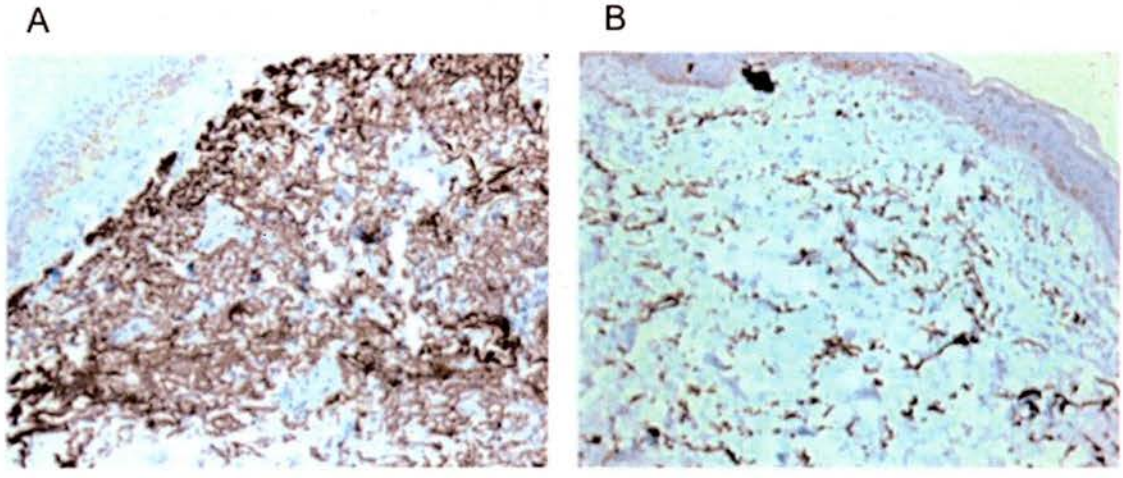

Figure 4.1: Immunohistochemistry in skin sections using anti elastin antibodies
Immunohistochemistry was performed on 3 micron sections of paraffin embedded skin with an human elastin-specific primary antibody and a dextran polymer secondary antibody stained with DAB and lightly counterstained with H&E. Panel A shows a slide with severe elastin degradation (sun-exposed; patient with COPD, aged 69, 55 pack years, FEV1%predicted 44%) and panel B with mild elastin degradation (sun-exposed; control subject, aged 71, 35 pack years, FEV1%predicted 124%) at 10x magnification.

4.3.2 Image processing and analysis

Images of each section were obtained using 10x magnification and processed with QCapture Pro software (Media Cybernetics, Bethesda, MD). Consecutive images were merged using Adobe Photoshop CS4 (San Jose, CA) to enable analysis of the entire section (Figure 4.2). As elastin fibres degrade, they thicken and occupy a greater relative area [Robert, 1988] which can be quantified to assess elastin degradation in the skin [Francès, 1991; Just, 2007]. We measured the area covered by elastin fibres in the reticular dermis using semi-automated quantification with ImageJ (NIH, Bethesda, MD) in a random order by the author, blinded to the subject identities. This was expressed as a percentage of the total area measured [ImageJ User Guide, 2011].

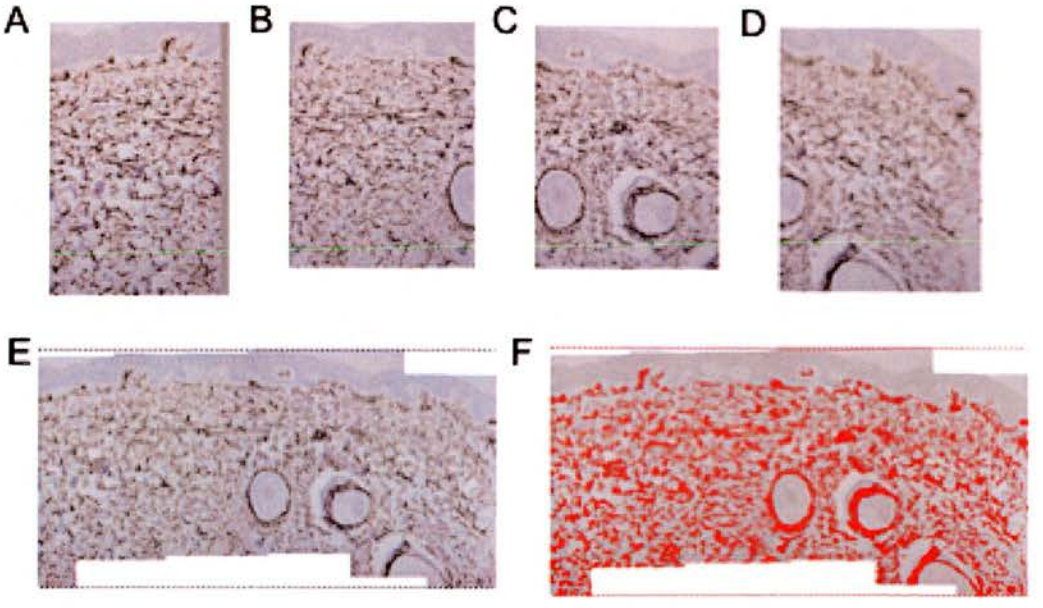

Figure 4.2 Image analysis.

Overlapping images were taken of each section at 10x magnification (panels A-D). Images were stitched to form a seamless panorama (panel E) and image J was used to select DAB-stained elastin fibres which calculated the percentage area of the reticular dermis occupied by elastin fibres (in red in panel F).

4.3.3 Protein and RNA extraction

Samples of skin tissue were homogenised in lysis buffer and an EDTA-free anti-protease cocktail (Roche, Welwyn Garden City, UK) using the Qiagen tissuelyser (Crawley, UK). Protein quantification was performed using a bicinchoninic acid (BCA) assay.

RNA was isolated from skin following homogenisation using the RNeasy Mini Kit (Qiagen, Crawley, UK). The steps employed a series of spin columns that bound RNA to a silica-based membrane and washed away contaminants. The purified RNA was eluted in water. All procedures were performed in an extractor fume hood in sterile conditions to ensure no contamination.

Quality and quantity of the purified RNA was checked using a spectrophotometer. Ratio of absorptions is the standard method to assess quality of the extracted RNA.

Absorption is measured at wavelengths of 260 nanometres (nm) and 280nm. These values are selected as RNA will absorb ultraviolet light most at 260nm and proteins will absorb strongly at 280 nm. A sample is considered uncontaminated if the ratio of 260/280 is greater than 2. The quantity of RNA is measured by multiplying the absorption at 260nm by 40. This is based on the assumption that at a concentration of 40 micrograms/ml, a purified RNA solution will have an optical density of 1. The average yield of RNA was 43ng/mcl and the quality of RNA was 2.2, consistent with high quality RNA.

4.3.4 Gene expression

Two-step real-time polymerase chain reaction (qRT-PCR) requires an initial process of reverse transcription of total RNA into cDNA using reverse transcriptase. The second stage is qPCR to establish a specific quantity of the gene of interest. This two-step method allows multiple real-time PCR reactions.

4.3.4.1 Reverse transcription

Complementary DNA (cDNA) is synthesised from a mRNA template (Figure 4.3). This single stranded DNA is more stable than mRNA and can be used for qPCR. Total RNA (0.1 micrograms) was reverse transcribed in a reaction mix containing 4 mcl of 5 x reaction buffer, 2 mcl dithiothreitol (DTT) (0.1 M), 0.5 mcl deoxynucleotides (dNTPs - 10 mM), 2 mcl of random hexamers, 0.5 mcl (200 U) Superscript II reverse transcriptase and 0.5ml (40U) RNase inhibitor (all Amersham, Buckinghamshire, UK). These cDNA samples were used to assess expression of MMP-2, -9, -12 and TIMP-1.

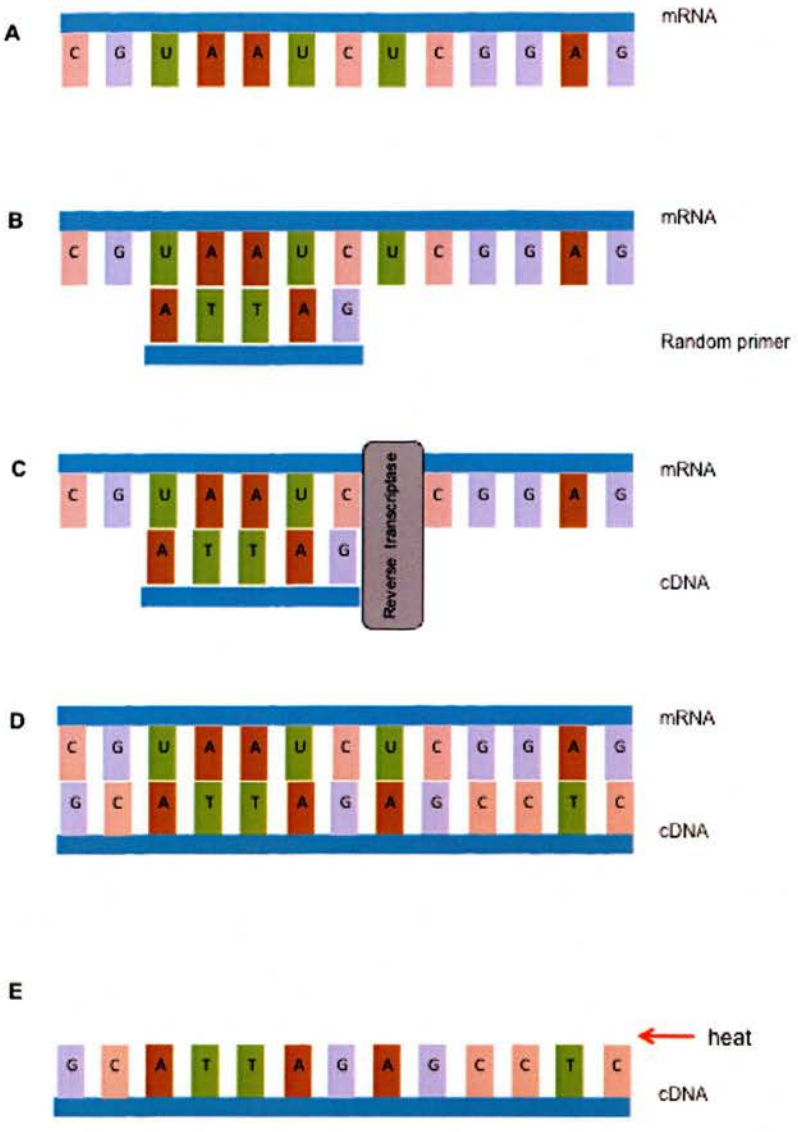

Figure 4.3 Reverse transcription.

mRNA is isolated from homogenised tissue (A). Random primers are introduced with reverse transcriptase (B and C) and a paired complimentary DNA (cDNA) strand is formed (D). cDNA is separated from the mRNA using heat (E).

4.3.4.2 Quantitative PCR (qPCR)

qPCR is a sensitive method for detection of mRNA expression. A pair of PCR primers is combined with a fluorogenic probe designed to bind to the DNA sequence between the primers (Figure 4.4). The reverse transcribed cDNA is mixed with DNA polymerase, dNTPs, a gene-specific forward primer, reverse primer and probe. The reactions take place in a thermal cycler which heats and cools the plate and performs fluorescence based absorption readings.

In this study, 0.1 microlitres of undiluted cDNA was used per reaction; the primer and probe sets were custom probes (University of Edinburgh); these predesigned primers are tested and standardised for reproducible expression analysis. Primer and cDNA were added to the TaqMan universal PCR master mix (Applied Biosystems, Carlsbad, USA) containing all the reagents for PCR. Experiments were performed in duplicate. Quantification of the PCR products was performed with an ABI prism 7500 (Applied Biosystems, Carlsbad, USA) using the relative standard curve method. cDNA that positively expresses the gene of interest was used to create a dilution series with arbitrary units. To ensure reproducibility, quantitative data were taken at a point in which each sample was in the exponential phase of amplification. At this phase of the reaction, there is doubling of DNA at each cycle and reagents are fresh and available to facilitate this. There is a slowing in subsequent linear and plateau phases as reagents are consumed due to exponential amplification. The threshold cycle is consistent for all samples and allows comparative quantification.

The mean quantity of target gene expression was determined from the generated standard curve. All samples were then normalised against an internal standard, reference gene 18S in all quantitative PCR reactions. All data are presented as a quotient relative to the control data.

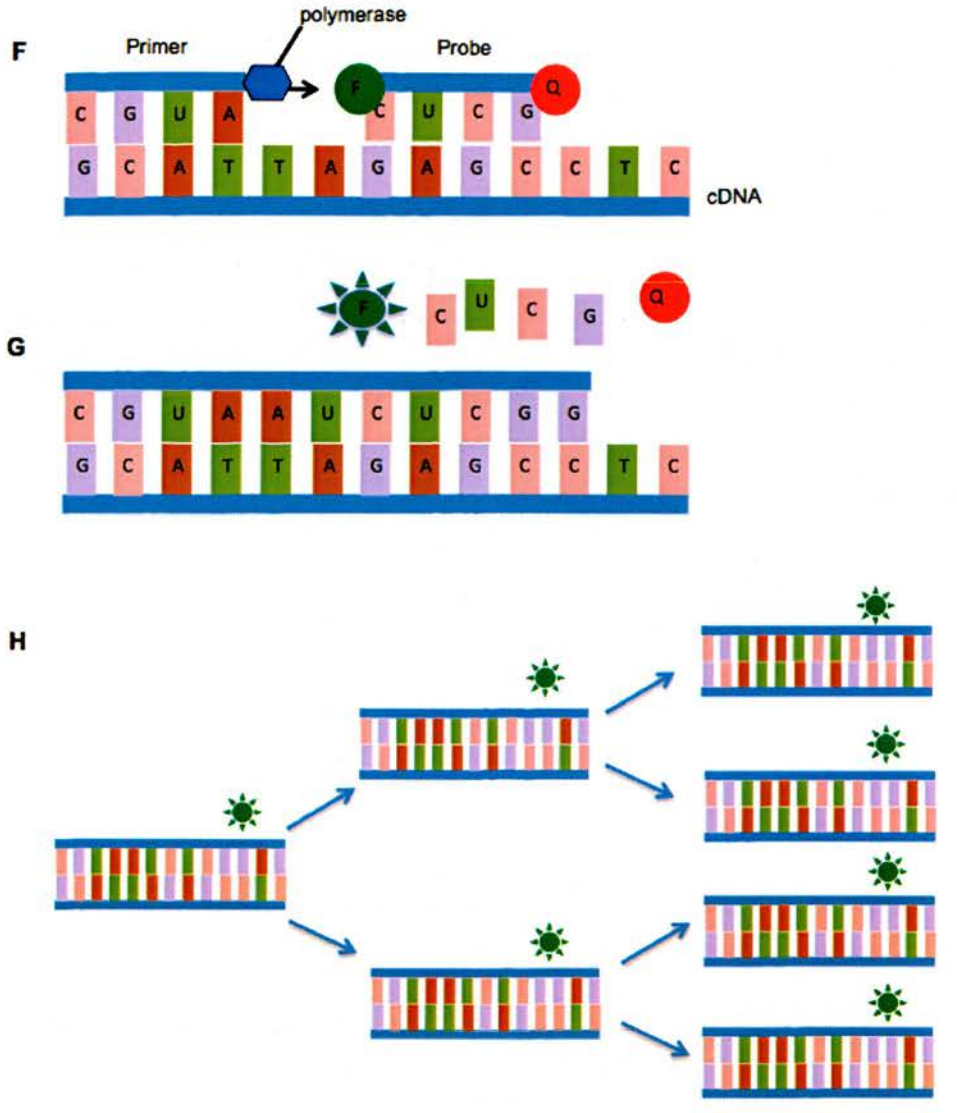

Figure 4.4 Polymerase chain reaction.

cDNA is mixed with gene specific primers and probes with fluorochrome attached (F). Polymerisation occurs, releasing the fluorochrome, detaching it from the quencher (G). Subsequent cycles result in doubling of DNA numbers exponentially and release of more fluorochromes (H).

4.3.5 Gelatin zymography

Zymography is a method by which proteolytic activity and quantity can be assessed in tissue extracts. It is often used to study matrix metalloproteinases and allowing investigators to assess MMP precursors (pro-MMP), MMP activity and total MMP (figure 4.5).

Sample separation was performed using precast 10% Tris-Glycine gels (7.5%), containing 0.1% gelatin (Invitrogen, Paisley, UK), in 2 × non-reducing sample buffer at 120V and Novex Tris Glycine SDS running buffer (Invitrogen, Paisley, UK). SDS was removed using zymogram renaturing buffer (Invitrogen, Paisley, UK) for 60 mins at room temperature. The gels were incubated overnight hours at 37°C in developing buffer (Invitrogen, Paisley, UK) and then stained with 0.1% Coomassie blue in 40% methanol and 10% acetic acid and destained until clear proteolytic bands appeared. Densitometry was performed using ImageJ (NIH, Bethesda, MD). Normalisation between gels was performed using 10 ng of recombinant MMP-2 (R&D Systems, Abingdon, UK) before adjusting for total protein.

Figure 4.5 Gelatin zymography.

Gel demonstrating distinct bands for proMMP-2 and active MMP-2 visible with only proMMP-9 present.

4.3.6 CT scanning

All fifteen healthy controls and thirteen subjects with COPD agreed to low-dose CT scanning performed at full inspiration using a 320-multidetector row CT scanner (Aquilion One, Toshiba Medical Systems, Nasushiobara, Japan). Non-contrast enhanced CT scans were obtained at 120kV, 100mAs during coached inspiratory breath-hold to total lung capacity. Images were reconstructed at 0.5-mm intervals with 0.5-mm thick slices using a FC-03 filter. The histogram of CT Hounsfield Units (HU) was corrected for the air offset in Toshiba CT scanners (about -985 HU instead of the nominal -1000 HU with the FC03 filter) using an extra-thoracic air calibration method [Stoel, 2008]. Emphysema was quantified using in-house software as percentage low attenuation voxels below -910 and -950 HU (%LAA-910 and %LAA-950) [Mair, 2009]. While the latter, higher threshold distinguishes well between those subjects with COPD and healthy controls, using the former, lower cut-off allows more uniform distribution of emphysema across this cohort of former smokers.

4.3.7 Arterial stiffness

Studies were conducted as described in Chapter 3 as per the Expert Consensus Document on Arterial Stiffness [Laurent, 2006], assessing carotid–femoral pulse wave velocity using a high-fidelity micromanometer (Millar Instruments, Houston, TX) and the SphygmoCorTM system (AtCor Medical, Sydney, Australia).

4.3.8 Data analysis

COPD and control groups were compared using Student's unpaired t-tests or an appropriate non-parametric alternative for variables not normally distributed. Associations between skin, post-bronchodilator spirometry, emphysema severity using %LAA-910 and arterial stiffness measures were explored using Pearson correlations and associations with emphysema severity using %LAA-950 were explored using Spearman's rank correlation. C-reactive protein was log-transformed for all analysis. All analyses were performed using SPSS version 18.0 (Chicago, IL) and Graphpad prism (La Jolla, CA). Statistical significance was taken at $p<0.05$.

4.4 Results

Men with COPD and controls were well matched for age and smoking history. Patients with COPD had a range of severity of airflow limitation (GOLD stage 1-4), were taking medication consistent with their disease severity and had considerably more emphysema than controls (Table 4.1).

Table 4.1: Subject characteristics

	COPD	CONTROL	p-value
n	16	15	
Age, years	65 (5.2)	64 (5.7)	0.83
Body mass index, kg/m^2	26.5 (3.8)	27.9 (3.8)	0.33
Cigarette smoke exposure, pack years*	36 (34-54)	34 (28-46)	0.58
Inhaled medications, number of subjects (%)			
Short acting beta agonist	15 (94%)		
Anticholinergic	10 (63%)		
Long acting beta agonist	2 (12%)		
Inhaled corticosteroid	1 (6%)		
ICS/LABA combination	13 (81%)		
Post bronchodilator spirometry, %			
FEV_1 % predicted	47.8 (18.5)	102.1 (10.2)	<0.001
FVC % predicted	87.0 (13.9)	100.9 (11.1)	0.005
FEV_1/FVC	41.6 (11.8)	79.2 (5.6)	<0.001
Emphysema severity			
%LAA-950	26.3 (10.3-42.4)	2.5 (1.1-3.8)	<0.001
%LAA-910	59.9 (43.4-64.9)	26.3 (10.3-42.5)	0.002
Aortic pulse wave velocity, m/s	10.4 (2.0)	8.5 (1.7)	0.009
hsCRP, mg/L*	2.1 (1.3-5.5)	1.0 (0.5-35)	0.04

Data presented as mean (standard deviation) or n (%), except * median (interquartile range). Groups compared using Student's t-test except for * where groups compared with Mann-Whitney U. ICS: inhaled corticosteroid; LABA: long acting beta agonist; %LAA-950: percentage low attenuation voxels below -950 Hounsfield units; %LAA-910: percentage low attenuation voxels below -910 Hounsfield units; hsCRP: high-sensitivity C-reactive protein

Elastin degradation was greater in sun-exposed skin (exposed) in patients with COPD in comparison to controls [mean (standard deviation); 43.5 (12.1)% vs 26.3 (6.9)%, $p<0.001$]. Elastin degradation was also greater in non-sun-exposed skin (non-exposed) in patients with COPD [22.4 (5.2)% vs 18.1 (4.3)%, $p=0.02$; Table 4.2; Figure 4.6].

MMP-9 mRNA expression and proMMP-9 protein concentrations were increased in the exposed skin of COPD patients in comparison to controls (Table 4.2). There was little detectable MMP-9 activity present in any subject. There was greater MMP-2 and TIMP-1 mRNA expression in the exposed skin of COPD patients, but these differences were not statistically significant. MMP-2 and -9 expression were positively associated with elastin degradation in exposed skin (MMP-2 $r=0.55$, $p=0.002$; MMP-9 $r=0.62$, $p<0.001$; Figure 4.7). Differences in MMP expression between COPD and controls were not seen in the non-exposed skin. There was little MMP-12 mRNA expression in the skin of any of the subjects.

In exploratory analyses, examining the whole cohort as a group of ex-smokers, we found that exposed and non-exposed skin elastin degradation were both related to FEV_1 (exposed $r=-0.56$, $p=0.001$, non-exposed $r=-0.45$, $p=0.01$) and to emphysema severity (%LAA-950: exposed $r=0.46$, $p=0.01$, non-exposed $r=0.54$, $p=0.003$). Elastin degradation in non-exposed skin was associated with aortic pulse wave velocity ($r=0.42$, 95%CI 0.06 to 0.68, $p=0.02$). An association of a smaller magnitude but in the same direction was found for exposed skin but it was not statistically significant ($r=0.19$, 95%CI -0.19 to 0.53, $p=0.32$). Aortic pulse wave velocity was associated with emphysema severity (%LAA-910: exposed $r=0.54$, $p=0.003$, non-exposed $r=0.50$, $p=0.007$; %LAA-950: exposed $r=0.46$, $p=0.01$, non-exposed $r=0.54$, $p=0.005$) and high-sensitivity C-reactive protein (hsCRP, $r=0.38$, $p=0.05$); hsCRP was not associated with cutaneous elastin degradation or emphysema. Age was neither associated with emphysema severity nor skin elastin degradation.

Table 4.2: Skin measurements

	COPD	CONTROL	p-value
Area of dermis occupied by elastin fibres, %			
Sun-exposed-skin	43.5 (12.1)	26.3 (6.9)	<0.001
Non-sun-exposed-skin	22.4 (5.2)	18.1 (4.3)	0.02
mRNA expression			
SES MMP-2	1.2 (0.2)	1.0 (0.2)	0.06
SES MMP-9	1.4 (0.5)	1.0 (0.3)	0.004
SES MMP-12	*Insufficient expression for analysis*		
SES TIMP-1	1.2 (0.2)	1.0 (0.2)	0.06
NSES MMP-2	1.0 (0.2)	1.0 (0.3)	0.6
NSES MMP-9	1.1 (0.8)	1.0 (0.7)	0.5
NSES MMP-12	*Insufficient expression for analysis*		
NSES TIMP-1	1.0 (0.2)	1.0 (0.2)	0.6
*Protein quantification, mg/mL**			
SES ProMMP-2	22.3 (16.7-24.7)	20.8 (16.5-33.5)	0.85
SES MMP-2 activity	13.6 (10.3-24.8)	22.7 (13.0-29.4)	0.25
SES Total MMP-2	33.3 (22.9-36.5)	29.9 (23.7-45.8)	0.93
SES ProMMP-9	11.7 (7.3-18.1)	7.7 (4.9-9.4)	0.02
NSES ProMMP-2	27.7 (19.5-37.6)	35.6 (28.6-47.6)	0.08
NSES MMP-2 activity	10.1 (6.4-15.2)	9.9 (8.4-13.3)	1.0
NSES Total MMP-2	17.7 (12.9-26.0)	23.5 (19.6-36.6)	0.06
NSES ProMMP-9	9.1 (6.1-11.5)	10.9 (8.9-12.2)	0.12

Data presented as mean (standard deviation) and analysed using Student's t-tests, except * median (interquartile range) analysed using Mann-Whitney U. mRNA expression calculated as a quotient relative to the control data. SES=sun-exposed skin; NSES=non-sun-exposed skin; MMP=matrix metalloproteinase, TIMP=tissue inhibitor of metalloproteinase.

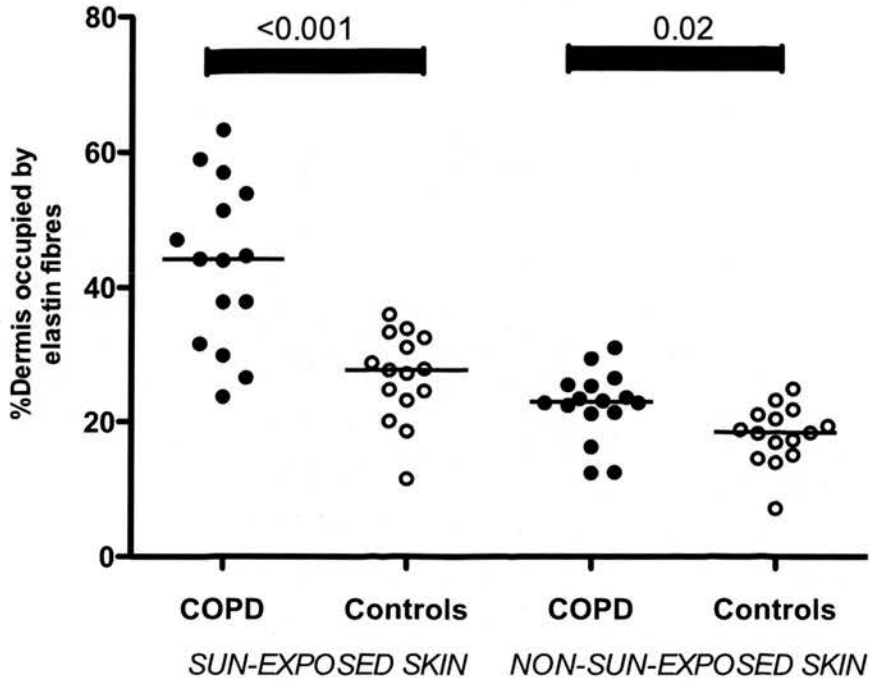

Figure 4.6: Patients with COPD have increased elastin degradation in both sun-exposed and non-sun-exposed skin.

Symbols represent individual values and the horizontal lines the means. Groups compared using Student's t-tests. One sun-exposed skin biopsy from a subject with COPD was unsuitable for analysis.

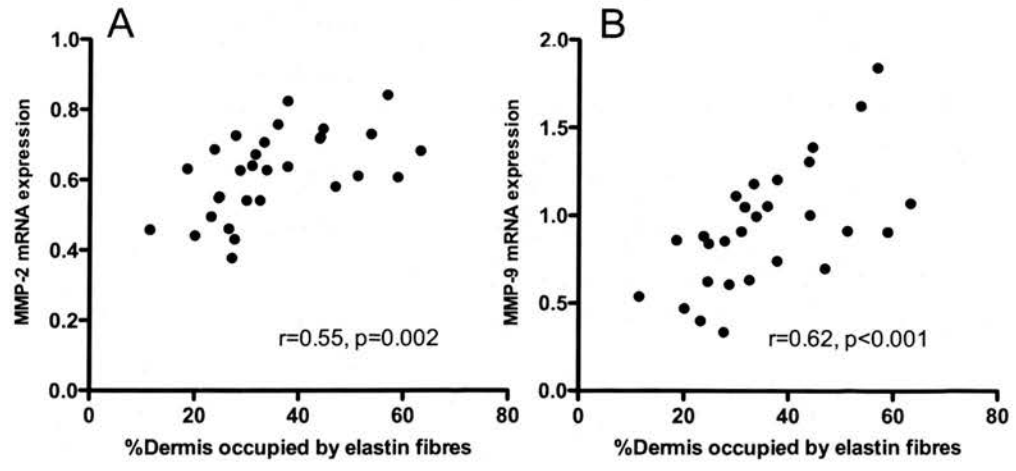

Figure 4.7: Sun-exposed skin elastin degradation is associated with both MMP-2 and MMP-9.

The Y-axis represents relative expression based on an internal standard. One subject with COPD and one control were below the lower limit of detection for MMP-9 expression.

4.5 Discussion

Patients with COPD have evidence of greater cutaneous elastin degradation than age and smoking-matched controls. Our findings represent evidence of enhanced degradation of elastin outside of the lung in COPD, and we believe that systemic elastin degradation may be a novel systemic feature of COPD. Furthermore, we have found increased expression of MMP-9 in the skin of patients with COPD, a protease implicated in the pathogenesis of emphysema, and these data suggest a role in systemic elastin degradation in COPD.

Elastin degradation in non-sun-exposed skin was associated with both emphysema severity and aortic pulse wave velocity. We speculate that the systemic elastin degradation demonstrated in the skin may also occur in other elastin-rich structures, such as the walls of large conduit arteries, as an explanation for the increased arterial stiffness in COPD.

Development of emphysema, arterial stiffness and elastin degradation are features of normal aging, reinforcing the importance of matching our groups for age. Our findings support the hypothesis that COPD is a disease characterised by accelerated aging. Recent manuscripts have suggested that the arterial stiffness associated with COPD may in part be due to increased vascular calcification, and that vascular calcification is associated with emphysema severity [Bolton, 2011; Dransfield, 2010; McAllister, 2011]. Indeed, this mirrors the medial elastocalcinosis seen in large vessels as a feature of normal aging, [Atkinson, 2008]. As the elastin in the vessel wall is degraded, the calcium content is thought to increase. Thus measures of vascular calcification in the aorta may also be a reflection of elastin degradation. In addition, as malnutrition is associated with elastin degradation in the lungs and vasculature [Riley, 1995] it is noteworthy that subjects with COPD had a similar mean body mass index to the controls,

Both the ATS/ERS consensus definition and the GOLD guidelines classify COPD as a disease with systemic consequences and extra-pulmonary effects [Celli, 2004b; Pauwels, 2001]. However, it is difficult determine whether this relationship is causal - that is, due to a direct consequence of COPD - or results from a combination of shared

risk factors for COPD and cardiovascular disease such as smoking, social deprivation and a sedentary lifestyle. We have performed carefully controlled studies comparing patients with COPD and controls matched for age and smoking history that suggest arterial stiffness is an independent systemic manifestation of COPD [Maclay, 2009]. Furthermore emphysema severity is closely related to arterial stiffness independent of cigarette smoking in patients with COPD [McAllister, 2007b]. Together these findings suggest a shared susceptibility to elastin degradation in the pulmonary and systemic tissues of COPD patients.

Vascular samples from conduit arteries are not readily available, however previous research on the dermatological manifestations of COPD suggested that skin may be a suitable surrogate for assessing systemic elastin degradation. Using visual categorisation of facial wrinkling, skin wrinkling has been associated with emphysema severity and lung function in a cohort of patients with COPD [Patel, 2006]. Our study provides the first direct evidence of increased elastin degradation in the skin in COPD patients, and importantly we are able to demonstrate that this observation is independent of cigarette smoke exposure. Two previous studies have reported increased cutaneous elastin degradation using biopsy specimens in cigarette smokers [Francès, 1991; Just, 2005], and have shown relationships with lung function [Just, 2005]. In the study by Francès et al, lung function was not measured and thus the potential contribution of lung disease to elastin degradation was not assessed in these smokers. Just et al reported an association with airflow limitation that was thought to be secondary to cumulative cigarette smoke exposure rather than a manifestation of lung disease.

In order to investigate potential mechanisms for increased systemic elastin degradation in COPD, we measured expression of mRNA coding for MMP-2, MMP-9, MMP-12 and TIMP-1 (the endogenous inhibitor of MMP-9). We selected these proteases specifically as they have been implicated in the pathogenesis of both COPD and arterial stiffness [Baraldo, 2007; Chung, 2009; Yasmin, 2005; Yasmin, 2006; Aldonyte, 2003]. MMP-9 mRNA expression was increased in patients with COPD, and this finding was supported by the presence of increased proMMP-9 concentrations (an inactive precursor of MMP-9) in the skin in COPD patients. Expression of MMP-2 and TIMP-1 was also higher in patients with COPD, but the

differences were not statistically significant. Previous work has shown increased MMP-2 in the peripheral lung of patients with early emphysema [Baraldo, 2007]. In addition, Aldonyte et al reported increased basal and LPS-stimulated release of MMP-9 from peripheral blood monocytes isolated from individuals with COPD in comparison with controls [Aldonyte, 2003]. Both MMP-2 and MMP-9 have also been implicated in the pathogenesis of arterial stiffness and atherosclerosis. MMP-2 is upregulated in the arteries of patients with chronic kidney disease, a condition associated with increased arterial stiffness, in comparison to matched donors.[Chung, 2009] In a healthy population, circulating MMP-9 levels are associated with arterial stiffness and polymorphisms in the MMP-9 gene predispose to arterial stiffness [Yasmin, 2005; Yasmin, 2006].

Expression of MMP-2 and MMP-9 mRNA were increased in the skin of patients with COPD. Taken together with previous work showing increased MMP-9 release from circulating monocytes [Aldonyte, 2003], these observations suggest systemic upregulation of proteases in COPD. The persistence of pulmonary and systemic inflammation long after the cigarette smoke stimulus has been removed has raised the possibility of the pathogenesis of COPD having an autoimmune component [Agusti, 2003]. Thus cell-mediated immunity may drive both pulmonary and systemic elastin degradation in emphysema [Lee, 2007]. An alternative hypothesis is that the systemic inflammation associated with COPD may increase production of MMPs from local inflammatory cells. Inflammatory mediators known to be increased in COPD, such as interleukin-8 and TNF-alpha, stimulate increased production of MMPs from neutrophils [Chakrabarti, 2005; Chakrabarti, 2006].

Protease/anti-protease imbalance is thought to play a key role in the pathogenesis of COPD and TIMP-1 is the anti-protease that inhibits MMP-9. However, although TIMP-1 expression was increased in COPD, the differences were not significant suggesting a protease/anti-protease imbalance causing elastin degradation is due to upregulation of MMP-9.

The novel finding of increased systemic elastin degradation in COPD is important for a number of reasons. Firstly, these observations provide further evidence of a role for proteases in the pathogenesis of COPD, and that they may contribute to extra-

pulmonary manifestations of the disease. Secondly, the upregulation of MMP-2 and -9 specifically is interesting as these proteases, known as gelatinase A and B, are zinc-dependent endopeptidases with similar structures and therefore may be potential targets for treatment [Visse, 2003]. Indeed, statins are reported to reduce MMP-2 and MMP-9 production by human vascular smooth muscle cells [Luan, 2003] and MMP-9 secretion by lung fibroblasts [Kamio, 2010] and macrophages [Bellosta, 1998]. Novel anti-inflammatory compounds such as p38 mitogen-activated protein kinase inhibitors specifically target activation and production of MMPs and have been shown to reduce MMP-9 release [Underwood, 2000]. Finally, the skin is a readily accessible tissue that may reflect the cumulative effects of COPD and may therefore provide further insights into the pathogenesis of the systemic manifestations of this complex disease.

Study limitations

Although there were differences in elastin degradation in both sun-exposed skin and non-sun-exposed skin, in non-sun-exposed skin there were no differences in the expression of proteases. There was generally reduced expression of MMPs in non-sun-exposed skin in comparison with sun-exposed skin and production of cutaneous MMPs in response to ultraviolet radiation is well described [Fisher, 1997]. Thus, it may be that sunlight exposure is a complementary factor which increases elastin degradation in susceptible skin tissue, unmasking differences in protease production between the patients with COPD and the healthy subjects. However, the pathophysiology of both COPD and vascular disease is complex and the exact inflammatory mechanisms are as yet unclear. Whilst we have specifically investigated cutaneous matrix metalloproteinases, it is important to acknowledge that other proteases may have a role in the pathogenesis of both vascular disease and the development of emphysema. Serine proteases (eg neutrophil elastase) have been implicated in both atherosclerosis and COPD in humans [Henriksen, 2008; Stockley, 2002] as have cysteine proteases (eg cathepsin K) in animal models [Lutgens, 2006; Golovatch, 2009]. Thus these other proteases may be involved in systemic elastin degradation in COPD.

We did consider measuring elastin degradation products, but our protein extraction methods were suitable to extract MMPs and not the relatively insoluble

desmosine/isodesmosine from samples. In any case, a consistent and reliable methodology for assessment of these proteins is yet to be established. Indeed, desmosine levels in the lung parenchyma are similar in patients with severe COPD and healthy donor lungs [Deslee, 2009]. Future studies could specifically assess desmosine levels, its relationship with elastin degradation and its prospective use as a local tissue biomarker.

Although we did exclude individuals on regular oral steroids, we were unable to adjust for inhaled corticosteroid use, taken by the majority of patients with COPD. Skin thinning, bruising and atrophy are recognised side effects of corticosteroid therapy. A small biopsy study suggested that inhaled corticosteroids may affect collagen synthesis [Autio, 1996], but in the largest study assessing the effects of long term inhaled corticosteroids on skin there was no effect on skin collagen or thickness [Haapasaari, 1998]. No specific effect of inhaled corticosteroids on elastin fibres has been described.

4.6 Conclusion

Patients with COPD have increased skin elastin degradation in comparison to age and smoking-matched controls and cutaneous elastin degradation was related to both emphysema severity and arterial stiffness. Furthermore, we identified upregulation of MMP-9 in the skin of COPD patients, which is associated with elastin degradation. Systemic elastin degradation due to increased proteolytic activity may represent a novel shared mechanism for the pulmonary, vascular and cutaneous features of COPD.

Chapter 5. Conclusions and future directions

5.1 Summary of thesis findings

Chronic obstructive pulmonary disease is now a condition recognised to have systemic consequences, effects and associations in addition to chronic lung inflammation and damage. Comorbidities contribute significantly to the morbidity and mortality associated with COPD and as such add to the burden this disease places on health services in the UK and abroad. With the increasing prevalence of COPD, there is a focus on both long-term strategies of prevention of disease with improved health education and the more medium-term strategies of reducing hospital admissions. This, at least in part, will need to address prevention and treatment of comorbid conditions.

It is well established that airflow obstruction, measured by FEV_1 is a risk factor for cardiovascular mortality. Several population-based studies have now shown that COPD is a risk factor for cardiovascular disease, independent of potential confounding factors including traditional risk factors such as smoking. In addition to the well-established traditional risk factors for cardiovascular disease, more mechanistic novel risk factors have been established which are thought to play an important role in the pathogenesis of cardiovascular disease.

The contribution of arterial walls and their structural components, along with the constituents of the blood have long been known to be factors causing development of atherothrombosis. The third arm of Virchow's triad is interruption of blood flow. A healthy endothelium allows small alterations in blood flow by regulation of vasomotion with release of vasodilators such as nitric oxide and prevention of thrombosis with release of the endogenous fibrinolytic tissue-plasminogen activator (t-Pa). Disruption of the endothelium is thought to be the primary step by which atherothrombosis occurs. Initial injury of the endothelium results in disruption of laminar flow, release of chemokines and initiation of an inflammatory cascade, activation of platelets and adhesion of circulating platelets, monocytes and platelet-monocyte aggregates to the denuded endothelium. Transmigration of monocytes and their differentiation into macrophages is known to be an early step in the formation of atherosclerotic plaques.

In addition to dysfunction of the endothelium, arterial stiffness is a global measure of vascular ill-health. Vascular compliance relies of the elastic properties particularly of the large conduit vessels. This compliance cushions the onset of systole, preventing end organ damage while contributing to blood delivery. Arterial stiffness increases with age and is an independent risk factor for both cardiovascular and all-cause mortality. Large artery stiffness is affected by their three main structural components: the endothelium, vascular smooth muscle and the extracellular matrix.

Cigarette smoking is known to cause activation of platelets, impairment of endothelial vasomotor and fibrinolytic function and increased arterial stiffness. We hypothesised that patients with COPD would have platelet activation, endothelial dysfunction and increased arterial stiffness contributing to their increased cardiovascular morbidity and mortality.

5.1.1 Patients with COPD have increased platelet activation that is further enhanced during exacerbations

In eighteen ex-smoking males with COPD and 16 ex-smokers matched for age and cigarette smoke exposure we assessed platelet activation. Using a sensitive measure of platelet activation, platelet-monocyte aggregation, we showed that patients with COPD have increased platelet activation. This is higher still during exacerbations of COPD. This is a mechanism by which the increased systemic inflammation associated with COPD may contribute to the increased cardiovascular risk, and a conceivable reason why patients with an exacerbation of COPD may be at further risk of cardiovascular events.

5.1.2 Patients with COPD have increased arterial stiffness that is not due to endothelial dysfunction

Measures of arterial stiffness and endothelial function were performed on eighteen ex-smoking males with COPD and 17 healthy ex-smokers matched for age and cigarette smoke exposure. With stringent inclusion and exclusion criteria in addition to matching for cigarette smoke exposure and age we sought demonstrate an effect of COPD itself rather than potential confounders. We confirmed increased arterial stiffness in COPD, but reported no difference in endothelial vasomotor function or fibrinolytic function. Thus the arterial stiffness associated with COPD is likely to be caused by dysfunction of an alternative structure in the arterial wall. Arterial stiffness is plausibly related to the increased cardiovascular risk associated with this condition.

5.1.3 Patients with COPD have evidence of systemic elastin degradation which may be due to increased expression of proteases

Previously published studies from our group had shown an association between CT-quantified emphysema severity and arterial stiffness. Thus we postulated that patients with COPD may have a shared susceptibility to elastin degradation in the lungs and arterial walls. Thus we measured elastin degradation in the skin of 16 men with COPD and 15 ex-smoking controls and related this to cutaneous matrix metalloproteinase expression, emphysema severity and aortic pulse wave velocity. There was increased cutaneous elastin degradation in the skin of COPD patients as well as increased expression of MMP-9. Skin elastin degradation was associated with both emphysema severity and arterial stiffness. These studies suggest that elastin degradation is both a local feature in the lungs in COPD causing emphysema, and a systemic effect causing arterial stiffness and skin wrinkling. Global upregulation of matrix metalloproteinases may represent a mechanism by which this occurs.

5.2 Future directions

5.2.1 Platelet activation

Following the conclusion that platelet activation may contribute to the increased cardiovascular risk associated with COPD, a prospective study addressing the use of platelet-monocyte aggregates as a prognostic marker in stable COPD and during exacerbations would allow evaluation of role of platelet activation as a potential mechanism.

The precise mechanism of increased platelet-monocyte aggregation in COPD remains unknown. Studies blocking binding sites between platelets and monocytes such as CD40, CD40 ligand, P-selectin glycoprotein ligand (PSGL)-1 and P-selectin would shed further light on the cell-surface receptor interactions that contribute to platelet activation in this condition. With this information, appropriate strategies for targeted alteration of platelet function in COPD would be possible. Platelet-monocyte aggregates form independently of the cyclooxygenase pathway and thus interventions other than aspirin are likely to be beneficial in this condition.

5.2.2 Arterial stiffness

Although we have circumstantial evidence of elastin degradation in large arteries in COPD, tissue to confirm this is considerably less accessible than skin. Cadaveric samples are becoming less available. In addition, operative samples of large arteries tend to only be available in vascular disease eg abdominal aortic aneurysm repair.

An alternative would be to use sections of mesenteric arteries resected during surgery for colonic carcinoma. In a significant number of resections there is no vascular involvement and thus the vessels remain intact. A large number of procedures are performed annually and given the prevalence of COPD in Scotland, a proportion of those attending will have airways disease. Thus comparing arterial elastin degradation with preoperative arterial stiffness measures and cutaneous elastin quantification would allow confirmation of findings of elastin degradation in larger arteries in

COPD and evaluation of the best, minimally invasive surrogate measure of arterial elastin.

5.2.3 Skin elastin degradation

Our novel finding of elastin degradation in the skin of COPD patients and the upregulation of MMPs raised a number of questions. In the first instance, it would be beneficial to try and establish the source of increased MMPs and whether this is due to local or circulating inflammatory cells. Further investigation of therapies that modify the production and/or effects of MMPs and in particular MMP-9 would add further insights, specifically looking at their systemic effects. In addition skin biopsies could be used as a less invasive alternative to examine the systemic effects of COPD as well as systemic effects of *novel therapies* in COPD.

5.3 Conclusions

In this thesis I have provided novel mechanisms by which cardiovascular risk may be increased in COPD. I describe increased platelet activation due to COPD, which may further contribute to increased cardiovascular risk. In addition to this, few studies have addressed the increased cardiovascular risk associated with *exacerbations* of COPD. It is plausible that exacerbations of COPD may precipitate or be precipitated by a cardiac event. As well as increased systemic inflammation, oxidative stress, hypoxia and haemodynamic stresses which may add to the cardiovascular risk, there is increased platelet activation during exacerbations of COPD.

Other groups have published manuscripts suggesting vascular dysfunction in COPD was associated with endothelial dysfunction. These earlier publications did not control well enough for cigarette smoking or perform optimum measures of vascular function. In a comprehensive assessment of vascular function using gold-standard methodology, I showed that, although smoking is known to cause endothelial dysfunction, COPD itself does not seem to cause impairment of endothelial function and thus the increased arterial stiffness associated with COPD is not due to endothelial dysfunction.

Finally, I describe a novel systemic effect of COPD: elastin degradation. In addition there is upregulation of cutaneous matrix metalloproteinases which may be a mechanism by which this occurs. This systemic effect may link the emphysema, arterial stiffness and skin wrinkling associated with COPD.

Publications arising from thesis

ORIGINAL ARTICLES

Maclay JD, McAllister DA, Mills NL, Paterson FP, Ludlam CA, Drost EM, Newby DE and MacNee W. Vascular dysfunction in chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine* 2009; 183:513-20

Maclay JD, McAllister DA, Johnstone S, Raftis J, McGuinness C, Deans A, Newby DE, Mills NL and MacNee W. Increased platelet activation in patients with stable and acute exacerbation of chronic obstructive pulmonary disease. *Thorax.* 2011; 66:769-74

Maclay JD, McAllister DA, Rabinovich R, Maxwell S, Hartland S, Connell M, Murchison J, Gray RD, Mills NL, and MacNee W. Systemic elastin degradation in chronic obstructive pulmonary disease (*submitted for publication*)

REVIEWS

Maclay JD, McAllister DA, MacNee W. Cardiovascular risk in chronic obstructive pulmonary disease. *Respirology* 2007; 12:634-41

MacNee W, Maclay JD, McAllister DA. Cardiovascular injury and repair in chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society* 2008; 5:824-33

References

Agustí A. Systemic effects of chronic obstructive pulmonary disease: what we know and what we don't know (but should). *Proc Am Thorac Soc.* 2007;4:522-5.

Agusti A, MacNee W, Donaldson K, et al. Hypothesis: does COPD have an autoimmune component? *Thorax.* 2003;58:832-4.

Albert M, Glynn R & Ridker P. Plasma concentration of C-reactive protein and the calculated framingham coronary heart disease risk score. *Circulation.* 2003;108:161-5.

Aldington S, Williams M, Nowitz M, et al. Effects of cannabis on pulmonary structure, function and symptoms. *Thorax.* 2007;62:1058-63.

Aldonyte R, Jansson L, Piitulainen E, et al. Circulating monocytes from healthy individuals and COPD patients. *Respir Med.* 2003;4:11.

Anderson HR, Spix C, Medina S, et al. Air pollution and daily admissions for chronic obstructive pulmonary disease in 6 European cities: results from the APHEA project. *Eur Respir J.* 1997;10:1064-71.

Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 Guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on practice guidelines. *Circulation.* 2007;116:E148-304.

Anderson TJ, Uehata A, Gerhard MD, et al. Close relation of endothelial function in the human coronary and peripheral circulations. *J Am Coll Cardiol.* 1995;26:1235-41.

Anthonisen NR, Connett JE, Enright PL, et al. Hospitalizations and mortality in the Lung Health Study. *Am J Respir Crit Care Med.* 2002;166:333-9.

Aoki M, Nata T, Morishita R, et al. Endothelial apoptosis induced by oxidative stress through activation of NF-{kappa}B: antiapoptotic effect of antioxidant agents on endothelial cells. *Hypertension.* 2001;38:48-55.

Armstrong PW, Baigrie RS, Daly PA, et al. Tissue plasminogen activator: Toronto (TPAT) placebo-controlled randomized trial in acute myocardial infarction. *J Am Coll Cardiol.* 1989;13:1469-76.

Ashitani J-I, Mukae H, Arimura Y, et al. Elevated plasma procoagulant and fibrinolytic markers in patients with chronic obstructive pulmonary disease. *Intern Med.* 2002;41:181-5.

Atkinson J. Age-related medial elastocalcinosis in arteries: mechanisms, animal models, and physiological consequences. *Journal of Applied Physiology.* 2008;105:1643-1651.

Atkinson JJ, Lutey BA, Suzuki Y, et al. The role of matrix metalloproteinase (MMP)-9 in cigarette smoke-induced emphysema. *Am J Respir Crit Care Med.* 2010;183:876-84.

Au DH, Curtis JR, Every NR, et al. Association between inhaled beta-agonists and the risk of unstable angina and myocardial infarction. *Chest.* 2002;121:846-51.

Autio P, Karjalainen J, Risteli L, et al. Effects of an inhaled steroid (budesonide) on skin collagen synthesis of asthma patients in vivo. *Am J Respir Crit Care Med.* 1996;153:1172-5.

Baraldo S, Bazzan E, Zanin ME, et al. Matrix metalloproteinase-2 protein in lung periphery is related to COPD progression. *Chest.* 2007;132:1733-40.

Barker DJ, Godfrey KM, Fall C, et al. Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. *BMJ.* 1991;303:671-5.

Barr RG, Mesia-Vela S, Austin JHM, et al. Impaired flow-mediated dilation is associated with low pulmonary function and emphysema in ex-smokers: the Emphysema and Cancer Action Project (EMCAP) study *Am J Respir Crit Care Med.* 2007;176:1200-7.

BD Biosciences. 2010;Introduction to flow cytometry [online]:Available at: http://www.bdbiosciences.com/support/training/self_paced.jsp [Accessed 25 January 2011].

Bellosta S, Via D, Canavesi M, et al. HMG-CoA reductase inhibitors reduce MMP-9 secretion by macrophages. *Arterioscler Thromb Vasc Biol.* 1998;18:1671-8.

Berger JS, Sanborn TA, Sherman W, et al. Effect of chronic obstructive pulmonary disease on survival of patients with coronary heart disease having percutaneous coronary intervention. *Am J Cardiol.* 2004;94:649-51.

Black LF & Kueppers F. Alpha1-antitrypsin deficiency in nonsmokers. *Am Rev Respir Dis.* 1978;117:421-8.

Boekholdt SM, Peters RJG, Hack CE, et al. IL-8 plasma concentrations and the risk of future coronary artery disease in apparently healthy men and women: the EPIC-Norfolk prospective population study. *Arterioscler Thromb Vasc Biol.* 2004;24:1503-8.

Bolton CE, Ionescu AA, Shiels KM, et al. Associated loss of fat-free mass and bone mineral density in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2004;170:1286-93.

Bolton CE, Mceniery CM, Raj V, et al. Aortic calcification, arterial stiffness and bone mineral density in patients with COPD. *Artery Research.* 2011;5:30-36.

Bolton CE, Stone MD, Edwards PH, et al. Circulating matrix metalloproteinase-9 and osteoporosis in patients with chronic obstructive pulmonary disease. *Chron Respir Dis.* 2009;6:81-7.

Boutouyrie P, Lacolley P, Girerd X, et al. Sympathetic activation decreases medium-sized arterial compliance in humans. *Am J Physiol.* 1994;267:H1368-76.

Burnett D, Chamba A, Hill SL, et al. Neutrophils from subjects with chronic obstructive lung disease show enhanced chemotaxis and extracellular proteolysis. *Lancet.* 1987;2:1043-6.

Burney and Jarvis. 2006;NHS Evidence - The Burden of COPD in the UK [online]:Available at : http://www.library.nhs.uk/RESPIRATORY/ViewResource.aspx?resID=187247 [Accessed January 23, 2011].

Bush DE, Jones CE, Bass KM, et al. Estrogen replacement reverses endothelial dysfunction in postmenopausal women. *Am J Med.* 1998;104:552-8.

Butterworth CE, Fielding JF, Finch CA, et al. Nutritional anaemias. Report of a WHO scientific group. *World Health Organ Tech Rep Ser.* 1968;405:5-37.

Calverley P, Anderson J, Celli B, et al. Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *N Engl J Med.* 2007;356:775-89.

Camilli AE, Robbins DR & Lebowitz MD. Death certificate reporting of confirmed airways obstructive disease. *Am J Epidemiol.* 1991;133:795-800.

Cataldo D, Munaut C, Noël A, et al. MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. *Int Arch Allergy Immunol.* 2000;123:259-67.

Celermajer D, Adams M, Clarkson P, et al. Passive smoking and impaired endothelium-dependent arterial dilatation in healthy young adults. *N Engl J Med.* 1996;334:150-4.

Celermajer DS, Sorensen KE, Gooch VM, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet.* 1992;340:1111-5.

Celermajer DS, Sorensen KE, Spiegelhalter DJ, et al. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *J Am Coll Cardiol.* 1994;24:471-6.

Celli B, Cote C, Marin J, et al. The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. *N Engl J Med.* 2004a;350:1005-1012.

Celli B, Decramer M, Kesten S, et al. Mortality in the 4-Year trial of tiotropium (UPLIFT) in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2009;180:948-55.

Celli BR, MacNee W & Force AET. Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper. *Eur Respir J.* 2004b;23:932-46.

Chakrabarti S & Patel KD. Regulation of matrix metalloproteinase-9 release from IL-8-stimulated human neutrophils. *J Leukoc Biol.* 2005;78:279-88.

Chakrabarti S, Zee JM & Patel KD. Regulation of matrix metalloproteinase-9 (MMP-9) in TNF-stimulated neutrophils: novel pathways for tertiary granule release. *J Leukoc Biol.* 2006;79:214-22.

Chambellan A, Chailleux E, Similowski T, et al. Prognostic value of the hematocrit in patients with severe COPD receiving long-term oxygen therapy. *Chest.* 2005;128:1201-8.

Chan NN, Vallance P & Colhoun HM. Endothelium-dependent and -independent vascular dysfunction in type 1 diabetes: role of conventional risk factors, sex, and glycemic control. *Arterioscler Thromb Vasc Biol.* 2003;23:1048-54.

Chen L, Einbinder E, Zhang Q, et al. Oxidative stress and left ventricular function with chronic intermittent hypoxia in rats. *Am J Respir Crit Care Med.* 2005;172:915-20.

Chen Q, Vazquez EJ, Moghaddas S, et al. Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem.* 2003;278:36027-31.

Chen Y. Genetics and pulmonary medicine.10: Genetic epidemiology of pulmonary function. *Thorax.* 1999;54:818-24.

Cho MH, Ciulla DM, Klanderman BJ, et al. Analysis of exonic elastin variants in severe, early-onset chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol.* 2009;40:751-5.

Chowienczyk PJ, Watts GF, Cockcroft JR, et al. Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. *Lancet.* 1992;340:1430-2.

Christie DJ, Kottke-Marchant K & Gorman RT. Hypersensitivity of platelets to adenosine diphosphate in patients with stable cardiovascular disease predicts major adverse events despite antiplatelet therapy. *Platelets.* 2008;19:104-10.

Chrysofakis G, Tzanakis N, Kyriakoy D, et al. Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD. *Chest.* 2004;125:71-6.

Chung AW, Yang HH, Kim JM, et al. Upregulation of matrix metalloproteinase-2 in the arterial vasculature contributes to stiffening and vasomotor dysfunction in patients with chronic kidney disease. *Circulation.* 2009;120:792-801.

Churg A, Wang R, Wang X, et al. Effect of an MMP-9/MMP-12 inhibitor on smoke-induced emphysema and airway remodelling in guinea pigs. *Thorax.* 2007;62:706-13.

Clini E, Bianchi L, Pagani M, et al. Endogenous nitric oxide in patients with stable COPD: correlates with severity of disease. *Thorax.* 1998;53:881-3.

Cohen AB & James HL. Reduction of the elastase inhibitory capacity of alpha 1-antitrypsin by peroxides in cigarette smoke: an analysis of brands and filters. *Am Rev Respir Dis.* 1982;126:25-30.

Cook S, Togni M, Schaub MC, et al. High heart rate: a cardiovascular risk factor? *Eur Heart J.* 2006;27:2387-93.

Corradi M, Montuschi P, Donnelly LE, et al. Increased nitrosothiols in exhaled breath condensate in inflammatory airway diseases. *Am J Respir Crit Care Med.* 2001;163:854-8.

Corretti MC, Anderson TJ, Benjamin EJ, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol.* 2002;39:257-65.

Cosio M, Ghezzo H, Hogg JC, et al. The relations between structural changes in small airways and pulmonary-function tests. *N Engl J Med.* 1978;298:1277-81.

Cosio MG, Majo J & Cosio MG. Inflammation of the airways and lung parenchyma in COPD: role of T cells. *Chest.* 2002;121:160-5S.

Cote C, Zilberberg MD, Mody SH, et al. Haemoglobin level and its clinical impact in a cohort of patients with COPD. *Eur Respir J.* 2007;29:923-9.

Cottin V, Fabien N, Khouatra C, et al. Anti-elastin autoantibodies are not present in combined pulmonary fibrosis and emphysema [letter]. *Eur Respir J.* 2009;33:219-221.

Curkendall SM, DeLuise C, Jones JK, et al. Cardiovascular disease in patients with chronic obstructive pulmonary disease, Saskatchewan Canada cardiovascular disease in COPD patients. *Ann Epidemiol.* 2006;16:63-70.

Curtis BM & O'Keefe JH. Autonomic tone as a cardiovascular risk factor: the dangers of chronic fight or flight. *Mayo Clin Proc.* 2002;77:45-54.

Dahl M, Vestbo J, Lange P, et al. C-reactive protein as a predictor of prognosis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2006;175:250-5.

Davì G, Basili S, Vieri M, et al. Enhanced thromboxane biosynthesis in patients with chronic obstructive pulmonary disease. The Chronic Obstructive Bronchitis and Haemostasis Study Group. *Am J Respir Crit Care Med.* 1997;156:1794-9.

Davì G & Patrono C. Platelet activation and atherothrombosis. *N Engl J Med.* 2007;357:2482-94.

De Blois J, Simard S, Atar D, et al. COPD predicts mortality in heart failure: the Norwegian Heart Failure Registry. *J Card Fail.* 2010;16:225-9.

Deanfield JE, Halcox JP & Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. *Circulation.* 2007;115:1285-95.

Del Maschio A, Evangelista V, Rajtar G, et al. Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents. *Am J Physiol.* 1990;258:H870-9.

Demedts IK, Morel-Montero A, Lebecque S, et al. Elevated MMP-12 protein levels in induced sputum from patients with COPD. *Thorax.* 2006;61:196-201.

Deslee G, Woods JC, Moore CM, et al. Elastin expression in very severe human COPD. *European Respiratory Journal.* 2009;34:324-331.

Devaraj S, Xu DY & Jialal I. C-reactive protein increases plasminogen activator inhibitor-1 expression and activity in human aortic endothelial cells: implications for the metabolic syndrome and atherothrombosis. *Circulation.* 2003;107:398-404.

DeWood MA, Spores J, Notske R, et al. Prevalence of total coronary occlusion during the early hours of transmural myocardial infarction. *N Engl J Med.* 1980;303:897-902.

Din J, Harding S, Valerio C, et al. Dietary intervention with oil rich fish reduces platelet-monocyte aggregation in man. *Atherosclerosis.* 2008;197:290-6.

Diodati JG, Cannon RO, 3rd, Epstein SE, et al. Platelet hyperaggregability across the coronary bed in response to rapid atrial pacing in patients with stable coronary artery disease. *Circulation.* 1992;86:1186-93.

Doshi SN, Naka KK, Payne N, et al. Flow-mediated dilatation following wrist and upper arm occlusion in humans: the contribution of nitric oxide. *Clin Sci.* 2001;101:629-35.

Dransfield M, Rowe S, Johnson J, et al. Beta-blocker use and the risk of death in hospitalized patients with acute exacerbations of COPD. *Thorax.* 2008;63:301-5.

Dransfield M, Washko G, Foreman M, et al. Gender differences in the severity of CT emphysema in COPD. *Chest.* 2007;132:464-70.

Dransfield MT, Huang F, Nath H, et al. CT emphysema predicts thoracic aortic calcification in smokers with and without COPD. *COPD.* 2010;7:404-10.

Eberly LE, Ockene J, Sherwin R, et al. Pulmonary function as a predictor of lung cancer mortality in continuing cigarette smokers and in quitters. *Int J Epidemiol.* 2003;32:592-9.

Egred M, Shaw S, Mohammad B, et al. Under-use of beta-blockers in patients with ischaemic heart disease and concomitant chronic obstructive pulmonary disease. *QJM.* 2005;98:493-7.

Eickhoff P, Valipour A, Kiss D, et al. Determinants of systemic vascular function in patients with stable COPD. *Am J Respir Crit Care Med.* 2008;178:1211-8.

Eisner MD, Balmes J, Yelin EH, et al. Directly measured secondhand smoke exposure and COPD health outcomes. *BMC Pulmonary Medicine.* 2006;6:12.

Feghali-Bostwick CA, Gadgil AS, Otterbein LE, et al. Autoantibodies in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2007;177:156-63.

Ferroni P, Basili S, Martini F, et al. Soluble P-selectin as a marker of platelet hyperactivity in patients with chronic obstructive pulmonary disease. *J Investig Med.* 2000;48:21-7.

Fichtlscherer S, Breuer S & Zeiher AM. Prognostic value of systemic endothelial dysfunction in patients with acute coronary syndromes: further evidence for the existence of the "vulnerable" patient. *Circulation.* 2004;110:1926-32.

Fijnheer R, Frijns CJ, Korteweg J, et al. The origin of P-selectin as a circulating plasma protein. *Thromb Haemost.* 1997;77:1081-5.

Fischer BM & Voynow JA. Neutrophil elastase induces MUC5AC gene expression in airway epithelium via a pathway involving reactive oxygen species. *Am J Respir Cell Mol Biol.* 2002;26:447-52.

Fisher GJ, Wang ZQ, Datta SC, et al. Pathophysiology of premature skin aging induced by ultraviolet light. *N Engl J Med.* 1997;337:1419-28.

Fletcher C & Peto R. The natural history of chronic airflow obstruction. *BMJ.* 1977;1:1645-8.

Foronjy R, Nkyimbeng T, Wallace A, et al. Transgenic expression of matrix metalloproteinase-9 causes adult-onset emphysema in mice associated with the loss of alveolar elastin. *Am J Physiol Lung Cell Mol Physiol.* 2008;294:L1149-57.

Francès C, Boisnic S, Hartmann DJ, et al. Changes in the elastic tissue of the non-sun-exposed skin of cigarette smokers. *Br J Dermatol.* 1991;125:43-7.

Freeman CM, Han MK, Martinez FJ, et al. Cytotoxic potential of lung CD8+ T Cells increases with chronic obstructive pulmonary disease severity and with in vitro stimulation by IL-18 or IL-15. *J Immunol.* 2010;184:6504-13.

Furchgott RF & Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.* 1980;288:373-6.

Galobardes B, McCarron P, Jeffreys M, et al. Association between early life history of respiratory disease and morbidity and mortality in adulthood. *Thorax.* 2008;63:423-9.

Gan WQ, Man SF, Senthilselvan A, et al. Association between chronic obstructive pulmonary disease and systemic inflammation: a systematic review and a meta-analysis. *Thorax.* 2004;59:574-80.

Gawaz M, Langer H & May AE. Platelets in inflammation and atherogenesis. *J Clin Invest.* 2005;115:3378-84.

Gianetti J, Parri MS, Sbrana S, et al. Platelet activation predicts recurrent ischemic events after percutaneous coronary angioplasty: a 6 months prospective study. *Thromb Res.* 2006;118:487-93.

Godtfredsen NS, Vestbo J, Osler M, et al. Risk of hospital admission for COPD following smoking cessation and reduction: a Danish population study. *Thorax.* 2002;57:967-72.

Golovatch P, Mercer BA, Lemaître V, et al. Role for Cathepsin K in emphysema in smoke-exposed guinea pigs. *Exp Lung Res.* 2009;35:631-645.

Goodman P, Agnew M, McCaffrey M, et al. Effects of the Irish smoking ban on respiratory health of bar workers and air quality in Dublin pubs. *Am J Respir Crit Care Med.* 2007;175:840-5.

Gottlieb SS, McCarter RJ & Vogel RA. Effect of beta-blockade on mortality among high-risk and low-risk patients after myocardial infarction. *N Engl J Med.* 1998;339:489-97.

Graat-Verboom L, Wouters EFM, Smeenk FWJM, et al. Current status of research on osteoporosis in COPD: a systematic review. *Eur Respir J.* 2009;34:209-18.

Griendling K & Ushio-Fukai M. Redox control of vascular smooth muscle proliferation. *J Lab Clin Med.* 1998;132:9-15.

Gurbel PA, Kereiakes DJ & Serebruany VL. Soluble P-selectin is not a surrogate marker for platelet P-selectin: evidence from a multicenter chest pain study group. *J Thromb Thrombolysis.* 2000;10:15-22.

Haapasaari K, Rossi O, Risteli J, et al. Effects of long-term inhaled corticosteroids on skin collagen synthesis and thickness in asthmatic patients. *Eur Respir J.* 1998;11:139-43.

Halcox JP, Schenke WH, Zalos G, et al. Prognostic value of coronary vascular endothelial dysfunction. *Circulation.* 2002;106:653-8.

Halonen JI, Lanki T, Yli-Tuomi T, et al. Urban air pollution, and asthma and COPD hospital emergency room visits. *Thorax.* 2008;63:635-641.

Han MK, Agusti A, Calverley PM, et al. Chronic obstructive pulmonary disease phenotypes: the future of COPD. *Am J Respir Crit Care Med.* 2010;182:598-604.

Hansell AL. Lies, damned lies and mortality statistics? *Thorax.* 2006;61:923-4.

Hansell AL, Walk JA & Soriano JB. What do chronic obstructive pulmonary disease patients die from? A multiple cause coding analysis. *Eur Respir J.* 2003;22:809-14.

Haq I, Chappell S, Johnson SR, et al. Association of MMP - 12 polymorphisms with severe and very severe COPD: A case control study of MMPs - 1, 9 and 12 in a European population. *BMC Medical Genetics.* 2010;11:7.

Harding S, Sarma J, Josephs D, et al. Upregulation of the CD40/CD40 ligand dyad and platelet-monocyte aggregation in cigarette smokers. *Circulation.* 2004a;109:1926-9.

Harding SA, Din JN, Sarma J, et al. Flow cytometric analysis of circulating platelet-monocyte aggregates in whole blood: methodological considerations. *Thromb Haemost.* 2007;98:451-6.

Harding SA, Din JN, Sarma J, et al. Promotion of proinflammatory interactions between platelets and monocytes by unfractionated heparin. *Heart.* 2006;92:1635-8.

Harding SA, Sommerfield AJ, Sarma J, et al. Increased CD40 ligand and platelet-monocyte aggregates in patients with type 1 diabetes mellitus. *Atherosclerosis.* 2004b;176:321-5.

Harrison D, Griendling K, Landmesser U, et al. Role of oxidative stress in atherosclerosis. *Am J Cardiol.* 2003;91:7-11.

Hartmann G, Tschop M, Fischer R, et al. High altitude increases circulating interleukin-6, interleukin-1 receptor antagonist and C-reactive protein. *Cytokine.* 2000;12:246-52.

Hautamaki RD, Kobayashi DK, Senior RM, et al. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science.* 1997;277:2002-4.

Hegewald MJ & Crapo RO. Socioeconomic status and lung function. *Chest.* 2007;132:1608-14.

Heindl S, Lehnert M, Criée CP, et al. Marked sympathetic activation in patients with chronic respiratory failure. *Am J Respir Crit Care Med.* 2001;164:597-601.

Heitzer T, Schlinzig T, Krohn K, et al. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation.* 2001;104:2673-8.

Heitzer T, Ylä-Herttuala S, Luoma J, et al. Cigarette smoking potentiates endothelial dysfunction of forearm resistance vessels in patients with hypercholesterolemia. Role of oxidized LDL. *Circulation.* 1996;93:1346-53.

Henriksen PA & Sallenave J-M. Human neutrophil elastase: mediator and therapeutic target in atherosclerosis. *Int J Biochem Cell Biol.* 2008;40:1095-100.

Hersh CP, Demeo DL & Silverman EK. National Emphysema Treatment Trial state of the art: genetics of emphysema. *Proc Am Thorac Soc.* 2008;5:486-93.

Hii SW, Tam JDC, Thompson BR, et al. Bullous lung disease due to marijuana. *Respirology.* 2008;13:122-7.

Hogg JC, Chu FSF, Tan WC, et al. Survival after lung volume reduction in chronic obstructive pulmonary disease: insights from small airway pathology. *Am J Respir Crit Care Med.* 2007;176:454-9.

Hole DJ, Watt GC, Davey-Smith G, et al. Impaired lung function and mortality risk in men and women: findings from the Renfrew and Paisley prospective population study. *BMJ.* 1996;313:711-5.

Hrafnkelsdottir T, Wall U, Jern C, et al. Impaired capacity for endogenous fibrinolysis in essential hypertension. *Lancet.* 1998;352:1597-8.

Huiart L, Ernst P & Suissa S. Cardiovascular morbidity and mortality in COPD. *Chest.* 2005;128:2640-6.

Humbert M, Morrell N, Archer S, et al. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol.* 2004;43:13-24.

Hunninghake GM, Cho MH, Tesfaigzi Y, et al. MMP12, lung function, and COPD in high-risk populations. *N Engl J Med.* 2009;361:2599-608.

Hunt SA, Abraham WT, Chin MH, et al. ACC/AHA 2005 Guideline update for the diagnosis and management of chronic heart failure in the adult: a report of the American College of Cardiology/American Heart Association Task Force on practice guidelines. *Circulation.* 2005;112:E154-235.

Ichikawa H, Flores S, Kvietys P, et al. Molecular mechanisms of anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. *Circ Res.* 1997;81:922-31.

Ichinose M, Sugiura H, Yamagata S, et al. Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. *Am J Respir Crit Care Med.* 2000;162:701-6.

ImageJ User Guide. 2011;National Institute of Health [online]:Available at: http://rsbweb.nih.gov/ij/docs/guide/userguide.html [Accessed on February 19, 2011].

Incalzi RA, Caradonna P, Ranieri P, et al. Correlates of osteoporosis in chronic obstructive pulmonary disease. *Respir Med.* 2000;94:1079-84.

Information Services Division (ISD) in Scotland. 2011;National Organisation for Health Information [online]:Available at: http://www.isdscotland.org/isd/CCC_FirstPage.jsp [Accessed 24 January 2011].

Ingebrigtsen T, Thomsen SF, Vestbo J, et al. Genetic influences on chronic obstructive pulmonary disease - A twin study. *Respir Med.* 2010;104:1890-5.

Ito I, Nagai S, Handa T, et al. Matrix metalloproteinase-9 promoter polymorphism associated with upper lung dominant emphysema. *Am J Respir Crit Care Med.* 2005;172:1378-82.

Iwamoto H, Yokoyama A, Kitahara Y, et al. Airflow limitation in smokers is associated with subclinical atherosclerosis. *Am J Respir Crit Care Med.* 2008;179:35-40.

Janoff A, Sloan B, Weinbaum G, et al. Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. *Am Rev Respir Dis.* 1977;115:461-78.

Jatoi NA, Jerrard-Dunne P, Feely J, et al. Impact of smoking and smoking cessation on arterial stiffness and aortic wave reflection in hypertension. *Hypertension.* 2007;49:981-5.

Jensen HH, Godtfredsen NS, Lange P, et al. Potential misclassification of causes of death from COPD. *Eur Respir J.* 2006;28:781-5.

Jensen-Urstad K & Johansson J. Gender difference in age-related changes in vascular function. *J Intern Med.* 2001;250:29-36.

Jiang R, Paik DC, Hankinson JL, et al. Cured meat consumption, lung function, and chronic obstructive pulmonary disease among United States adults. *Am J Respir Crit Care Med.* 2007;175:798-804.

John M, Lange A, Hoernig S, et al. Prevalence of anemia in chronic obstructive pulmonary disease: comparison to other chronic diseases. *Int J Cardiol.* 2006;111:365-70.

Johnstone MT, Creager SJ, Scales KM, et al. Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *Circulation.* 1993;88:2510-6.

Joseph JE, Harrison P, Mackie IJ, et al. Increased circulating platelet-leucocyte complexes and platelet activation in patients with antiphospholipid syndrome, systemic lupus erythematosus and rheumatoid arthritis. *Br J Haematol.* 2001;115:451-9.

Just M, Monsó E, Ribera M, et al. Relationships between lung function, smoking and morphology of dermal elastic fibres. *Exp Dermatol.* 2005;14:744-51.

Just M, Ribera M, Monsó E, et al. Effect of smoking on skin elastic fibres: morphometric and immunohistochemical analysis. *Br J Dermatol.* 2007;156:85-91.

Kamath S, Blann AD, Caine GJ, et al. Platelet P-selectin levels in relation to plasma soluble P-selectin and beta-thromboglobulin levels in atrial fibrillation. *Stroke.* 2002;33:1237-42.

Kameda K, Matsunaga T, Abe N, et al. Correlation of oxidative stress with activity of matrix metalloproteinase in patients with coronary artery disease: Possible role for left ventricular remodelling. *Eur Heart J.* 2003;24:2180-5.

Kamio K, Liu XD, Sugiura H, et al. Statins inhibit matrix metalloproteinase release from human lung fibroblasts. *Eur Respir J.* 2010;35:637-46.

Karayama M, Inui N, Suda T, et al. Antiendothelial cell antibodies in patients with COPD. *Chest.* 2010;138:1303-8.

Kelleher CM, Silverman EK, Broekelmann T, et al. A functional mutation in the terminal exon of elastin in severe, early-onset chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol.* 2005;33:355-62.

Kjensli A, Falch JA, Ryg M, et al. High prevalence of vertebral deformities in COPD patients: relationship to disease severity. *Eur Respir J.* 2009;33:1018-1024.

Klinkhardt U, Bauersachs R, Adams J, et al. Clopidogrel but not aspirin reduces P-selectin expression and formation of platelet-leukocyte aggregates in patients with atherosclerotic vascular disease. *Clin Pharmacol Ther.* 2003;73:232-41.

Kohansal R, Martinez-Camblor P, Agusti A, et al. The natural history of chronic airflow obstruction revisited: an analysis of the Framingham offspring cohort. *Am J Respir Crit Care Med.* 2009;180:3-10.

Koike T, Kitajima S, Yu Y, et al. Human C-reactive protein does not promote atherosclerosis in transgenic rabbits. *Circulation.* 2009;120:2088-94.

Labella F & Lindsay WG. The structure of human aortic elastin as influenced by age. *J Gerontol.* 1963;18:111-8.

Landmesser U & Drexler H. Oxidative stress, the renin-angiotensin system, and atherosclerosis. *Eur Heart J Suppl.* 2003;5:A3-7.

Lang N, Gumundsdottir I, Boon N, et al. Marked impairment of protease-activated receptor type 1-mediated vasodilation and fibrinolysis in cigarette smokers: smoking, thrombin, and vascular responses in vivo. *J Am Coll Cardiol.* 2008;52:33-9.

Lapperre TS. Relation between duration of smoking cessation and bronchial inflammation in COPD. *Thorax.* 2006;61:115-21.

Lattimore J-DL, Wilcox I, Nakhla S, et al. Repetitive hypoxia increases lipid loading in human macrophages-a potentially atherogenic effect. *Atherosclerosis.* 2005;179:255-9.

Laurent S, Cockcroft J, Van Bortel L, et al. Expert consensus document on arterial stiffness: methodological issues and clinical applications. *Eur Heart J.* 2006;27:2588-605.

Lawall H, Hoffmanns W, Hoffmanns P, et al. Prevalence of deep vein thrombosis (DVT) in non-surgical patients at hospital admission. *Thromb Haemost.* 2007;98:765-70.

Lee HM, Le H, Lee BT, et al. Forced vital capacity paired with Framingham Risk Score for prediction of all-cause mortality. *Eur Respir J.* 2010;36:1002-6.

Lee SH, Goswami S, Grudo A, et al. Antielastin autoimmunity in tobacco smoking-induced emphysema. *Nat Med.* 2007;13:567-9.

Lee YW, Kühn H, Hennig B, et al. IL-4-induced oxidative stress upregulates VCAM-1 gene expression in human endothelial cells. *J Mol Cell Cardiol.* 2001;33:83-94.

Libby P, Ridker PM & Maseri A. Inflammation and atherosclerosis. *Circulation.* 2002;105:1135-43.

Libby P & Theroux P. Pathophysiology of coronary artery disease. *Circulation.* 2005;111:3481-8.

Lieberman EH, Gerhard MD, Uehata A, et al. Estrogen improves endothelium-dependent, flow-mediated vasodilation in postmenopausal women. *Ann Intern Med.* 1994;121:936-41.

Lind L. Arterial compliance influences the measurement of flow-mediated vasodilation, but not acetylcholine-mediated forearm blood flowThe prospective investigation of the vasculature in uppsala seniors (PIVUS) study. *Atherosclerosis.* 2007;190:212-5.

Lind L. Arterial compliance and endothelium-dependent vasodilation are independently related to coronary risk in the elderly: the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study. *Clin Physiol Funct Imaging.* 2008;28:373-7.

Lomas D & Parfrey H. α1-Antitrypsin deficiency• 4: Molecular pathophysiology. *Thorax.* 2004;59:529-35.

London GM, Blacher J, Pannier B, et al. Arterial wave reflections and survival in end-stage renal failure. *Hypertension.* 2001;38:434-8.

Luan Z, Chase A & Newby A. Statins inhibit secretion of metalloproteinases-1, -2, -3, and -9 From vascular smooth muscle cells and macrophages. *Arterioscler Thromb Vasc Biol.* 2003;23:769-75.

Luc G, Bard J-M, Juhan-Vague I, et al. C-reactive protein, interleukin-6, and fibrinogen as predictors of coronary heart disease: the PRIME Study. *Arterioscler Thromb Vasc Biol.* 2003;23:1255-61.

Luisetti M, Sturani C, Sella D, et al. MR889, a neutrophil elastase inhibitor, in patients with chronic obstructive pulmonary disease: a double-blind, randomized, placebo-controlled clinical trial. *Eur Respir J.* 1996;9:1482-6.

Luksha L, Agewall S & Kublickiene K. Endothelium-derived hyperpolarizing factor in vascular physiology and cardiovascular disease. *Atherosclerosis.* 2009;202:330-44.

Lundbäck B, Lindberg A, Lindström M, et al. Not 15 but 50% of smokers develop COPD?--Report from the Obstructive Lung Disease in Northern Sweden Studies. *Respir Med.* 2003;97:115-22.

Lutgens E, Lutgens SPM, Faber BCG, et al. Disruption of the cathepsin K gene reduces atherosclerosis progression and induces plaque fibrosis but accelerates macrophage foam cell formation. *Circulation.* 2006;113:98-107.

Ly HQ, Kirtane AJ, Murphy SA, et al. Association of platelet counts on presentation and clinical outcomes in ST-elevation myocardial infarction (from the TIMI Trials). *Am J Cardiol.* 2006;98:1-5.

Mackenzie IS, Wilkinson IB & Cockcroft JR. Assessment of arterial stiffness in clinical practice. *QJM.* 2002;95:67-74.

Maclay JD, McAllister DA & Macnee W. Cardiovascular risk in chronic obstructive pulmonary disease. *Respirology.* 2007;12:634-41.

Maclay JD, McAllister DA, Mills NL, et al. Vascular dysfunction in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2009;180:513-20.

MacNee W. Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease. *Proc Am Thorac Soc.* 2005;2:50-60.

MacNee W, Maclay J & Mcallister D. Cardiovascular injury and repair in chronic obstructive pulmonary disease. *Proc Am Thorac Soc.* 2008;5:824-33.

Mair G, Miller JJ, Mcallister D, et al. Computed tomographic emphysema distribution: relationship to clinical features in a cohort of smokers. *Eur Respir J.* 2009;33:536-542.

Man W, Kemp P, Moxham J, et al. Skeletal muscle dysfunction in COPD: clinical and laboratory observations. *Clin Sci.* 2009;117:251-64.

Mannino DM. Underdiagnosed chronic obstructive pulmonary disease in England: new country, same story. *Thorax.* 2006;61:1032-4.

Mannino DM, Thorn D, Swensen A, et al. Prevalence and outcomes of diabetes, hypertension and cardiovascular disease in COPD. *Eur Respir J.* 2008;32:962-9.

Martinez F, Curtis J, Sciurba F, et al. Sex sifferences in severe pulmonary emphysema. *Am J Respir Crit Care Med.* 2007;176:243-52.

Mascarenhas J, Lourenco P, Lopes R, et al. Chronic obstructive pulmonary disease in heart failure. Prevalence, therapeutic and prognostic implications. *Am Heart J.* 2008;155:521-5.

Mathew JP, Fontes ML, Tudor IC, et al. A multicenter risk index for atrial fibrillation after cardiac surgery. *JAMA.* 2004;291:1720-9.

McAllister D, Maclay J, Cheuk S, et al. Acute coronary events during acute exacerbations of chronic obstructive pulmonary disease. *European Respiratory Society Conference.* 2007a:[abstract].

McAllister DA, Maclay JD, Mills NL, et al. Arterial stiffness is independently associated with emphysema severity in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2007b;176:1208-14.

McAllister DA, MacNee W, Duprez D, et al. Pulmonary function is associated with distal aortic calcium not proximal aortic distensibility: MESA Lung Study. *COPD.* 2011;8:71-8.

McEniery CM, Wallace S, Mackenzie IS, et al. Endothelial function is associated with pulse pressure, pulse wave velocity, and augmentation index in healthy humans. *Hypertension.* 2006;48:602-8.

Michelson AD, Barnard MR, Krueger LA, et al. Evaluation of platelet function by flow cytometry. *Methods.* 2000;21:259-70.

Michelson AD, Barnard MR, Krueger LA, et al. Circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface p-selectin: studies in baboons, human coronary intervention, and human acute myocardial infarction. *Circulation.* 2001;104:1533-7.

Miller MR, Hankinson J, Brusasco V, et al. Standardisation of spirometry. *Eur Respir J.* 2005;26:319-38.

Mills NL, Miller JJ, Anand A, et al. Increased arterial stiffness in patients with chronic obstructive pulmonary disease: a mechanism for increased cardiovascular risk. *Thorax.* 2008;63:306-11.

Mills NL, Tornqvist H, Gonzalez MC, et al. Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. *N Engl J Med.* 2007;357:1075-82.

Mills NL, Törnqvist H, Robinson SD, et al. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. *Circulation.* 2005;112:3930-6.

Modena MG, Bonetti L, Coppi F, et al. Prognostic role of reversible endothelial dysfunction in hypertensive postmenopausal women. *J Am Coll Cardiol.* 2002;40:505-10.

Monnink SH, van Haelst PL, van Boven AJ, et al. Endothelial dysfunction in patients with coronary artery disease: a comparison of three frequently reported tests. *J Investig Med.* 2002;50:19-24.

Murray CJ & Lopez AD. Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet.* 1997;349:1498-504.

Newby D, Wright R, Labinjoh C, et al. Endothelial dysfunction, impaired endogenous fibrinolysis, and cigarette smoking - A mechanism for arterial thrombosis and myocardial infarction. *Circulation.* 1999;99:1411-5.

Newby DE, McLeod AL, Uren NG, et al. Impaired coronary tissue plasminogen activator release is associated with coronary atherosclerosis and cigarette smoking: direct link between endothelial dysfunction and atherothrombosis. *Circulation.* 2001;103:1936-41.

Newby DE, Wright RA, Ludlam CA, et al. An in vivo model for the assessment of acute fibrinolytic capacity of the endothelium. *Thromb Haemost.* 1997;78:1242-8.

Noguera A, Batle S, Miralles C, et al. Enhanced neutrophil response in chronic obstructive pulmonary disease. *Thorax.* 2001;56:432-7.

Noguera A, Busquets X, Sauleda J, et al. Expression of adhesion molecules and G proteins in circulating neutrophils in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 1998;158:1664-8.

Nowak D, Kasielski M, Pietras T, et al. Cigarette smoking does not increase hydrogen peroxide levels in expired breath condensate of patients with stable COPD. *Monaldi Arch Chest Dis.* 1998;53:268-73.

Nunez B, Sauleda J, Anto JM, et al. Anti-tissue antibodies are related to lung function in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2011;183:1025-31.

O'Donnell R, Breen D, Wilson S, et al. Inflammatory cells in the airways in COPD. *Thorax.* 2006;61:448-54.

O'Rourke MF, Pauca A & Jiang XJ. Pulse wave analysis. *Br J Clin Pharmacol.* 2001;51:507-22.

Ogawa E, Nakano Y, Ohara T, et al. Body mass index in male patients with COPD: correlation with low attenuation areas on CT. *Thorax.* 2009;64:20-5.

Ohara T, Hirai T, Muro S, et al. Relationship between pulmonary emphysema and osteoporosis assessed by CT in patients with COPD. *Chest.* 2008;134:1244-9.

Oliver JJ, Webb DJ & Newby DE. Stimulated tissue plasminogen activator release as a marker of endothelial function in humans. *Arterioscler Thromb Vasc Biol.* 2005;25:2470-9.

Orozco-Levi M, Garcia-Aymerich J, Villar J, et al. Wood smoke exposure and risk of chronic obstructive pulmonary disease. *Eur Respir J.* 2006;27:542-6.

Osman LM, Douglas JG, Garden C, et al. Indoor air quality in homes of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2007;176:465-72.

Panza JA, Quyyumi AA, Brush JE, et al. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med.* 1990;323:22-7.

Paredi P, Kharitonov S, Leak D, et al. Exhaled ethane, a marker of lipid peroxidation, is elevated in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2000;162:369-73.

Parimon T, Chien JW, Bryson CL, et al. Inhaled corticosteroids and risk of lung cancer among patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2007;175:712-9.

Parr D, White A, Bayley D, et al. Inflammation in sputum relates to progression of disease in subjects with COPD: a prospective descriptive study. *Respir Res.* 2006;7:136.

Patel BD, Coxson HO, Pillai SG, et al. Airway wall thickening and emphysema show independent familial aggregation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2008;178:500-5.

Patel BD, Loo WJ, Tasker AD, et al. Smoking related COPD and facial wrinkling: is there a common susceptibility? *Thorax.* 2006;61:568-71.

Pauwels RA, Buist AS, Calverley PM, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med.* 2001;163:1256-76.

Perticone F, Ceravolo R, Pujia A, et al. Prognostic significance of endothelial dysfunction in hypertensive patients. *Circulation.* 2001;104:191-6.

Pierrou S, Broberg P, O'Donnell RA, et al. Expression of genes involved in oxidative stress responses in airway epithelial cells of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2007;175:577-86.

Practico D, Basili S, Vieri M, et al. Chronic obstructive pulmonary disease is associated with an increase in urinary levels of isoprostane F2alpha-III, an index of oxidant stress. *Am J Respir Crit Care Med.* 1998;158:1709-14.

Prescott E, Godtfredsen N, Vestbo J, et al. Social position and mortality from respiratory diseases in males and females. *Eur Respir J.* 2003;21:821-6.

Pretorius M, Rosenbaum DA, Lefebvre J, et al. Smoking impairs bradykinin-stimulated t-PA release. *Hypertension.* 2002;39:767-71.

Price LC, Lowe D, Hosker HS, et al. UK National COPD Audit 2003: Impact of hospital resources and organisation of care on patient outcome following admission for acute COPD exacerbation. *Thorax.* 2006;61:837-42.

Pryor WA & Stone K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Ann N Y Acad Sci.* 1993;686:12-27.

Rahman I, Morrison D, Donaldson K, et al. Systemic oxidative stress in asthma, COPD, and smokers. *Am J Respir Crit Care Med.* 1996;154:1055-60.

Ramírez-Venegas A, Sansores RH, Pérez-Padilla R, et al. Survival of patients with chronic obstructive pulmonary disease due to biomass smoke and tobacco. *Am J Respir Crit Care Med.* 2006;173:393-7.

Ramos CL, Huo Y, Jung U, et al. Direct demonstration of P-selectin- and VCAM-1-dependent mononuclear cell rolling in early atherosclerotic lesions of apolipoprotein E-deficient mice. *Circ Res.* 1999;84:1237-44.

Rautaharju P, Warren J, Jain U, et al. Cardiac infarction injury score: an electrocardiographic coding scheme for ischemic heart disease. *Circulation.* 1981;64:249-56.

Reboul C, Tanguy S, Gibault A, et al. Chronic hypoxia exposure depresses aortic endothelium-dependent vasorelaxation in both sedentary and trained rats: involvement of L-arginine. *J Appl Physiol.* 2005;99:1029-35.

Rentrop KP, Feit F, Sherman W, et al. Serial angiographic assessment of coronary artery obstruction and collateral flow in acute myocardial infarction. Report from the second Mount Sinai-New York University Reperfusion Trial. *Circulation.* 1989;80:1166-75.

Ridker P, Danielson E, Fonseca F, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med.* 2008;359:2195-207.

Ridker P, Rifai N, Stampfer M, et al. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation.* 2000a;101:1767-72.

Ridker PM, Cushman M, Stampfer MJ, et al. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med.* 1997;336:973-9.

Ridker PM, Hennekens CH, Buring JE, et al. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med.* 2000b;342:836-43.

Riley DJ & Thakker-Varia S. Effect of diet on lung structure, connective tissue metabolism and gene expression. *J Nutr.* 1995;125:1657S-1660S.

Robert C, Lesty C & Robert AM. Ageing of the skin: study of elastic fiber network modifications by computerized image analysis. *Gerontology.* 1988;34:291-6.

Robinson SD, Ludlam CA, Boon NA, et al. Endothelial fibrinolytic capacity predicts future adverse cardiovascular events in patients with coronary heart disease. *Arterioscler Thromb Vasc Biol.* 2007;27:1651-6.

Sabit R, Bolton CE, Edwards PH, et al. Arterial stiffness and osteoporosis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2007;175:1259-65.

Safar ME, Levy BI & Struijker-Boudier H. Current perspectives on arterial stiffness and pulse pressure in hypertension and cardiovascular diseases. *Circulation.* 2003;107:2864-9.

Salisbury AC, Reid KJ & Spertus JA. Impact of chronic obstructive pulmonary disease on post-myocardial infarction outcomes. *Am J Cardiol.* 2007;99:636-41.

Salpeter S, Ormiston T & Salpeter E. Cardioselective beta-blockers for chronic obstructive pulmonary disease. *Cochrane Database Syst Rev.* 2005:Art. No.: CD003566.

Sarma J, Laan CA, Alam S, et al. Increased platelet binding to circulating monocytes in acute coronary syndromes. *Circulation.* 2002;105:2166-71.

Savransky V, Nanayakkara A, Li J, et al. Chronic intermittent hypoxia induces atherosclerosis. *Am J Respir Crit Care Med.* 2007;175:1290-7.

Schindler TH, Hornig B, Buser PT, et al. Prognostic value of abnormal vasoreactivity of epicardial coronary arteries to sympathetic stimulation in patients with normal coronary angiograms. *Arterioscler Thromb Vasc Biol.* 2003;23:495-501.

Schols AM, Slangen J, Volovics L, et al. Weight loss is a reversible factor in the prognosis of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 1998;157:1791-7.

Schönhofer B & Köhler D. Prevalence of deep-vein thrombosis of the leg in patients with acute exacerbation of chronic obstructive pulmonary disease. *Respiration.* 1998;65:173-7.

Schuerholz T, Keil O, Wagner T, et al. Hydrocortisone does not affect major platelet receptors in inflammation in vitro. *Steroids.* 2007;72:609-13.

Schumacher A, Liebers U, John M, et al. P-selectin glycoprotein ligand-1 (PSGL-1) is up-regulated on leucocytes from patients with chronic obstructive pulmonary disease. *Clin Exp Immunol.* 2005;142:370-6.

Schwartz AG & Ruckdeschel JC. Familial lung cancer: genetic susceptibility and relationship to chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2006;173:16-22.

Scottish Public Health Observatory. 2010a;Chronic obstructive pulmonary disease (COPD): mortality data [online]:Available at: http://www.scotpho.org.uk/home/Healthwell-beinganddisease/copd/copd_data/copd_mortality.asp [Accessed 23 January 2011].

Scottish Public Health Observatory. 2010b;Chronic obstructive pulmonary disease (COPD): deprivation data. Deprivation and secondary care [online]:Available at: http://www.scotpho.org.uk/home/Healthwell-beinganddisease/copd/copd_data/copd_deprivation.asp [Accessed 24 January 2011].

Seagrave J, Barr EB, March TH, et al. Effects of cigarette smoke exposure and cessation on inflammatory cells and matrix metalloproteinase activity in mice. *Exp Lung Res.* 2004;30:1-15.

Seemungal TA, Donaldson GC, Bhowmik A, et al. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2000;161:1608-13.

Sekine Y, Kesler KA, Behnia M, et al. COPD may increase the incidence of refractory supraventricular arrhythmias following pulmonary resection for non-small cell lung cancer. *Chest.* 2001;120:1783-90.

Shapiro SD, Goldstein NM, Houghton AM, et al. Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice. *Am J Pathol.* 2010;163:2329-35.

Shifren A & Mecham RP. The stumbling block in lung repair of emphysema: elastic fiber assembly. *Proc Am Thorac Soc.* 2006;3:428-33.

Shih H, Webb C, Conway W, et al. Frequency and significance of cardiac arrhythmias in chronic obstructive lung disease. *Chest.* 1988;94:44-8.

Shimbo D, Muntner P, Mann D, et al. Endothelial dysfunction and the risk of hypertension: the multi-ethnic study of atherosclerosis. *Hypertension.* 2010;55:1210-6.

Sidney S, Sorel M, Quesenberry CP, et al. COPD and incident cardiovascular disease hospitalizations and mortality: Kaiser Permanente Medical Care Program. *Chest.* 2005;128:2068-75.

Silverman EK, Chapman HA, Drazen JM, et al. Genetic epidemiology of severe, early-onset chronic obstructive pulmonary disease. Risk to relatives for airflow obstruction and chronic bronchitis. *Am J Respir Crit Care Med.* 1998;157:1770-8.

Similowski T, Agustí A, Macnee W, et al. The potential impact of anaemia of chronic disease in COPD. *Eur Respir J.* 2006;27:390-6.

Sin DD & Man SF. Why are patients with chronic obstructive pulmonary disease at increased risk of cardiovascular diseases? The potential role of systemic inflammation in chronic obstructive pulmonary disease. *Circulation.* 2003;107:1514-9.

Sin DD, Wu L & Man SFP. The relationship between reduced lung function and cardiovascular mortality: a population-based study and a systematic review of the literature. *Chest.* 2005;127:1952-9.

Skwarski K, Morrison D, Barratt A, et al. Effects of hypoxia on renal hormonal balance in normal subjects and in patients with COPD. *Respir Med.* 1998;92:1331-6.

Smeeth L, Thomas SL, Hall AJ, et al. Risk of myocardial infarction and stroke after acute infection or vaccination. *N Engl J Med.* 2004;351:2611-8.

Smith BS, Williamson N & McConkey B. Dermal connective tissue in patients with chronic obstructive airways disease. *Lancet.* 1967;1:341-3.

Sorlie PD, Kannel WB & O'Connor G. Mortality associated with respiratory function and symptoms in advanced age. The Framingham Study. *Am Rev Respir Dis.* 1989;140:379-84.

Stănescu D, Sanna A, Veriter C, et al. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax.* 1996;51:267-71.

Stenborg A, Kalimo H, Viitanen M, et al. Impaired endothelial function of forearm resistance arteries in CADASIL patients. *Stroke.* 2007;38:2692-7.

Stern D, Morgan W, Wright A, et al. Poor airway function in early infancy and lung function by age 22 years: a non-selective longitudinal cohort study. *Lancet.* 2007;370:758-64.

Stewart AD, Millasseau SC, Kearney MT, et al. Effects of inhibition of basal nitric oxide synthesis on carotid-femoral pulse wave velocity and augmentation index in humans. *Hypertension.* 2003;42:915-8.

Stockley RA. Neutrophils and the Pathogenesis of COPD. *Chest.* 2002;121:151S-155.

Stoel BC & Connell M. Quantitative analysis of emphysema in 3D using MDCT: correction of air offset in Toshiba scanners. *Eur J Radiol.* 2008;65:235-6.

Strack PR, Waxman L & Fagan JM. Activation of the multicatalytic endopeptidase by oxidants. Effects on enzyme structure. *Biochemistry.* 1996;35:7142-9.

Suissa S, Assimes T & Ernst P. Inhaled short acting beta agonist use in COPD and the risk of acute myocardial infarction. *Thorax.* 2003;58:43-6.

Suissa S, Hemmelgarn B, Blais L, et al. Bronchodilators and acute cardiac death. *Am J Respir Crit Care Med.* 1996;154:1598-602.

Svanes C, Sunyer J, Plana E, et al. Early life origins of chronic obstructive pulmonary disease. *Thorax.* 2010;65:14-20.

Tennant PWG, Gibson GJ & Pearce MS. Lifecourse predictors of adult respiratory function: results from the Newcastle Thousand Families Study. *Thorax.* 2008;63:823-30.

Tetley TD. Macrophages and the pathogenesis of COPD. *Chest.* 2002;121:156-9S.

Thaulow E, Erikssen J, Sandvik L, et al. Blood platelet count and function are related to total and cardiovascular death in apparently healthy men. *Circulation.* 1991;84:613-7.

Thomson AJ, Drummond GB, Waring WS, et al. Effects of short-term isocapnic hyperoxia and hypoxia on cardiovascular function. *J Appl Physiol.* 2006;101:809-16.

Tillin T, Chambers J, Malik I, et al. Measurement of pulse wave velocity: site matters. *J Hypertens.* 2007;25:383-9.

Tockman M, Pearson J, Fleg J, et al. Rapid decline in FEV1. A new risk factor for coronary heart disease mortality. *Am J Respir Crit Care Med.* 1995;151:390-8.

Torzewski M, Rist C, Mortensen RF, et al. C-reactive protein in the arterial intima: role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis. *Arterioscler Thromb Vasc Biol.* 2000;20:2094-9.

Truelsen T, Prescott E, Lange P, et al. Lung function and risk of fatal and non-fatal stroke. The Copenhagen City Heart Study. *Int J Epidemiol.* 2001;30:145-51.

Tsuji H, Venditti FJ, Manders ES, et al. Reduced heart rate variability and mortality risk in an elderly cohort. The Framingham Heart Study. *Circulation.* 1994;90:878-83.

Turino GM. Proteases in COPD: a critical pathway to injury. *Chest.* 2007;132:1724-5.

Underwood DC, Osborn RR, Bochnowicz S, et al. SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. *Am J Physiol Lung Cell Mol Physiol.* 2000;279:L895-902.

Vaidyula VR, Boden G & Rao AK. Platelet and monocyte activation by hyperglycemia and hyperinsulinemia in healthy subjects. *Platelets.* 2006;17:577-85.

van Gestel YRBM, Hoeks SE, Sin DD, et al. COPD and cancer mortality: the influence of statins. *Thorax.* 2009;64:963-7.

van Gestel YRBM, Hoeks SE, Sin DD, et al. Impact of cardioselective beta-blockers on mortality in patients with chronic obstructive pulmonary disease and atherosclerosis. *Am J Respir Crit Care Med.* 2008;178:695-700.

Vanhoutte P. Ageing and endothelial dysfunction. *Eur Heart J Suppl.* 2002;4:A8-17.

Varraso R, Fung TT, Hu FB, et al. Prospective study of dietary patterns and chronic obstructive pulmonary disease among US men. *Thorax.* 2007;62:786-91.

Vedam H, Phillips C, Wang D, et al. Short-term hypoxia reduces arterial stiffness in healthy men. *Eur J Appl Physiol.* 2009;105:19-25.

Venugopal SK, Devaraj S, Yuhanna I, et al. Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. *Circulation.* 2002;106:1439-41.

Verhoeven GT, Wijkhuijs AJ, Hooijkaas H, et al. Effect of an inhaled glucocorticoid on reactive oxygen species production by bronchoalveolar lavage cells from smoking COPD patients. *Mediators Inflamm.* 2000;9:109-13.

Verma S, Li S-H, Badiwala MV, et al. Endothelin antagonism and interleukin-6 inhibition attenuate the proatherogenic effects of C-reactive protein. *Circulation.* 2002;105:1890-6.

Vestbo J, Prescott E, Almdal T, et al. Body mass, fat-free body mass, and prognosis in patients with chronic obstructive pulmonary disease from a random population sample: findings from the Copenhagen City Heart Study. *Am J Respir Crit Care Med.* 2006;173:79-83.

Visse R & Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res.* 2003;92:827-39.

Vlachopoulos C, Aznaouridis K & Stefanadis C. Clinical appraisal of arterial stiffness: the Argonauts in front of the Golden Fleece. *Heart.* 2006;92:1544-50.

Vlachopoulos C, Aznaouridis K & Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness. *J Am Coll Cardiol.* 2010;55:1318-27.

Volterrani M, Scalvini S, Mazzuero G, et al. Decreased heart rate variability in patients with chronic obstructive pulmonary disease. *Chest.* 1994;106:1432-7.

Vrieze A, Greef MHG, Wýkstra PJ, et al. Low bone mineral density in COPD patients related to worse lung function, low weight and decreased fat-free mass. *Osteoporos Int.* 2007;18:1197-1202.

Walter RE, Wilk JB, Larson MG, et al. Systemic inflammation and COPD: The Framingham Heart Study. *Chest.* 2008;133:19-25.

Wang TJ, Gona P, Larson MG, et al. Multiple biomarkers for the prediction of first major cardiovascular events and death. *N Engl J Med.* 2006;355:2631-9.

Welle I, Eide GE, Gulsvik A, et al. Pulmonary gas exchange and educational level: a community study. *Eur Respir J.* 2004;23:583-8.

Widlansky ME, Gokce N, Keaney JF, et al. The clinical implications of endothelial dysfunction. *J Am Coll Cardiol.* 2003;42:1149-60.

Wilkinson IB, Franklin SS & Cockcroft JR. Nitric oxide and the regulation of large artery stiffness: from physiology to pharmacology. *Hypertension.* 2004;44:112-6.

Wilkinson IB, MacCallum H, Cockcroft JR, et al. Inhibition of basal nitric oxide synthesis increases aortic augmentation index and pulse wave velocity in vivo. *Br J Clin Pharmacol.* 2002;53:189-92.

Wilkinson IB & Webb DJ. Venous occlusion plethysmography in cardiovascular research: methodology and clinical applications. *Br J Clin Pharmacol.* 2001;52:631-46.

Willemse BW, ten Hacken NH, Rutgers B, et al. Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. *Eur Respir J.* 2005;26:835-45.

Williams B, Lacy PS, Thom SM, et al. Differential impact of blood pressure-lowering drugs on central aortic pressure and clinical outcomes: principal results of the Conduit Artery Function Evaluation (CAFE) study. *Circulation.* 2006;113:1213-25.

Willum-Hansen T, Staessen JA, Torp-Pedersen C, et al. Prognostic value of aortic pulse wave velocity as index of arterial stiffness in the general population. *Circulation.* 2006;113:664-70.

Wilson DG, Bellamy MF, Ramsey MW, et al. Endothelial function in Marfan syndrome: selective impairment of flow-mediated vasodilation. *Circulation.* 1999;99:909-15.

Wilson DO, Weissfeld JL, Balkan A, et al. Association of radiographic emphysema and airflow obstruction with lung cancer. *Am J Respir Crit Care Med.* 2008;178:738-44.

Witte D, Vandergraaf Y, Grobbee D, et al. Measurement of flow-mediated dilatation of the brachial artery is affected by local elastic vessel wall properties in high-risk patients. *Atherosclerosis.* 2005;182:323-30.

Wood AM, De Pablo P, Buckley CD, et al. Smoke exposure as a determinant of autoantibody titre in 1-antitrypsin deficiency and COPD. *Eur Heart J.* 2011;37:32-8.

World Health Statistics. 2008;Geneva:World Health Organisation, 2008.

Wouters EFM. Local and systemic inflammation in chronic obstructive pulmonary disease. *Proc Am Thorac Soc.* 2005;2:26-33.

Wright JL, Farmer SG & Churg A. Synthetic serine elastase inhibitor reduces cigarette smoke-induced emphysema in guinea pigs. *Am J Respir Crit Care Med.* 2002;166:954-60.

Yasmin, McEniery CM, O'Shaughnessy KM, et al. Variation in the human matrix metalloproteinase-9 gene is associated with arterial stiffness in healthy individuals. *Arterioscler Thromb Vasc Biol.* 2006;26:1799-805.

Yasmin, McEniery CM, Wallace S, et al. Matrix metalloproteinase-9 (MMP-9), MMP-2, and serum elastase activity are associated with systolic hypertension and arterial stiffness. *Arterioscler Thromb Vasc Biol.* 2005;25:372.

Yasmin, McEniery CM, Wallace S, et al. C-reactive protein is associated with arterial stiffness in apparently healthy individuals. *Arterioscler Thromb Vasc Biol.* 2004;24:969-74.

Yeh ET, Anderson HV, Pasceri V, et al. C-reactive protein: linking inflammation to cardiovascular complications. *Circulation.* 2001;104:974-5.

Yoshida T & Tuder RM. Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. *Physiol Rev.* 2007;87:1047-82.

Young RP, Hopkins R & Eaton TE. Forced expiratory volume in one second: not just a lung function test but a marker of premature death from all causes. *Eur Respir J.* 2007;30:616-22.

Zhai G, Valdes AM, Cherkas L, et al. The interaction of genes and smoking on forced expiratory volume: a classic twin study. *Chest.* 2007;132:1772-7.

Zieman SJ, Melenovsky V & Kass DA. Mechanisms, pathophysiology, and therapy of arterial stiffness. *Arterioscler Thromb Vasc Biol.* 2005;25:932-43.

Zvezdin B, Milutinov S, Kojicic M, et al. A postmortem analysis of major causes of early death in patients hospitalized with COPD exacerbation. *Chest.* 2009;136:376-80.